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Molekulární mechanismus cirkadiánních hodin a jejich synchronizace v trávicím systému potkana

Molecular mechanism of the circadian clock and its entrainment within the rat digestive system

Dizertační práce

Vedoucí závěrečné práce: PharmDr. Alena Sumová, DSc.

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 27.04.2015 Lenka Polidarová

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LIST OF ABBREVIATIONS

AMPK	adenosine monophosphate-activated protein kinase
ANOVA	analysis of variance
ANS	autonomic nervous system
B2M	β-2-microglobulin
Ccgs	clock-controlled genes
CDK1/cycB1	cyclin-dependent kinase1/cyclinB1 complex
cDNA	complementary deoxyribonucleic acid
CK1δ, ε	casein kinase 1 delta, epsilon
CT	circadian time
DBP	albumin D-site binding protein
DD	constant dark, dark/dark
DL	reversed light/dark, dark/light
DMEM	Dulbecco´s modified Eagle´s medium
DNA	deoxyribonucleic acid
E	embryonic day
E4BP4	E4 promoter-binding protein 4
FAA	food-anticipatory activity
FCS	fetal calf serum
FEO	food-entrainable oscilator
GI	gastrointestinal
GIT	gastrointestinal tract
HAT	histone acetyltransferase
LD	light/dark
LL	constant light, light/light
mRNA	messenger ribonucleic acid
NAMPT	nicotinamide phosphoribosyltransferase
NAD^+/NADH	oxidized/reduced nicotinamide adenine dinucleotide
OD	optical density
P	postnatal day
PARbZIP	proline and acidic amino acid-rich basic leucine zipper
PBS	phosphate buffer saline

PGC1α	peroxisome proliferator-activated receptor-γ coactivator 1α
PPARα,γ	peroxisome proliferator-activated receptor-α, γ
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RF	restricted feeding regime
SCN	suprachiasmatic nuclei
SEM	standard error of the mean
SHR	spontaneously hypertensive rats
VIP	vasoactive intestinal peptide

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1 ABSTRACT

The circadian system controls the timing of behavioral and physiological processes in most organisms with a period of about 24 h. In mammals, the circadian system consists of the central oscillator in the suprachiasmatic nuclei (SCN) and peripheral oscillators located in numerous organs such as the liver, heart, lung, muscles, intestines etc. Peripheral oscillators are cell-autonomous, they could also work independently of the SCN entrained by a feeding cycle. The misalignment of the endogenous timekeeping system, due to e.g. irregular daily schedule or shiftwork, may lead to the development of severe diseases including sleep disorders, gastrointestinal (GI) problems and various types of cancer. Therefore, understanding the molecular mechanism of the circadian clock may facilitate the treatment of diseases caused by malfunction of the circadian system.

In my PhD thesis, I focused on the determination and synchronization of circadian clocks within the rat digestive system and on their development during ontogenesis. Moreover, the circadian system of a rat strain with pathology, i.e., spontaneously hypertensive rat (SHR) was also studied.

We identified the circadian clocks in the individual parts of the intestine and ascertained that these clocks are mutually synchronized with the phase-delay along the cranio-caudal axis of the alimentary tract.

Our data further showed that the GI clocks differ in their resistance to disruption by prolonged exposure to constant light. Distorted rhythmicity may be restored by a restricted feeding regime (RF) with various efficiency in individual GI tissues. The colonic clock seems to be more sensitive to changes in external conditions than the hepatic and duodenal clock.

The rhythmic expression of clock genes in the colon developed gradually during the postnatal ontogenesis, with changes in their mutual phasing and amplitudes until an adult-like state at postnatal day 30. Prenatally, the maternal circadian phase may modulate further development of the colonic clock. Postnatally, the presence/absence of rhythmic maternal care affected the phasing of the clock gene expression profiles in the colon.

Finally, we proved that the organization of the circadian system in SHR differs to that of control normotensive Wistar rats. These changes may result in poor temporal control of gene expression in peripheral organs. Moreover, SHR were more sensitive to feeding challenge at behavioral level which correlated with the responsiveness of their hepatic clock. The potential role of *Bmal2* gene in the adaptation of the hepatic clock to the RF was suggested.

ABSTRAKT

Cirkadiánní systém řídí načasování behaviorálních a fyziologických procesů většiny organismů s periodou zhruba 24 h. U savců se cirkadiánní systém skládá z centrálního oscilátoru v suprachiasmatických jádrech hypothalamu (SCN) a z periferních oscilátorů uložených v mnoha orgánech jako jsou játra, srdce, plíce, svaly, střeva atd. Periferní oscilátory jsou autonomní, mohou fungovat nezávisle na SCN a být seřizovány změnou doby příjmu potravy. Narušení vnitřního časového systému v důsledku např. nepravidelného režimu nebo práce na směny může vést k rozvoji nejrůznějších onemocnění, např. spánkových poruch, trávicích problémů a různých druhů nádorových onemocnění. Pochopení fungování molekulárního mechanismu cirkadiánních hodin může usnadnit léčbu onemocnění způsobených poruchami cirkadiánního systému.

Ve své disertační práci jsem se zaměřila na identifikaci a synchronizaci cirkadiánních hodin v trávicím systému potkana a na jejich vývoj během ontogeneze. Kromě toho byl také studován cirkadiánní systém kmene potkana vykazující patologii, tzn. spontánně hypertenzního potkana (SHR).

Podařilo se nám identifikovat cirkadiánní hodiny v jednotlivých částech střeva a zjistili jsme, že tyto hodiny jsou vzájemně synchronizovány s fázovým zpožděním ve směru kranio-kaudální osy trávicího traktu.

Naše data dále ukazují, že hodiny v gastrointestinálním traktu (GIT) se liší v odolnosti k narušení vlivem vystavení dlouhodobému stálému světlu. Narušená rytmicita může být obnovena změnou doby příjmu potravy (RF) s různou účinností v jednotlivých gastrointestinálních (GI) tkáních. Cirkadiánní hodiny v tlustém střevu se zdají být více citlivé na změny vnějších podmínek v porovnání s hodinami v játrech a dvanáctníku.

Rytmická exprese hodinových genů v tlustém střevu se vyvíjí postupně během postnatální ontogeneze prostřednictvím změn v jejich vzájemném nastavení fází a amplitudě až do stavu shodného s dospělými jednici ve věku 30 dnů. Prenatálně může mateřská cirkadiánní fáze modulovat budoucí vývoj střevních hodin mláďat. Postnatálně poté přítomnost/nepřítomnost rytmické mateřské péče ovlivňuje fázování exprese hodinových genů v tlustém střevu.

Nakonec jsme prokázali odlišnosti v organizaci cirkadiánního systému SHR v porovnání s kontrolními normotenzními potkany kmene Wistar. Tyto změny mohou vést ke špatnému časovému řízení genové exprese v periferních orgánech. Kromě toho byli SHR na úrovni chování více citliví na změnu doby příjmu potravy, což korelovalo se zvýšenou citlivostí jejich jaterních hodin. Výsledky naznačily potenciální roli genu *Bmal2* v adaptaci jaterních hodin na RF.

2 INTRODUCTION

Almost all organisms from procaryotes to humans evolved the endogenous timekeeping system (= the circadian clock) as an evolutionary adaptation in order to anticipate daily regular changes in external environment. To fulfill this role, the circadian clock drives physiological, metabolic and behavioral processes of the body, namely the sleep/wake cycle, rhythms in activity, temperature, hormone and enzyme secretions etc., with a period of about 24 h and coordinates them to the appropriate time of a day.

The circadian rhythms persist even in constant nonperiodic conditions in which free-run with an intrinsic, genetically determined period tau. Tau is species-specific and varies approximately around 24 h (Aschoff 1960). Under natural conditions, the endogenous period of the circadian clock is entrained to the 24-h solar day mainly by the light/dark cycle, but also by nonphotic cues such as physical activity, food intake, social factors and chemical substances (reviewed in Hastings et al. 1998, Challet 2007).

At the cellular level, the general principle of the molecular clockwork is based on a set of clock genes together interlinked into autoregulatory feedback loops of transcription and translation to produce circadian oscillations in order to control e.g. cell cycle divisions, DNA damage responses, metabolism, endocrine functions and epigenetic regulations (reviewed in Takahashi et al. 2008).

Aberrant circadian rhythms could lead to defects in the regulation of these processes. A major consequence of the modern lifestyle is the disruption of circadian rhythms that may contribute to the development of several pathological states including sleep disturbances, metabolic diseases and tumorigenesis. Epidemiological studies have revealed a direct link between shiftwork and some cardiovascular, metabolic, GI (Scheer et al. 2009) and mental disorders (Driesen et al. 2011; reviewed in Vogel et al. 2012), as well as some types of cancer (Mormont et al. 2000; Schernhammer et al. 2001; Viswanathan et al. 2007; Knutsson et al. 2013). Disruption of circadian rhythms was also associated with changes in tumor growth in animal models (Filipski et al. 2002; Fu et al. 2002). Additionally, it seems that so called chronotherapy, which is based on the administration of drugs at a specific time of a day in order to minimize toxicity and maximize effect, may improve the efficacy of the treatment of various

diseases (Filipski et al. 1999; Granda et al. 2001, Granda et al. 2002; Mormont & Lévi 2003; Lévi 2006).

Elucidating the mechanisms of how the circadian system interacts with the internal milieu and external conditions may help to minimize health risk factors and to facilitate the treatment of human clock-related pathological states.

3 OVERVIEW OF THE LITERATURE

3.1 Hierarchy of the circadian system

In mammals, the timekeeping system has a complex architecture composed of the central pacemaker in the SCN of the hypothalamus (Ralph et al. 1990) and subsidiary clocks in nearly every body cell of multiple tissues, such as the liver, heart, lungs, kidneys and muscles (Balsalobre et al. 1998; Sakamoto et al. 1998; Yamazaki 2000; Nagoshi et al. 2004; Yamamoto et al. 2004; Yoo et al. 2004; Nishide et al. 2006). The circadian system consists of three integral parts: input pathways that receive environmental cues (zeitgebers) and relay them to the central oscilator; the central oscillator that maintains circadian time and generates output rhythms; and output pathways that manifest the rhythms via the control of various metabolic, physiological and behavioral processes (reviewed in Balsalobre 2002; Silver & Schwartz 2006).

Each rodent SCN is composed of 10.000 neurons which themselves represent mutually synchronized individual circadian oscillators (Welsh et al. 1995). Apart from the SCN, peripheral clocks in mammalian tissues maintain circadian rhythms and modulate transcription factors in a paracrine fashion to regulate tissue-specific gene expression (reviewed in Dibner et al. 2010; Mohawk et al. 2012). The central clock ensures the synchrony of the endogenously generated rhythms with external environmental conditions and keeps the mutual synchrony of local oscillators within the entire organism via neural and humoral signals (Balsalobre 2000; McNamara et al. 2001; Terazono et al. 2003; Kalsbeek et al. 2004; Guo et al. 2005; Reddy et al. 2007; Pezük et al. 2012). The precise mechanism of interaction between the SCN and the peripheral clocks is unknown.

The knowledge of the circadian system hierarchy helps us to study the fundamental properties of each level of organization of the mammalian timekeeping system from cell-autonomous molecular oscillations to tissue-specific properties, to the interaction of central and peripheral oscillators, and ultimately, to the overt daily rhythms of behavior observed in animals and humans.

3.2 Synchronization of the circadian system

Although the molecular clocks in both the SCN and peripheral tissues are self-sustained and cell autonomous, the individual clocks must be regularly entrained to external cues to generate the appropriate 24-h rhythms, or otherwise, they lose their mutual coherence (reviewed in Reppert & Weaver 2002). In mammals, the peripheral clocks are entrained by the master pacemaker in the SCN. The SCN is directly entrained by the light/dark cycle (LD) and conveys this information to oscillators in other brain regions and in peripheral tissues (reviewed in Welsh et al. 2010). There are likely multiple entraining pathways, but the main entraining factors that relay this information and participates in the resetting of the peripheral clocks has not been identified yet.

An organism may become arrhythmic due to a disturbance of the circadian system. In this situation, SCN signaling to the peripheral clocks is lacking. This arrhythmicity may arise from prolonged exposure to constant light (LL) or irregular and frequent shifting of the LD cycle. In humans, such situations may develop due to long-term shiftwork, frequent crossing of multiple time zones, or an irregular life style (reviewed in Rajaratnam & Arendt 2001). Such conditions are considered risk factors for the development of many diseases of civilization, including GI disorders (reviewed in Hoogerwerf 2009).

While the LD cycle is the dominant cue for the SCN clock, the timing of food intake appears to be important in resetting the phase of clock gene expression in peripheral tissues (Oishi et al. 2004; Goh et al. 2008; Wu et al. 2008), indicating that metabolic signaling from ingested food might be a driving force for the generation of various peripheral rhythms. Under natural conditions, the timing of locomotor activity and food intake is controlled by the SCN. However, when access to food is limited only to a specific time of a day (the restricted feeding regime), the peripheral clocks, e.g. in the liver, heart, kidney, lung and intestines, adjust their phases to the timing of food availability, while the SCN clock remains unaffected and continues running in phase with the external LD cycle (Damiola et al. 2000; Hara et al. 2001; Stokkan et al. 2001; Hoogerwerf et al. 2007; Sládek et al. 2007). Under the RF regime in laboratory conditions, animals soon develop food-anticipatory activity (FAA) in advance of food availability (Mistlberger 1994; Davidson et al. 2001), including increases in locomotor activity, body temperature (Challet et al. 1997), corticosterone levels (Honma et al.

1983), and plasma levels of ketone body and free fatty acid (Escobar et al. 1998). This phenomenon is thought to be driven by another SCN-independent oscillator, the so called food-entrainable oscilator (FEO), whose existence and localization have not been proven yet (Davidson et al. 2003). It seems that under the challenge of the RF regime, signaling from the SCN competes with food, such that the timing of food availability dominates signaling about the LD cycle from the SCN. This mechanism, which uncouples the phases of peripheral clocks and the SCN clock, may ensure vital adaptation to abrupt changes in food availability while maintaining synchrony with environmental time (see Fig. 1).

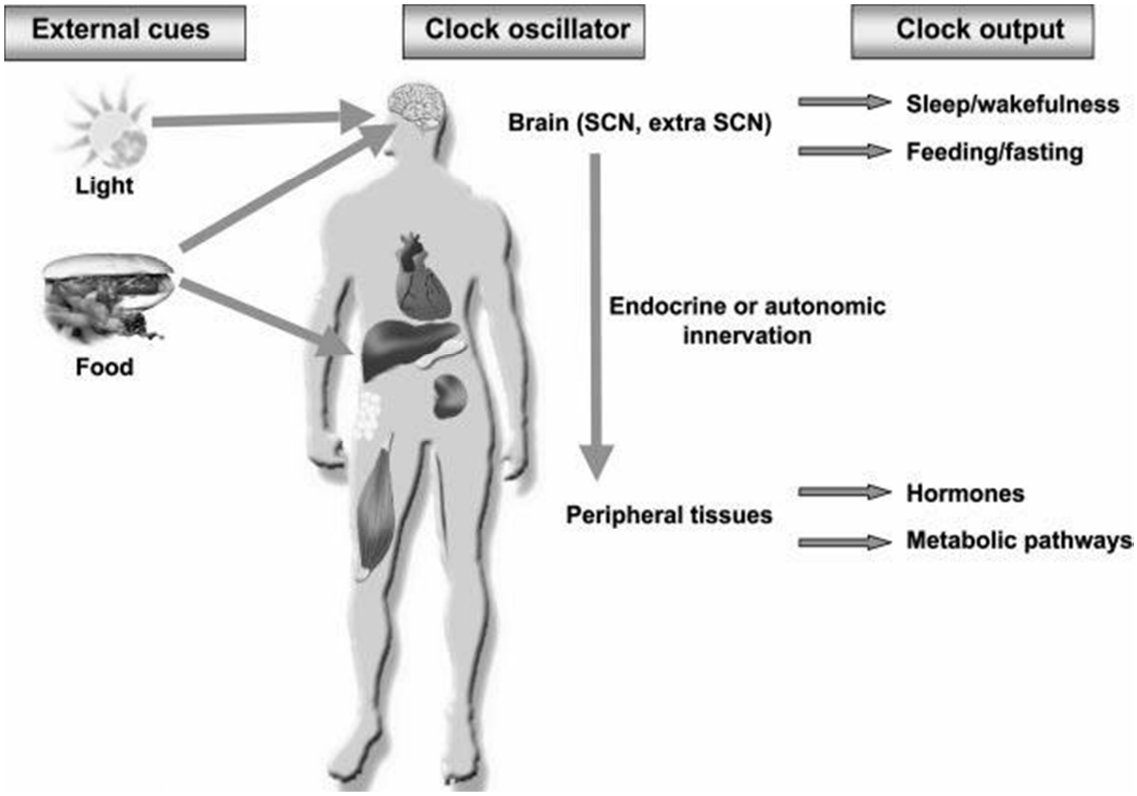

Fig. 1: Synchronization of the circadian system by external cues.
Light is the dominant environmental cue for the SCN. In turn, the SCN maintain circadian synchrony of the peripheral clocks via both neuronal and/or humoral signals. Peripheral oscillators may also be entrained by food intake independent of the SCN. Circadian synchrony within the body is reflected in robust rhythmic behavioral and physiological output rhythms in feeding, sleep/wakefulness, hormone secretion and metabolic processes. For more details see chapter 3.2 (adapted from Maury et al. 2010).

3.3 Molecular mechanism

The mammalian circadian molecular oscillator model encompasses our current understanding of the circadian control of gene expression within individual cells throughout the body. In this model, core circadian clock genes (*Per1*, *Per2*, *Cry1*, *Cry2*, *Rev-erbα*, *Rorα*, *Bmal1* and *Clock*) and their protein products form a

transcriptional/translational feedback loops that are responsible for the generation and regulation of 24-h rhythmicity (reviewed in Takahashi et al. 2008) (see Fig. 2).

CLOCK and BMAL1 proteins heterodimerize and induce transcription of genes containing E-box cis-regulatory sequences in their promoters, including *Per* and *Cry* genes (King et al. 1997; Gekakis 1998; Hogenesch et al. 1998; Bunger et al. 2000). PER and CRY proteins then form a complex in the cytoplasm that translocates to the nucleus and subsequently represses transcription of their own and other genes by direct inhibition of CLOCK/BMAL1. Thus, the CLOCK/BMAL1 heterodimer forms the positive, or transactivating component in this loop, while the PER/CRY complex acts as the negative, or transinhibiting component (Shearman et al. 1997; Kume et al. 1999; Vitaterna et al. 1999). To start a new transcription cycle, the activity of CLOCK/BMAL1 complex needs to be restored through the proteolytic degradation of PER and CRY proteins. *Bmal2* gene is a paralog of *Bmal1* (Ikeda et al. 2000), and its expression from a constitutively expressed promoter can restore the clock and metabolic phenotypes of *Bmal1*-knockout mice (Shi et al. 2010).

In addition, accessory regulatory loops interconnected with the core loop add robustness and stability to the clock mechanism and provide additional layers of control. The first of these accessory loops involves members of the large nuclear receptor family, *Ror* and *Rev-erb* genes, which are regulated by CLOCK/BMAL1-mediated activation through E-boxes in their promoters. Both of these proteins are transcription factors that bind to the *Bmal1* promoter at REV-ERBα and RORα response elements. RORα activates *Bmal1* transcription, whereas REV-ERBα inhibits it (Preitner et al. 2002; Triqueneaux et al. 2004; Akashi and Takumi 2005). The RORα-mediated activation of *Bmal1* transcription is enhanced by peroxisome proliferator-activated receptor-γ (PPARγ) coactivator 1α (PGC1α) (Liu et al. 2007). Members of the proline and acidic amino acid-rich basic leucine zipper (PARbZIP) transcription factor family, including the activator D-box binding protein (DBP) and the repressor E4 promoter-binding protein 4 (E4BP4) act via D-box elements in target genes to form a second accessory feedback loop (Mitsui et al. 2001; Ueda et al. 2005; Ohno et al. 2007).

Posttranslational modifications of the core clock components, such as phosphorylation, ubiquitination, sumoylation and acetylation/deacetylation, play a crucial role in establishing the 24-h rhythm of the circadian clock. Some of these modifications are essential for clock function, while others only fine-tune rhythms.

Previous works revealed that casein kinases δ and ε (CK1δ/ε) -mediated phosphorylation regulates PER nuclear localization and its ability to inhibit CLOCK/BMAL1-mediated transcription and promotes its ubiquitin-dependent degradation via the proteasome (Lee et al. 2001; Akashi et al. 2002; Eide et al. 2005). Adenosine monophosphate-activated protein kinase (AMPK) was shown to modulate the stability of CRY protein by phosphorylation, which stimulates the direct binding of FBXL3 ubiquitin ligase to CRY and its subsequent targeting for proteolytic degradation (Busino et al. 2007; Lamia et al. 2010). Sumoylation of BMAL1 regulates its stability (Cardone et al. 2005), whereas acetylation of BMAL1 enhances its interaction with CRY (Hirayama et al. 2007).

Another level of circadian machinery regulation is achieved through chromatin remodelling. Circadian promoters have oscillations in histone modifications, such as acetylation/deacetylation and methylation. Interestingly, CLOCK has been shown to possess intrinsic histone acetyltransferase (HAT) activity (Doi et al. 2006). This suggests that CLOCK, while activating transcription with its partner BMAL1, may rhythmically acetylate histones at target genes and thereby participate in chromatin remodelling. CLOCK also acetylates nonhistone substrates, including its partner BMAL1 (Hirayama et al. 2007).

The circadian clock controls the expression of nicotinamide phosphoribosyltransferase (NAMPT), a key rate-limiting enzyme in the salvage pathway of nicotinamide adenine dinucleotide (NAD^+) biosynthesis (Nakahata et al. 2009). The transcriptional activity of CLOCK/BMAL1 is sensitive to the cellular NAD^+/NADH ratios, its activity is thus intimately tied to a cell redox state (Rutter et al. 2001). Therefore, circadian clock-mediated oscillations in NAD^+/NADH metabolism may in turn feedback to influence the timing of the circadian clock at the cellular level (Nakahata et al. 2009).

The aforementioned general clock mechanism drives rhythmic expression of a large number of genes, so called clock-controlled genes (ccgs), involved in the control of various regulatory pathways and provides thus a circadian output to regulate physiology (Bass & Takahashi 2010; Asher & Schibler 2011). Microarray studies have shown that approximately 10-15 % of all mammalian transcripts exhibit circadian oscillations in a tissue-specific manner (Akhtar et al. 2002; Duffield et al. 2002; Panda et al. 2002). It is important to emphasize that many ccgs are crucial for the regulation of

metabolic and physiological output functions and, conversely, some of the output products can also feedback to the clock machinery (reviewed in Brown & Schibler 1999).

It has recently been shown that microRNAs also participate in the control of posttranscriptional clock output pathways (reviewed in Cheng & Obrietan 2007; O'Neill & Hastings 2007).

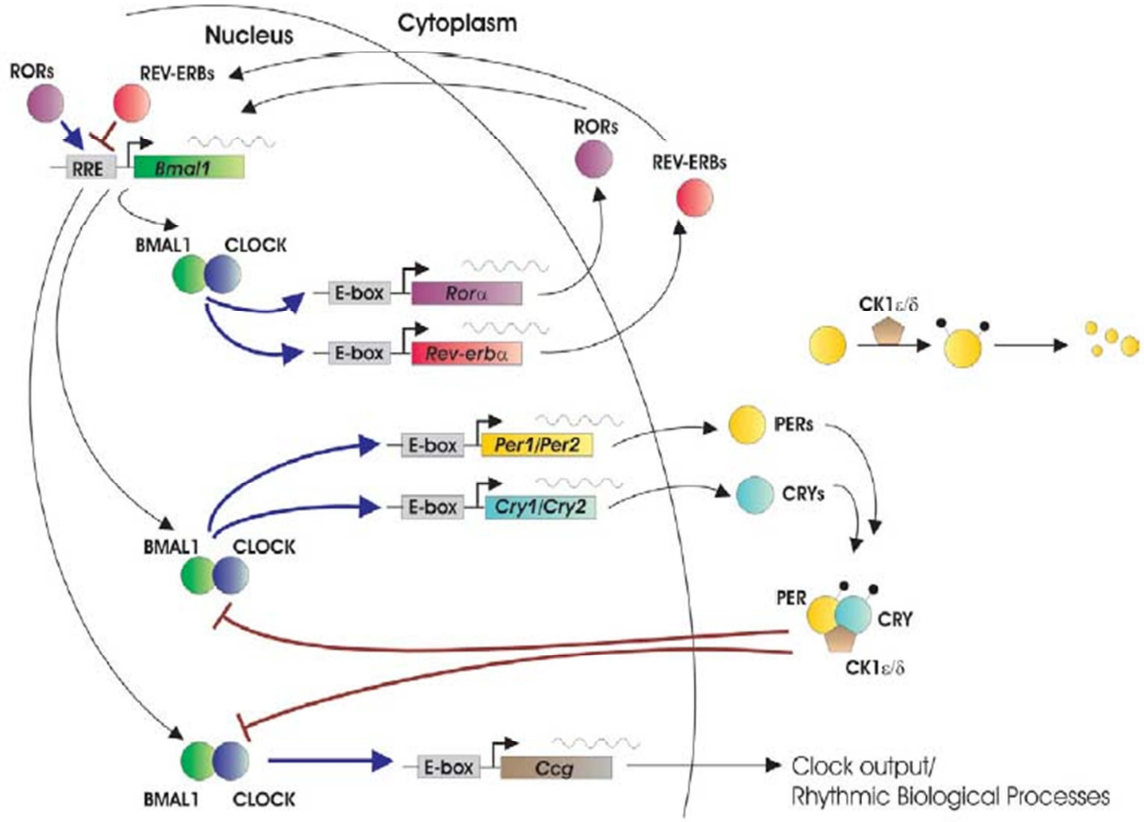

Fig. 2: Simplified scheme of the molecular mechanism of the circadian clock in mammals.

In this model, core circadian clock genes (Per1, Per2, Cry1, Cry2, Rev-erbα, Rorα, Bmal1 and Clock) form a transcriptional/translational feedback loops that are responsible for the generation of 24-h rhythmicity. CLOCK/BMAL1 heterodimers induce transcription of Per, Cry, Rev-erbα, Rorα and ccgs. Phosphorylated complexes of PER and CRY bind to CLOCK/BMAL1 heterodimers and repress transcription of their own and other genes. Additional feedback loops including Rev-erbα and Rorα and post-translational modifications fine-tune precision of the clock mechanism. For more details see chapter 3.3 (adapted from Ko & Takahashi 2006).

3.4 Circadian clock in the gastrointestinal tract

Circadian rhythms play an important role in the regulation of the digestive system of many organisms. The GIT exhibits daily rhythms in many physiological processes such as gut motility, digestion, absorption, activity of mucosal enzymes and transporters, cell proliferation and regeneration (reviewed in Scheving et al. 2000; Hoogerwerf 2006; Hussain & Pan 2009; Karpowicz et al. 2013). These rhythms are driven by food intake but persist even during food deprivation which indicates the existence of the endogenous circadian oscilator (Scheving et al. 1984; Froy et al. 2005). The presence of the clock genes has been demonstrated in various parts of the GIT in different species such as the rat, mouse small/large intestine and human oral mucosa and intestinal epithelium (Bjarnason et al. 1999; Davidson et al. 2003; Pardini et al. 2005; Froy & Chapnik 2007; Hoogerwerf et al. 2007, Sládek et al. 2007). This suggests a possible role of clock genes in the regulation of various GIT functions through the modulation of organ-specific ccgs. Approximately, 3,7 % of distal colonic genes are expressed rhythmically (Hoogerwerf et al. 2008). Daily variations seem to be regulated by two pathways. The first pathway could be a consequence of a passive response to cyclically available gut luminal signals from food intake. The second pathway may use anticipatory mechanisms to prepare the intestine for the expected presence of these signals before their exposure to luminal content. Or, they may result from a combination of both mechanisms (Tavakkolizadeh et al. 2001; Pan et al. 2002; Pan & Hussain 2009; Stearns et al. 2009). The SCN likely synchronize the GI clocks via multiple neuronal and humoral pathways including the autonomic nervous system (ANS) (Kalsbeek et al. 2004; Guo et al. 2005) and glucocorticoids (Balsalobre 2000; Terazono et al. 2003; Reddy et al. 2007; Pezük et al. 2012). The colonic clock might be also entrained by the feeding regime *per se* (Hoogerwerf et al. 2007; Sládek et al. 2007). Disruption of circadian rhythms, which occurs with long-term shiftwork and jet lag, has been associated with symptoms of disordered GI (Costa 1996; Karlsson et al. 2003; Caruso et al. 2004). This suggests a close relationship between circadian rhythms, GIT homeostasis and diseases. Therapies based on optimization of food intake may help to cure these diseases.

3.5 Circadian regulation of the cell cycle in the gastrointestinal tract

In the GIT, the proliferation rhythms exhibit a cranio-caudal gradient, with the proliferation rhythms of the distal part lagging behind those of the proximal part (Scheving et al. 1979, Scheving et al. 1980). Rhythms in proliferation are phase-locked relative to day-time so that the S and M phases of the cell cycle occur only at a specific time of a day. The mechanisms evolved in order to separate processes from DNA-damaging UV light and from oxidative stress (Matsuo et al. 2003; Chen & Mcknight 2007). The intestinal epithelium is a continually self-renewing differentiating tissue whose cells originate from the stem cells located in the base of the intestinal crypts. During differentiation, cells migrate up the crypt-villus axis, undergo apoptosis and are extruded into the intestinal lumen. The cells continue to proliferate or differentiate depending on cell cycle regulation (Marra et al. 1994).

Several lines of evidence suggest the existence of a crosstalk between the circadian clock and the cell cycle via the temporal control of molecules that control cell cycle progress (see Fig. 3). CLOCK/BMAL1 heterodimers may control the cell cycle through the repression of *c-Myc* (G0/G1 transition) and *p21* (G1/S transition), and through the activation of *Wee1* (G2/M transition) and the multifunctional tumor suppressor gene *p53* (Fu et al. 2002; Matsuo et al. 2003; Gréchez-Cassiau et al. 2008; Mullenders et al. 2009). WEE1 is a kinase that phosphorylates and inactivates the cyclin-dependent kinase1/cyclinB1 complex (CDK1/cycB1) to control the G2/M transition. *Wee1* was found to be an E-box containing ccg in the mouse liver (Matsuo et al. 2003). The expression of some cyclins in the human oral mucosa and rectal epithelium undergoes circadian variation as well (Bjarnason et al. 1999; Griniatsos et al. 2006).

Disruption of circadian homeostasis by mutations in certain circadian clock components results in alterations in cell proliferation, apoptosis, DNA damage and metabolism that in some cases predispose to cancer. *Per2* gene has unique tumor suppressor functions. *Per2*-mutant mice are predisposed to oncogenic transformation following γ-radiation (Fu et al. 2002). Mating these mice with polyp formation-prone $Apc^{Min/+}$ mice increases the frequency of polyp formation in intestinal mucosa (Wood et al. 2008; Yang et al. 2009a). The *Clock* mutation and decreased *Per2* and *Per3*

expressions have also been found in human tumors including colorectal cancer (Zeman et al. 2008; Alhopuro et al. 2010; Wang et al. 2012). The oncogenic role of *Wee1* was supported by its significant changes in expression during colorectal tumorigenesis (Soták et al. 2013). Together, these data emphasize the coupling of cell proliferation and the circadian clock in the GIT.

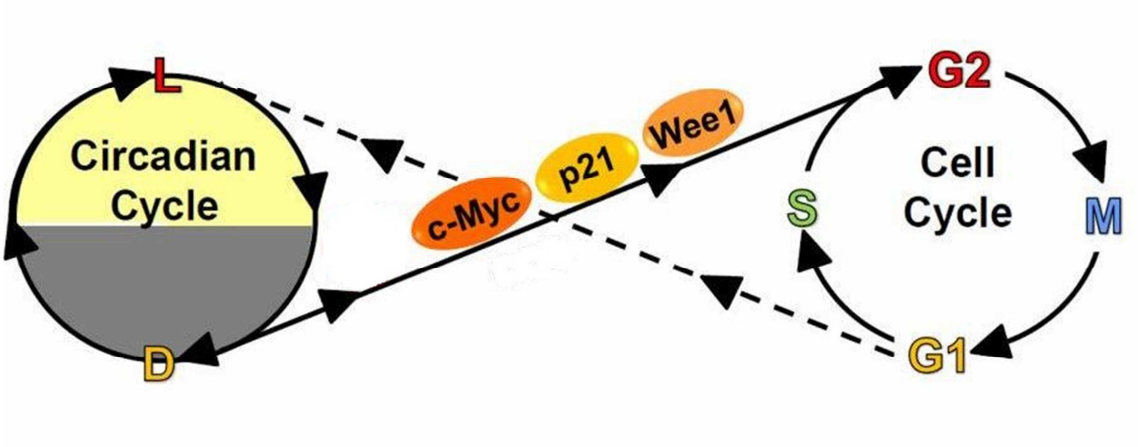

Fig. 3: Coupling the circadian clock to the cell cycle.
The circadian clock directly drives circadian transcription of ccgs Wee1, c-Myc and p21, which in turn modulate the circadian activity of the regulators (Cdk/cyclin complexes) of the cell cycle. Hypothetically, the cell cycle may influence the circadian cycle by halting transcription during mitosis, thus causing a phase shift of the circadian rhythm. For more details see chapter 3.5 (adapted from Sancar et al. 2010).

3.6 Ontogenesis of the circadian system

The circadian system of the rat develops gradually during the prenatal and postnatal ontogenesis depending on the maturation and functional relevance of tissues and organs (reviewed in Weinert 2005; Sumová et al. 2006). The GIT undergoes major morphological and functional modifications which are apparent from the prenatal period until weaning (reviewed in Pácha 2000). Investigation of the evolution of circadian rhythmicity considers the development of individual oscillators and their intrinsic rhythms and development of entraining pathways enabling synchronization.

Neurogenesis of the SCN is completed at embryonic day 18 (E18), but the morphological maturation continues until postnatal day 10 (P10). Only sparse synapses

among the SCN neurons could be observed during the prenatal and early postnatal period. Thus, the multilevel intercellular coupling web of synapses important for complete functionality of the adult SCN clock is probably not yet evolved during the prenatal period. The number of synapses gradually increases until about P10 (Moore & Bernstein 1989), when the master clock achieves adult-like state and becomes fully functional (Sládek et al. 2004).

The peripheral clocks begin to exhibit individual rhythmicity independent of each other at various developmental stages (Yamazaki et al. 2009). In the rat heart, the rhythmic expression of clock genes begins between P2 and P5 (Sakamoto et al. 2002). In the rat liver, clock genes expression rhythms occur at P2 and further develop until weaning (Sládek et al. 2007). Thus, the first appearance of molecular oscillations is tissues-specific.

In the prenatal and early postnatal period, the developing circadian clock is synchronized with the external LD cycle dominantly by maternal neuronal and humoral cues. During the early postnatal stages, the peripheral clocks are likely set or driven by maternal behavior and feeding because the undeveloped SCN is not able to synchronize them. Later postnatally, the LD synchronization via the SCN gradually prevails maternal signaling (reviewed in Sumová et al. 2012). In contrast to the SCN clock, which is already set to the proper phase with the external LD cycle after the birth (Sládek et al. 2004; Kováčiková et al. 2006), the phases of peripheral clocks including the liver and heart shift during the postnatal period of life (Sakamoto et al. 2002; Sládek et al. 2007). This phenomenon is likely related to changes in timing of food intake. As during lactation, pups of nocturnal animals are breast fed during the day-time and then start to consume the solid food preferably during the night after weaning. During the first weeks of life, the setting of peripheral clocks by the feeding regime may overcome the entrainment by the undeveloped SCN (Sládek et al. 2007; reviewed in Sumová et al. 2012) (see Fig. 4). The mechanisms of how a mother entrains her pups are not fully elucidated, but candidate pathways including maternal care, feeding regime and melatonin signaling have been suggested (reviewed in Sumová et al. 2006; Sumová et al. 2012, Houdek et al. 2014). Development of the colonic circadian clock has not been studied yet.

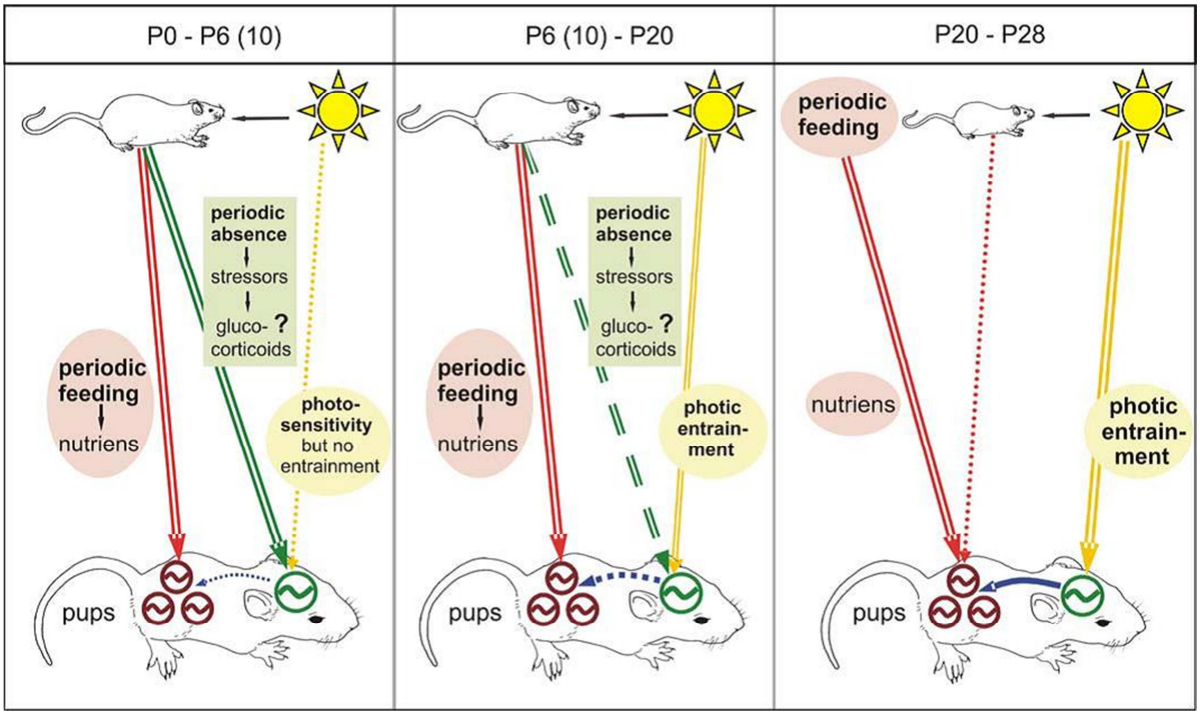

Fig 4: Signals entraining the central and peripheral clocks during three developmental periods after birth in rodents.

P0-P6 (10): Pups are fully dependent on maternal care. Periodic absence of the mother might entrain molecular oscillations within the pup's SCN. Periodic breast feeding and maternal care entrain molecular oscillations in the peripheral clocks. The photic entrainment of the newborn pup´s SCN does not occur yet.

P6 (10)-P20: Pups start to be partially independent of their mothers. Significance of maternal absence as an entraining cue of the pup's SCN is lost, the pup's SCN begin to be entrained by photic stimuli. Therefore, the pup's SCN may start to control the peripheral clocks. Apart from the maternal day-time feeding, pups begin gradually to forage themselves during the night-time and molecular oscillations of peripheral clocks shift accordingly.

P20-P28: Pups become completely independent of their mothers. Similarly to adults, the SCN clock is entrained dominantly by photic cues and peripheral clocks by nocturnal feeding regime. The SCN may entrain molecular oscillations in peripheral clocks either directly or rather indirectly via entraining the feeding regime with the external day-time. For more details see chapter 3.6 (adapted from Sumová et al. 2006).

3.7 Spontaneously hypertensive rats

SHR, which are normotensive at birth and develop sustained hypertension between the 3th and 6th month of life, are a widely studied animal model of hypertension and metabolic syndrome (Pravenec et al. 2004). The cardiovascular and metabolic functions are both temporally controlled by the endogenous timekeeping system. Therefore, SHR can be used to study the interference of these diseases with the circadian system. Moreover, Woon et al. recently identified several polymorphisms in the SHR *Bmal1* promoter, which are associated with hypertension and metabolic syndrome, that suggests a potential relationship between the circadian system and the SHR pathological phenotype (Woon et al. 2007). Previous results suggested that the temporal control of physiological processes may be affected in SHR. SHR exhibit aberrant sleeping patterns (Carley et al. 2000; Kuo et al. 2004), dampened amplitudes of diurnal rhythms in blood pressure (Lemmer et al. 1993) and elevated expression of vasoactive intestinal peptide (VIP) crucial for a high amplitude circadian rhythmicity in the SCN (Peters et al. 1994), which could contribute to the spectrum of SHR pathophysiology. At the level of peripheral clocks, tissue-specific differences in clock gene expressions were also found in SHR. The clock gene and ccg expression rhythms exhibited increased amplitudes in the heart, but not in the aorta of SHR compared to Wistar Kyoto rats (Naito et al. 2003). SHR exhibit the attenuation in the diurnal variation of feeding behavior which is caused by a significant increase in food intake during the light period and by a mild decrease during the dark period. Altered expression levels of some hypothalamic neuropeptides affecting feeding behavior and energy balance as well as circulating nutrients and hormones secreted from metabolic tissues were also found in SHR. Therefore, SHR have disordered diurnal control of feeding circuits at both the behavioral and molecular levels (Cui et al. 2011). Moreover, fifteen metabolism-related proteins were identified that display different levels of expression in the liver of SHR and confirm thus the impact of hypertension on hepatic physiology (Svoboda & Kawaja 2012). Factors regulating both circadian and metabolic systems exhibit significant overlap and disruption to one leads to reciprocal disturbances in the other (Marcheva et al. 2010). Regular food intake may contribute to normalize disrupted physiological functions connected with the malfunction of the circadian system, e.g. the RF regime may restore diurnal rhythm in blood pressure,

clock and metabolism-related gene expressions in cardiovascular tissues of SHR (Cui et al. 2011). SHR are supposed to be hyperresponsive to environmental stimuli (Williams et al. 2000), therefore studies regarding sensitivity of SHR to metabolic challenge may help to moderate the pathology of this strain.

4 AIMS OF THE THESIS

1. **Identification of the circadian clocks and their phase relationship within the individual parts of the rat gut**

The objective of the study was to identify the circadian clock within the individual parts of the rat gut and to ascertain whether clocks in the upper part of the gut (duodenum) are synchronized with those in the lower part (colon). Moreover, we aimed to detect the expression of the cell cycle kinase *Wee1* in the upper and lower parts of the gut as a marker of putative clock-controlled rhythms.

2. **Synchronization of the rat circadian clocks within the digestive system in conditions of disturbed signalization from the SCN**

The aim of the study was to determine the extent to which disturbance in the SCN signaling via prolonged exposure to LL affects circadian rhythms in the liver, duodenum, and colon, as well as to determine whether and to what extent food intake may restore rhythmicity in these individual parts of the digestive system. To assess the functional state of the hepatic and intestinal clocks, the ability of these peripheral clocks to drive rhythms of ccgs *Wee1* and *Dbp* was investigated.

3. **Development and entrainment of circadian oscillations in the rat colon during ontogenesis**

We aimed to find out when and how the circadian clock in the colon develops during the ontogenesis between the fetal stage at E20 and P30 when weaning is completed. We also studied whether maternal cues and/or the developing pup´s SCN may influence the ontogenesis of the colonic clock. To address this question, cross-foster study and the manipulation with maternal lighting and feeding conditions were performed.

4. Characterization of the circadian system of SHR and comparison of its sensitivity to temporal changes in the feeding regime with the normotensive Wistar rat strain

In the first part of our study, we wanted to ascertain whether the functional state of the circadian system of a rat strain exhibiting complex pathophysiological symptoms involving spontaneous hypertension and an abnormal metabolic state, i.e. in SHR, differs from that of control Wistar rats.

In the second part of the study, we aimed to compare sensitivity of the circadian system to the RF regime between SHR and Wistar rats.

We analyzed the function of the circadian system of SHR in its complexity, at the behavioral level and at the level of molecular mechanism in the liver and colon. The potential impact of the SHR phenotype on the circadian control of metabolic pathways was estimated by daily profiles of metabolism-relevant gene expression.

5 METHODS

5.1 Experimental animals

Prior to the experiments, adult Wistar rats (BioTest s.r.o., Konarovice, Czech Republic) and SHR/Ola (Institute of Physiology, Academy of Sciences of the Czech Republic) were fed *ad libitum* and maintained at a temperature of 23 ± 2 °C under LD 12:12 (12 h of light, 12 h of darkness). 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.

In the first experiment concerning the identification of the circadian clock within the individual parts of the gut, the animals were released into constant darkness (DD) on the day of the experiment in order to avoid the masking effect of the light. To determine the daily profiles in gene expression, the animals were sampled every 4 h throughout the whole 24-h circadian cycle. In a separate experiment, the animals were sampled every 1 h during the previous light phase of the LD cycle.

To unravel the impact of LL and the effect of the RF regime on the circadian clock in studied tissues, the animals were exposed to LL for 30 days and fed *ad libitum* or they were exposed to the RF regime (when food was provided only for 6 h, i.e., between 09:00 and 15:00 h) for the last 14 days of the LL-exposure interval. To determine the daily gene expression profiles in both groups, five animals were sampled every 4 h throughout the entire 24-h circadian cycle on the 30^{th} day in LL, starting at the time corresponding to lights-on of the previous LD regime.

In the ontogenetic study, pregnant rats were maintained individually in cages and the day of delivery was designated P0. Sampling was performed at E20, P2, P10, P20, and P30. The pups remained with their mothers throughout the entire experiment in spite of the fact that around P21, weaning was completed, and thus, the pups sampled at P30 were already feeding themselves independent of their mothers. On each day of sampling, pregnant rats or pups with their mothers were released into DD and sampled every 4 h during the 24-h interval. In the cross-foster experiment, rats were maintained either in LD 12:12 or in a reversed dark/light cycle (DL 12:12). The pregnant rats were housed as described above. On the day of delivery, entire litters of pups born to mothers in LD 12:12 were transferred to foster mothers in DL 12:12 and in parallel, entire litters

of pups born to mothers in DL 12:12 were transferred to foster mothers maintained in LD 12:12. Cross-fostering was performed within 12 h of birth. At P10, pups reared by foster mothers in LD 12:12 and DL 12:12 were sampled in 4-h intervals during the entire 24-h cycle. To ascertain the effect of LL and maternal RF regime, the pregnant rats were exposed to LL from conception. At P1, the mothers with their pups were divided into two groups. One group of mothers was fed *ad libitum*, and the other group had temporally restricted access to food, so that the food was present for 6 h per the light part of a day. Pups born to mothers from both groups were sampled at P10 and P20 every 4 h during the 24-h cycle in LL.

In the SHR study, locomotor activity in LD and DD and feeding behavior of SHR and Wistar rats were monitored during the period leading to the sampling. In the second part of the SHR study, rats of both strains were maintained under LD 12:12 with free access to food and water for 16 days. During the next 10 days in LD 12:12, the animals were subjected to the RF protocol. To determine the daily profiles in gene expression, rats of both strains were sampled as described in the first experiment.

5.2 Locomotor activity and feeding monitoring

Rats were maintained individually in cages equipped with infrared movement detectors attached above the center of the cage top. 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 the visualization of data. The resulting data, including calculations of the chi-square periodograms with $p < 0.001$, were analyzed using ClockLab toolbox (Actimetrics, Illinois, USA).

To analyze the feeding activities of SHR and Wistar, the rats were maintained in cages equipped with motion-sensitive video cameras, apart from the aforementioned infrared detectors. The cameras were connected to a video recorder. The time the rats spent by feeding throughout the 24-h period was counted by two independent observers who were blind to the experimental procedure. In the LL study, total time in minutes spent feeding was cumulated for the subjective day and subjective night on the 2^{nd}, 15^{th} and 29^{th} days.

5.3 *In situ* hybridization

The daily profiles of the levels of *Per1, Per2, Bmal1 and Rev-erbα* mRNA were measured by *in situ* hybridization method to detect the presence of rhythm in gene expression and its phase in the pup and adult SCN.

The pups or adult rats were killed by rapid decapitation. Brains 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 *Per1, Per2, Bmal1* and *Rev-erbα* 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 at 60°C for 20 h. 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).

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.

5.4 Real-time qRT-PCR

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

The total RNA was extracted by homogenization from the liver and by sonication from the colon and subsequently purified using the RNeasy Mini kit (Qiagen, Valencia, USA) according to the manufacturer's instructions. RNA concentrations were determined by spectrophotometry at 260 nm, and the RNA quality was assessed by electrophoresis on a 1.5% agarose gel. Moreover, the integrity of randomly selected samples of total RNA was tested using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). Then, the total RNA was reverse transcribed using the SuperScript VILO cDNA synthesis kit (Invitrogen, Carlsbad, USA) with random primers. The resulting cDNAs were used as templates for qRT-PCR. Diluted cDNA was amplified on a LightCycler 480 (Roche, Basel, Switzerland) using the Express SYBR

GreenER qPCR SuperMix Universal kit (Invitrogen) and the corresponding primers (sequences were published previously (Sládek et al. 2007; Sládek et al. 2012)). Relative quantification was achieved using a standard curve and subsequently normalizing the gene expression to a housekeeping gene *β2-microglobulin* (B2M).

5.5 Immunohistochemistry

To compare the spatial distribution of BMAL1-immunoreactive cells in the colon of SHR and controls, 12-mm-thick coronal sections of colon were cut, mounted on slides, fixed in 4% paraformaldehyde in PBS and processed for immunohistochemistry using the standard avidin-biotin method with diaminobenzidine as the chromogen (Vector Laboratories, Peterborough, UK) as described elsewhere (Sládek et al. 2007). The BMAL1 antibody was raised against the C-terminal 15 residues of mBMAL1 (GLGGPVDFSDLPWPL) using the Sigma-Aldrich custom peptide antibody service and was characterized previously (Reddy et al. 2005). As controls for background staining, parallel sections were treated simultaneously through the immunohistochemical procedure without incubation with the specific primary antibody.

5.6 Western blot

Samples of liver were removed, frozen in dry ice and homogenized in CellLytic MT extraction reagent with Protease inhibitor cocktail (both from Sigma-Aldrich) using SilentCrusher S (Heidolph, Schwabach, Germany). Protein concentration was determined by Bradford assay (Thermo Scientific, MA, USA). Total protein was mixed with NuPAGE LDS Sample buffer and Sample reducing reagent, denatured and separated with protein ladder on NuPAGE Bis-Tris 4-12% premade gel using NuPAGE MOPS SDS running buffer with antioxidant according to the manufacturer's instructions. The protein was transferred by electroblotting in NuPAGE transfer buffer onto a nitrocellulose membrane according to the manufacturer's instructions. The membrane was blocked in StartingBlock T20 blocking buffer and then incubated with a primary antibody against BMAL2 (both from Sigma-Aldrich). The membrane was then

washed and incubated with a secondary anti-rabbit HRP-conjugate antibody (Promega, Madison, USA). The membrane was then incubated with SuperSignal West Femto Chemiluminescent substrate (Pierce, USA) and immunoreactive bands were detected using a cooled camera system. Photographs of blots were imported into the ImageJ (NIH, USA) software, where the optical density of immunoreactive bands corresponding to the predicted protein size of BMAL2 was quantified relative to β-ACTIN internal standard.

5.7 Cell culture and real-time bioluminescence monitoring

Primary rat fibroblasts were prepared from adult Wistar and SHR rat skeletal muscle. Minced fragments were incubated in collagenase in DMEM with penicillin-streptomycin and gentamycin (all from Sigma-Aldrich). Digestion was stopped with fetal calf serum (FCS) (Sigma-Aldrich). The suspension was plated in fresh DMEM containing FCS, Glutamax-I CTS (Gibco, Carlsbad, USA) and antibiotics. The fibroblasts emigrating from tissue pieces were allowed to reach 80% confluence, trypsinized and then grown in DMEM (FCS, Glutamax, antibiotics) until spontaneously immortalized cells emerged. These cells were used for experiments between passages 5–12. Fibroblasts were transfected after reaching 70-80% confluence by *Bmal1-dLuc* (*Bmal1* promoter in destabilized luciferase-containing plasmid) (Sato et al. 2006) (Mr. M. H. Hastings, MRC-LMB, UK). On the day of the experiment, the cells were either synchronized with forskolin or horse serum (both from Sigma-Aldrich) for 1 h or left untreated. The medium was then replaced with a recording medium containing luciferin-EF (Promega). Dishes were placed into the Lumicycle (Actimetrics) for luminescence recording. Both the Wistar and SHR fibroblasts were transfected and recorded simultaneously. The transfections and recordings were repeated and assessed in 2 to 3 independent experiments for each rat strain and condition.

5.8 Statistical analysis

Locomotor activity and the differences between subjective day-time and subjective night-time feeding were evaluated by Student's t test, with $p < 0.05$ being required for significance.

The data for the 24-h profiles of studied gene expression levels were depicted either as individual values or expressed as the mean ± SEM for each group. The 24-h gene expression 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.

Moreover, the 24-h profiles were fitted with single cosine curves (Nelson et al. 1979) defined by the equation Y = mesor + [amplitudecos(2π[X-acrophase]/wavelength)] with a constant wavelength of 24 h. The least squares regression method was applied using Prism 5 software (GraphPad, La Jolla, USA). The amplitude, acrophase and coefficient of determination R^2 (i.e., the goodness of fit) were calculated. The acrophases and amplitudes of the profiles were compared by Student's t test and $p < 0.05$ was required for significance.

For quantitative analysis of the bioluminiscence data software package supplied with Lumicycle (Actimetrics) was used.

For more detailed information, please see the attached publications.

6 LIST OF PUBLICATIONS

Publications discussed in the PhD thesis:

1. **Identification of the circadian clocks and their phase relationship within the individual parts of the rat gut**

POLIDAROVÁ L., SOTÁK M., SLÁDEK M., PÁCHA J., SUMOVÁ A., 2009. Temporal gradient in the clock gene and cell-cycle checkpoint kinase *Wee1* expression along the gut. *Chronobiology Int*. 26(4): 607-20, IF 4,028

2. **Synchronization of the rat circadian clocks within the digestive system in conditions of disturbed signalization from the SCN**

POLIDAROVÁ L., SLÁDEK M., SOTÁK M., PÁCHA J., SUMOVÁ A., 2011. Hepatic, duodenal, and colonic circadian clocks differ in their persistence under conditions of constant light and in their entrainment by restricted feeding. *Chronobiology Int*. 28(3): 204-15, IF 4,028

3. **Development and entrainment of circadian oscillations in the rat colon during ontogenesis**

POLIDAROVÁ L., OLEJNÍKOVÁ L., PAUŠLYOVÁ L., SLÁDEK M., SOTÁK M., PÁCHA J., SUMOVÁ A., 2014. Development and entrainment of the colonic circadian clock during ontogenesis. *Am J Physiol Gastrointest Liver Physiol*. 306(4): G346-56, IF 3,65

4. **Characterization of the circadian system of SHR and comparison of its sensitivity to temporal changes in the feeding regime with the normotensive Wistar rat strain**

SLÁDEK M., POLIDAROVÁ L., NOVÁKOVÁ M., PARKANOVÁ D., SUMOVÁ A., 2012. Early chronotype and tissue-specific alterations of circadian clock function in spontaneously hypertensive rats. *PLoS One* 7(10): e46951, IF 4,09

POLIDAROVÁ L., SLÁDEK M., NOVÁKOVÁ M., PARKANOVÁ D., SUMOVÁ A., 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* 8(9): e75690, IF 4,09

Publications that are not discussed in the PhD thesis:

SLÁDEK M., RYBOVÁ M., JINDRÁKOVÁ Z., ZEMANOVÁ Z., POLIDAROVÁ L., MRNKA L., O'NEILL J., PÁCHA J., SUMOVÁ A., 2007. Insight into the circadian clock within rat colonic epithelial cells. *Gastroenterology*. 133(4): 1240-9. IF 12,455

SOTÁK M., POLIDAROVÁ L., MUSÍLKOVÁ J., HOCK M., SUMOVÁ A., PÁCHA J., 2011. Circadian regulation of electrolyte absorption in the rat colon. *Am J Physiol Gastrointest Liver Physiol*. 301(6): G1066-74, IF 3,431

NOVÁKOVÁ M., POLIDAROVÁ L., SLÁDEK M., SUMOVÁ A., 2011. Restricted feeding regime affects clock gene expression profiles in the suprachiasmatic nucleus of rats exposed to constant light. *Neuroscience* 197: 65-71, IF 3,389

SOTÁK M., POLIDAROVÁ L., ERGANG P., SUMOVÁ A., PÁCHA J., 2013. An association between clock genes and clock-controlled cell cycle genes in murine colorectal tumors. *Int J Cancer* 132(5): 1032-41, IF 5,444

HOUDEK P., POLIDAROVÁ L., NOVÁKOVÁ M., MATĚJŮ K., KUBÍK Š., SUMOVÁ A., 2015. Melatonin administered during the fetal stage affects circadian clock in the suprachiasmatic nucleus but not in the liver. *Dev Neurobiol*. 75(2): 131-44, IF 4,423

Reviews:

SUMOVÁ A., BENDOVÁ Z., SLÁDEK M., EL-HENNAMY R., MATĚJŮ K., POLIDAROVÁ L., SOSNIYENKO S., ILLNEROVÁ H., 2008. Circadian molecular clocks tick along ontogenesis. *Physiol Res*. 57 Suppl 3: S139-48, IF 1,555

SUMOVÁ A., SLÁDEK M., POLIDAROVÁ L., NOVÁKOVÁ M., HOUDEK P., 2012. Circadian system from conception till adulthood. *Prog Brain Res*. 199: 83-103, IF 3,040

7 RESULTS

7.1 Identification of the circadian clocks and their phase relationship within the individual parts of the rat gut

For detailed information and figures, please see the attached publication

POLIDAROVÁ L., SOTÁK M., SLÁDEK M., PÁCHA J., SUMOVÁ A., 2009. Temporal gradient in the clock gene and cell-cycle checkpoint kinase *Wee1* expression along the gut. *Chronobiology Int*. 26(4): 607-20, IF 4,028

The first goal was to determine whether clock genes *Per1*, *Per2*, *Bmal1*, and *Rev-erbα* are expressed rhythmically within all studied parts of the rat intestine, i.e. the duodenum, jejunum, ileum, and distal colon. 24-h profiles of the clock gene mRNA exhibited significant circadian rhythms in all studied intestinal parts. The profiles of each individual clock gene in all parts of the gut had similar waveforms. However, a closer inspection revealed that the daily expression profiles in the two most distal parts of the intestine might differ, the profiles of clock gene expression within the distal colon seemed phase-delayed compared to the profiles in the duodenum.

Therefore, we performed an exact comparison between the phases of the circadian rhythms in the expression of studied clock genes in the different parts of the gut by a detailed analysis of the expression as determined every 1 h during the previous light phase of the LD cycle. The levels of *Per1* mRNA were first elevated at circadian time 9 (CT9) in the duodenum, at CT8 in the jejunum, at CT10 in the ileum and at CT12 in the colon. The levels of *Per2* mRNA first increased at CT8 in the duodenum and jejunum and at CT12 in the ileum and colon. *Rev-erbα* mRNA levels began to decrease at CT12 in the duodenum, did not change significantly in the jejunum, and began to rise at CT6 in the ileum and at CT8 in the colon. *Bmal1* mRNA levels first declined at CT4 in the duodenum and jejunum and descended for the first time at CT6 in the ileum and at CT10 in the colon. Altogether, the data demonstrate that the timing of the rise or decline in clock-gene expression differs between the individual parts of the gut, with the rise or decline occurring earlier in the duodenum than in the distal colon. Therefore, the circadian clock in the upper part of the gut, as represented by the

duodenum, is advanced relatively to the clock in its lower part, as represented by the distal colon.

Afterwards, we ascertained whether the clock-driven gene rhythmicity manifests similar differences in phase along the cranio-caudal axis of the gut. As the duodenum and distal colon were found to be the regions with the most apparent differences in the phases of clock-gene expression profiles, we studied the profiles in expression of *Wee1* in these two parts of the gut. We found that the 24-h profiles of *Wee1* mRNA levels exhibit a significant circadian rhythm within the duodenal as well as the colonic epithelium. In the duodenum, *Wee1* mRNA levels rose for the first time between CT5 and CT6 and further between CT9 and CT10 and between CT10 and CT11. In the colon, *Wee1* mRNA levels declined from CT4 to CT7. The data thus demonstrate that the *Wee1* gene is expressed rhythmically within the duodenum and distal colon. The phase of the rhythm in *Wee1* expression in the duodenum is advanced to that in the colon which correlates with similar phase differences of clock gene expression.

7.2 Synchronization of the rat circadian clocks within the digestive system in conditions of disturbed signalization from the SCN

For detailed information and figures, please see the attached publication

POLIDAROVÁ L., SLÁDEK M., SOTÁK M., PÁCHA J., SUMOVÁ A., 2011. Hepatic, duodenal, and colonic circadian clocks differ in their persistence under conditions of constant light and in their entrainment by restricted feeding. *Chronobiology Int*. 28(3): 204-15, IF 4,028

First, we monitored the effect of LL on the locomotor activity and feeding behavior of rats. After releasing the rats into LL, their locomotor activity started to run with a period of 25.5 ± 0.3 h during the first 14 days of LL; however, this rhythmicity was gradually lost. After 30 days in LL, the locomotor activity of all rats fed *ad libitum* became completely arrhythmic. Similarly to locomotor activity, feeding behavior of rats maintained in LL also gradually lost circadian rhythmicity.

The exposure of rats maintained under LL conditions and then exposed to the RF regime during the last 14 days entrained the locomotor activity rhythm immediately, the

rats became active mostly during the time of food availability. As expected, they also exhibited FAA and became active shortly before the time when food was provided. After the beginning of the RF, the endogenous period of 25.5 ± 0.3 h disappeared, and only food-entrained behavior was detected. Due to the experimental design, feeding could obviously occur only during the interval of 6 h when food was available and, therefore, it was not monitored.

As a next step, we measured the expression of the clock genes and ccgs, namely *Per1*, *Per2*, *Bmal1*, *Rev-erbα*, *Wee1* and *Dbp* in the liver, duodenum and colon of rats maintained in LL and fed *ad libitum*. In contrast to the complete behavioral arrhythmicity found in all animals after 30 days in LL, the gene expression profiles in the liver, duodenum and colon were not completely abolished. Importantly, the degree of persistence of circadian rhythms in clock gene expression depended on the gene and tissue studied. In the liver and colon, levels of *Per1*, *Per2*, *Bmal1*, and *Wee1* mRNAs did not exhibit circadian rhythmicity. In contrast, levels of *Rev-erbα* and *Dbp* mRNAs exhibited significant circadian rhythms that were in phase in both tissues. In the duodenum, all the studied gene expression profiles, with the exception of *Wee1*, exhibited circadian rhythms. The circadian rhythm in the expression of *Bmal1* was in antiphase to the rhythms in *Per1*, *Per2*, *Rev-erbα*, and *Dbp*.

Finally, we assessed the effect of the RF under LL on the studied gene expression profiles. Exposure of animals maintained under LL and then exposed to RF showed restored circadian rhythms in gene expression within the liver, duodenum, and colon. However, the degree of the restoration differed between these individual parts of the digestive system. In the liver and duodenum, the expression profiles of all studied genes were significantly rhythmic. In the colon, only expression profiles of *Per1*, *Bmal1* and *Rev-erbα* were significantly rhythmic. In contrast to the profiles observed in the liver and duodenum, the expression profiles of the clock gene *Per2* and the ccgs *Wee1* and *Dbp* did not exhibit significant circadian rhythms in the colon.

7.3 Development and entrainment of circadian oscillations in the rat colon during ontogenesis

For detailed information and figures, please see the attached publication

POLIDAROVÁ L., OLEJNÍKOVÁ L., PAUŠLYOVÁ L., SLÁDEK M., SOTÁK M., PÁCHA J., SUMOVÁ A., 2014. Development and entrainment of the colonic circadian clock during ontogenesis. *Am J Physiol Gastrointest Liver Physiol*. 306(4): G346-56, IF 3,65

To ascertain how the circadian clock in the colon develops from the prenatal to early postnatal period until weaning, daily profiles of *Per1*, *Per2*, *Rev-erbα*, *Cry1*, *Bmal1*, and *Clock* mRNA were detected at E20, P2, P10, P20 and P30. In the fetal colon at E20, significant circadian rhythms were found for the daily profiles of all clock genes, but not *Clock*. After birth at P2, only the *Per1*, *Per2*, and *Cry1* genes were expressed rhythmically, but *Rev-erbα* and *Bmal1* lost their prenatally expressed rhythms. *Clock* expression remained constitutive. At P10, significant circadian rhythms were revealed for the expression of *Per1*, *Per2*, *Rev-erbα*, and *Bmal1*; the daily profiles of *Cry1* and *Clock* expression did not exhibit circadian rhythm. At P20, all studied clock genes with the exception of *Clock* were expressed rhythmically, although *Per1* and *Bmal1* with rather atypical waveforms. At P30, only the *Rev-erbα*, *Cry1*, *Bmal1* and *Clock* expression profiles exhibited circadian rhythms. From comparison of developmental changes of the individual clock gene expression profiles between E20 and P30 it became apparent that the rhythms underwent remarkable shifting in their phases. They moved from a narrow cluster at the fetal stage to a mutual phase-relationship at P20 - P30, which is a prerequisite for the functional circadian clock. Apart from phases, the amplitudes of rhythms in clock gene expression had tendencies to increase with age.

Because the above described data demonstrate significant changes in the phases and amplitudes of the circadian rhythms of expression of the studied clock genes in the colon during the developmental periods, we aimed to elucidate whether the change in the relative relationship between prenatal conditions and postnatal maternal nurturing would affect the setting of these phases. To test this hypothesis, pups were reared since birth by a foster mother who was entrained to an opposite LD cycle than their own

mother (groups LD/DL and DL/LD), and clock gene expression profiles were detected in pups at P10 in the SCN and colon. In the SCN of pups from both experimental groups, *Per1*, *Per2*, *Rev-erbα,* and *Bmal1* were expressed with high amplitudes and in the same phases relative to the LD cycle. In the colon, only *Per2*, *Rev-erbα,* and *Bmal1* were expressed rhythmically in both experimental groups. Similarly to the SCN, the acrophases of the rhythmic profiles in DL/LD and LD/DL groups did not significantly differ. To determine whether the reversed LD cycle and related maternal breast feeding of pups by foster mothers affected the phasing of the circadian clock in the colon at P10, the clock gene expression profiles were compared with those determined under conditions when pups were reared by their original mothers and maintained on LD 12:12 during the entire experiment (LD/LD). The prenatal history of the maternal lighting conditions significantly affected the phasing of the *Per2* and *Rev-erbα* expression rhythms in the colon within the first 10 days of postnatal life. The phase of the *Bmal1* expression rhythm was unaffected. From comparison of the amplitudes between all three experimental conditions, it appeared that the amplitude of the *Rev-erbα* rhythm was lower and the amplitude of *Per2* was higher in pups reared by foster mothers. The amplitude of *Bmal1* did not significantly change. The results revealed that the temporal reversal of maternal care and a LD cycle during the first 10 days after birth affected the phasing of the *Per2* and *Rev-erbα* expression rhythms in the colon, but not in the SCN.

To distinguish between the contribution of signaling from the maternal feeding regime and signaling from the developing pup´s SCN to postnatal colonic clock entrainment, the clock gene expression profiles were studied at P10 and P20 in the SCN and colons of pups born to mothers maintained under LL since the beginning of their pregnancy, and then since delivery fed *ad libitum* or exposed to the RF. In the pup´s SCN, exposure to LL reduced rhythmicity in *Per2*, *Rev-erbα,* and *Bmal1* expression in a gene- and age-dependent manner. Imposing the circadian rhythm on maternal behavior by the RF weakly affected *Per2* gene expression in the SCN at P10, and the effect was lost at P20. The pup´s SCN was, thus, likely unable to produce a rhythmic signal. In the colon of pups at P10, exposure to LL abolished the rhythm in the expression of *Per2*, whereas *Per1*, *Rev-erbα,* and *Bmal1* were expressed rhythmically in the same phase. Similarly, at P20, the acrophases of the rhythmic *Per2*, *Rev-erbα,* and *Bmal1* profiles did not significantly differ among each other. The exposure of mothers maintained in

LL to the RF since delivery affected the colonic expression profiles in a gene- and age-dependent manner. At P10, all studied clock genes were rhythmically expressed and were phase-shifted relative to the profiles in pups born to mothers fed *ad libitum*. At P20, all studied clock gene expression profiles were rhythmic under the RF similar to the situation at P10. At both ages, *Rev-erbα* and *Bmal1* profiles were mutually in antiphase, which was in contrast to the situation in which mothers maintained in LL were fed *ad libitum*. From a comparison of the effects of maternal RF on the SCN and colon, it appears that maternal RF was able to synchronize and entrain the clock in the pup´s colons independently of their SCN clock.

7.4 Characterization of the circadian system of SHR and comparison of its sensitivity to temporal changes in the feeding regime with the normotensive Wistar rat strain

For detailed information and figures, please see the attached publications

SLÁDEK M., POLIDAROVÁ L., NOVÁKOVÁ M., PARKANOVÁ D., SUMOVÁ A., 2012. Early chronotype and tissue-specific alterations of circadian clock function in spontaneously hypertensive rats. *PLoS One* 7(10): e46951, IF 4,09

POLIDAROVÁ L., SLÁDEK M., NOVÁKOVÁ M., PARKANOVÁ D., SUMOVÁ A., 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* 8(9): e75690, IF 4,09

7.4.1 Characterization of the circadian system of SHR

First of all, we recorded the locomotor activity to evaluate the functional state of the central clock of rats in both strains. Analysis of representative actograms revealed that the free-running period in DD was significantly shorter in SHR than Wistar rats. The overall activity under both LD and DD conditions did not differ between both rat strains, but the circadian rhythm in locomotor activity exhibited significantly lower amplitude in SHR compared with Wistar rats. Under LD conditions, the control rats

became active at the time of the lights-off, whereas the SHR activity increased already 3 h before the light offset.

Further, daily profiles of *Per1*, *Per2*, *Rev-erbα* and *Bmal1* mRNA levels were determined by *in situ* hybridization in the SCN of SHR and Wistar rats maintained under LD 12:12. All of the profiles exhibited high-amplitude circadian rhythms with significantly earlier acrophases in SHR. Therefore, the clock gene expression rhythms in the SCN of SHR were significantly advanced, but not suppressed, compared to those of controls.

Next, the daily profiles in expression of *Per1*, *Per2*, *Cry1*, *Rev-erbα*, *Bmal1* and *Bmal2* in the liver and colon were compared between SHR and controls maintained under LD 12:12. In the liver, none of the clock gene expression profiles of SHR differed from control rats. In contrast, in the colon the acrophases of *Per2, Rev-erbα* and *Bmal1* expression profiles were phase advanced and the amplitudes of all of the rhythms were decreased in SHR compared to control rats. The suppression did not seem to be due to the reduction in the number of clock cells in the colon of SHR because the spatial distribution and number of clock cells, as determined by BMAL1 immunohistochemistry, did not differ from Wistar rats. These data demonstrate significant tissue-specific differences between the clock gene expression profiles in SHR and Wistar rats.

Comparison of the phases between the central clock in the SCN and peripheral clocks revealed that in both strains, the hepatic and colonic clocks were delayed to the SCN by approximately 6–8 h, depending on the gene and strain studied. Apparently, the hepatic clock was in the same phase in SHR and controls, whereas the SCN and colonic clock of SHR were phase advanced compared to controls. Consequently, the mutual phase relationship between the SCN and peripheral clocks in SHR differed from that observed in controls.

The daily expression profiles of ten ccgs and clock-related genes involved in metabolic pathways, namely *Dbp, Wee1, E4bp4, Nampt, Pparα, Pparγ, Pgc1α, Hdac3, Hif1α* and *Ppp1r3c*, were determined in the liver and colon of SHR and Wistar rats, which were maintained under LD 12:12. In the liver, significant circadian rhythms in the expression of all studied genes were detected, with the exception of *Hdac3* in both strains, and *Hif1α* in SHR only. In the colon, all gene expression profiles exhibited circadian rhythms, with the exception of *Ppp1r3c* and *Pgc1α* in both strains and *Pparα*

in SHR only. Further analysis demonstrated that whereas in the liver, the differences in the gene expression profiles between both strains were negligible or marginal, in the colon most of these profiles were significantly different, being phase-advanced (*Dbp, Wee1, E4bp4, Hif1α*) and/or suppressed (all but *Pparα*) in SHR relative to controls.

Then, the endogenous period of the peripheral clock was measured in spontaneously immortalized fibroblasts isolated from SHR and Wistar rats transfected with *Bmal1-dLuc* circadian reporter. The resulting periods showed no significant differences between either entraining conditions or strains. The data suggest that the peripheral clocks likely run with the same period in both rat strains *in vitro*.

7.4.2 Sensitivity of the circadian system of SHR to temporal changes in the feeding regime

First of all, the effect of RF on locomotor and feeding behavior was measured in SHR and Wistar rats under conditions of feeding *ad libitum* and subsequently the RF. Total activity levels did not significantly differ between both rat strains and both feeding conditions. The RF affected the distribution of activity during the 24-h period. The rats became active during the time of food presence and also during a certain interval prior to feeding, i.e., they exhibited FAA. The data demonstrate that SHR develop FAA much earlier than Wistar rats and that FAA was higher in SHR compared with Wistar rats. Moreover, a detailed inspection of actograms from rats maintained under LD 12:12 and subjected to the RF revealed that the exposure to RF shifted the nocturnal activity to the time of food presence in SHR, whereas it did not affect the timing of nocturnal activity in Wistar rats.

As a next step, the effect of RF on the SCN clock was studied. Daily profiles of *Per2, Rev-erbα* and *Bmal1* expression were detected in the SCN of SHR maintained under LD 12:12 and fed *ad libitum* or subjected to the RF. The expression profiles of all of the studied clock genes exhibited significant circadian rhythms and did not differ in their amplitudes under either experimental condition. Only the acrophase of *Bmal1* expression rhythm was delayed under the RF compared to *ad libitum* conditions.

We compared the daily profiles of clock gene expression in the liver and colon of each rat strain under *ad libitum* feeding conditions and the RF. In the liver, the exposure to RF phase-advanced the clock gene expression profiles of SHR and Wistar

rats. Importantly, in Wistar rats, the expression profiles under the RF were significantly suppressed compared with *ad libitum* feeding in all studied clock genes, with the exception of *Bmal2*. The acrophase of the *Bmal2* expression profile under *ad libitum* conditions was delayed compared with the acrophase of the *Bmal1* profile under the RF and was very close to the phase of the *Bmal1* profile under *ad libitum* feeding conditions. In contrast to Wistar rats, the *Bmal2* expression profile in SHR under *ad libitum* feeding was expressed rhythmically, and under the RF, the acrophase was significantly phase-advanced compared with *ad libitum* feeding. Moreover, in SHR the acrophases of *Bmal1* and *Bmal2* expression profiles were approximately the same, and the synchronization was maintained under both *ad libitum* and RF conditions. In contrast to Wistar rats, the rhythmic expression under the RF was either not suppressed or even upregulated in SHR. In the colon, the exposure of SHR and Wistar rats to the RF phase-advanced the clock gene expression profiles in both strains as expected. In Wistar rats and SHR, the expression profiles of *Bmal2* in the colon did not exhibit circadian variation under *ad libitum* nor RF conditions and did not differ under either condition. Moreover, the data demonstrate that in contrast to the liver, the RF either does not affect or rather suppresses the rhythmicity of the colonic clock in both rat strains.

The clock gene expression profiles in the liver and colon under the RF were compared to detect the responsiveness of the hepatic and colonic clocks of both strains to feeding challenge. The results revealed that the RF regime phase-advanced the peripheral clocks of SHR more than those of Wistar rats. In the liver, the expression profiles of *Per2* and *Bmal2* were upregulated in SHR compared with Wistar rats, whereas in the colons of SHR the profiles were down-regulated. Among all the studied clock genes, *Bmal2* exhibited the most significant differences in its daily expression profiles under the RF between both strains and tissues. Moreover, the mutual phasing between *Bmal1* and *Bmal2* differed between the two rat strains. In the liver, the *Bmal2* profile was significantly delayed compared with *Bmal1* in Wistar rats, however, the profiles of these two paralogs were approximately in the same phase in SHR. In the colon, the *Bmal2* expression was arrhythmic in both strains and significantly down-regulated in SHR compared with Wistar rats.

Apart from *Bmal2* mRNA, the daily profiles of BMAL2 protein levels were compared in the liver of Wistar rats and SHR subjected to the RF. The data revealed

that both strains differed in timing of the maximal BMAL2 levels. Whereas in Wistar rats the peak appeared during the end of the subjective night, in SHR the peak was shifted to the subjective day.

Because the data described above suggest the higher sensitivity of the peripheral circadian clocks to the RF in SHR compared with Wistar rats, the expression profiles of genes that are either under direct circadian control or whose protein products interact with the core clockwork (*Wee1, Dbp, E4bp4, Nampt, Pparα, Pparγ, Pgc1α, Prkab2, Hdac3, Hif1a and Ppp1r3c*) were examined in the liver and colon of both rat strains under RF conditions. In the liver of SHR, the daily expression profiles of the studied genes under the RF (apart from *Wee1* and *Prkab2*) were either suppressed or did not differ when compared with those in Wistar rats. In accordance with the above described suppression of the colonic clock in SHR under the RF regime, the daily profiles of the majority of the clock- and metabolism-related genes studied herein were also suppressed in SHR compared with Wistar rats.

8 DISCUSSION

8.1 Identification of the circadian clocks and their phase relationship within the individual parts of the rat gut

Our results demonstrate circadian rhythms in the expression of the canonical clock genes *Per1*, *Per2*, *Rev-erbα*, and *Bmal1* within the rat epithelium of the duodenum, jejunum, ileum, and distal colon, as well as circadian rhythms in the expression of the putative clock-driven gene *Wee1* in the duodenum and distal colon. Detailed examination of the exact time when the expression of the individual clock gene increased or decreased during the previous light period in the LD cycle led to the discovery that the circadian clocks are phased differently along the proximal-distal axis of the gut, with the clock in the duodenum significantly advanced to the clock in the distal colon.

In all tissues studied, the phase of the rhythms of individual clock gene expression agreed with the current model of the molecular clockwork (Reppert & Weaver 2002; Ko & Takahashi 2006; Dardente & Cermakian 2007). Under the same conditions, circadian rhythms of clock gene expression were recently detected within the rat (Sládek et al. 2007) and mouse (Hoogerwerf et al. 2007) colon as well as in the mouse stomach (Hoogerwerf et al. 2007) and jejunum (Froy & Chapnik 2007). Our current data provide evidence of the presence of the multiple gastrointestinal circadian clocks and their mutual phases within the same experimental arrangement. Altogether, our data, along with those from previous studies, clearly demonstrate that all separate parts of the gastrointestinal tract along the cranio-caudal axis of the intestine harbor functional peripheral circadian clocks similar to that previously described in the liver (Damiola et al. 2000; Stokkan et al. 2001).

The expression of *Wee1* was previously found to be under transcriptional regulation by the CLOCK/BMAL1 complex in the liver (Matsuo et al. 2003). The phase-advanced rhythm of *Wee1* expression in the duodenum compared to the rhythm in the colon was correlated with the phasing of the clock gene expression rhythms. This indicates that in the intestine, the *Wee1* gene is likely also under transcriptional control of the CLOCK/BMAL1 complex. Our data support the hypothesis that the circadian clock may control the cycle of cell division within the duodenal and colonic crypt

epithelium. The discovery of such periodic temporal regulation of the cell cycle along the cranio-caudal axis of the gut is in good agreement with previous data that indicate a considerable phase shift of cell proliferation between the most proximal (oral epithelium) and most distal (rectum) parts of the GIT in rodents and humans (Scheving et al. 1972; Scheving et al. 1978; Fujimoto et al. 1992; Bjarnason & Jordan 2002; Tsunada et al. 2003).

The data thus provide evidence that there is a difference in the phasing of circadian clocks and cell cycles in the duodenum and distal colon and support the hypothesis that circadian clocks control the timing of cell division within epithelial cells of different parts of the intestine.

8.2 Synchronization of the rat circadian clocks within the digestive system in conditions of disturbed signalization from the SCN

We ascertained that the circadian clocks in the GIT may be disrupted by exposure to LL and that the RF regime can synchronize these disrupted clocks. The effect of LL and RF differed between the clocks in the liver, duodenum and colon.

Due to exposure to LL for 4 weeks, the rats exhibited complete behavioral and feeding arrhythmicity. The arrhythmicity was likely due to the effect of LL directly on the SCN. Exposure to LL did not compromise the ability of individual SCN cells to oscillate but desynchronized these cells among each other (Ohta et al. 2005). Under such conditions, the SCN was likely not able to generate synchronized circadian signals to the rest of the body and SCN-driven overt rhythms, such as locomotor activity and related food intake, were abolished. It is generally supposed that due to the lack of SCN signaling, the individual circadian clocks in the peripheral cells desynchronize. In our study, in spite of the absence of behavioral rhythmicity due to the exposure of rats fed *ad libitum* to a prolonged period of LL, peripheral rhythms in the GIT were partially preserved. The degree of the preservation was dependent on the section of the GIT; it was higher in the duodenum where the expression of all the studied clock genes, i.e., *Per1*, *Per2*, *Rev-erbα*, and *Bmal1*, as well as the clock-controlled gene *Dbp*, remained rhythmic, than in the colon and liver, where expression of only two genes, i.e., *Rev-erbα* and *Dbp*, exhibited rhythmicity. This indicates that at least in the duodenum, rhythmical

cues other than those that are known to be behavior-related might be important for the internal coordination of the cellular clocks. It is plausible to speculate that exposure to LL might not compromise all SCN output pathways to the same degree, and, therefore, some rhythmical cues derived from the SCN may still synchronize the peripheral clocks which may differ in their sensitivity to the cue. Despite the fact that the rhythms in some peripheral tissues still persisted in LL, the peripheral clocks were likely not completely functional, as they were not able to drive transcription of the ccg *Wee1*. It supports the hypothesis that lost or reduced SCN signaling may affect the cell cycle kinetics of intestinal cells. Similarly, other clock-driven rhythms, e.g., rhythms in physiological functions such as plasma lipid homeostasis and the expression of small intestinal and hepatic triglyceride transfer protein, a key protein in lipoprotein production, are abolished when rats and mice are kept in LL (Pan & Hussain 2007).

Restricted food availability with a periodicity of 24 h was reported to entrain behavioral activity in rats (Coleman et al. 1982; Challet et al. 2003), mice (Abe et al. 1989; Sharma et al. 2000), and hamsters (Mistlberger 1994). In our study, the RF restored behavioral and feeding rhythms lost due to exposure to LL. Moreover, the RF synchronized and/or restored the rhythmicity of all the studied clock genes in the duodenum and liver, whereas in the colon only the expression of *Per1*, *Rev-erbα*, and *Bmal1*, but not *Per2*, became rhythmic. Importantly, the hepatic and duodenal, but not the colonic clocks were functional, because they were able to drive the expression of the ccgs *Wee1* and *Dbp* and thus control tissue-specific functions. In contrast, the colonic clock remained malfunctional. This was not simply because the colonic clock was not sensitive to the RF, as our group previously demonstrated that under the LD regime, when the SCN clock was not disturbed, the colonic clock could be fully entrained by RF (Sládek et al. 2007).

The data of this study suggest that under LL, the RF regime differed in its ability to entrain the circadian clocks in different parts of the gut, because it was able to fully synchronize the duodenal but only weakly the colonic clock. We hypothesize that within the colonic clock, signaling from the SCN competes with food as an entraining cue, whereas in the duodenum the food-related entrainment seems to dominate in LL. Our findings might also imply that under situations when the circadian system was disorganized due to exposure to LL, the synchronized temporal regulation of cell division in the colonic epithelium was prevented and not restored by the RF regime.

Loss of temporal regulation of cell division is often correlated with the development of cancer (Gery et al. 2006; Chen-Goodspeed & Lee 2007; Wood et al. 2008; Yang et al. 2009b).

8.3 Development and entrainment of circadian oscillations in the rat colon during ontogenesis

We demonstrated that circadian rhythms in individual clock gene expression were already detectable in the colons of 20-day-old fetuses. The amplitudes of the rhythms were rather low with the exception of *Rev-erbα*. This finding was in accordance with our previous study on ontogenesis of the clock in the rat liver, in which *Rev-erbα* was also found to exhibit high-amplitude rhythm in expression at E20 (Sládek et al. 2007). The presence of the circadian rhythmicity in most of the canonical clock genes at E20 might suggest that a functional circadian clock already operates before birth in the colon. However, the peaks of all of the individual clock gene expression rhythms are set to a narrow temporal window during the 24-h cycle. Thus, the phasing of all studied clock gene expression rhythms at the same time of day may reflect a situation in which fetal clock gene expression is directly induced and driven by rhythmical signal from their mother. After birth at P2, only the *Per1*, *Per2*, and *Cry1* from all the studied genes were found to be rhythmically expressed. In this period, the pup´s colon is exposed to a rhythmic supply of maternal milk, which may likely entrain, but apparently not induce, rhythmicity. At P10, significant rhythmicity was again detectable for *Per1*, *Per2*, *Rev-erbα*, and *Bmal1*. At this developmental stage, the individual clock gene rhythms peaked at different phases during the 24-h cycle, suggesting that the functional colonic clock may already begin to operate and/or the maternal breast feeding regime is able to entrain the clock. At P20, all studied genes with the exception of *Clock* were expressed rhythmically. At this developmental stage, the pups are already sighted and exhibit nocturnal behavioral activity, which is driven by their SCN (Sumová et al. 2012). In addition to day-time breast feeding, they may begin to consume solid food during the night-time. Finally, at P30, *Rev-erbα* and *Bmal1* exhibited high-amplitude rhythms, *Cry1* and *Clock* had low-amplitude rhythms, and *Per1* and *Per2* were not expressed rhythmically. Importantly, at this developmental stage, the phasing of the clock genes in the colon well corresponded to that in adults

(Sládek et al. 2007; Sládek et al. 2012), reflecting the fact that at this stage, the weaning is completed and the colon is solely entrained by the nocturnal feeding regime. In addition, the amplitudes of the *Rev-erbα* and *Bmal1* expression rhythms at P30 were higher compared with the earlier developmental stages, suggesting that the colonic circadian clock is fully matured at P30.

The phase reversal of the LD cycle performed prenatally (DL/LD) and postnatally (LD/DL) had the same effect on the SCN and colonic profiles in 10-day-old pups. In accordance with previous data for pups at P10 (Sládek et al. 2004; Kováčiková et al. 2006), the SCN exhibited significant and high-amplitude rhythms in the expression of the studied clock genes, which were already fully entrained by the external actual LD cycle, and thus, their phases corresponded to those in adult animals (Oishi et al. 1998; Sládek et al. 2007). In the colons of 10-day-old fostered pups, *Per2*, *Rev-erbα*, and *Bmal1* were expressed rhythmically and no rhythm in *Per1* expression was detected. The fostering selectively affected the phasing of *Per2* and *Rev-erbα*, which differed by ~ 4-6 h compared with the nonfostering conditions. Interestingly, for both groups of fostered pups (LD/DL and DL/LD), an antiphase relationship between *Rev-erbα* and *Bmal1* was already established, which was not yet the case for 10-day-old pups reared by their own mothers maintained in the same LD cycle throughout gestation and breast feeding. Thus, the data unexpectedly suggest that the manipulation of the timing of breast feeding in nocturnal animals might affect entrainment of the developing clock. Moreover, the clock gene expression profiles in the colon and SCN of 10-day-old pups were in the same phase. In contrast, in adults, the phase of the colonic clock significantly lags behind the SCN (Sládek et al. 2012). The simultaneous phasing of the central and colonic clocks supports the hypothesis that by P10, the colonic clock is still entrained by maternal behavior and related feeding regime rather than signaling from the pup´s SCN, which would otherwise set a phase-delayed rhythm.

Previously, we showed that the maintenance of pregnant rats on LL during the entire gestation period leads to a situation in which the maternal SCN clock is unable to synchronize the fetal SCN (Nováková et al. 2010). In accordance with this finding, no oscillations or only faint oscillations in clock gene expression were detected in the SCN of pups at P10 and P20, which was thus, likely unable to drive the colonic clock. Imposing the RF after delivery to mothers maintained in LL affected the pup´s SCN profiles only slightly at P10, and no effect was present at P20. Therefore the maternal

RF during breast feeding appeared to be a weak or ineffective entraining cue for the pup´s SCN. In the colons of pups reared by *ad libitum*-fed mothers in LL, *Rev-erbα*, and *Bmal1* expression retained circadian rhythmicity, but they both peaked at the same phase. Imposing the RF to mothers shifted the *Rev-erbα* and *Bmal1* rhythms in the pup´s colons, so that they were expressed in an antiphase, suggesting a functional clock. Thus, whereas the breast feeding was unable to synchronize the SCN, it was a strong entraining signal for the colonic clock. The developmental phase shift of the colonic clock observed between P10 and P20 appears to be independent of signaling from the pup´s SCN.

Our data demonstrate for the first time the ontogenetic maturation of the colonic circadian clock from the fetal stage until weaning. Our findings also suggest a molecular mechanism of how the clock is entrained by maternal breast feeding and propose a SCN-independent developmental switch for the colonic clock from a maternal-dependent to maternal-independent stage.

8.4 Characterization of the circadian system of SHR and comparison of its sensitivity to temporal changes in the feeding regime with the normotensive Wistar rat strain

8.4.1 Characterization of the circadian system of SHR

Wistar rats, the original background strain for SHR, represent a well-established rat model that does not have any cardiovascular or metabolic pathology and has also been widely used in chronobiological studies. Therefore, using Wistar rats as a control strain allowed us to use all the extensive general knowledge on basic properties of the circadian system in healthy rats and compare it with the data obtained from SHR.

Behavioral analysis revealed the shorter free-running period of SHR maintained under constant conditions which means that the SCN clock, which controls the behavioral rhythm, runs faster in SHR relative to control rats. Previous studies, using wheel running rhythm, also reported shorter endogenous period in SHR compared to WKY rats under constant conditions (Rosenwasser & Plante 1993; Peters et al. 1994). Also under the LD cycle, SHR started to be active earlier, already before the lights-off.

The advanced onset of activity correlated with advanced profiles of clock gene expression in the SHR SCN. However, the phase advance was unlikely simply due to the shorter period in SHR, because the phase advance was much larger (in hours) than would be expected due to the first day of free-running (in minutes). Therefore, it is likely that the different entrainment of the behavioral rhythm in SHR arises from a different phase angle of entrainment of the SCN clock by the LD cycle. Indeed, a difference in light sensitivity of the circadian system light pulses in SHR compared to WKY rats, has been suggested (Peters et al. 1994).

In contrast to differences in circadian periods of behavioral activity rhythms in SHR and controls *in vivo*, no variance in periods or amplitudes between both strains were detected during various entraining conditions in fibroblasts, thereby suggesting that the *in vitro* SHR molecular core clockwork in periphery was not compromised. Therefore, our data strongly suggest that the polymorphisms in the SHR *Bmal1* promoter (Woon et al. 2007) likely do not influence the core molecular clockwork in the fibroblasts.

Clock gene expression profiles in the hepatic and colonic peripheral clocks exhibited significant tissue-specific differences between SHR and Wistar rats. The phases and amplitudes of the hepatic clocks did not differ between both strains, whereas the colonic clocks were phase advanced and suppressed in SHR compared to controls. Importantly, the suppression of the rhythms in the colon was likely not due to differences in the spatial distribution of colonic cells containing circadian clock between SHR and controls, which was demonstrated by detection of BMAL1-immunopositive cells. In the colon, genes represent canonical clock components that receive rhythmical signals mostly from the SCN (*Per2*) or are solely part of the peripheral clockwork (*Rev-erbα* and *Bmal1*) were phase advanced and suppressed. Thus, reduced SCN signaling as well as suppressed rhythmicity of the peripheral colonic clock was suggested in SHR. The dampened amplitude of the colonic clock in SHR is in accordance with the above mentioned hypothesis of a weaker signaling from the central clock to the periphery. However, this effect seems to be tissue-specific because, in contrast to the colon, the hepatic clock of SHR was not suppressed. Similarly to our data, tissue-specific differences in clock gene expression in the liver and heart of SHR were detected (Cui et al. 2011). Our data show that in SHR the liver clock was not advanced to the same extent as that in the SCN compared with Wistar rats. This might

suggest involvement of different pathways mediating the SCN signal to the liver and colon.

In order to ascertain the functional properties of the circadian clock in the liver and colon of SHR and control rats, we selected several clock- and metabolism-related genes and analyzed their daily expression profiles. The selected genes were i) clock-controlled (*Dbp*, *E4bp4*) and clock-related (*Hif1a*) transcription factors that regulate various metabolic pathways, ii) nuclear receptors (*Pparα, Pparγ*) and their co-activators (*Pgc1α*) that regulate metabolism, and iii) enzymes involved in posttranslational modification (*Wee1*, *Ppp1r3c*, *Hdac3*) and NAD^+ biosynthesis (*Nampt*). In the liver, there were either negligible or only marginal differences detected in the gene expression profiles between SHR and controls, whereas these differences were profound in the colon. Most of the studied profiles differed significantly, and were phase-advanced and/or suppressed in the colon of SHR compared to controls. This is in accordance with our results concerning the clock gene expression profiles in the liver and colon. A relationship between the anomalies in the temporal control of circadian and metabolic transcriptome is highly suggested. However, it is difficult to determine the cause and effect relationship because an impaired SHR clock may directly and indirectly influence metabolism and, at the same time, systemic changes in SHR metabolism may feedback on the clock mechanism.

Our data revealed unexpected differences in the organization of the circadian system in SHR and Wistar rats, which might potentially contribute to the metabolic phenotype of the rat strain.

8.4.2 Sensitivity of the circadian system of SHR to temporal changes in the feeding regime

Our results demonstrate that SHR are behaviorally more sensitive to situations in which food availability is restricted to an improper time of day, developing earlier and stronger FAA than control Wistar rats. Whereas the restriction of food availability phase-shifted the nocturnal locomotor activity of the SHR, it only temporally redistributed this activity into two bouts in Wistar rats. A similar effect of the RF on the locomotor activity of Wistar rats has been observed previously (Carneiro & Araujo 2012). The results suggest that whereas in Wistar rats the behavioral activity is

entrained by the RF as well as by the external LD conditions, in SHR, the RF dominates the entraining cue.

The phasing of the clock gene expression profiles in the SCN of SHR is not affected by the RF, which is in good agreement with our previous findings in Wistar rats (Sládek et al. 2007) as well as with findings in all other species studied so far. Thus, the RF-induced behavioral phase shift in SHR was not mediated by the SCN.

The RF has been widely recognized as a strong entraining signal to some peripheral clocks, including those in the liver (Damiola et al. 2000; Hara et al. 2001; Stokkan et al. 2001) and colon (Hoogerwerf et al. 2007; Sládek et al. 2007). In the present study, the RF significantly phase advanced the daily profiles of clock gene expression in both peripheral tissues according to the time of food presentation in both rat strains. The colonic clock responded to the RF in a very similar manner in both rat strains. However, obvious strain-dependent differences in the response to the RF were detected in the liver. Whereas in Wistar rats the RF suppressed the oscillation of clock gene expression in the liver, in SHR the oscillation of the hepatic clock was facilitated. The most striking difference between these two rat strains was found in the effect of the RF on the temporal control of the *Bmal2* mRNA profiles. In Wistar rats, the *Bmal2* expression did not exhibit circadian variation under *ad libitum* conditions. Under the RF, *Bmal2* became expressed rhythmically with a very low amplitude and in the same phase as *Bmal1* under the *ad libitum* feeding conditions. In contrast, in SHR, *Bmal2* was expressed with a low amplitude already under *ad libitum* conditions, and the amplitude of the rhythm increased and was significantly phase advanced under the RF. Importantly, in SHR, both *Bmal* paralogs were in the same phase under the RF. These data demonstrate the higher sensitivity of the *Bmal2* gene to the RF in the liver of SHR. Indeed, daily temporal regulation of BMAL2 protein levels seemed also to differ between SHR and Wistar rats under the RF. Our hypothesis that *Bmal2* plays a role in mediating the interaction between the clock and metabolism is further supported by a recent finding that the constitutive expression of *Bmal2* rescues the rhythmicity of the locomotor activity and oxygen consumption in *Bmal1*-knockout mice (Shi et al. 2010; Shi & Zheng 2013). Several polymorphisms associated with metabolic syndrome were identified in the SHR *Bmal1* promoter (Woon et al. 2007). It is possible that this deficiency is compensated by the higher *Bmal2* sensitivity in SHR.

We used *Wee1*, *Dbp*, *E4bp4* and *Nampt* genes as markers of conventional E-box driven rhythmicity to confirm the effect of the RF on the circadian clock in SHR. *Wee1* expression corresponded to an enhanced and phase-advanced rhythmicity of the hepatic clock in SHR. For other genes, the advance was subtle and the amplitude was only slightly decreased. The expression profile of none of these genes did differ between the two rat strains under *ad libitum* feeding (Sládek et al. 2012). The results also demonstrated that under the RF, the expression profiles of some studied genes in the liver of SHR were suppressed or did not differ compared with those in Wistar rats. This might be because these genes are not driven by the clock but rather by changes in the metabolic state. Thus, the data suggest that the RF impacts the metabolic state in the liver of SHR differently than in Wistar rats, with the most obvious difference in the suppression of *Pgc1α* and *Pparα* expression. These changes in gene expression might have a broad impact on the transcription of variety of genes, including those whose products feed back on the circadian clock mechanism.

The results show significant differences in the sensitivity of the circadian system of SHR to metabolic challenge compared with controls at both behavioral and gene expression levels. The RF exerted a gene-, tissue- and strain-specific impact on the temporal regulation of the expression of genes that are either driven by the clock or whose protein products enable communication between the clock and metabolism.

9 CONCLUSIONS

1. **Identification of the circadian clocks and their phase relationship within the individual parts of the rat gut**

We demonstrated that circadian clocks within the rat duodenum, jejunum, ileum, and distal colon exhibit differences in their phases, such that the clocks in the upper part of the intestine are phase-advanced compared to the clocks in the lower part. The timing of the expression of a cell-cycle regulator *Wee1* is correlated with the phase of the core clockwork within the duodenum and distal colon.

2. **Synchronization of the rat circadian clocks within the digestive system in conditions of disturbed signalization from the SCN**

Unexpectedly, peripheral rhythms in the digestive system were partially preserved in conditions with disturbed signalization from the SCN due to prolonged exposure to LL. The degree of preservation was higher in the duodenum than in the liver and colon. The RF fully resynchronized the duodenal and hepatic clocks while the colonic clock remained malfunctional.

3. **Development and entrainment of circadian oscillations in the rat colon during ontogenesis**

The circadian clock in the colon developed gradually during the ontogenesis. Adult-like state with high-amplitude and mutually phased rhythms in clock gene expression is achieved around P30. The maternal circadian phase during pregnancy may modulate further development of the pup´s colonic clock. Postnatally, the presence or absence of rhythmic maternal nurturing affected the phasing of clock gene expression profiles in the colon.

4. Characterization of the circadian system of SHR and comparison of its sensitivity to temporal changes in the feeding regime with the normotensive Wistar rat strain

SHR exhibited a shorter period of rhythm in locomotor activity in constant conditions and phase-advanced rhythms in locomotor activity and expression of clock genes in the SCN in LD conditions. The change of the central clock phasing relative to the external LD cycle might lead to its impaired signaling to the peripheral organs. This internal desynchrony may contribute to a weakening in the temporal control of gene expression in the colon.

Under the RF regime, SHR developed earlier and stronger FAA and their locomotor activity was completely phase reset by feeding regime. The RF regime had similar effects on the colon in both rat strains, but it affected the hepatic clock of SHR differently. The potential role of *Bmal2* gene in adaptation of the hepatic clock of SHR to the RF was suggested.

10 SUMMARY

In my PhD thesis, I focused on the circadian clocks in the rat digestive system, mainly on their localization, entrainment and development. Moreover, the functional state of the circadian system of the strain with pathological phenotype, i.e. SHR, was studied.

Our data underline the existence of a mutual relationship between the circadian system, cell cycle and metabolism. It seems that changes in one system may affect the functioning of the other.

The colonic clock seems to be more sensitive to changes in cycles of external lighting conditions and food intake, which in case of their disruption might contribute to the incidence of various GIT diseases.

Information about maturation of the colonic clock during ontogenesis may facilitate our understanding of the basis of GI syndromes, which occur in preterm newborns. Our findings stress the importance of regular feeding and light regime during development.

Altered circadian system of SHR may potentially contribute to the metabolic pathology of this strain. SHR could be used as a valuable model of human disorders originating in poor synchrony of the circadian system with external conditions.

Together, the data may contribute to the understanding of various diseases associated with disturbances of the circadian system, such as metabolic syndrome and colorectal cancer.

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12 SUPPLEMENT: PUBLICATIONS