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SYNCHRONIZACE CIRKADIÁNNÍCH HODIN POTKANA BĚHEM ONTOGENEZE A V DOSPĚLOSTI

SYNCHRONIZATION OF CIRCADIAN CLOCK IN RAT DURING ONTOGENESIS AND IN ADULTHOOD

Dizertační práce

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ABSTRACT

The circadian system temporally controls behavioral and physiological processes in most organisms so that they change during the day and night with a period of about 24 h. It is an evolutionary adaptation to anticipate periodic changes in environment on the Earth. In mammals, the circadian system consists of the central pacemaker in the suprachiasmatic nuclei (SCN) of hypothalamus and of oscillators located in numerous peripheral organs and tissues. At the molecular level, the circadian clock is based on the rhythmic expression of so called clock genes. The ontogenetic development of the circadian system is a gradual process and the most important changes undergo during the late embryonic and early postnatal stage. Many behavioral, hormonal and metabolic signals provided by the mother are considered to be involved in circadian clock synchronization during early ontogenesis. The mechanisms of the entrainment are not fully known yet. The aim of this thesis was to study the development of the circadian clock and its entrainment via maternal signals and to compare the development of circadian rhythms in two model rat strains – Wistar rat and spontaneously hypertensive rat (SHR).

Firstly, we described the ontogenetic maturation of the Wistar rat circadian clock in the colon from the fetal stage until weaning. Our findings suggest a molecular mechanism of how the colonic clock is entrained by maternal breast-feeding and propose a developmental switch from the maternal-dependent to maternal-independent stage. We found that an adult-like state was achieved around postnatal day 20.

Then, we compared the development of the circadian systems in Wistar rat and SHR and revealed significant differences in the dynamics of the SHR circadian system development and its sensitivity to changes in maternal-feeding regime. In the SCN and liver of SHR, the development of high-amplitude expression rhythm of canonical clock gene *Bmal1* was delayed. We also detected significant differences in maternal behavior between SHR and Wistar rats with a less frequent maternal care in SHR, which may be a factor contributing to the atypical development of the SHR circadian clocks during postnatal ontogenesis. To test this hypothesis, we performed cross-strain fostering set of experiments results of which revealed that the altered care provided by SHR mother worsened the entrainment of the central clock with the light/dark cycle in Wistar rat pups. The presumably better maternal care, provided by a Wistar rat mother to SHR pups, improved amplitude of the SCN-driven rhythms and their entrainment to external cues in adulthood. The peripheral

clocks in the liver and colon responded more robustly to the cross-strain fostering and the response was not present in pups reared by a foster mother of the same rat strain.

Finally, we found out that in Wistar rat pups, combination of daily maternal stress with the mild arousal caused by pups manipulation increased their plasma-levels of glucocorticoids and shifted the rhythm of expression of clock gene *Bmal1* in the SCN. This effect was completely blocked by administration of the glucocorticoid receptor antagonist. In contrast, in SHR pups, maternal stress itself was able to shift the phase of the *Bmal1* expression rhythm in the SCN but this effect was probably not mediated via the glucocorticoid-dependent mechanism.

Key words: circadian clock, suprachiasmatic nuclei, ontogenesis, glucocorticoids

ABSTRAKT

Cirkadiánní systém řídí behaviorální a fyziologické procesy u většiny organismů, tak aby byly seřízeny s vnějším podmínkami s periodou přibližně 24 hodin. Tento systém vznikl jako evoluční adaptace umožňující předvídat periodické změny prostředí na Zemi. U savců se cirkadiánní systém skládá z centrálního oscilátoru v suprachiasmatických jádrech (SCN) hypotalamu a oscilátorů v periferních orgánech a tkáních a na molekulární úrovni je řízen mechanismem rytmické exprese tzv. hodinových genů. Ontogeneze cirkadiánního systému je postupný proces a nejdůležitějšími změnami prochází během pozdní embryonální a rané postnatální fáze života. Na synchronizaci cirkadiánních hodin během časné ontogeneze se pravděpodobně podílí mnoho behaviorálních, hormonálních a metabolických signálů zprostředkovaných matkou. Přesné mechanismy mateřské synchronizace nejsou dosud plně objasněny. Cílem této práce bylo studium vývoje cirkadiánních hodin a jejich synchronizace prostřednictvím mateřských signálů u dvou modelových kmenů potkana - Wistar a spontánně hypertenzního potkana (SHR).

Popsali jsme ontogenetický vývoj cirkadiánních hodin v tlustém střevě mláďat potkana kmene Wistar od embryonálního věku až do odstavu. Naše výsledky naznačují možný molekulární mechanismus synchronizace hodin v tlustém střevě mláďat prostřednictvím kojení a ukazují vývojový přechod od mateřské synchronizace k synchronizaci na matce nezávislé. Zjistili jsme, že vývoj hodin v tlustém střevě dosáhne úrovně funkčního stavu dospělých jedinců ve dvacátý den po narození.

Dále jsme porovnali vývoj cirkadiánního systému u potkanů Wistar a SHR a odhalili významné rozdíly v dynamice vývoje cirkadiánních hodin u kmene SHR a jeho odlišnou citlivost na změny v příjmu potravy matky. V SCN a játrech SHR byl opožděn vývoj amplitudy exprese kanonického hodinového genu *Bmal1*. Také jsme zjistili významné rozdíly v chování matek SHR a Wistar, s méně intenzivní mateřskou péčí u matek SHR, což může být faktorem přispívajícím k atypickému vývoji cirkadiánních hodin u tohoto kmene. Abychom tuto hypotézu potvrdili, provedli jsme řadu tzv. cross-fostering experimentů. Zjistili jsme, že rozdílná péče poskytovaná SHR matkou negativně ovlivnila synchronizaci centrálních hodin u mláďat potkana Wistar. Mateřská péče poskytovaná Wistar matkou mláďatům SHR naopak zlepšila rytmy řízené SCN a zlepšila synchronizaci s vnějším prostředím u těchto zvířat v dospělosti. Mnohem výrazněji než centrální hodiny ovlivnila

péče matky jiného kmene synchronizaci hodin v játrech a tlustém střevě. Pokud byla mláďata vychovávaná náhradní matkou stejného kmene, tento efekt jsme nepozorovali.

V dalším experimentu jsme testovali vliv každodenního vystavení matek stresu zároveň s mírným stresem způsobeným manipulací mláďat. Tato kombinace zvýšila hladiny glukokortikoidů v krvi mláďat potkana Wistar a způsobila fázový posun v rytmu exprese genu *Bmal1* v jejich SCN. Tento efekt byl zcela vyrušen podáním antagonisty glukokortikoidních receptorů. U mláďat SHR už samotné stresování matek způsobilo fázový posun v rytmu exprese genu *Bmal1* v SCN, tento účinek však pravděpodobně nebyl zprostředkován pouze glukokortikoidy.

Klíčová slova: cirkadiánní hodiny, suprachiasmatická jádra, ontogeneze, glukokortikoidy

LIST OF ABBREVIATIONS

AMPK	adenosine monophosphate-activated protein kinase
ANOVA	analysis of variance
AVP	arginine vasopressin
B2M	β-2-microglobulin
CaMK	calcium/calmodulin-dependent protein kinase
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CK1δ, ε	casein kinase 1 delta, epsilon
CREB	calcium/cAMP response element binding protein
CREs	calcium/cAMP response elements
cRNA	complementary RNA
CT	circadian time
DBP	diastolic blood pressure
DD	constant dark, dark/dark
DL	reversed light/dark, dark/light
dmSCN	dorsomedial suprachiasmatic nucleus
DNA	deoxyribonucleic acid
E	embryonic day
GABA	γ-Aminobutyric acid
GC	glucocorticoids
GR	glucocorticoid receptor
GRP	gastrin-releasing peptide
HPA	hypothalamic–pituitary–adrenal
HR	heart rate
IGL	intergeniculate leaflet
ipRGC	intrinsically photosensitive retinal ganglion cells
ir	immunoreactivity
LD	light/dark regime
LL	constant light, light/light
MAP	mean arterial pressure
MAPK	mitogen-activated protein kinase
mRNA	messenger ribonucleic acid

OD	optical density
P	postnatal day
PACAP	pituitary adenylate cyclase-activating polypeptide
PBS	phosphate buffer saline
PKA	protein kinase A
RF	restricted feeding regime
RHT	retino-hypothalamic tract
RT-qPCR	quantitative reverse transcription polymerase chain reaction
SBP	systolic blood pressure
SCN	suprachiasmatic nuclei
SEM	standard error of the mean
SD	standard deviation
SHR	spontaneously hypertensive rats
TTFLs	transcriptional-translational feedback loops
VIP	vasoactive intestinal peptide
vlSCN	ventrolateral suprachiasmatic nucleus
WKY	Wistar Kyoto rat

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1 INTRODUCTION

Most of the known organisms - from cyanobacteria to humans - possess a system capable of "measuring" time. The system allows them the better adaptation to periodically changing conditions on the Earth that are caused not only by the alternation of day and night, but also by the alternation of seasons. Therefore, most of the behavioral and physiological processes in the mammalian organism exhibit daily oscillations. These rhythmic processes are controlled by the internal time-keeping system. This system consists of an endogenous clock that run with the period of about one day and, therefore, is called the circadian clock (from Latin “circa diem”, approximately one day). The clock is regularly entrained with a solar day that lasts exactly 24 h by external cues, which are called "Zeitgebers" (from German "time giver").

1.1 Circadian clock in mammals

In mammals, the time-keeping system is a hierarchical system which consists of the central pacemaker in the suprachiasmatic nuclei (SCN) in the hypothalamus and numerous oscillators in other regions of brain and peripheral organs and tissues (Ralph *et al.*, 1990; Abe *et al.*, 2001; Balsalobre, 2002; Lamont *et al.*, 2005; Sládek *et al.*, 2007a). For its proper function the synchronization of individual oscillators with external conditions and with each other is necessary.

1.1.1 Suprachiasmatic nuclei

The SCN harbor the principal clock (Ralph *et al.*, 1990) that drives daily rhythms in sleep and wakefulness and other physiological and metabolic functions of the body to optimize them relative to day/night cycles (for review see (Mistlberger, 2005).

SCN are paired nuclei, placed above the optic chiasma. In rodents, each nucleus consists of 8000 to 10000 neurons. According to its morphology and function, the nucleus can be divided into two parts: the dorsomedial part (dmSCN), called the shell, and the ventrolateral part (vlSNC), called the core. There are mutual synapses connecting these two parts (Leak *et al.*, 1999). The shell is known for arginine vasopressin production (AVP), whereas the presence of vasoactive intestinal peptide (VIP) is typical for the core. Most of the neurons in the SCN produce γ-Aminobutyric acid (GABA) as a neurotransmitter (van den Pol, 1980; Abrahamson and Moore, 2001; Albus *et al.*, 2005).

The paired nuclei are composed of autonomous cellular oscillators which are coupled via web of synapses to produce a robust rhythmic signal (Welsh *et al.*, 1995; Aton and Herzog, 2005).

1.1.2 Other oscillators

Discovery of the molecular mechanism that underlies the clock (see **chapter 1.2**) enabled to reveal circadian clocks in other parts of the mammalian body. These clocks are present not only in different parts of the brain but also in various peripheral organs and tissues.

Circadian oscillations were detected for instance in other hypothalamic nuclei, in thalamic nuclei, amygdala, bulbus olphactorius of thelencephalone, cerebellum etc. (Abe *et al.*, 2001; Lamont *et al.*, 2005). In the retina, the circadian clock was detected in the inner nuclear layer as well as in the photoreceptor layer (Tosini *et al.*, 2007). Furthermore, cones in human eye exhibit a circadian rhythm in bioelectric potential (Danilenko *et al.*, 2011).

Rhythms in gene expression were detected in the liver, kidney, spleen, pancreas, heart, skeletal muscle, thyroid gland, adrenal, intestine (Balsalobre *et al.*, 1998; Yamamoto *et al.*, 2004; Sládek *et al.*, 2007b) and other tissues. These peripheral clocks generate autonomous oscillations, but they are regularly entrained by signals from the SCN (Guo *et al.*, 2006).

1.2 Molecular mechanism of circadian clock

At the molecular level, the circadian clock is driven by the rhythmic expression of so called „clock genes“, which form transcriptional-translational feedback loops (TTFLs) (Dunlap, 1999; Reppert and Weaver, 2001; Okamura *et al.*, 2002; Hastings and Herzog, 2004). It is a self-regulating system temporally controlling clock genes transcription and proteins translation. Following genes are involved in the TTFLs – three *Period* genes (*Per1,2,3*), two *Cryptochrome* genes (*Cry1,2*), *Clock*, *Bmal1*, *Casein kinase 1 epsilon*, *Rev-erbα* (also called *Nr1d1*) and *Rora.* The core mechanism of the circadian cycle generation is based on the mutual interaction of the protein products of the clock genes, i.e., the positive and negative elements forming the TTFLs switching gene expression on and off with a circadian period. Heterodimers formed of CLOCK and BMAL1 are positive elements that bind to the promoters of other clock genes (including *Per1, Per2*, *Cry1, Cry2*, and *Rev-erbα*) and switch on their expression. PER1, PER2, CRY1, and CRY2 are negative elements that accumulate in the cytoplasm and, with a delay controlled by their post-translational modifications, are translocated as a heterodimer into the nucleus, where they bind to

CLOCK-BMAL1 and inhibit its activity. REV-ERBα is also a negative element, which binds to the promoter of *Bmal1* gene and inhibits its transcription. Consequently, the *Bmal1* gene is expressed roughly in antiphase relative to other clock genes. Approximately 24 h after the activation of clock gene transcription, the inhibitory PER-CRY complex is degraded, and the cycle starts again as CLOCK-BMAL1 begins to activate gene expression (Albrecht, 2004) (**Fig. 1**).

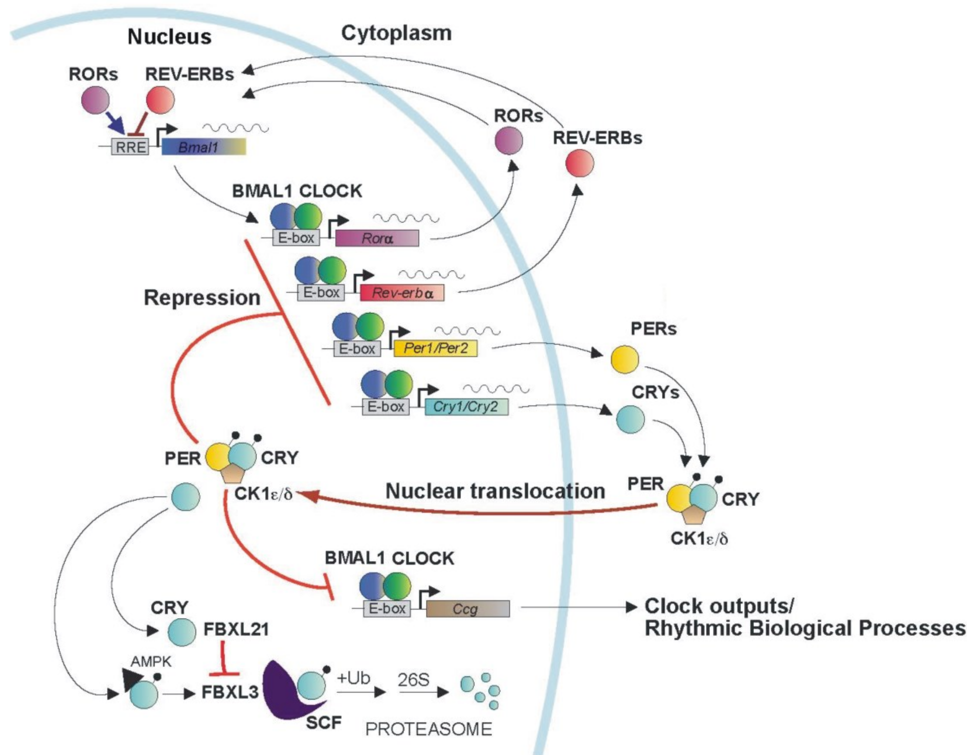


Fig. 1: Simplified model of the molecular clock mechanism in mammals. The mechanism includes positive and negative feedback loops. CLOCK-BMAL1 heterodimers activate transcription of *Per*, *Cry* and *Rev-erb*. The phosphorylated PER-CRY complex binds to the CLOCK-BMAL1 heterodimers and thereby inhibits the transcription of its genes. The additional loop involves the inhibition of *Bmal1* transcription by REV-ERBα and the activation of its transcription via RORA (adapted from Ko and Takahashi, 2006).

Post-translational modifications and degradation of clock proteins are important for the accuracy and robustness of the molecular clockwork. Casein kinases Ck1ε and Ck1δ play a key role in post-translational modifications of clock protein stability. They phosphorylate PER proteins which further modify the ability of PER-CRY complex to enter the nucleus. Moreover, phosphorylation of PER results in its rapid degradation, which is dependent on the ubiquitin-proteasome pathway (Lee *et al.*, 2001; Akashi *et al.*, 2002). 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 (Lamia *et al.*, 2009)

Transcription of numerous genes involved in the regulation of physiological processes is controlled by the circadian clock. These genes are called “clock-controlled genes” and are responsible for the manifestation of output rhythms as they are involved in various pathways regulating the metabolism, cell cycle or immune response (reviewed in Reppert and Weaver, 2001).

1.3 Ontogenetic development of circadian system

The largest body of knowledge on ontogenesis of the circadian system comes from rodent studies. The process of development of the circadian clock proceeds gradually during ontogenesis and the most dramatic changes occur during the late embryonic and early postnatal period (**Fig. 2**). The morphological development of the SCN is paralleled by the gradual development of the molecular clock robustness, manifested by a gradual increase in the clock gene expression rhythm amplitudes from no rhythmicity at embryonic day 19 (E19) to high-amplitude rhythms at postnatal day 10 (P10) (Sládek *et al.*, 2004; Kováčiková *et al.*, 2006; Houdek and Sumová, 2014). The development of entrainment of the circadian system with external environment is crucial for its proper function.

This thesis studies the development of the circadian clock of two rat strains, namely the commonly used Wistar rat and the spontaneously hypertensive rat (SHR).

The morphological development of the SCN was predominantly examined in Wistar rat (Moore, 1991). The length of gestation is 22 to 23 days. Neurogenesis of the SCN occurs between E14 and E17 (Ifft, 1972). The SCN originates from a specialized zone of the ventral diencephalic primordial epithelium. The neurons of the shell appear in E15 and E16, whereas core neurons are formed in E16 and E17. Neurogenesis is completed by E18, but the gradual maturation of the neuronal morphology continues until P10.

SCN synaptogenesis is mostly a postnatal process. At E19, the number of synapses is minimal and it slowly rises until P4. The quantity of synapses increases rapidly between P4 and P10, when the density of synapses reaches the same level as in adults (Moore and Bernstein, 1989).

The development of the SCN rhythmicity was originally examined by measuring the output rhythms and then, after discovery of mammalian clock genes, by observing the daily rhythms in clock gene expressions. The development of the metabolic activity in SCN cells

during early ontogenesis was determined by C^{14}-labeled deoxyglucose uptake. In rat, the difference between day and night levels of metabolic activity of SCN cells can be observed before birth between E19 and E22 (Reppert and Schwartz, 1984). The rhythm in the electric activity of SCN neurons was recorded first at E22 and its amplitude increased until P14 (Shibata and Moore, 1987). Later on, with discovery of the mammalian clock genes, the development of SCN rhythmicity was examined via following the development of rhythmic clock gene expressions. The study of Sládek et al., examined daily profiles of clock gene expression in the SCN at three developmental stages – E19, P3 and P10 (Sládek *et al.*, 2004). The age E19 was chosen because at that time the SCN neurogenesis is already finished, P3 represents the age at which the number of synapses is still low and P10 is the stage when synaptogenesis is completed (Moore and Bernstein, 1989). Result of this study demonstrated that at E19, the expression of all studied clock genes (*Per1*, *Per2*, *Cry1*, *Bmal1* and *Clock*) was already detectable, but the expression was not rhythmic. The corresponding proteins (PER1, PER2, CRY1) were not detected at this age. The rhythms in clock gene expression are detectable at E20-21 (Ohta *et al.*, 2002; Kováčiková *et al.*, 2006; Houdek and Sumová, 2014). Amplitudes of the rhythmic expression of genes *Per1*, *Per2*, *Bmal1* and *Cry1* increased during postnatal stage up to P10, when it attained the same level as in adults. (Sládek *et al.*, 2004; Kováčiková *et al.*, 2006).

The above mentioned findings indicate that rhythms in clock genes expression in the rat SCN evolve gradually until P10.

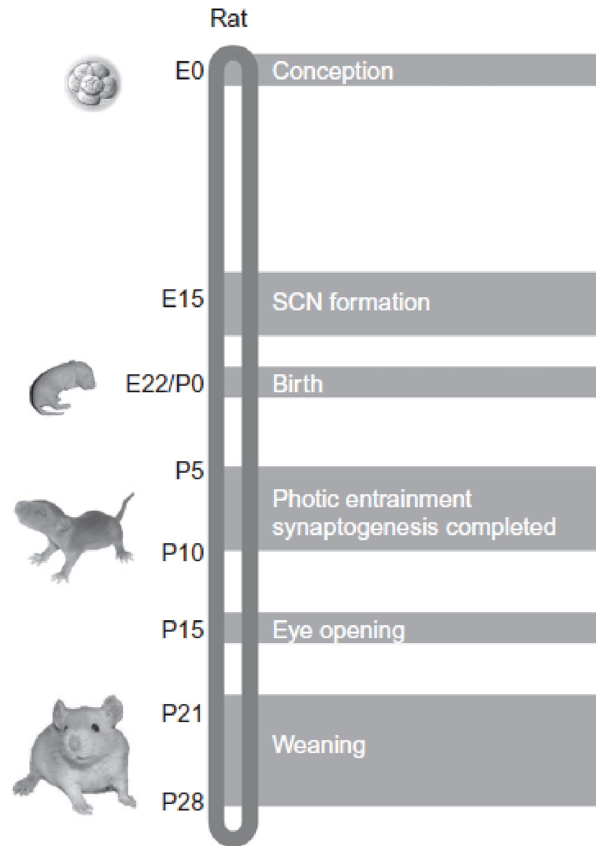


Fig. 2: Schematic diagram of the main circadian milestones of the rat ontogenesis. Vertical bar represents a timescale from embryonic day (E) 0 till postnatal day (P) 28 (adapted from Sumova *et al.*, 2012).

1.4 Entrainment of the SCN

1.4.1 Photic entrainment

The entrainment of the SCN with the external conditions is enabled via several input signals. The main synchronizing cue for the adult SCN is the light and regular changes of its intensity during the day and night. The information about the external light conditions is transmitted from retina to the ventrolateral part of the SCN via neuronal pathways.

Retino-hypothalamic tract (RHT) is a mono-synaptic pathway which leads directly from the retina to the SCN. The RHT is formed by axons of a subpopulation of photosensitive retinal ganglion cells (ipRGC) (Berson *et al.*, 2002), which are able to relay the photic signal to the SCN even in the absence of rods and cones. The neurotransmitter of this pathway is glutamate (Ruby *et al.*, 2002; Semo *et al.*, 2003).

Geniculo-hypothalamic tract (GHT) is a poly-synaptic pathway, connecting the retina with the SCN via the intergeniculate leaflet (IGL) of the thalamus. The neurotransmitter released by neurons from the IGL to the vlSCN is neuropeptide Y (Card and Moore, 1989;

Moore and Card, 1994). The light signals are integrated through this pathway with non-photic cues, which are transmitted to the thalamus from other parts of the brain (Harrington, 1997).

Third pathway that leads to the SCN, but conveys non-photic signals, is a *raphe-hypothalamic pathway*, which connects raphe nuclei to the SCN and uses serotonin as its neurotransmitter (for review see Lehman and Boer, 2000).

Upon the light stimulation of ipRGC, the information is transmitted to the vlSCN and activates the cascade of events that induce gene expression. The light pulse stimulates release of glutamate and pituitary adenylate cyclase-activating polypeptide (PACAP) on synapses in the SCN. Neuropeptide PACAP has a modulatory role and enhances the effect of glutamate (Michel *et al.*, 2006). Glutamate depolarizes the membrane which leads to the calcium release and the activation of kinases such as calcium/calmodulin-dependent protein kinase (CaMK), mitogen-activated protein kinase (MAPK) and protein kinase A (PKA). These kinases phosphorylate the transcription factor CREB (calcium/cAMP response element binding protein) (Ginty *et al.*, 1993; Obrietan *et al.*, 1998). Phosphorylated CREB binds calcium/cAMP response elements (CREs) and induces transcription of several genes including *c-fos* and *Per1* (Shigeyoshi *et al.*, 1997; Field *et al.*, 2000). **The light-mediated induction of *Per* gene expression in the SCN is considered as the most important step in the molecular mechanism, which sets the central clock in adults** (Albrecht *et al.*, 2001)**.**

The exposure to constant light (LL, light-light) causes the disruption of circadian rhythms and a gradual loss of rhythmicity in mammals (Pittendrigh and Daan, 1976; Nováková *et al.*, 2011). The arrhythmicity under LL conditions is probably caused by mutual desynchronization among individual SCN neurons, as the ability of individual cells to generate rhythms is not affected (Ohta *et al.*, 2005).

1.4.2 Entrainment of the SCN in newborn rats

In adulthood, the circadian clock is dominantly entrained by the light, but during early ontogenesis, other synchronizing cues, especially maternal, are involved. The mechanism of this entrainment has not been elucidated yet, but behavioral, hormonal and metabolic signals are likely involved. The mechanisms considered to be involved in newborns synchronization are the rhythms in maternal behavior and food intake. Involvement of food intake-mediated synchronization can be studied by applying the restricted feeding regime (RF), which is an experimental approach when animals have access to food for only a limited time at

a particular part of the day. In pregnant female Wistar rats with disrupted circadian system due to the exposure to LL, the transmission of rhythmic maternal signals to the fetal SCN is abolished and the rhythms in the expression of clock genes in the newborns SCN are impaired. If these mothers are exposed to the RF, their rhythmic behavior is restored. Moreover, under these conditions the rhythms in clock gene expressions in the SCN of newborn pups are presented and are synchronized with the time of maternal feeding. However, if these pregnant rats are kept under a light/dark 12:12 (LD) regime and their central clock is thus fully functional, the exposure to the RF does not entrain the fetal clock in the SCN (Nováková *et al.*, 2010). Therefore, this signaling pathway seems to be redundant in Wistar rats and has an importance only if the circadian system of the mother is altered.

The importance of maternal synchronization decreases gradually with the maturation of pups' circadian clock (Shimoda *et al.*, 1986; Viswanathan, 1999). The rat SCN is sensitive to the photic cues immediately after birth (Matějů *et al.*, 2009). The clock of pups becomes to be synchronized by the light at the age of P5 (Duncan *et al.*, 1986), but it is not fully entrainable by changes in the light/dark regime until the age of P6 (Ohta *et al.*, 2002). In adult animals, the sensitivity of the circadian clock to the light is restricted to the period of subjective night (so called "gating mechanism"). The gating mechanism is needed for SCN synchronization by photic stimulation and it is not present immediately after birth. This mechanism evolves gradually until P10 (Matějů *et al.*, 2009).

According to existing studies, the process of the photic synchronization development is completed at the age of P10 (reviewed in Sumova *et al.*, 2012).

1.5 Spontaneously hypertensive rat – general characteristics and its circadian clock

Wistar rats represent a model of animals, whose circadian system is considered as normally fully functional. Spontaneously hypertensive rat (SHR) is the animal model which was bred from the Wistar rat strain by selecting rats that spontaneously developed hypertension (Okamoto and Aoki, 1963). The blood pressure increase starts approximately on the 5th to 6th week after birth when in adults reaches the levels from 180 to 200 mmHg. The cardiovascular pathologies, such as the cardiac hypertrophy, occur between the 40th and 50th week of life (Conrad *et al.*, 1995).

Apart from the elevated blood pressure, SHRs exhibit other pathological symptoms such as insulin resistance and glucose intolerance (Pravenec *et al.*, 2004). Another well known fact is that SHRs differ to normotensive rat strains in their sensitivity to stress. Young SHRs

at the age of 3-6 weeks exhibit not only a higher basal plasma level of corticosterone, but their hypothalamic–pituitary–adrenal (HPA) axis is significantly more sensitive compared to Wistar Kyoto (WKY) rats (Häusler *et al.*, 1983; Kenyon *et al.*, 1993). SHRs also exhibit sleep disturbances, compared to WKY animals (Carley *et al.*, 1996).

Studies comparing the circadian system in SHRs with normotensive control rats revealed significant differences between these rat strains. In general, amplitudes of daily oscillations in various functions, for example the rhythm in blood pressure (Lemmer *et al.*, 1993) or the rhythm in the spontaneous locomotor activity (Sládek *et al.*, 2012) are significantly lower in SHRs compared with Wistar rats. The clock gene expressions in peripheral tissues and organs, such as in the heart, liver, adipose tissue or colon also differ (Cui *et al.*, 2011; Sládek *et al.*, 2012). Another important feature of SHRs is that they exhibits a significantly shorter endogenous period in its free-running locomotor activity and, therefore, their central oscillator in the SCN runs faster in constant conditions compared to that of control Wistar rat (Sládek *et al.*, 2012). In standard light/dark conditions (LD12:12), Wistar rats begin to be active at the time of lights off, whereas SHRs begin to be active significantly earlier which is caused by phase advances of their SCN clock compared to Wistar rat (Sládek *et al.*, 2012). Peters et al. found that the level of VIP, which plays the important role in SCN neurons synchronization, was significantly higher in SHRs compared to that in WKY. The level of another important SCN neuropeptide gastrin-releasing peptide (GRP) did not differ in these two rat strains (Peters *et al.*, 1994).

Moreover, the circadian system of adult SHR is significantly more sensitive to the RF exposure (Polidarová *et al.*, 2013). The higher sensitivity is detectable in SHR at behavioral level as well as at the level of the clock gene expression in the peripheral tissues (Polidarová *et al.*, 2013). Furthermore, the regular food intake may contribute to normalization of disrupted physiological functions related to the malfunction of the circadian system, e.g. the RF regime may restore the diurnal rhythm of blood pressure, clock and metabolism-related gene expressions in cardiovascular tissues of SHR (Cui *et al.*, 2011). The SHR may thus serve as a valuable model of human circadian disorders originating from the poor synchrony of the circadian system with the external light/dark regime (Sládek *et al.*, 2012).

Before this thesis was completed, the ontogenetic development of the circadian system of SHR had been studied only at the systemic level. The results of the studies suggested a difference between maternal behavior of SHR and WKY rats. The milk production in SHR mothers is lower than in WKY mothers and SHR pups therefore require more frequent feeding. When the litters of pups are switched between the mothers of a different strain, the

SHR pups reared by WKY mother show significantly lower blood pressure, compared to SHR pups reared by their original mother, whereas the heart rate does not differ between the two groups. In WKY pups reared by SHR mother both the blood pressure and heart rate remain unchanged in adulthood (Gouldsborough and Ashton, 1997).

1.6 Corticosterone

In adult rat, the basal plasma corticosterone levels exhibit a circadian periodicity with low concentration in the morning and high values at the beginning of the night (Critchlow *et al.*, 1963; Dunn *et al.*, 1972). In addition, the circadian pattern of circulating levels of glucocorticoids consists of a series of ultradian pulses of varying amplitudes and with the frequency of approximately one pulse per h (Lightman and Conway-Campbell, 2010). Similar rhythms of glucocorticoid levels are also evident in the subcutaneous tissue and hippocampus. These rhythms are synchronized with those in blood (Qian *et al.*, 2012). Furthermore, the plasma level of corticosterone has been found to correlate with its level in the breast milk (Yorty *et al.*, 2004; Brummelte *et al.*, 2010).

1.7 Restricted feeding regime and its impact on animals

In rats, the change in feeding regime due to the RF exposure is accompanied by increased plasma corticosterone levels (Belda *et al.*, 2005; Stamp *et al.*, 2008). This effect is even more pronounced when the food is available only during the light phase of the day, when rats are inactive and the peak in corticosterone plasma levels occurs at the expected time of feeding (Krieger, 1974; Belda *et al.*, 2005). Under standard *ad libitum* feeding conditions, the plasma levels of corticosterone in rat exhibit circadian rhythmicity with its peak during the beginning of their locomotor activity, which in nocturnal animals occurs at the beginning of the night (Cheifetz, 1971). The food restriction to the light phase of the day causes the daytime peak in corticosterone plasma levels rhythm preceding the expected time of feeding (Krieger, 1974). In adult animals, the RF regime uncouples the central and peripheral clocks because it causes the phase shift in clock gene expressions in peripheral organs but not in the SCN (Hara *et al.*, 2001).

1.8 Glucocorticoid receptors in the SCN

Glucocorticoid receptors belong to the family of nuclear receptors which are widely expressed in all tissues and organs. In adult Wistar rats, these receptors are present in various

parts of the brain, but they are absent in the SCN (Rosenfeld *et al.*, 1988; Aurélio Balsalobre *et al.*, 2000). In earlier study, a presence of immunopositive cells for glucocorticoid receptors was confirmed in the SCN of neonatal Wistar rats (Rosenfeld *et al.*, 1988). The study reported that during ontogenesis the number of glucocorticoid receptors in the SCN decreases reaching the minimal levels in 20-day-old pups and disappearing in adults. The presence and distribution of the glucocorticoid receptors in the SCN of SHRs has not been determined yet.

1.9 Mifepristone

Mifepristone (RU486) is a commonly used antagonist of progesterone and glucocorticoid receptors that competes for the binding site with progesterone or corticosterone/cortisol, respectively. Structurally, mifepristone is 11β dimethyl-amino-phenyl derivate of norethindrone (Cadepond F et al. 1997; Mahajan & London 1997). Its usage is widely described in adult rats and in human as well. The doses commonly used in adult rats are 4 – 10 mg per kg subcutaneously (Velíšek and Vathy, 2005; Carrillo-Martínez *et al.*, 2011). Pharmacokinetics of mifepristone in human has been extensively investigated and it was found that it reaches maximal plasma levels 1 h after the administration and its half-life is about 20 h. Effective plasma level persists until 48 h after administration (Lähteenmäki *et al.*, 1987). Usually, single doses of 100 to 300 μg were administered to newborn pups (Csaba and Inczefi-Gonda, 2000; Csaba and Karabélyos, 2001). However, in some studies, mifepristone was administered to pups repeatedly for a few days, for example for 3 days (Weinstein *et al.*, 1992).

From the data summarized above it appears that the ontogenetic development of the circadian system is a complex process during which the clocks are sensitive to various maternal cues. These cues entrain the circadian clocks before they become sensitive to the light.

2 AIMS OF THE THESIS

The general goals of the thesis are as follows:

- To compare the ontogenetic development **of the circadian system in pups of Wistar rat and SHR.**
- To examine whether **the altered maternal care** affects the development of the circadian clock in pups of these two rat strains.

Specifically, these aims were addressed in 4 projects, which were focused on:

2.1 Ontogenetic development of circadian clock in colon of Wistar pups

The aim of the first study was to ascertain when and how the circadian clock in the colon develops during the perinatal period and whether maternal cues and/or the developing pup SCN may influence the ontogenesis of the colonic clock. Daily profiles of clock gene expressions were determined in the colon since embryonic day 20 (E20) through postnatal days (P) 2, 10, 20, and 30 when weaning is completed. It was not known whether and how is the developing colonic clock entrained by the maternal feeding regime during the early postnatal period when the pups are fully dependent on maternal breast-feeding. To answer this question, the cross-fostering study and the manipulation with maternal lighting and feeding conditions were performed.

2.2 Development of circadian system in SHR pups

This study was aimed at the elucidation of how the SHR circadian system develops during ontogenesis and to assess its sensitivity to changes in the maternal feeding regime. Clock gene expression rhythms were determined in the SCN, liver and colon during postnatal ontogenesis from birth until P30. We tested the hypothesis that the circadian system in SHR differs to that in control animals already during ontogenesis. We considered maternal behavior and sensitivity of the pup’s circadian system to maternal behavior as a plausible cause of the difference. According to this hypothesis we examined the sensitivity of the developing SHR circadian system to changes in maternal behavior; we shifted the maternal-feeding regime and examined the functional state of the SHR circadian system at two critical developmental ages at P1 and at P10.

2.3 Comparison of sensitivity to maternal stress in Wistar and SHR pups

In these experiments we tested a hypothesis that during a critical period shortly after birth the developing SCN clock may be affected by elevated corticosterone levels in mothers due to their exposure to the stressful stimulus. We compared that effect in two rat strains with the different sensitivity to stress, namely SHR and Wistar rat. The mothers of both strains were exposed to stress by food restriction immediately after delivery. Pups of both strains were either untreated or injected daily by the vehiculum or glucocorticoid receptor antagonist. The impact of the combination of maternal stress and manipulation with pups on the developing circadian clock was observed.

2.4 Impact of foster mother on molecular circadian clock in rat pups and its long-lasting effect in adulthood

The differences in circadian and maternal behavior between SHR and Wistar rat validated in our previous studies provided us with the unique opportunity to study the impact of altered maternal care on the developing circadian clocks in rat pups which are reared since birth by a foster mother of a different rat strain. Clock gene expression profiles in the SCN, liver and colon were determined, and, furthermore, the locomotor activity and blood pressure were examined in adults that were exposed to cross-strain fostering procedure early after birth. We hypothesized that altered maternal care of the foster mother could affect the development of the circadian system in pups and may last until adulthood.

3 MATERIALS, EXPERIMENTAL PROTOCOLS AND METHODS

3.1 Animals

Male and female SHR/Ola and Wistar:Han rats (Institute of Physiology, Academy of Sciences of the Czech Republic) were maintained at a temperature of 21 ± 2°C in a light-dark regime depending on the experimental protocol (see below) with free access to water and feeding regime according to each experimental protocol (see below). Light was provided by overhead 40-W fluorescent tubes, and illumination was about 150 lx, depending on the cage position in the animal room. In experiments where rats were maintained in LD12:12 (12 h of light and 12 h of darkness), the lights were turned on at 06:00 h and off at 18:00 h. The time at which the lights were turned on was designated as ***Zeitgeber time*** (**ZT**) 0, and the time at which the lights were turned off was designated as ZT12. The female rats were examined for vaginal smears to determine the day of the estrus. Then, they were mated with males and on the next morning, their vaginal smears were checked. In case of sperm positivity, the pregnant rats were maintained individually in cages.

All experiments were approved by the Animal Care and Use Committee of the Institute of Physiology in agreement with the Animal Protection Law of the Czech Republic, the European Community Council directives 86/609/EEC. All efforts were made to minimize the suffering of the animals.

3.2 Experimental groups and procedures

3.2.1 Project 1 – Ontogenetic development of circadian clock in colon of Wistar pups

This project included three separate experiments. First, we examined the dynamics of the ontogenetic development of the colonic circadian clock. Then, we tested the influence of constant light and maternal restricted feeding on the developing circadian clock in the SCN and in the colon. Finally, we determined the impact of the foster mother which is kept on reverse light-dark regime than the original mother.

3.2.1.1 Experimental protocol – Experiment 1

Female Wistar rats were maintained under LD12:12 and mated with males. The female rats were then checked for sperm presence in their vaginal smears and the day when they were found to be sperm-positive was designated as embryonic day 0 (E0). Pregnant rats were maintained individually in cages, and the day of delivery, which was designated postnatal

day 0 (P0), was monitored via infrared-sensitive video cameras attached above the cage. 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 with solid food independently of their mothers. During the entire experiment, the mothers and pups had free access to food and water. On each day of sampling, pregnant rats or pups with their mothers were released into the constant darkness, and sampling was performed in dim red light (<1 lux). The time of the previous lights-on was designed as ***circadian time*** *0* (**CT**0), and sampling was performed from CT4 until CT24; for clarity of presented data, the value at CT24 was replotted as CT0. On E20, one pregnant rat was sacrificed every 4 h during the 24 h, and colons were collected from three fetuses at each time point. At postnatal ages P2, P10, P20, and P30, the colons were also sampled every 4 h during the 24 h from three pups at each time point. Pups were euthanized by decapitation under deep anesthesia, which occurred within < 1–2 min (thiopental injection, 50 mg per kg i.p.), and the sampling of colons was performed as described below.

3.2.1.2 Experimental protocol – Experiment 2

Female Wistar rats were maintained under LD12:12, mated with males, and days E0 and P1 were designated, as described above. Beginning at E0, pregnant rats were maintained individually in cages and exposed to constant light (LL), so that on E0, the lights were not switched off at 18:00 as usual and remained on during the entire experiment. After delivery, the pups were kept with their mothers until the end of the experiment. 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 (restricted feeding regime, RF), so that the food was present for 6 h per day between 09:00 and 15:00. Pups born to mothers from both groups were sampled at P10 and P20 every 4 h during 24 h in LL. Five pups per each time point were euthanized, and brains and colons were collected as described below.

3.2.1.3 Experimental protocol – Experiment 3

Male and female Wistar rats maintained in LD12:12, as described above, were divided into two groups. The first group remained in the previous LD12:12 with lights on from 06:00 to 18:00, and the other group was transferred into a reversed dark-light cycle (DL 12:12) with lights on from 18:00 to 06:00. The animals of both groups had free access to food and water during the entire experiment. After 4 weeks on either LD12:12 or DL12:12, vaginal

smears of females from both experimental groups were sampled daily to determine the day of estrus when females were mated with males. Days E0 and P0 were determined as described above. Pregnant rats from both groups were individually housed in cages equipped with infrared-sensitive video cameras attached above the cage top to monitor the exact time of delivery. On the day of delivery, entire litters of pups born to mothers in LD12:12 were transferred to foster mothers in DL12:12 and in parallel, entire litters of pups born to mothers in DL12:12 were transferred to foster mothers maintained in LD12:12.

The cross-fostering was performed within 12 h after birth (for scheme of the experimental design, see **Fig. 3**). At P10, pups reared by foster mothers in LD12:12 and DL12:12 were sampled in 4-h intervals during the entire 24-h cycle. During the dark phase, sampling was performed with the aid of dim red light (<1 lux). The time of the previous lights-on was designed as CT0, and sampling was performed from CT4 to CT24; for clarity of presented data, the value at CT24 was replotted as CT0. The time when pups were sampled was expressed in real time to emphasize the actual LD regime, as well as in CT (CT0 corresponded with the time of the previous lights-on). Pups were killed by decapitation under deep anesthesia (thiopental injection, 50 mg per kg i.p.), and samples of the brain and colon were collected as described below. At each time point, all of the litters were sampled. For analyses of clock gene expression profiles, five pups per each time point were assessed.

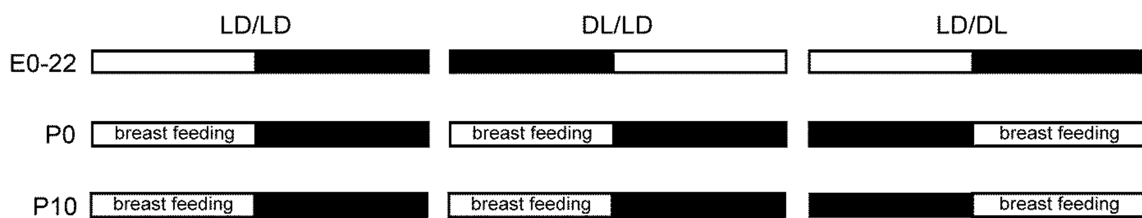


Fig. 3: Scheme of the cross-fostering experiment. Pregnant rats at embryonic day E0 through E22 (E0–E22) were maintained under a light-dark regime with lights on from 06:00 to 18:00 (LD) or under a reversed regime with lights on from 18:00 to 06:00 (DL). On the day of delivery (P0), the light-dark regime was either unchanged (LD/LD) or reversed to the opposite regime (LD/DL and DL/LD). At P10, the pups were sampled in regular intervals throughout the 24 h, and daily profiles of clock gene expression were determined.

3.2.2 Project 2 – Development of circadian system in SHR pups

Experimental protocol

Female rats of both SHR and Wistar rat strain were maintained under LD12:12 and mated with males as described above. Pregnant rats were maintained individually in cages. Since E0 till the end of the experiment (when the pups were sacrificed at P1 or P10), one group of pregnant rats was fed *ad libitum* and the other group was exposed to the restricted feeding regime (RF), which means that the rats were provided with food for only 6 h during the daytime (between ZT3 and ZT9). Access to drinking water was not limited. The day of delivery, which was designated P0, was monitored via infrared-sensitive video cameras attached above the cage. In another experiment, sampling of pups born to mothers fed *ad libitum* was performed on P1, P10, P20 and P30. The pups remained with their mothers throughout the entire experiment in spite of the fact that around P21–P25 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 constant darkness, and sampling was performed in dim red light (51 lux). The time of the previous lights on was designed as CT0 and sampling was performed from CT0 till CT24; in case of the profiles in SHR pups at P1–30, the sampling was performed from CT4 till CT24 and the value CT24 was re-plotted as CT0. The brain, liver and colon were collected every 4 h during the 24 h. From 3 to 5 pups were sampled at each time point. Pups were sacrificed by decapitation under deep anesthesia, which occurred within approximately 1–2 min (i.p. injection of thiopental, 50 mg/kg).

3.2.3 Project 3 - Comparison of sensitivity to maternal stress in Wistar and SHR pups

Experimental protocol

For both rat strains maintained as described above, the females were mated with males and the pregnant rats were housed individually in cages. After delivery, which was designated postnatal day 0 (P0), the dams and their pups of each strain were maintained in LD12:12 and divided into four groups (for experimental scheme, see **Fig. 4**). For Wistar (W) and SHR rats, the dams were either fed *ad libitum* and their pups were left untreated as controls **(group 1-W; group 1-SHR)** or they were subjected to the restricted feeding regime (RF) since delivery till the end of the experiment. The RF means that mothers had food available only for 4 h of the light phase since ZT4 till ZT8, i.e., between 10:00 and 14:00. Access to drinking water was not restricted. Pups of each litter of the RF exposed mothers

were divided into three groups, i.e., pups were left untreated (**group 2-W**; **group 2-SHR**), or they were treated every day with vehicle injection (0.03 ml ethanolic saline; s.c.) (**group 3-W**; **group 3-SHR**) or mifepristone (8 mg per kg in 0.03 ml ethanolic saline; s.c.) (**group 4-W**; **group 4-SHR**). The pups of groups 3 and 4 were treated at ZT7, i.e., 1 h prior to the food removal to their mothers and they were returned to their mothers immediately after the injection; the treatment was repeated for three subsequent days (P1 - P3). At P4, the dams and pups of both strains were released into constant darkness and the pups were sacrificed in dim red light (<1 lx) by rapid decapitation. The time of the previous lights on was designed as CT0 and the sampling was performed every 4 h from CT0 till CT24. For both rat strains, each of the above described groups (group 1 - 4) consisted of 35 pups (from litters of 7 - 8 dams) and at each time point 4 to 6 pups were sampled to collect the brains. The brains were immediately frozen on dry ice and were kept at -80° C. They were processed according to the method used (see below). The blood samples were collected from the tail vein at ZT4 from mothers of both rat strains which were either fed *ad libitum* (controls) or exposed to RF. At the same time, trunk blood samples were collected from pups of both rat strains that were reared by *ad libitum* fed mothers or RF-exposed mothers and left intact or injected s.c. with vehicle 15 min earlier. For detection of glucocorticoids (GC), blood from 2 (occasionally 3) pups was pooled per each sample. The plasma samples were processed for the detection of GC levels (see below).

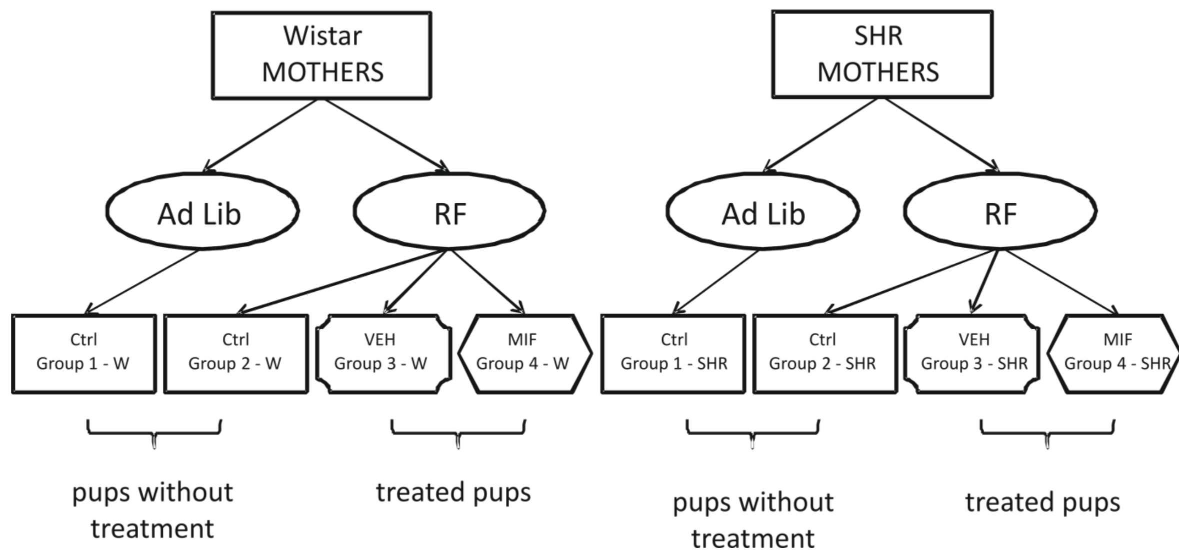


Fig. 4: Experimental scheme. Wistar rat and SHR mothers were fed ad libitum (ad lib) or exposed to stressful stimuli, i.e., a restricted feeding regime (RF). For each rat strain, the pups of mothers fed ad libitum (Group 1-W, Group 1-SHR) were left intact and served as controls (INTACT), and the pups of mothers exposed to RF were either untreated and served as controls (UNTREATED) (Group 2-W, Group 2-SHR), injected with vehicle (VEH) (Group 3-W, Group 3-SHR) or injected with mifepristone (MIF) (Group 4-W, Group 4-SHR). For more details, see MATERIALS AND METHODS.

3.2.4 Project 4 – Impact of foster mother on molecular circadian clock in rat pups and its long-lasting effect in adulthood

Experimental protocol

The female rats were mated with males and, in cases of sperm positivity in their vaginal smears they were maintained individually in cages. After delivery, which was designated postnatal day 0 (P0), the dams and their pups were maintained in LD12:12 and divided into 4 groups (for the experimental scheme, see Fig. 5). The pups of the control group were left undisturbed and were reared by their own mother throughout the lactation period until they were sacrificed during 24 h at P10 or P30 (for details, see below). The other groups of pups were exposed to fostering procedures which meant that on P1, they were transferred to a foster mother that was either of the different rat strain (cross-strain fostering), i.e. the Wistar rat pups were reared by the foster SHR mother, and the SHR pups were reared by the foster Wistar rat mother, or of the same rat strain (intra-strain fostering), i.e. the Wistar rat pups were reared by the foster Wistar rat mother and the SHR pups were reared by the foster SHR mother. Pups reared by foster mothers were sampled at P10 and/or P30 as described for the control group. Another group of pups was exposed to the foster mother of the different strain but was returned to their own biological mother at P10 that reared them until P30 when they were sampled (cross-strain return). Additionally, the pups of groups control and cross-

strain fostering groups were weaned at P30 and maintained under LD12:12 and their locomotor activity and blood pressure and heart rate were monitored when they were 1 - 2 and 6 - 8 months old.

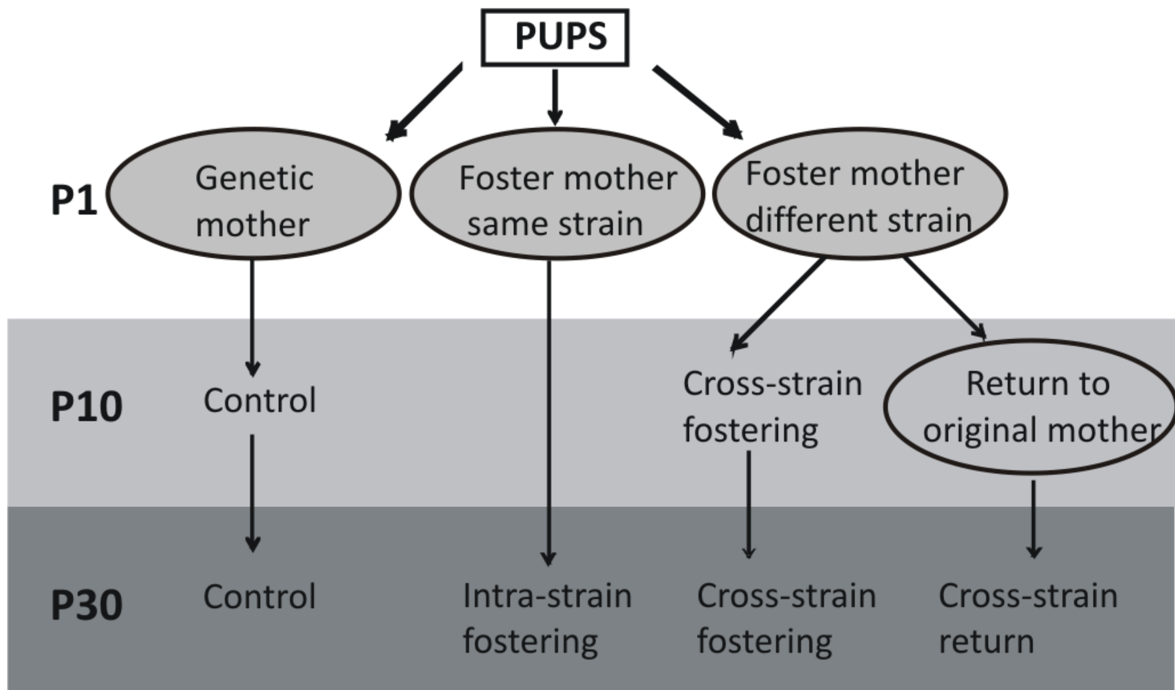


Fig. 5: Cross-fostering experimental scheme. The newborn rat pups were reared either by their genetic mother (control) or exposed to the fostering procedure which means that on the first postnatal day (P1), they were transferred to a foster mother. The foster mother of the same rat strain (intra-strain fostering) or of the different rat strain (cross-strain fostering) reared the pups until weaning (P30), or the pups were exposed to cross-strain fostering till P10 and then returned to their original mother for the rest of the lactation period (until P30).

3.3 Tissue sampling

3.3.1 Brain, liver and colon

Immediately after removal, the brains were frozen on dry ice and kept at -80°C. The brains were sectioned throughout the entire rostrocaudal extent of the SCN into five series of 12µm thick slices in an alternating order. The sections were further processed for *in situ* hybridization or laser microdissection and RT-qPCR to determine gene expression profiles in the SCN. Colons of fetuses and pups were dissected from the coecum to the rectum, and the longitudinal piece of the tissue was immersed in RNA later stabilization reagent (Qiagen, Valencia, CA). Dissected samples of the liver were immersed in RNAlater as well. All

samples were stored at 4°C for no longer than 1 week prior to the isolation of total RNA and subsequent RT-qPCR.

3.3.2 Blood sampling

Blood was sampled into the tubes coated by EDTA, centrifuged and collected plasma was immediately frozen and stored at -20°C until processing.

3.4 Methods

3.4.1 In situ hybridization

The cDNA fragments of rat *rPer1* (980 bp; 581-1561; GenBank AB_002108), *rPer2* (1512 bp; 369-1881; GenBank NM_031678), *rBmal1* (841 bp; 257-1098; GenBank AB012600) and *rRev-erbα* (1109bp; 558-1666; GenBank BC062047) were used as templates for the in vitro transcription of cRNA probes. The probes were labeled using ^{35}S-UTP, and the in situ hybridizations were performed as described previously (Sumová *et al.*, 2003). The brain sections were hybridized for 20 h at 58-61°C, depending on the probe. Following a post-hybridization wash, the sections were dehydrated in ethanol and dried. Finally, the slides were exposed to BIOMAX MR film (Kodak, USA) for 10-14 days and developed using the ADEFO-MIX-S developer and ADEFOFIX fixer (Adefo-Chemie, Germany) in an automatic film processing machine (Protec, Germany). Thereafter, the sections were processed for cresyl violet staining to localize the SCN position. Autoradiographs of the sections were analyzed using an image analysis system (Image Pro, Olympus, USA) to detect the relative optical density (OD) of the specific hybridization signal in the area of the SCN. For comparison between the experimental groups, the data were normalized to the highest value of each of the daily profile and expressed as the mean ± SEM.

3.4.2 Immunohistochemistry

The coronal sections of brain containing the SCN were mounted on slides and fixed in 4% paraformaldehyde in PBS. The slides were processed for immunohistochemistry using the GR polyclonal primary antiserum (M-20, Santa Cruz Biotechnology, USA) and the standard avidin-biotin method with diaminobenzidine as the chromogen (Vector Laboratories, UK), as described elsewhere (Sumová *et al.*, 2002). As controls for background staining, parallel sections were treated simultaneously through the immunohistochemical procedure without incubation with the specific primary antibody.

3.4.3 RNA isolation and real-time RT-qPCR

3.4.3.1 Laser dissected SCN samples

Total RNA was isolated using the RNeasy Micro kit (Qiagen, USA) according to the manufacturer's instructions. Isolated RNA samples were immediately reverse-transcribed into cDNA using the SuperScript VILO cDNA Synthesis Kit (Invitrogen, USA). The cDNA samples were analyzed by RT-qPCR on a ViiA7 Real-Time PCR System (Life Technologies, USA) using 5x HOT FIREPol Probe qPCR Mix Plus (Solis Biodyne, Estonia) and TaqMan Gene Expression Assays (Life Technologies, USA) specific for rat gene *Nr3c1* (cat. no. Rn00561369_m1). Expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Rat GAPDH Endogenous Control cat. No. 4352338, Applied Biosystems, USA) was measured in a duplex reaction to normalize the mRNA concentrations. A single RT-qPCR reaction was performed in a final volume of 20 µl; target gene probes and the GAPDH probe were dye-labeled with FAM (6-carboxyfluorescein) and VIC (4,7,2′-trichloro-7′-phenyl-6-carboxyfluorescein) fluorescent dyes, respectively. The ΔΔCt method was used for the quantification of relative cDNA concentration. Samples containing the laser-dissected hippocampus from adult animals were used for comparison (100%) of the relative expression level in the SCN of 4-day-old pups. All samples were assayed in the same RT-qPCR run.

3.4.3.2 Liver and colon samples

Total RNA was extracted from the colon and liver by homogenization with MagnaLyser Green Beads (Roche Diagnostics) (E20 and P2) and sonication (P10, P20, and P30) and subsequently purified using RNeasy mini kit (Qiagen, USA), according to the manufacturer's instructions. RNA concentrations were determined by spectrophotometry at 260 nm, and 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, USA). The RT-qPCR method used to detect the clock genes was described previously (Sládek *et al.*, 2007a). Briefly, 1µg of total RNA was reverse transcribed using the SuperScript VILO cDNA synthesis kit (Invitrogen, USA) with random primers. The resulting cDNAs were used as templates for RT-qPCR. Diluted cDNA was amplified on Light-Cycler480 (Roche, Switzerland) using the Express SYBR GreenER qPCR SuperMix (Invitrogen, USA) and corresponding intron-spanning primers (Sládek *et al.*, 2012). Melting curve analysis was performed at the end of each run to verify specificity. Relative quantification was achieved by generating a dilution curve from a common standard

in each run and subsequently normalizing the gene expression to β_2-*microglobulin* (B2M), which has been used as a housekeeping gene previously (Sládek *et al.*, 2007a). Its expression was stable throughout the day and did not vary between the analyzed tissues.

3.4.4 Corticosterone levels in plasma

The plasma samples were extracted with diethyl ether, purified with solid phase extraction technique with methanol and concentrated using a speed-vac (Sosvorova *et al.*, 2015). LC-MS/MS was performed using an QTRAP 5500 (AB Sciex, Canada) connected to the UPLC UltiMate3000 RSLC (Thermo, USA). Chromatographic separation was carried out on Kinetex C18 2.6 μm (100 × 2.1 mm) column (Phenomenex, USA) at the flow rate 0.4 ml/min at 30 °C. Mobile phases consisted of water (solvent A) and acetonitrile (solvent B). The following gradient was employed (all steps linear): 0 min, 85:15 (A:B); 1.5 min, 75:25; 3.5 min, 75:25; 9 min, 5:95; 10 min, 85:15 and at 10 min stop. The mass spectrometer was operating in the positive electrospray mode using multiple-reaction monitoring transitions (corticosterone 347.3 > 121.0 m/z quantifier ion, 347.3 > 97.1 m/z qualifier ion; 11-dh-corticosterone 345.2 > 121.1 m/z quantifier ion, 345.2 > 241.9 m/z qualifier ion).

3.4.5 Direct measurements of blood pressure and heart rate

The heart rate (HR) and blood pressure, namely mean arterial pressure (MAP), systolic blood pressure (SBP) and diastolic blood pressure (DBP), were measured. The polyethylene catheter (PE50) was inserted into the left carotid artery under isoflurane anesthesia (5 % induction and 2-3 % maintenance; Forane, AbbVie, USA) and filled with heparinized saline, tunneled subcutaneously and exteriorized in the interscapular region. One day after the surgical procedures, the experiments were carried out in conscious rats kept in small transparent cages as described previously (Behuliak *et al.*, 2015). The animals were allowed to stabilize for a period of 30 min before the measurement. The arterial catheter was connected to a pressure transducer (MLT0380/D, ADInstruments, Australia) that was placed at the heart level of rat. The signal from pressure transducer connected to bridge amplifier (QUAD Bridge, ADInstruments, Australia) was digitalized with a computer based monitoring PowerLab system (PowerLab/8SP, ADInstruments, Australia) and recorded with a sampling rate of 400 s^{-1} (Hz) by LabChart software (ADInstruments Ltd, Australia). HR was derived from arterial pressure signal as the reciprocal of pulse interval (PI) which was computed as the interval between two consecutive systolic peaks.

3.4.6 Recording of behavior

3.4.6.1 Maternal behavior

Mothers were monitored by infra-red cameras attached above the cage top and connected to a video recorder. The time when a mother was present in the nest with their pups was recorded each 1 h on the 1st, 3rd, 6th and 11th day after delivery and analyzed by an observer blind to the experimental procedure.

3.4.6.2 Locomotor activity recording

The pups were weaned at P30 and housed individually to monitor the locomotor activity immediately after weaning and again at the age of 6 months. Rats were maintained individually in cages equipped with infrared movement detectors, which were attached above the center of the top of the cage. A circadian activity monitoring system (Dr. H.M. Cooper, INSERM, France) was used to measure activity each minute. The data were analyzed using the ClockLab toolbox (Actimetrics, USA). Double-plotted actograms and chi-squared periodograms were generated to evaluate the activity and calculate its period and amplitude (power of the period estimation). For more details see (Houdek *et al.*, 2016).

3.5 Statistical analysis

To confirm the **presence of circadian rhythmicity** in gene expression profiles, the following two criteria were selected: One-way ANOVA revealed significant differences among the levels of gene expression at individual time points (with high and low levels grouped in opposite phases), and at the same time a cosine analysis confirmed that the data significantly fit the cosine curve. To perform the cosinor analysis, the data were fit with two alternative regression models to distinguish between rhythmic and non-rhythmic expression: either a horizontal straight line (null hypothesis) or a single cosine curve (experimental hypothesis), defined by the equation Y = mesor + (amplitude * cos (2 * π * (X-acrophase) / wavelength)) with a constant wavelength of 24 h. The extra sum-of-squares F-test was used for comparison, and the cosine curve parameters were calculated unless the p value exceeded 0.05. The results of cosinor analyses are summarized in tables attached.

The **differences between** gene expression rhythms of **two experimental groups** were compared by two-way ANOVA (effect of time, groups and interaction); the results are summarized in tables attached where needed. Post hoc analyses were performed using Šidák's multiple comparisons test; $p<0.05$ was required for significance. The significant

differences between the individual profiles detected by post hoc analyses are shown in the Results.

Amplitude (i.e., the difference between the peak and the mean value of a cosine curve), acrophase (i.e., the phase angle of the peak of a cosine curve) and coefficient of determination R^2 (i.e., goodness of fit) were compared where applicable. The differences in amplitudes and acrophases between the profiles at individual developmental stages in SHR (P1–P30) were analyzed by one-way ANOVA. The differences in acrophases and amplitudes between SHR and Wistar rats were evaluated by Student's t-test and where applicable, the test was corrected for multiple comparisons. The results of the Student's t-test are expressed as p value (level of significance), with $p<0.05$ required for significance.

The differences in the locomotor activity (i.e. the values of the total 24 h activity, the activity determined during 3 h intervals and the food anticipatory activity) were evaluated by two-way ANOVA followed by Šidák's multiple comparisons test with $p<0.05$ required for significance.

The differences in corticosterone levels, heart rates and blood pressure were compared between the experimental groups by two-way ANOVA.

All of the statistical calculations were performed using Prism 6 software (GraphPad, La Jolla, USA).

4 LIST OF PUBLICATIONS

Publications discussed in the PhD thesis

4.1 Ontogenetic development of circadian clock in colon of Wistar pups

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

4.2 Development of circadian system in SHR pups

OLEJNÍKOVÁ L., POLIDAROVÁ L., PAUŠLYOVÁ L., SLÁDEK M., SUMOVÁ A., 2015.
Diverse development and higher sensitivity of the circadian clocks to changes in maternalfeeding regime in a rat model of cardio-metabolic disease.
Chronobiology Int. 32(4), 531–547, IF 3.54

4.3 Comparison of sensitivity to maternal stress in Wistar and SHR pups

OLEJNÍKOVÁ L., POLIDAROVÁ L., SUMOVÁ A., 2017.
Stress affects expression of the clock gene Bmal1 in the suprachiasmatic nucleus of neonatal rats via glucocorticoid-dependent mechanism.
Acta Physiol (Oxf). 2017 Dec 20. doi: 10.1111/apha.13020. [Epub ahead of print], IF 4.867

4.4 Impact of foster mother on molecular circadian clock in rat pups and its long-lasting effect in adulthood

OLEJNÍKOVÁ L., POLIDAROVÁ L., BEHULIAK M., SLÁDEK M., SUMOVÁ A.
Circadian alignment in a foster mother improves the offspring's pathological phenotype.
Journal Physiol. Under revision, IF 5.037

5 RESULTS

5.1 PROJECT 1 - ONTOGENETIC DEVELOPMENT OF CIRCADIAN CLOCK IN COLON OF WISTAR PUPS

5.1.1 Development of clock gene expression profiles and their phasing in rat colon

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 (**Fig. 6A**). The data were assessed by one-way ANOVA and cosine analysis, as described in MATERIALS AND METHODS, and the results of cosine analysis are summarized in **Table 1**.

The significant circadian rhythms for the daily profiles of *Per1*, *Per2*, *Rev-erbα*, *Cry1*, and *Bmal1* expression (but not *Clock*) were revealed in the fetal colon at E20 (**Fig. 6A**). However, cosine analysis revealed that acrophases of all of the rhythmic profiles were accumulated close to each other into a narrow interval of the circadian cycle (**Fig**. **6B**).

At P2, only the *Per1*, *Per2*, and *Cry1* genes were expressed rhythmically similarly to the fetal stage, but *Rev-erbα* and *Bmal1* lost their prenatally expressed rhythms and *Clock* expression remained constitutive (**Fig. 6A**). While acrophase of *Cry1* expression profile did not shift after birth, the circadian rhythms of *Per1* and *Per2* expression were advanced by 3 h compared with the fetal rhythms (**Fig. 6B**).

The significant circadian rhythms were determined for the expression of *Per1*, *Per2*, *Rev-erbα*, and *Bmal1* at P10; the daily expression of *Cry1* and *Clock* was arrhythmic (**Fig. 6A**). Acrophases of clock gene expression shifted remarkably; compared with P2, *Per1* was advanced by 7 h and *Per2* by 4,5 h compared with E20, *Rev-erbα* shifted by 11 h and *Bmal1* by nearly 10 h. Therefore, at P10, the phases of rhythm of the clock gene expression spread across the circadian cycle with *Per1* and *Per2* peaking during the first half of the subjective day, *Bmal1* peaking in the middle, and *Rev-erbα* at the end of the subjective night (**Fig. 6B**).

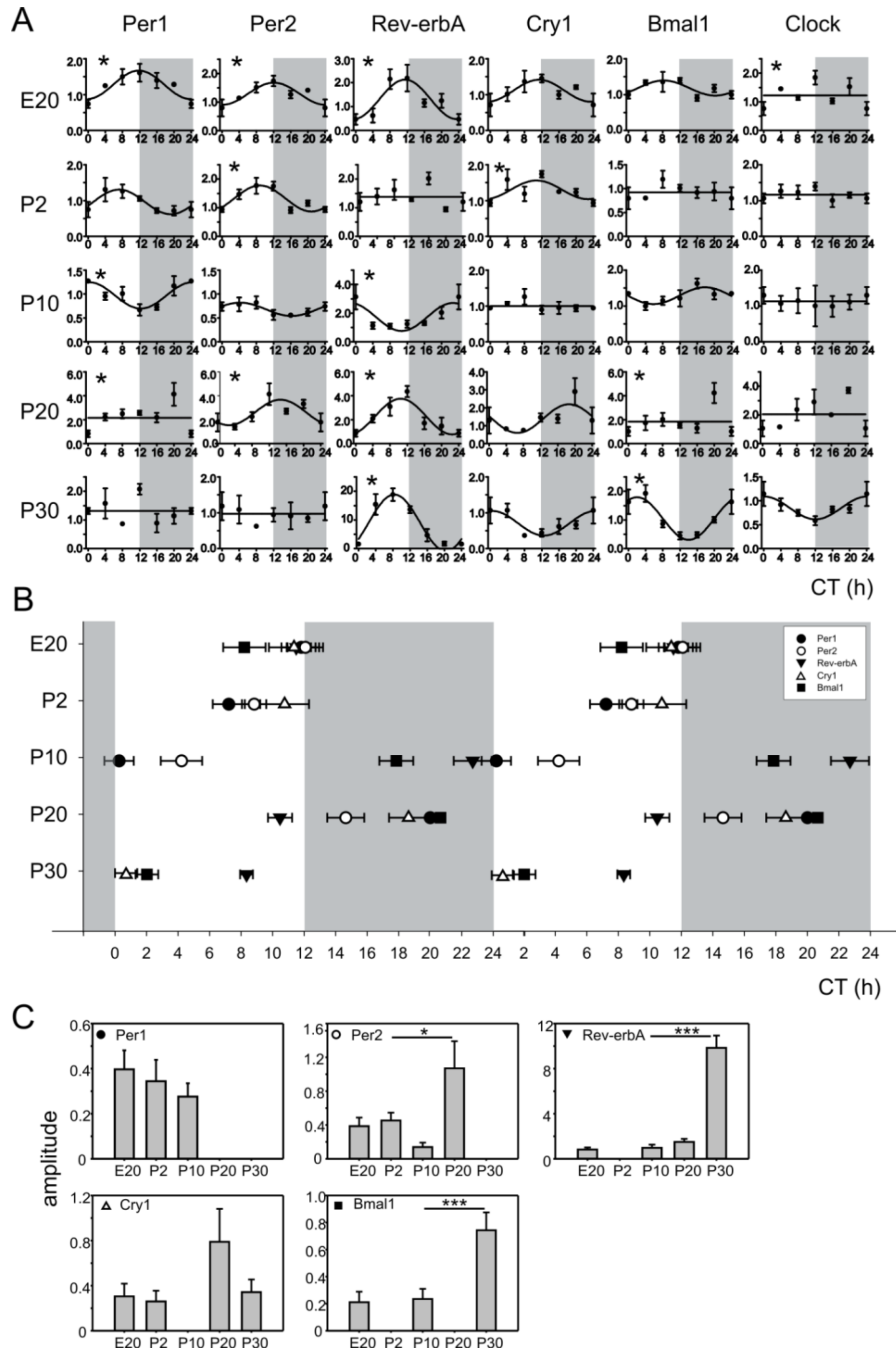


Fig. 6: **Daily profiles of clock gene expression in the colon during ontogenesis**.
(A): **levels of *Per1*, *Per2*, *Rev-erbα*, *Cry1*, *Bmal1*, and *Clock*** mRNA at E20, P2, P10, P20 and P30. Time is expressed as circadian time (CT); CT0 corresponds to the time of lights-on on the previous LD cycle (for clarity, the gray areas depict the dark phase). Each time point represents the mean ± SEM of three animals. Asterisks depict the profiles in which one-way ANOVA revealed significant differences among the time points.
(B): **acrophases** (± SEM) of the expression rhythms at E20, P2, P10, P20, and P30 to compare between the developmental changes in the peaks of the rhythmical clock gene expression profiles as shown in A. For the *Per1* and *Bmal1* expression profiles at P20, the timing of the peak levels instead of acrophases is depicted. Time is expressed as circadian time (CT). For better comparison, the data are double-plotted.
(C): **amplitudes** (± SEM) of the rhythmical clock gene expression profiles shown in B are depicted for each clock gene (with exception of *Clock*). Amplitude values are missing for developmental stages in which the profiles did not exhibit circadian rhythms. Significant differences between amplitudes at the individual developmental stages are indicated by the asterisk (*$p < 0.05$ and ***$p < 0.001$).

Colon Age	Cosinor data	*Per1*	*Per2*	*Rev-erbα*	*Cry1*	*Bmal1*	*Clock*
E20	Acro ± SEM	11.81 ± 0.92	12.07 ± 1.13	11.50 ± 0.94	11.36 ± 1.59	8.21 ± 1.35	-
	Amp ± SEM	0.397 ± 0.084	0.386 ± 0.101	0.826 ± 0.180	0.305 ± 0.113	0.211 ± 0.078	-
	R^2	0.555	0.450	0.540	0.289	0.291	0.217
	p	0.0007	0.0046	0.0009	0.0466	0.0453	0.1109
P2	Acro ± SEM	7.22 ± 1.02	8.83 ± 0.77	-	10.76 ± 1.55	-	-
	Amp ± SEM	0.344 ± 0.095	0.453 ± 0.093	-	0.260 ± 0.095	-	-
	R^2	0.447	0.575	0.136	0.293	0.150	0.142
	p	0.0087	0.0005	0.2685	0.044	0.2315	0.2527
P10	Acro ± SEM	0.26 ± 0.933	4.22 ± 1.3	22.70 ± 1.2	-	17.85 ± 1.08	-
	Amp ± SEM	0.276 ± 0.059	0.139 ± 0.052	0.975 ± 0.280	-	0.235 ± 0.075	-
	R^2	0.544	0.291	0.404	0.172	0.352	0.041
	p	0.0008	0.0455	0.0095	0.1836	0.0202	0.6851
P20	Acro ± SEM	-	14.64 ± 1.17	10.47 ± 0.77	18.63 ± 1.24	-	-
	Amp ± SEM	-	1.070 ± 0.321	1.493 ± 0.274	0.798 ± 0.292	-	-
	R^2	0.187	0.385	0.625	0.294	0.069	0.282
	p	0.1728	0.0125	0.0001	0.0435	0.5249	0.0506
P30	Acro ± SEM	-	-	8.35 ± 0.41	0.50 ± 1.4	2.02 ± 0.72	0.07 ± 1.3
	Amp ± SEM	-	-	9.842 ± 1.087	0.343 ± 0.112	0.743 ± 0.132	0.238 ± 0.074
	R^2	0.030	0.073	0.822	0.343	0.642	0.365
	p	0.7635	0.5079	< 0.0001	0.0228	< 0.0001	0.0168

Table 1: Cosinor analysis of clock gene expression profiles in the colon during ontogenesis. Acro (acrophase) in circadian time (CT); Amp (amplitude); R^2 (coefficient of determination).

At the age of P20, *Per2* and *Rev-erbα* expression profiles were rhythmical. *Per1* and *Bmal1* expression exhibited atypical waveforms with only narrow peaks, and thus, the data did not fit in the cosine curves; however, one-way ANOVA revealed significant differences between the time points (*Per1*: F_5=4.648, p=0.016; *Bmal1*: F_5=4.687; p=0.013) (see METHODS for details on the circadian rhythm criteria). The *Clock* gene expression was arrhythmical (**Fig. 6A**). The shifts in the phases of the circadian expression profiles were observed again comparing to those in P10, so that the phases were almost reversed. *Per1* and *Per2* were now peaking during the first and second half of the subjective night, respectively, and *Cry1* peaked in the middle of the subjective night. The *Rev-erbα* and *Bmal1* expression profiles were in an anti-phase, peaking at the end of the subjective day and during the second half of the subjective night, respectively (**Fig. 6B**).

At P30, only the *Rev-erbα*, *Cry1*, and *Bmal1* (and also *Clock*) expression profiles exhibited circadian rhythms (**Fig. 6A**). The rhythm in the *Rev-erbα* expression peaked during the second half of the subjective day and those in *Cry1* and *Bmal1* expression peaked during the early subjective day (**Fig. 6B**).

Comparing the developmental changes of the individual clock gene expression profiles between E20 and P30, it appeared that the rhythms underwent significant shifting in their phases, moving from a narrow cluster at the fetal period to a mutual phase at P20–P30, which is equivalent to that present in adult rats and generally considered as essential for a functional molecular clockwork. Not only acrophases, but also amplitudes of the individual clock gene expression changed during the development (**Fig. 6C**). Although only a partial comparison of amplitudes at the developmental stages was possible due to the absence of rhythms in the gene expression at some of the studied ages, from the available data, it seems evident that amplitudes tended to increase with age, which could specifically be observed for *Per2* (one-way ANOVA; F_3=5.028; p=0.003), *Rev-erbα* (one-way ANOVA; F_3=56.152; p<0.001), and *Bmal1* (one-way ANOVA; F_2=9.296; p<0.001). The expression of *Clock* became rhythmic only at P30.

5.1.2 Effect of maternal feeding behavior on clock gene expression in colon during development

Since we determined significant changes in the phases and amplitudes of expression profiles of the studied clock genes in the colon during the ontogenetic development between E20 and P10 and also between P10 and P20-P30, the next experiments were aimed at elucidating whether the change in the relative relationship between prenatal conditions and the postnatal maternal care would affect the setting of these phases and amplitudes.

5.1.3 Cross-fostering study

To examine whether a reversed maternal activity and related feeding regime after delivery may influence phase and amplitude of the clock gene expression profiles in the colon, pups were raised since birth by a foster mother who was entrained to an inverted LD regime than their genetic mother. *Per1*, *Per2*, *Rev-erbα* and *Bmal1* expression profiles were determined in the SCN and colon of 10-day-old pups (**Fig. 7**). Results from cosinor analysis of the expression profiles are summarized in **Table 2**.

At P10, in the SCN in both experimental groups, high-amplitude rhythms of *Per1*, *Per2*, *Rev-erbα* and *Bmal1* genes were detected. Their acrophases did not significantly differ under LD/DL and DL/LD conditions suggesting that the rhythms were in the same phase relative to the LD cycle (**Table 2**).

The expression profiles of *Per2*, *Rev-erbα* and *Bmal1* were rhythmical in the colon of 10-day-old pups, in both experimental groups. Comparable to the SCN, acrophases of the rhythmic profiles in DL/LD and LD/DL did not significantly change (**Table 2**). To ascertain 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 of 10-day-old pups, acrophases of the clock gene expression profiles were compared with those determined under conditions when pups were reared by their original mothers and maintained on LD12:12 during the entire experiment (LD/LD) (for data see **Figs. 6A, B** and **7**; **Tables 1** and **2**). Acrophase of the *Per2* expression profile was at CT 10.09 ± 1.05 and CT 8.84 ± 0.66 under LD/DL and DL/LD, respectively, but it was at CT 4.22 ± 1.31 under LD/LD. Acrophase of the *Rev-erbα* expression profile was at CT 3.13 ± 0.95 and CT 3.39 ± 0.67 under LD/DL and DL/LD, respectively; however, it was at CT 22.70 ± 1.21 under LD/LD. Thus, 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. In contrast, the

phase of the *Bmal1* expression rhythm was unaffected because acrophases were at CT 17.85 ± 1.08, CT 18.52 ± 0.54, and CT 18.58 ± 0.74 under LD/LD, LD/DL, and DL/LD, respectively. From comparison of amplitudes between all three experimental conditions (for data, see **Tables 1** and **2**), we found out that amplitude of the *Rev-erbα* rhythms declined in pups reared by foster mothers (LD/LD vs. DL/LD: one-way ANOVA; F_2=9.189; p<0.001), while amplitude of *Per2* rhythms increased (LD/LD vs. DL/LD: one-way ANOVA; F_2=6.245; p=0.009), and amplitude of *Bmal1* expression did not significantly change (LD/LD vs. DL/LD vs. LD/DL: one-way ANOVA; F_2=1.573; p=0.143). Therefore, the results showed 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.

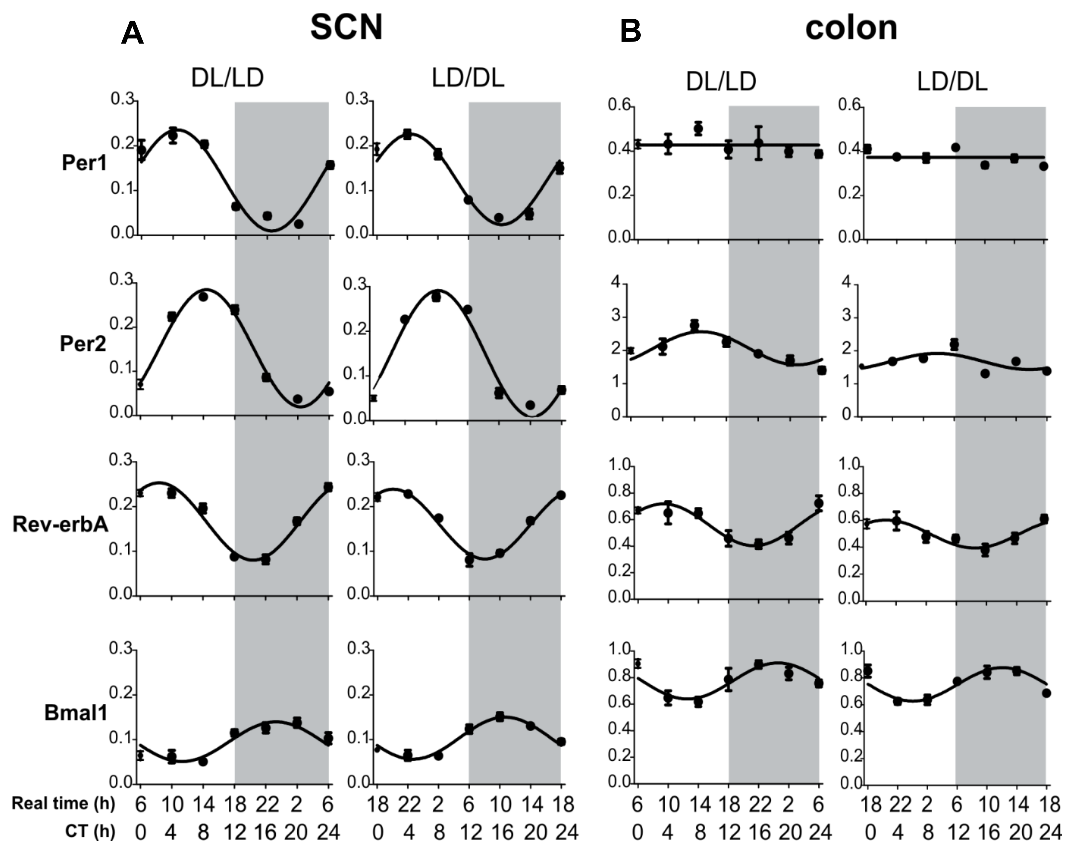


Fig. 7: The effect of cross-fostering on the daily profiles of clock gene expression in the pup SCN (A) and colon (B). The *Per1*, *Per2*, *Rev-erb*α and *Bmal1* expression profiles at P10. Pups reared by a foster mother maintained in an opposing light-dark regime compared with their original mother - LD/DL, pups born to mothers entrained to LD and reared since birth by foster mothers entrained to DL; DL/LD, pups born to mothers entrained to DL and reared since birth by foster mothers entrained to LD. For the experimental scheme, please see **Fig. 3** and MATERIALS AND METHODS. Time is expressed in real time (h) and circadian time (CT); the gray areas depict the dark phase. Each time point represents the mean ± SEM from five animals.

A. Cosinor analysis of clock gene expression profiles in the SCN.

Mother regime pre/post	Cosinor data	*Per1*	*Per2*	*Rev-erbα*	*Bmal1*
LD/DL	Acro ± SEM	22.40 ± 0.23 (CT4.40 ± 0.23)	2.25 ± 0.16 (CT8.25 ± 0.16)	20.41 ± 0.22 (CT2.05 ± 0.22)	10.68 ± 0.32 (CT16.68 ± 0.32)
	Amp ± SEM	0.101 ± 0.006	0.141 ± 0.006	0.078 ± 0.004	0.047 ± 0.004
	R^2	0.885	0.946	0.913	0.785
	p	< 0.0001	< 0.0001	< 0.0001	< 0.0001
DL/LD	Acro ± SEM	10.58 ± 0.26 (CT4.58 ± 0.26)	14.40 ± 0.15 (CT8.40 ± 0.15)	8.05 ± 0.23 (CT2.41 ± 0.23)	23.32 ± 0.51 (CT17.32 ± 0.51)
	Amp ± SEM	0.113 ± 0.009	0.133 ± 0.006	0.087 ± 0.005	0.044 ± 0.007
	R^2	0.849	0.949	0.902	0.583
	p	< 0.0001	< 0.0001	< 0.0001	< 0.0001

B. Cosinor analysis of clock gene expression profiles in the colon.

Mother regime pre/post	Cosinor data	*Per1*	*Per2*	*Rev-erbα*	*Bmal1*
LD/DL	Acro ± SEM	-	4.09 ± 1.05 (CT10.09 ± 1.1)	21.13 ± 0.95 (CT3.13 ± 0.95)	12.52 ± 0.54 (CT18.52 ± 0.54)
	Amp ± SEM	-	0.280 ± 0.072	0.090 ± 0.023	0.126 ± 0.020
	R^2	0.046	0.325	0.334	0.548
	p	0.4707	0.0019	0.0015	< 0.0001
DL/LD	Acro ± SEM	-	14.84 ± 0.66 (CT8.84 ± 0.66)	9.39 ± 0.67 (CT3.39 ± 0.67)	0.58 ± 0.74 (CT18.58 ± 0.74)
	Amp ± SEM	-	0.499 ± 0.087	0.158 ± 0.028	0.136 ± 0.030
	R^2	0.072	0.514	0.495	0.395
	p	0.3021	< 0.0001	< 0.0001	0.0003

Table 2: Cosinor analysis of SCN (A) and colonic (B) clock gene expression profiles in pups at P10 raised by foster mothers. Acro (acrophase) in real time (h) and circadian time (CT); Amp (amplitude); R^2 (coefficient of determination); LD/DL pups born to mothers maintained on LD12:12 and raised since birth by a foster mother on DL12:12; DL/LD pups born to mothers maintained on DL12:12 and raised since birth by the foster mother on LD12:12.

5.1.4 Maternal food restriction

As a next step, the expressions of clock genes were examined at the age of P10 and P20 in the SCN and colons of pups whose mothers were kept under LL since the beginning of their pregnancy and then, since delivery, fed either *ad libitum* or exposed to RF. The aim of this experiment was to distinguish between the contribution of signaling from the maternal feeding regime and signaling from the developing pup's SCN to the postnatal colonic clock synchronization. The summarized results from cosinor analysis of the clock gene expression profiles are attached in **Tables 3** and **4**.

AGE	Cosinor data	*Per2*		*Rev-erbα*		*Bmal1*	
		AL	RF	AL	RF	AL	RF
P10	Acro ± SEM	-	0.98 ± 1.14	19.42 ± 0.93	18.56 ± 0.48	-	-
	Amp ± SEM	-	0.032 ± 0.008	0.022 ± 0.006	0.029 ± 0.004	-	-
	R^2	0.059	0.306	0.309	0.613	0.059	0.132
	p	0.3782	0.0029	0.0027	< 0.0001	0.3782	0.1038
P20	Acro ± SEM	-	-	-	-	-	6.37 ± 0.79
	Amp ± SEM	-	-	-	-	-	0.010 ± 0.002
	R^2	0.117	0.151	0.085	0.120	0.004	0.364
	p	0.1357	0.0731	0.2526	0.1306	0.9466	0.0007

Table 3: **Cosinor analysis of clock gene expression profiles in the SCN** of pups at P10 and P20. The 10-day-old pups (P10) and 20-day-old pups (P20) were born to mothers that were maintained under constant light since the beginning of their gestation and fed *ad libitum* (AL) or exposed to restricted feeding (RF) since delivery.

AGE	Cosinor data	*Per1*		*Per2*		*Rev-erbα*		*Bmal1*	
		AL	RF	AL	RF	AL	RF	AL	RF
P10	Acro ± SEM	16.97 ± 1.21	23.38 ± 1.12	-	23.53 ± 1.01	18.00 ± 1.13	12.96 ± 1.06	16.27 ± 1.17	1.93 ± 1.20
	Amp ± SEM	0.226 ± 0.079	0.357 ± 0.094	-	0.402 ± 0.094	0.158 ± 0.053	0.090 ± 0.023	0.209 ± 0.069	0.233 ± 0.070
	R^2	0.202	0.327	0.094	0.377	0.219	0.344	0.225	0.270
	p	0.0269	0.0027	0.217	0.0008	0.0193	0.0018	0.0169	0.0088
P20	Acro ± SEM	-	11.06 ± 1.07	16.87 ± 0.95	14.60 ± 0.92	15.92 ± 0.93	2.13 ± 0.63	17.35 ± 0.83	17.21 ± 0.57
	Amp ± SEM	-	0.654 ± 0.166	0.569 ± 0.157	1.318 ± 0.311	0.423 ± 0.108	0.748 ± 0.118	0.971 ± 0.236	1.435 ± 0.237
	R^2	0.142	0.334	0.292	0.370	0.334	0.572	0.346	0.543
	p	0.0861	0.0018	0.004	0.0008	0.0019	< 0.0001	0.0011	< 0.0001

Table 4: Cosinor analysis of clock gene expression profiles in the colon of pups at P10 and P20. The 10-day-old pups (P10) and 20-day-old pups (P20) were born to mothers that were maintained under constant light since the beginning of their gestation and fed AL or exposed to RF since delivery.

The exposure to LL caused reduction of rhythmicity in expression of *Per2*, *Rev-erbα* and *Bmal1* in the SCN of pups in a gene- and age-dependent manner (**Fig. 8A** and **Table 3**). At P10, in pups born to mothers which were fed *ad libitum*, only the expression of *Rev-erbα* gene was rhythmic with low amplitude and the *Per2* and *Bmal1* expression profiles did not exhibit circadian rhythms. In 20-day-old pups, none of the studied clock genes was expressed rhythmically. In pups whose mothers were exposed to RF, the expression of *Per2* and *Rev-erbα* was rhythmic with low amplitude at P10, and only a very low-amplitude rhythm was present for *Bmal1* at P20. The phase of *Rev-erbα* expression was not significantly affected by maternal RF. Therefore, imposing the circadian rhythm on maternal behavior by RF weakly affected the *Per2* gene expression in the SCN at P10 and this effect was lost at P20. The pup SCN was, thus, likely unable to produce a rhythmic signal.

In the colon of pups at P10, the exposure to LL did not abolish the rhythms in the expression of *Per1*, *Rev-erbα*, and *Bmal1*, whereas *Per2* was not expressed rhythmically

(**Fig. 8B** and **Table 4**). However, in contrast with the colonic clock gene expression rhythms in pups of the same age maintained under LD12:12 (see **Fig. 6B**, **Table 1**), rhythms in *Per1*, *Rev-erbα* and *Bmal1* were all about in the same phase in pups maintained under LL, because their acrophases did not significantly differ (one-way ANOVA; F_2=0.553; *p*=0.577). Similarly, in pups maintained in LL and sampled at P20 (**Fig. 8B** and **Table 4**), acrophases of the rhythmic *Per2*, *Rev-erbα*, and *Bmal1* profiles did not significantly differ among each other (one-way ANOVA; F_2=0.647; *p*=0.526). The expression of *Per1* was not rhythmic (**Fig. 8B** and **Table 4**).

The exposure of mothers maintained in LL to RF since delivery affected the colonic expression profiles in a gene- and age-dependent manner. At P10, all studied clock genes were rhythmically expressed (**Fig. 8B** and **Table 4**) and were phase shifted relative to the profiles in pups born to mothers fed *ad libitum* because acrophases significantly differed between both groups (two-way ANOVA; *Per1*: *p*<0.001; *Rev-erbα*: *p*=0.007; *Bmal1*: *p*<0.0001). In contrast with the situation in which mothers were fed *ad libitum*, the *Rev-erbα* and *Bmal1* profiles were mutually in anti-phase under RF conditions (two-way ANOVA; *p*<0.001). In 20-day-old pups, all studied clock gene expression profiles were rhythmic under RF conditions similar to the situation at P10 (**Fig. 8B** and **Table 4**). The *Rev-erbα* expression rhythm was phase shifted compared with the profile under *ad libitum* conditions (two-way ANOVA; *Rev-erbα*: *p*<0.0001), but the *Per2* and *Bmal1* expression rhythms were in approximately the same phase (two-way ANOVA; *Per2*: *p*=0.146 and *Bmal1*: *p*=0.999). At P20, the *Rev-erbα* and *Bmal1* profiles were mutually in anti-phase (two-way ANOVA, *p*<0.001).

Under *ad libitum* and RF conditions, amplitudes of the rhythms in expression of the studied clock genes increased between P10 and P20 (for data, see **Table 4**). Specifically, under *ad libitum* conditions, the increase was significant for the *Bmal1* expression (two-way ANOVA; *p*<0.05) and under RF, it was significant for *Rev-erbα* and *Bmal1* (two-way ANOVA; both *p*<0.001). Furthermore, amplitude of *Rev-erbα* expression rhythm was significantly higher under RF than under *ad libitum* conditions at P20 (two-way ANOVA; *Rev-erbα*: *p*<0.05).

It appears that maternal RF was able to synchronize and entrain the clock in pup colons independent of their SCN clock.

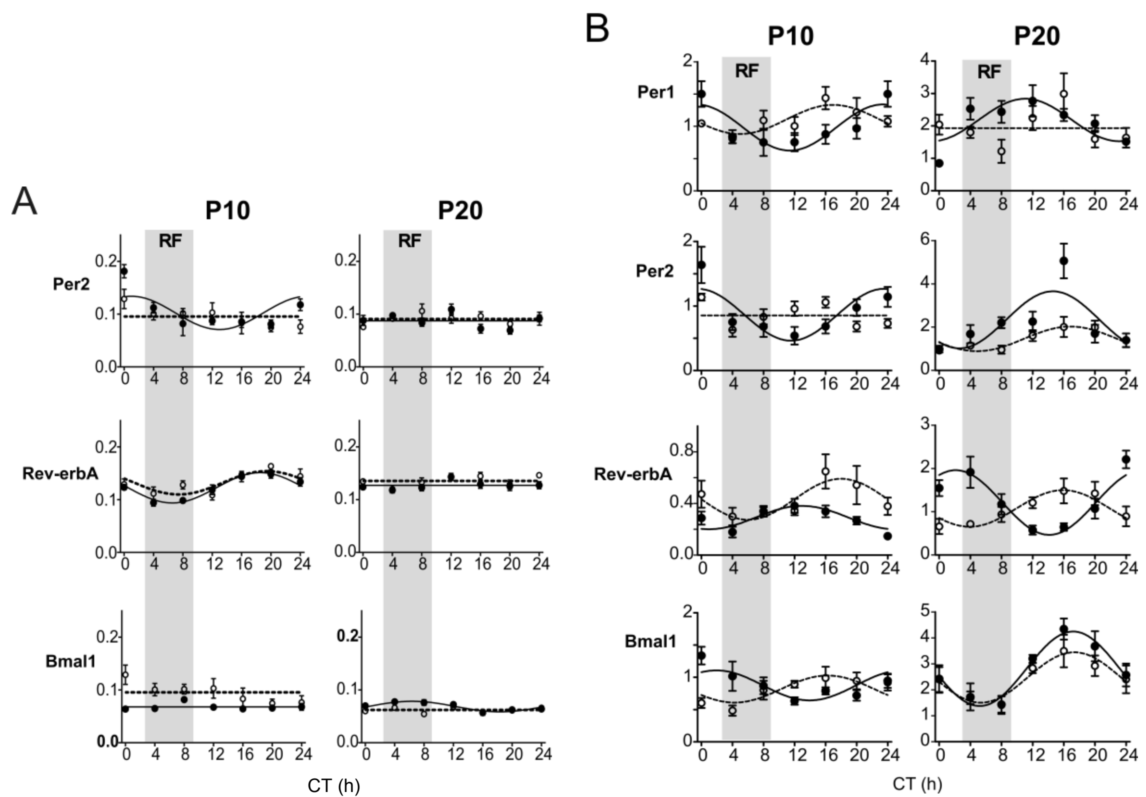


Fig. 8: **Effect of the exposure to constant light and maternal restricted feeding on the daily profiles of clock gene expression in the pup SCN and colons.** Daily expression profiles of *Per2*, *Rev-erbα* and *Bmal1* in the SCN **(A)** and *Per1*, *Per2*, *Rev-erbα* and *Bmal1* in the colon **(B)** at P10 and P20. Pups were maintained with their mothers under constant light since E0. Since P0, the mothers were fed *ad libitum* (open circles; dashed line) or exposed to restricted feeding (RF) (solid circles; full line). The timing of food availability is depicted by the gray areas. For more details, see MATERIALS AND METHODS. Time is expressed as circadian time (CT). Each time point represents the mean ± SEM from five animals.

5.2 PROJECT 2 - DEVELOPMENT OF CIRCADIAN SYSTEM IN SHR PUPS

5.2.1 Maternal behavior of SHR

Mothers with their pups were monitored by video cameras during the first part of the lactation period to detect how much time during the day they stayed in the nest with their pups. The video records of one Wistar rat and one SHR were analyzed immediately after delivery for 20 h (**Fig. 9A**) and thereafter during 24-h intervals on the 3rd, 6th and 11th day (**Fig. 9B**).

From comparisons between the maternal behavior of both strains directly after delivery, it appears that mothers of both strains remained in the nest during almost the entire light phase of a day. However, during the dark phase, the SHR mother seemed to spend less time in the nest compared to the Wistar rat, and consequently, the total time that the mothers were present in the nest during the 20 h after delivery appeared to be lower in SHR (87.27 %) than Wistar rat (99.3 %) (**Fig. 9A**). A similar trend was still apparent on the 3rd day after delivery, when the SHR also spent less time in the nest (74.6 %) compared with the Wistar rat (83.4 %). The difference was not obvious on the 6th day (67.3 % and 62.5 % for SHR and Wistar rat, respectively) and on the 11th day (34.8 % and 40.1 % for SHR and Wistar rat, respectively) (**Fig. 9B**).

The data showed an obvious trend that mothers to spend less time in the nest during the postnatal period, mainly during the nighttime. Moreover, it seems that SHR mothers may leave their newborn pups more often than Wistar rats during the first 3 days after delivery.

5.2.2 Development of the SCN clock in SHR

Daily profiles of *Per1*, *Per2*, *Rev-erbα* and *Bmal1* expression were determined in the SCN of SHR pups at P1, P10, P20 and P30 (**Fig. 10**). The expression levels at each time point represent mean ± SEM of three animals and the small error bars indicate a very low variance of clock gene expression in the SCN among the individual pups. All studied genes at all developmental stages were expressed rhythmically (results of cosinor analysis for *Per1*, *Per2* and *Rev-erbα*: $p<0.001$, for *Bmal1* at P1: $p=0.0085$); however, the *Bmal1* rhythm at P1 was shallow because, in contrast to other genes, one-way ANOVA did not confirm the significant effect of time (**Fig. 10A**). Acrophases of these profiles did not exhibit significant changes in the course of postnatal development.

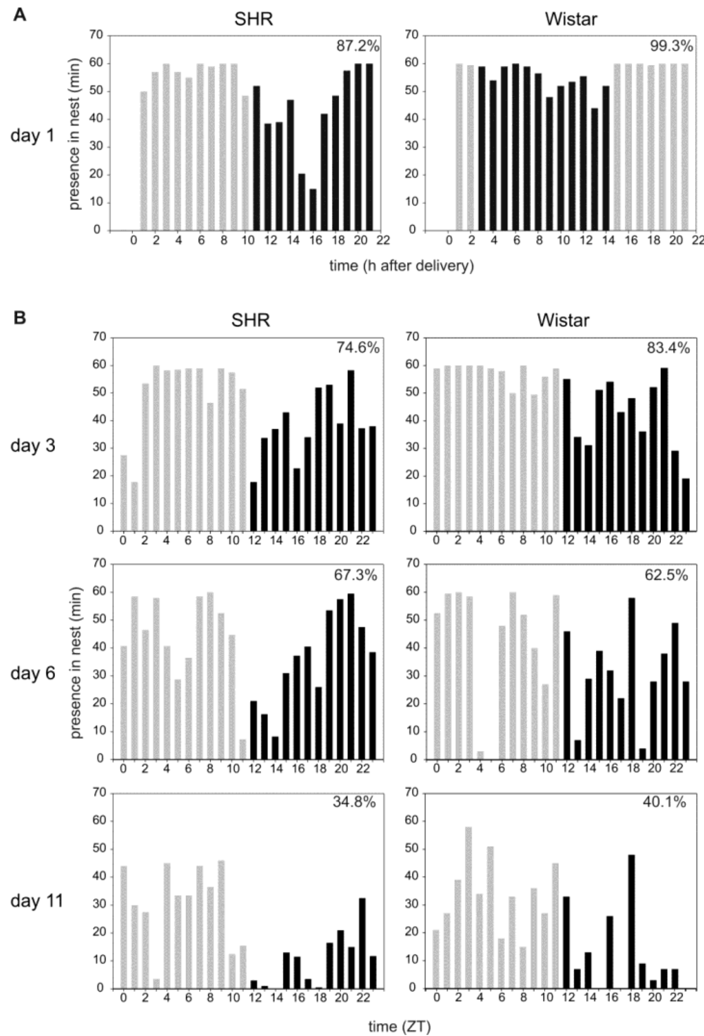


Fig. 9: Presence of the SHR and Wistar rat mothers in the nest. Duration of mothers' presence in the nest with their pups was recorded and expressed in 1-h bins. The maternal behavior was recorded during **(A)** the first 20 h after delivery and **(B)** the 24 h on the 3rd, 6th and 11th day after delivery. Time is expressed as hours after delivery (A) or as time of a day in Zeitgeber time (ZT) (B). The gray columns represent behavior during the light phase and the black columns during the dark phase of a day. Numbers in the upper right corner of the graphs represent % of the total time of the recording interval for which the mothers stayed in the nest.

During the postnatal development, amplitudes of the *Per1*, *Per2* and *Rev-erbα* expression rhythms (**Fig. 10B**) increased between P1 and P10 but did not change between P10 and P20. And thereafter, they either remained the same until P30 (*Per2*) or even declined compared with P20 or P10 (*Per1* and *Rev-erbα*). The dynamics of the *Bmal1* expression rhythm development were delayed compared with the other studied genes because amplitude did not increase significantly between P1 and P10, as opposed to the other clock gene expression rhythms; amplitude increased slightly at P20 and more significantly only at P30.

The delayed development of the *Bmal1* expression rhythm found in the SCN of SHR was previously not observed in Wistar rats (Sládek *et al.*, 2004). Therefore, in this study,

daily *Per2*, *Rev-erbα* and *Bmal1* expression profiles were compared between SHR and Wistar rat pups at P10 (**Fig. 11A**), i.e. at the postnatal age when the significant amplitude elevation, compared with P1, was present in all studied genes in Wistar rats (Sládek *et al.*, 2004) but not in SHR (this study, **Fig. 10**). Acrophases of the studied clock gene expression profiles did not differ significantly between both strains (**Fig. 11B**). Amplitudes of *Per2* and *Bmal1* expression profiles were significantly lower in SHR compared to Wistar rats (*Per2*: p=0.0334; *Bmal1*: p=0.0335), whereas amplitude of the *Rev-erbα* profile was higher in SHR (p=0.0024) (**Fig. 11C**).

The mRNA levels in Wistar rats and SHR were not assayed in the same *in situ* hybridization assay; nevertheless, variations in the background binding of the hybridization probes between these two assays did not seem to account for the observed differences in the rhythm characteristics. Altogether, these results suggest that in SHR, the dynamics of development of the *Bmal1* expression rhythm during early ontogenesis might be delayed compared with Wistar rats.

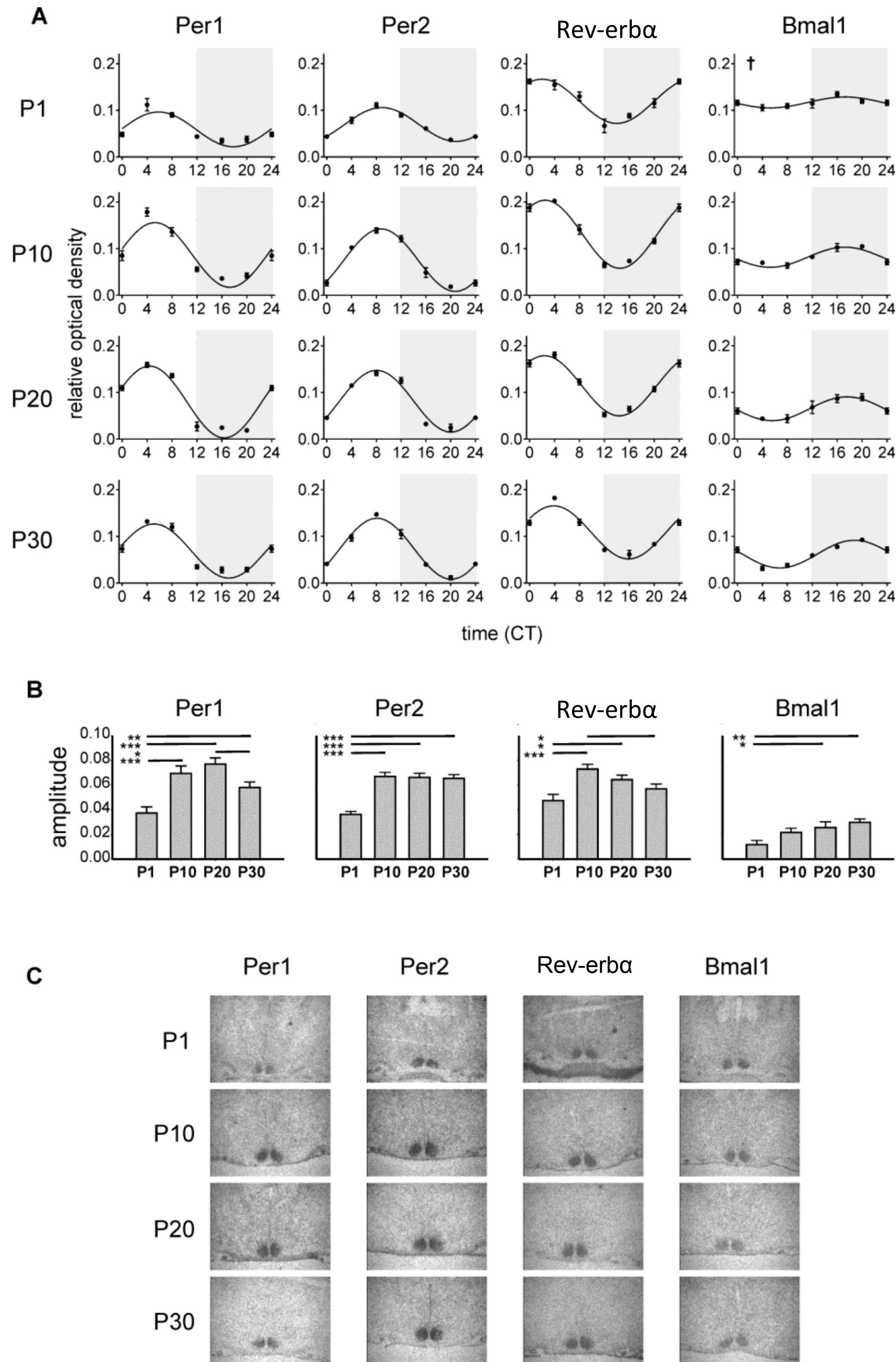


Fig. 10: Daily clock gene expression profiles in the SCN of SHR pups during ontogenesis.
(A) Daily expression profiles of clock gene *Per1*, *Per2*, *Rev-erbα* and *Bmal1* in the SCN at P1, P10, P20 and P30. Time is expressed as circadian time (CT), the gray area on each graph corresponding to the interval of darkness in the previous LD cycle. Data are expressed as the mean ± SEM of three animals.
(B) Comparison between amplitudes of the cosine curves depicted in (A) for each gene at developmental stages P1, P10, P20 and P30. Results of one-way ANOVA comparison are depicted as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.
(C) Representative autoradiographs of the hypothalamic sections of rat pups at P1, P10, P20 and P30 demonstrating the expression of *Per1*, *Per2*, *Rev-erbα* and *Bmal1* in the SCN (dark spots). Selected autoradiographs correspond to circadian time (CT) with the highest hybridization signal, i.e. CT 4 for *Per1* and *Rev-erbα*, CT 8 for *Per2* and CT 20 for *Bmal1*.

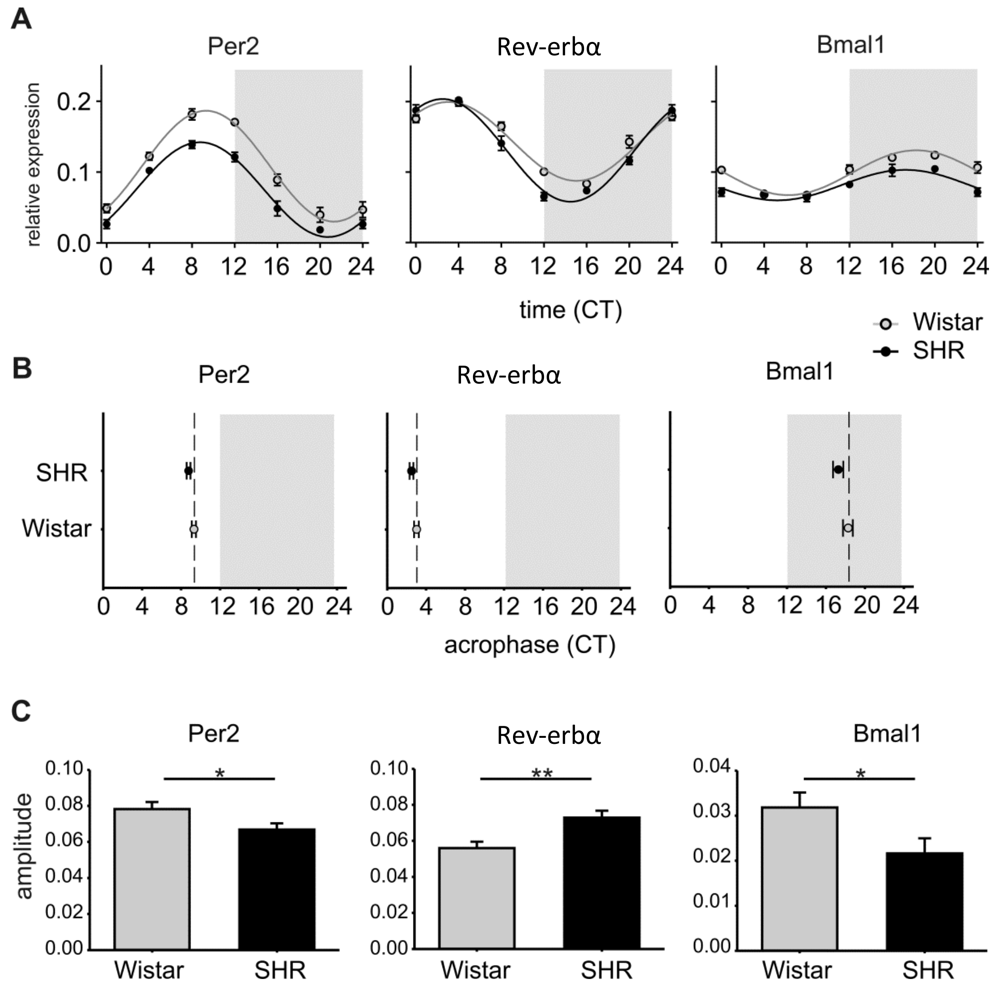


Fig. 11: Comparison of clock gene expression profiles in the SCN of 10-day-old SHR and Wistar rat pups.

(A) Daily profiles of *Per2*, *Rev-erbα* and *Bmal1* expression were determined by *in situ* hybridization in the SCN of SHR (black dots and black line) and Wistar rat (gray dots and gray line) pups. Data are expressed as the mean ± SEM of five animals.

(B) Comparison between acrophases of the cosine curves for each gene in SHR (black dots) and Wistar rat (gray dots) pups depicted in (A). No significant differences between acrophases in both rat strains were detected.

(C) Comparison between amplitudes of the cosine curves depicted in (A) for each gene in SHR and Wistar rat pups. Results of Student's t-test are depicted as * $p<0.05$, ** $p<0.01$.

5.2.3 Development of peripheral clocks in SHR

Daily profiles of *Per1*, *Per2*, *Rev-erbα* and *Bmal1* mRNA levels were determined in the liver (**Fig. 12**) and colon (**Fig. 13**) of SHR pups at P1, P10, P20 and P30. The results of cosinor analysis are summarized in **Table 5**.

5.2.3.1 Development of SHR hepatic clock

For *Per1*, *Per2* and *Rev-erbα* expression profiles (**Fig. 12A**) cosinor analysis confirmed circadian rhythms at all postnatal ages, with the exception of *Per1* at P30. For *Bmal1*, the

rhythm was significant at P1 and P30, whereas it was only very shallow at P10 (cosinor analysis revealed a borderline significant rhythm and one-way ANOVA did not confirm the significant effect of time) and even absent at P20 (neither cosinor analysis nor one-way ANOVA were significant). Acrophases of the expression rhythms changed substantially during the interval from P1 to P30 (**Fig. 12B**). Interestingly, dynamics of the change were gene-specific.

At P1, the *Per1*, *Per2* and *Rev-erbα* expressions peaked during the subjective night, whereas the *Bmal1* expression peaked during the subjective day. At P10, acrophases of all studied genes shifted relative to P1, so that *Per1*, *Per2* and *Rev-erbα* expression levels peaked during the subjective day (P1 vs. P10, $p<0.001$ for all genes). However, whereas the *Per1*, *Per2* and *Rev-erbα* rhythms shifted to the anti-phase between P1 and P10, the *Bmal1* rhythm dampened significantly and shifted only moderately, if at all. At P20, acrophases of the *Per1* and *Per2* rhythms shifted back to the subjective night (P10 versus P20 for both genes $p<0.001$), i.e. to a position close to that at P1. In contrast, the *Rev-erbα* rhythm shifted only moderately ($p<0.001$), and its acrophase remained in the subjective day, as it was at P10. *Bmal1* expression profiles were not rhythmic at P20. Finally, between P20 and P30, acrophases of *Per2* and *Rev-erbα* rhythms did not shift ($p=0.08$ and $p=0.199$, respectively) and remained at the subjective night and subjective day, respectively. The *Bmal1* became expressed rhythmically at P30 and acrophase of the high-amplitude rhythm was approximately opposite compared with acrophase at P1 ($p<0.001$).

These results demonstrate that during ontogenesis, the phase of rhythmic expression in the liver of SHR pups phase-reverses between P1 and P10 for *Per1*, *Per2* and *Rev-erbα*, and the phase-reversal is final for *Rev-erbα*, but not for *Per1* and *Per2*. The *Bmal1* expression rhythm adjustment is delayed compared to the other genes because it proceeds via significant dampening of the rhythm around P10–P20 to the apparent phase-reversal by P30.

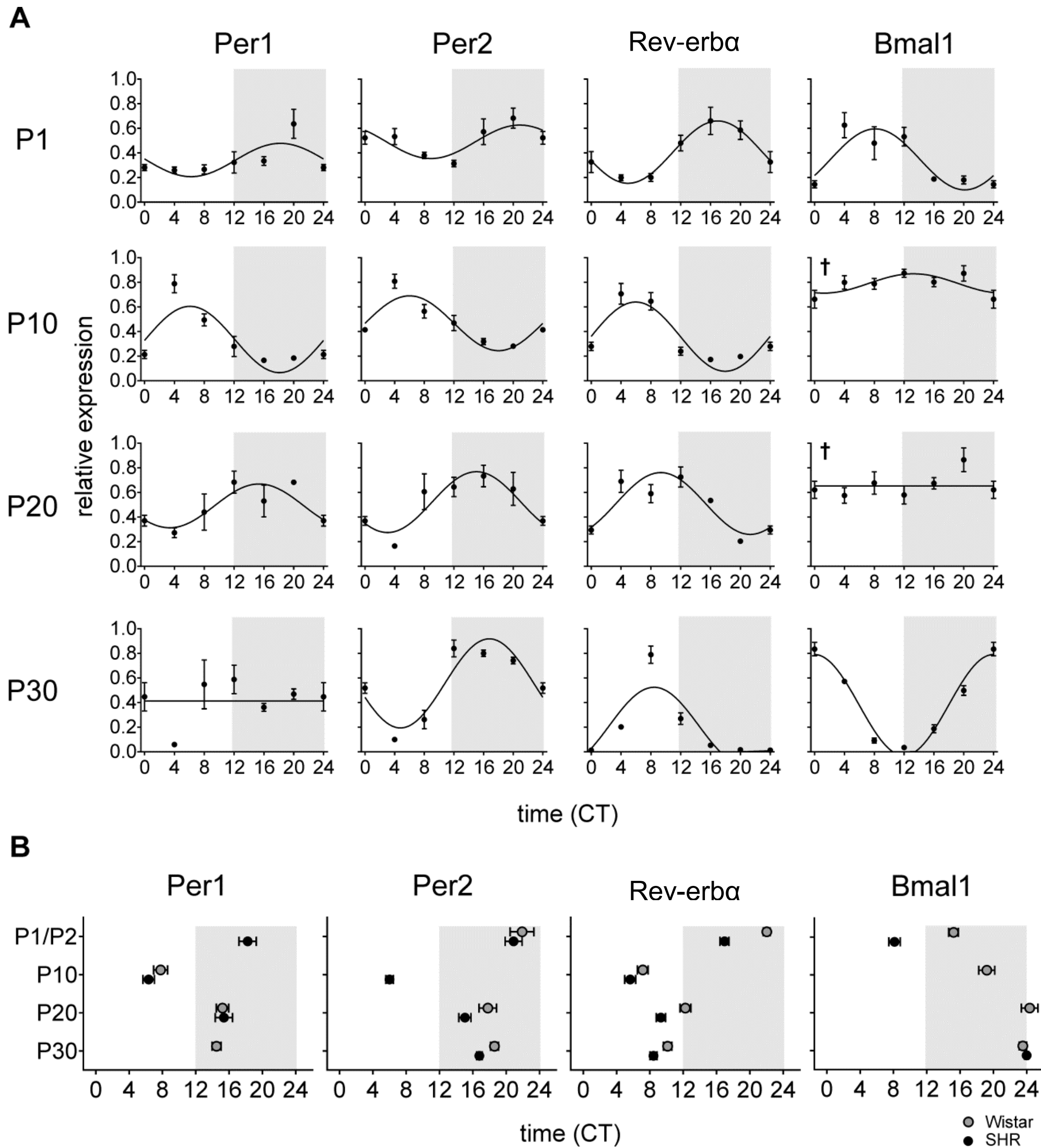


Fig. 12: Daily clock gene expression profiles in the SHR liver during ontogenesis.
(A) Daily profiles of clock gene *Per1*, *Per2*, *Rev-erbα* and *Bmal1* in the liver at P1, P10, P20 and P30. Time is expressed as circadian time (CT), the shaded area on each graph corresponds to the interval of darkness in the previous LD cycle. Data are expressed as the mean ± SEM of five animals. Symbol † in the upper left corner of the graphs depicts the profiles where one-way ANOVA did not confirm a significant effect of time.
(B) Acrophases of the cosine curves depicted in (A) for each gene at developmental stages P1, P10, P20 and P30 (black dots). For comparison between SHR and Wistar rats, the gene expression profiles previously detected in Wistar rats (Sládek *et al.*, 2007a) were analyzed with cosinor analysis and acrophases of the rhythmic gene expression profiles are shown (gray dots). Symbols are missing when cosinor analysis did not reveal significant fits. Acrophases are depicted in circadian time (CT) as a mean ± SEM. Results of one-way ANOVA comparison are presented in the text.

	age	*Per1*	*Per2*	*Rev-erbα*	*Bmal1*
LIVER	**P1**	0.0113	0.0027	< 0.0001	< 0.0001
	P10	< 0.0001	< 0.0001	< 0.0001	0.0468
	P20	0.0025	< 0.0001	< 0.0001	n.s.
	P30	n.s.	< 0.0001	< 0.0001	< 0.001
COLON	**P1**	0.0100	0.0021	< 0.0001	0.0012
	P10	0.0002	< 0.0001	< 0.0001	< 0.0001
	P20	0.0003	< 0.0001	< 0.0001	< 0.0001
	P30	0.0131	< 0.0001	< 0.0001	< 0.0001

Table 5: Results of cosinor analysis (significance, *p*) of clock gene expression profiles in the liver and colon of SHR pups at the postnatal age P1, P10, P20 and P30. The daily profiles are depicted in **Figs. 12** and **13**.

5.2.3.2 Development of SHR colonic clock

For gene expression profiles in the colon of SHR pups (**Fig. 13A**), cosinor analysis confirmed circadian rhythms for all genes at all studied postnatal ages (**Table 5**); however, the rhythm was shallow for *Per1* at P1 and P30 (one-way ANOVA did not confirm a significant effect of time).

At P1, the expression levels of *Per1* and *Per2* peaked in the middle of the subjective day, *Rev-erbα* at the end of the subjective night, and *Bmal1* during the first part of the subjective night. Acrophases of the expression rhythms in the colon changed between P1 and P30 in a gene-specific manner (**Fig. 13B**). Between P1 and P10, only *Rev-erbα* rhythms (but not the *Per1*, *Per2* and *Bmal1* rhythms) phase shifted significantly ($p<0.001$). Between P10 and P20, *Per1*, *Per2* and *Rev-erbα* rhythms shifted ($p<0.001$ for all genes) and at P20 they peaked during the first part of the subjective night (*Per1*, *Per2*) or late in the subjective day (*Rev-erbα*). In contrast, the *Bmal1* rhythm at P20 still did not phase shift significantly compared with P10 or P1. Finally at P30, the *Per1*, *Per2* and *Rev-erbα* rhythms remained in phase with those at P20 or shifted only moderately and peaked during the first part of the subjective night and the second part of the subjective day, respectively. The *Bmal1* rhythm

shifted significantly for the first time at P30 compared with the previous developmental stages ($p<0.001$) so that acrophase occurred at the end/beginning of the subjective night/day.

Therefore, in the colon of SHR pups, *Per1*, *Per2* and *Rev-erbα* rhythms changed their phases with different dynamics compared with the liver; only the *Rev-erbα* rhythm shifted between P1 at P10, whereas the *Per1* and *Per2* acrophases changed later (only at P20). However, similarly in both tissues, resetting of the *Bmal1* mRNA rhythm was delayed compared with the other studied genes because the rhythm shifted only after P20.

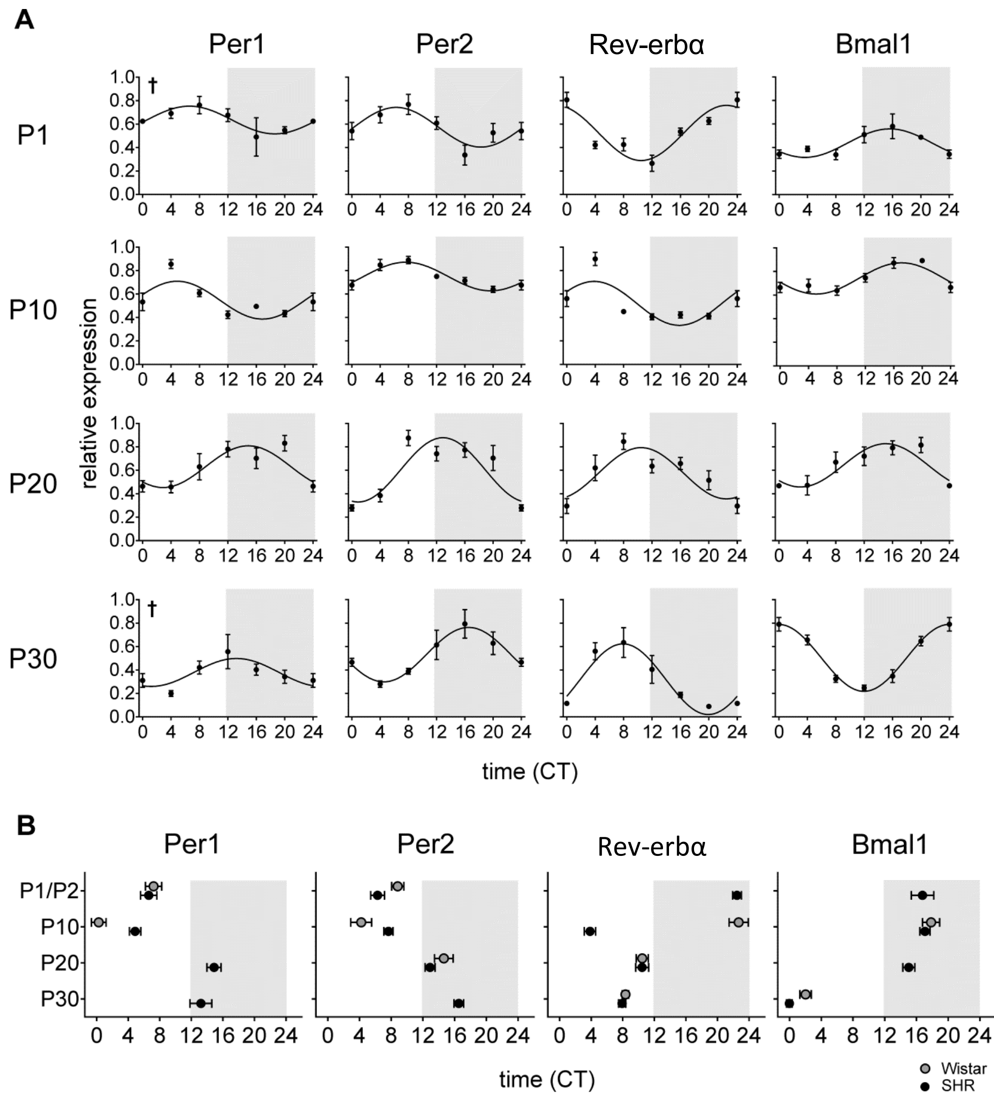


Fig. 13. **Daily clock gene expression profiles in the SHR colon during ontogenesis.** For description to **(A),** see the legend in **Fig. 12**.
(B) Acrophases of cosine curves depicted in (A) for each gene at developmental stages P1, P10, P20 and P30 (black dots). For comparison between SHR and Wistar rats, acrophases of the gene expression profiles detected previously in Wistar rats (Polidarová *et al.*, 2014) are shown (gray dots). Symbols are missing when cosinor analysis did not reveal significant fits. Acrophases are depicted in circadian time (CT) as the mean ± SEM. Results of one-way ANOVA comparison are presented in the text.

5.2.4 Comparison between development of peripheral clocks in SHR and Wistar rats

As described above, the detailed analysis of the peripheral clock ontogenesis in SHR revealed that adjustment of the *Bmal1* expression profile in the liver proceeded via amplitude suppression during the postnatal period P10–P20. This was in contrast to the previously described development of the peripheral clocks in Wistar rats (Sládek *et al.*, 2007a; Polidarová *et al.*, 2014). In order to limit repetition of the previous studies in Wistar rats to the minimal extend, in this study we compared the *Bmal1* and *Rev-erbα* clock gene expression profiles of SHR and Wistar rat liver and colon only at P10 (**Fig. 14**). The age was chosen because according to the results of this study in SHR and the previously published result in Wistar rat, the profiles of these two clock genes seemed different between both rat strain peripheral tissues. The comparison was performed by detection of the gene expression in the same RT-qPCR assay. In the liver (**Fig. 14**, left column) of 10-day-old Wistar rats, *Rev-erbα* expression was rhythmic in both rat strains and peaked at CT 7.1 ± 0.6 in Wistar rats and at CT 5.6 ± 0.7 in SHR; the profile in SHR was thus moderately phase-advanced compared with that in Wistar rats ($p<0.001$). *Bmal1* expression was significantly rhythmic in Wistar rats (acrophase at CT 19.2 ± 1.0), but in SHR the expression was arrhythmic and generally down-regulated. In the colon (**Fig. 14**, right column), the *Rev-erbα* expression was also rhythmic in both rat strains and peaked at CT 2.0 ± 0.9 in Wistar rats and at CT 4.2 ± 1.1 in SHR ($p<0.001$). The *Bmal1* expression was rhythmic and in the same phase in the colon of Wistar rats and SHR (CT 18.0 ± 0.8 and CT 18.0 ± 0.9, respectively); however, in SHR the expression levels were lower than those in Wistar rats.

Altogether, the data revealed differences in the regulation of the clock gene expression in SHR compared with Wistar rats at P10. Notably, the expression of *Bmal1* was constitutive over 24 h in the liver of SHR and its expression levels were decreased throughout the day both in the liver and colon of SHR compared to Wistar rats. For comparison between SHR and Wistar rats at other developmental stages, the data from our previous studies are provided in **Figs. 12B** and **13B**. For the hepatic clock in Wistar rats, the data from our previous study (Sládek *et al.*, 2007a) were fitted with cosine curves and acrophases are depicted in **Fig. 13B**. For results on the colonic clock, the data from our previous study (Polidarová *et al.*, 2014) are depicted in Fig. **12B**. From the comparison between both rat strains in the liver it appears that whereas *Per1* and *Per2* profiles developed similarly, there were significant differences in the development of *Rev-erbα* and *Bmal1* profiles. For the

colon, no significant differences were obvious from the available data. The results are discussed in detail in DISCUSSION.

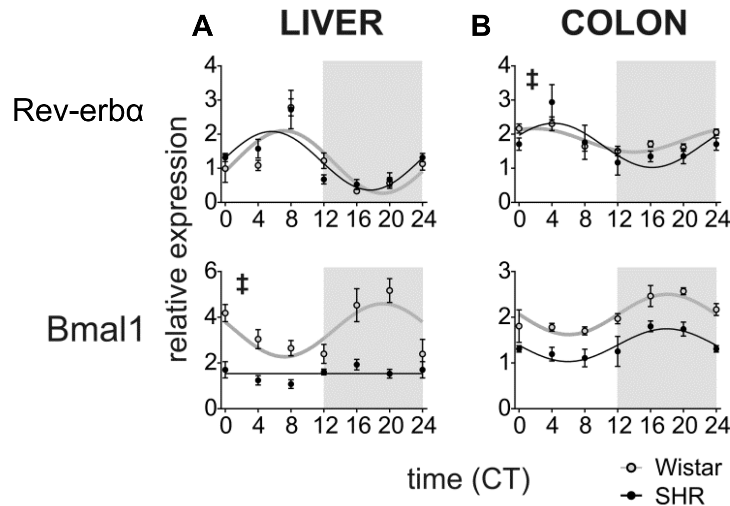


Fig. 14. Comparison of clock gene expression profiles in the liver (A) and colon (B) of 10-day-old SHR and Wistar rat pups. Relative levels of the *Rev-erbα* and *Bmal1* expression in SHR (black dots and black lines) and Wistar rat (gray dots and gray lines) pups were determined in the same RT-qPCR assay in both tissues. Time is expressed as circadian time (CT), the shaded area on each graph corresponds to the interval of darkness in the previous LD cycle. Data are expressed as the mean ± SEM of five animals. Symbols in the upper left corner of the graphs ‡ depict the profiles where one-way ANOVA did not confirm a significant effect of time for profiles in SHR (for profiles in Wistar rats, the effect was significant).

5.2.5 Effect of maternal-feeding regime on developing circadian system in SHR

SHR pups born to mothers fed *ad libitum* (controls) or exposed to RF were sampled at P1 (to detect whether maternal RF has any effect on the fetal clocks) and at P10 (to detect whether extension of maternal RF to the postnatal period has an effect on the development of the clocks). The daily gene expression profiles were detected in the SCN (*Per2*, *Rev-erbα*, *Bmal1*), and in the liver and colon (*Per1*, *Per2*, *Rev-erbα*, *Bmal1*, *Bmal2*); at each time point, five animals were sampled and data are expressed as the mean ± SEM (**Fig. 15**).

A

SCN P1

Per2 Rev-erbα Bmal1 †

relative o.d.

SCN P10

Per2 Rev-erbα Bmal1

P1

LIVER COLON

P10

LIVER COLON

Per1

Per2 ‡

Rev-erbα

Bmal1 ‡ †‡

Bmal2 †‡ †‡

relative expression

time (CT)

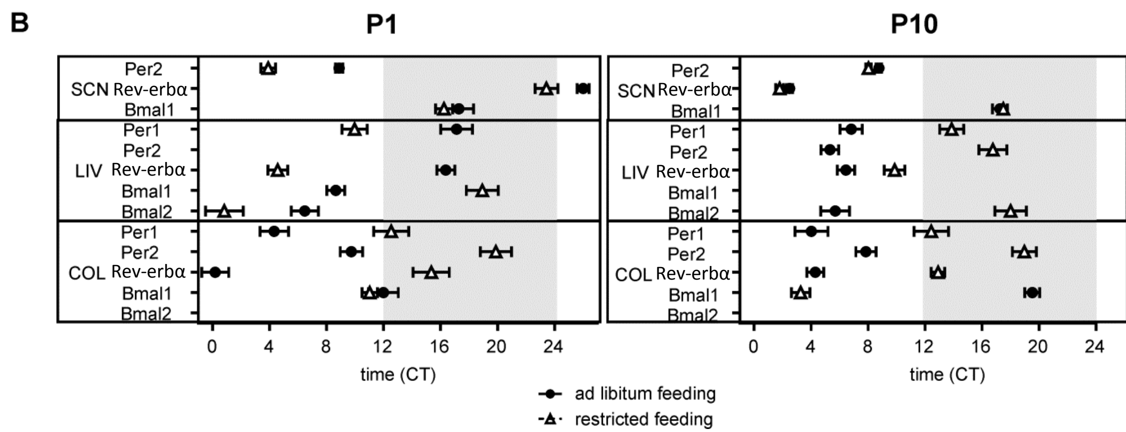


Fig. 15: **Effect of maternal restricted feeding regime on daily clock gene expression profiles in the SCN, liver and colon of SHR pups during ontogenesis.** Mothers were fed *ad libitum* (AL) (black dots, full lines) or were exposed to restricted feeding (RF) (open triangles, dashed lines) since the beginning of gestation.
(A) Daily profiles of clock gene expression in SHR pups at P1 (left column) and P10 (right column) in the SCN, liver and colon which were born to and were nursed by *ad libitum* fed mothers (black dots and black lines) and mothers exposed to RF (opened triangles, dashed lines). Time is expressed as circadian time (CT), the shaded area on each graph corresponded to the interval of darkness in the previous LD cycle. Data are expressed as the mean ± SEM of five animals. Symbols in the upper left corner of the graphs depict the profiles where one-way ANOVA did not confirm a significant effect of time in pups born to *ad libitum* fed mothers (†) and mothers exposed to RF (‡).
(B) Comparison of acrophases from the cosine curves in the SCN, liver and colon between the control pups, which were born to and reared by mothers fed *ad libitum* (black dots) and the pups of mothers exposed to RF (open triangles). Acrophases are depicted in circadian time (CT) as the mean ± SEM. Results of one-way ANOVA comparison are presented in the text.

5.2.6 Maternal restricted feeding during prenatal period

In the SCN of 1-day-old SHR pups (**Fig. 15A**; P1), *Per2*, *Rev-erbα* and *Bmal1* were expressed rhythmically under both feeding conditions, i.e. *ad libitum* and RF (ad lib: *Per2*, *Rev-erbα* $p<0.001$, *Bmal1* $p=0.0085$; RF: all genes $p<0.001$). However, the *Bmal1* rhythm was only shallow because in pups raised by *ad libitum* fed mothers the one-way ANOVA did not confirm a significant effect of time. From the comparison between acrophases of the rhythms (**Fig. 15B**; P1; SCN), it appeared that, whereas *Per2* and *Rev-erbα* expression profiles of pups born to mothers on RF were significantly phase advanced compared with those born to control mothers (*Per2*: $p<0.001$; *Rev-erbα*: $p=0.0055$), the *Bmal1* rhythm was not noticeably phase shifted ($p=0.3875$) likely because it was only very shallow under both feeding conditions. The data demonstrate that the SCN clock of SHR fetuses was considerably sensitive to maternal cues related to changes in the maternal feeding regime and the maternal RF phase-reset the *Per2* and *Rev-erbα* rhythms.

In the liver (**Fig. 15A**; P1), *Per1*, *Rev-erbα*, *Bmal1* and *Bmal2* expression exhibited circadian rhythms in 1-day-old pups born to mothers fed *ad libitum* (*Per1*: $p=0.0237$; *Rev-erbα*: $p<0.001$; *Bmal1*: $p<0.001$; *Bmal2*: $p=0.0048$) as well as to those exposed to RF (*Per1*: $p=0.0002$; *Rev-erbα*: $p=0.0002$; *Bmal1*: $p=0.021$; *Bmal2*: $p=0.0093$). Although the *Per2* expression profile exhibited a low-amplitude rhythm in the previous experiment (**Fig. 12**), only one-way ANOVA but not cosinor analysis revealed a significant effect, and thus the profiles under both conditions of this experiment did not meet the criteria for significant rhythms (*ad libitum*: $p=0.0987$; RF: $p=0.5994$). The *Bmal1* rhythm in the liver of pups born to mothers exposed to RF was significantly suppressed because one-way ANOVA did not reveal a significant effect of time. Comparison of acrophases from profiles in pups born to

mothers fed *ad libitum* and exposed to RF (**Fig. 15B**; P1; LIV) revealed that maternal RF significantly phase shifted all of the rhythmic profiles ($p<0.001$ for *Per1*, *Rev-erbα*, *Bmal1*; $p=0.0009$ for *Bmal2*). In fact, *Per1* and *Bmal2* rhythms were phase-advanced, and the *Rev-erbα* and *Bmal1* rhythms were even completely phase-reversed in the liver of pups born to mothers on RF compared to those born to *ad libitum* fed mothers.

In the colon (**Fig. 15A**; P1), *Per1*, *Per2*, *Rev-erbα* and *Bmal1* expression exhibited circadian rhythms in pups at P1 born to *ad libitum* fed mothers (*Per1*: $p=0.0051$; *Per2*: $p<0.001$; *Rev-erbα*: $p=0.0003$; *Bmal1*: $p=0.0016$) as well as to those exposed to RF (*Per1*: $p=0.0042$; *Per2*: $p=0.0103$; *Rev-erbα*: $p=0.0232$; *Bmal1*: $p<0.001$). The *Bmal2* expression was not rhythmic under either conditions (*ad libitum*: $p=0.2122$; RF: $p=0.2852$). The phases of *Per1*, *Per2* and *Rev-erbα* rhythms (**Fig. 15B**; P1) were significantly phase shifted in the colon of pups born to mothers exposed to RF compared with those born to control mothers ($p<0.001$ for all genes); however, the *Bmal1* rhythm was not affected ($p=0.4246$). Therefore, the exposure of pregnant SHR maintained on LD12:12 to RF during gestation had the significant effect on the fetal circadian system; both the central SCN clock and the peripheral clocks in the liver and colon were phase shifted at P1 due to the change in the maternal feeding regime.

5.2.7 Maternal restricted feeding during early postnatal period

In the SCN of 10-day-old SHR pups (**Fig. 15A**; P10), *Per2*, *Rev-erbα* and *Bmal1* were expressed rhythmically under both *ad libitum* feeding and RF conditions ($p<0.001$ for all genes and both conditions). The *Per2* and *Rev-erbα* expression rhythms were phase advanced in the SCN of pups reared by RF exposed mothers compared to *ad libitum* fed mothers (*Per2*: $p=0.0106$; *Rev-erbα*: $p=0.0444$); however, although significantly different between both groups, these phase shifts were too small to have any physiological relevance. The *Bmal1* rhythm was not shifted ($p=0.7025$) (**Fig. 15B**; P1). Therefore, in contrast to P1, the effect of maternal RF on the SCN at P10 was much smaller, if present.

In the liver of SHR pups at P10 (**Fig. 15A**; P10), *Per1*, *Per2*, *Rev-erbα* and *Bmal2* were expressed rhythmically under both *ad libitum* feeding and RF conditions (*Per1*: $p=0.0007$ and $p=0.0002$; *Per2*: $p<0.001$ and $p=0.0055$; *Rev-erbα*: $p<0.001$ and $p<0.001$; *Bmal2*: $p=0.0098$ and $p=0.0154$, respectively); the *Bmal1* profile did not meet the criteria for a significant circadian rhythm under any conditions (*ad libitum*: $p=0.2573$; RF: $p=0.3816$). The exposure of mothers to RF had a different effect on the hepatic clock of pups at P10

compared with that at P1 (**Fig. 15B**; P10). At P10, *Per1* and *Rev-erbα* rhythms of pups reared by RF exposed mothers were phase shifted compared with *ad libitum* fed mothers ($p<0.001$ and $p=0.0006$, respectively), and *Per2* and *Bmal2* rhythms were completely phase-reversed ($p<0.001$ for both genes).

In the colon of 10-day-old pups (**Fig. 15A**; P10), *Per1*, *Per2*, *Rev-erbα* and *Bmal1* expression exhibited circadian rhythms in pups born to mothers fed *ad libitum* and mothers exposed to RF (*Per1*: $p=0.0148$ and $p=0.0056$; *Per2*: $p<0.001$ and $p=0.0016$; *Rev-erbα*: $p<0.001$ and $p<0.001$; *Bmal1*: $p<0.001$ and $p<0.001$, respectively); however, *Bmal2* expression was not rhythmic under either conditions ($p=0.3267$ and RF: $p=0.2167$, respectively).

To compare sensitivity of the circadian system to maternal RF between SHR and Wistar rat pups, mothers of both strains were subjected to RF and the profiles of *Bmal1* and *Rev-erbα* expression in the SCN and liver of their 10-day-old pups were determined together in the same assay to provide a quantitative comparison of the gene expression profiles between both strains (**Fig. 16A**). The results in SHR pups confirmed the data presented in **Figs. 14** and **15**. In the SCN of SHR and Wistar rats at P10, *Rev-erbα* and *Bmal1* expression exhibited significant circadian rhythms ($p<0.001$ for both genes and rat strains) and, apparently, no differences between acrophases of these rhythms in both strains were detected (*Rev-erbα*: CT 1.8 ± 0.3 and CT 2.6 ± 0.2; *Bmal1*: CT 17.2 ± 0.6 and CT 17.5 ± 0.3; for Wistar rats and SHR, respectively). Therefore, the phases of the SCN clocks were similar in both rat strains. In the liver of pups raised by RF exposed mothers, *Rev-erbα* expression was rhythmic in both rat strains ($p<0.001$), and the peak was phase advanced ($p<0.001$) in Wistar rats (CT 6.9 ± 0.8) compared with that in SHR (CT 11.2 ± 0.8). The *Bmal1* expression was rhythmic in Wistar rats ($p=0.0131$) but constitutive in SHR ($p=0.3046$). In order to compare the response of the hepatic *Rev-erbα* rhythm to the maternal RF between both rat strains, the rhythms in pups reared by mothers fed *ad libitum* and exposed to RF were determined. From the comparison of acrophases of these *Rev-erbα* profiles (**Fig. 16B**), it appeared that maternal RF did not affect the phase of *Rev-erbα* rhythm in Wistar rats but it significantly shifted the phase in SHR ($p<0.001$). These data clearly demonstrated significant strain-specific differences in the response to maternal RF in the hepatic but not the SCN clock of 10-day-old pups.

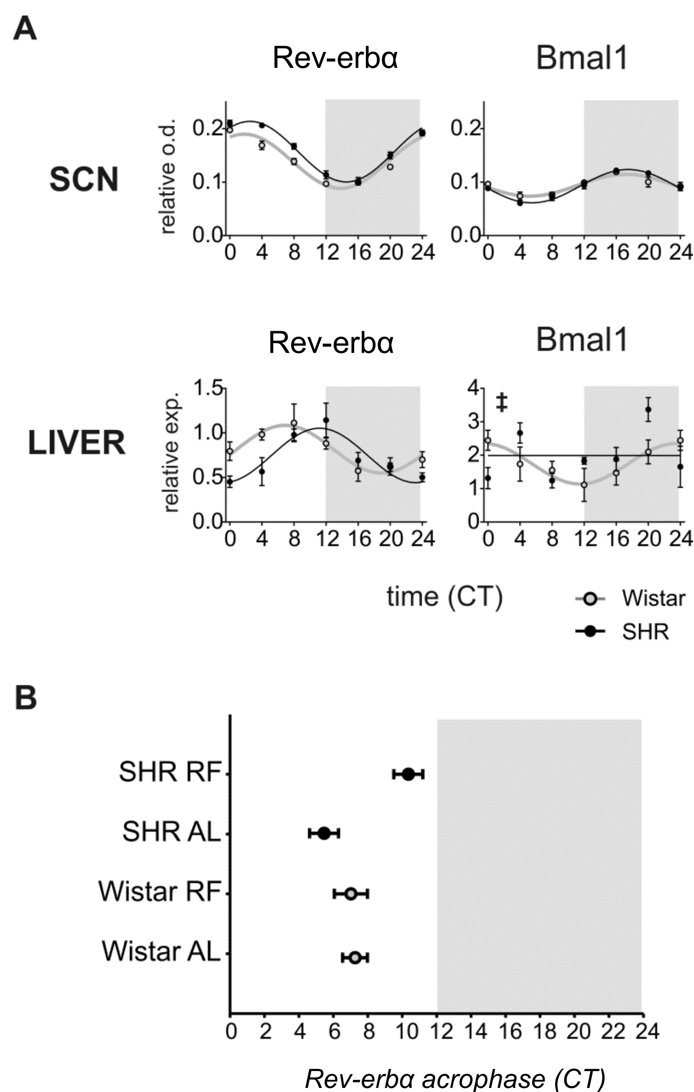
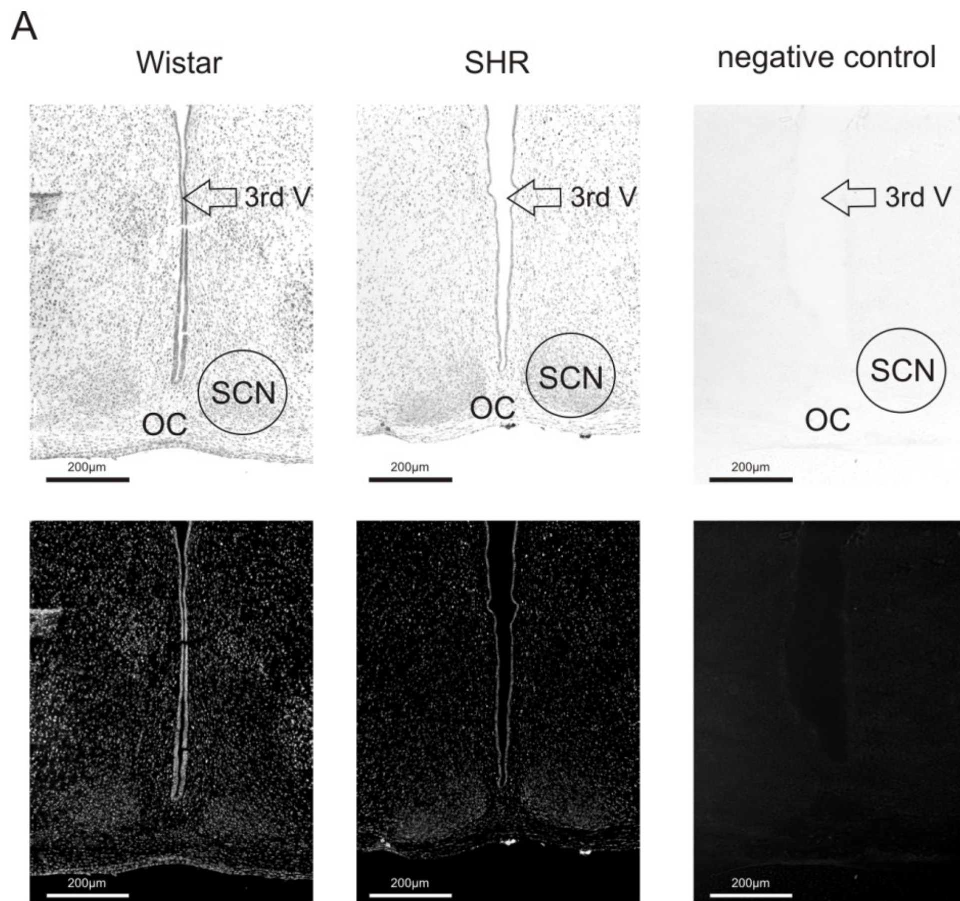


Fig. 16. (A) Comparison of daily profiles of *Rev-erbα* and *Bmal1* expression in the SCN and liver of 10-day-old SHR and Wistar rat pups born to mothers maintained in LD12:12 and exposed to restricted feeding regime (RF) since beginning of gestation. The y-axes represent the relative optical density (relative o.d.) in the SCN and relative expression (relative exp.) in the liver. For further details, see the legend in **Fig. 15**.
(B) Comparison of acrophases of the *Rev-erbα* expression profiles in 10-day-old SHR (black dots) and Wistar rats (gray dots) born to mothers fed *ad libitum* (AL) or exposed to RF. Acrophases are expressed as the mean ± SEM in circadian time (CT), the shaded area depicts the interval of the subjective night.

5.3 PROJECT 3 – COMPARISON OF SENSITIVITY TO MATERNAL STRESS IN WISTAR AND SHR PUPS

5.3.1 The SCN of 4-day-old rat pups contains GRs

The presence of the GR in the SCN has not been properly assessed in Wistar rat pups and has even not been studied in SHR pups. There is only one study published in 1988 reporting the presence of GR immunoreactivity (-ir) in the SCN of neonatal Wistar rats. Therefore, to provide a background for the proposed GR-mediated modulation of the pups' SCN clock, we determined the presence of GRs in the SCN of 4-day-old pups of both rat strains at the mRNA and protein levels (**Fig. 17**). Specific GR-ir was detected throughout the hypothalamus, including in the SCN, in both rat strains (**Fig. 17A**). In order to quantitatively assess the GR levels selectively within the SCN, the pups' SCN were isolated using a laser dissection technique for detection of *Nr3c1* mRNA (= GR mRNA) levels. The SCN samples were assayed via RT-qPCR together with samples of the hippocampus (a brain structure known to contain high GR levels) that were laser-dissected from adult animals, and the SCN mRNA levels were expressed as a percent of the hippocampal levels (**Fig. 17B**). The results suggested that the level of *Nr3c1* mRNA in the SCN relative to the hippocampus was lower in Wistar rats (64.8 ± 12.7 %; n=5) than in SHR (96.0 ± 27.4 %; n=4), but the difference did not pass the significance threshold (p=0.0556). The results confirmed the presence of relevant levels of GRs in the SCN of 4-day-old pups in both rat strains, and the expression levels in SHR tended to be higher than in Wistar rats.



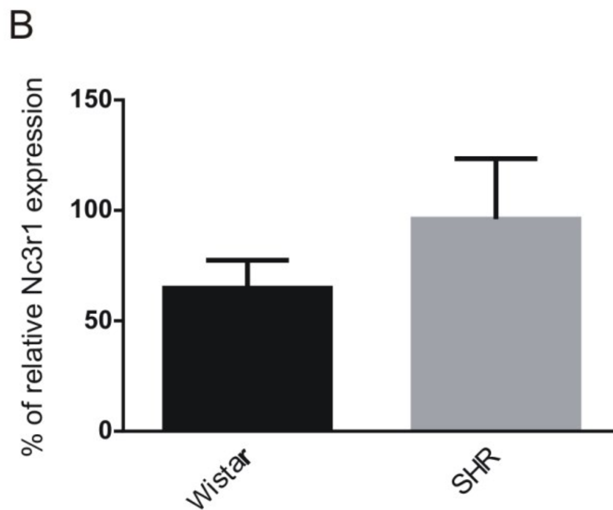


Fig. 17: Glucocorticoid receptors detected in the SCN of 4-day-old pups at the protein and mRNA levels. (**A)** Representative hypothalamic sections containing the SCN of Wistar rat and SHR pups processed for immunohistochemistry to detect the GR-ir cells. For each rat strain, the light and dark field microscopy image is depicted in the upper and lower sections, respectively. SCN - suprachiasmatic nuclei, 3rd V - 3rd ventricle, OC - optic chiasma.
(B) The relative levels of *Nc3r1* mRNA in the laser-dissected samples of the SCN from 4-day-old Wistar and SHR pups expressed as the percent of levels detected in samples that contained the adult hippocampus (100 %).

5.3.2 Effect of RF on GC plasma levels in mothers

To confirm the effect of RF on GC plasma levels in lactating mothers, we compared GC levels in the plasma of mothers that were either fed *ad libitum* and left untreated (controls) or exposed to RF for 4 days after delivery (**Fig. 18A**); the GC levels were detected 15 min before the food was provided on RF. The levels in control Wistar rats and SHR mothers (117.5 ± 19.7 $ng.ml^{-1}$ and 47.8 ± 26.1 $ng.ml^{-1}$, respectively) increased due to RF (184.7 ± 34.3 $ng.ml^{-1}$ and 192.6 ± 11.8 $ng.ml^{-1}$, respectively), and the rise in GC levels was significant in Wistar rats as well as in SHR (p=0.0145 and p<0.0001, respectively). The relative increase in SHR (approx. 4 times) was significantly higher than in Wistar rats (approx. 1.6 times) (two-way ANOVA, effect of strain: p=0.0262), which was due to a difference in the basal (p=0.0033) but not the RF-affected (p=0.6554) GC levels. The results demonstrated that the exposure of lactating mothers of both rat strains to RF induced an increase in plasma CS levels in anticipation of food availability during the daytime.

5.3.3 Plasma GC levels in pups are affected by maternal stress and s.c. injections

The effect of experimental conditions on the plasma GC levels in pups of all groups and both rat strains was detected (**Fig. 18B**). Injections/manipulation of pups reared by untreated mothers did not increase plasma GC levels in Wistar rat (65.5 ± 22.7 vs. 71.3 ± 22.8 $ng.ml^{-1}$; p=0.9959) and in SHR pups (108.6 ± 28.4 vs. 117.3 ± 23.2 $ng.ml^{-1}$; p=0.9072). The exposure of mothers to RF per se also did not affect GC levels in Wistar rat pups (65.5 ± 22.7 vs. 77.5 ± 8.1 $ng.ml^{-1}$; p=0.9959) and SHR pups (108.6 ± 28.4 vs. 130.3 ± 36.5 $ng.ml^{-1}$; p= 0.9485). However, injections/manipulation of pups reared by RF-exposed mothers significantly elevated their plasma GC levels that rose up to 340.9 ± 98.1 $ng.ml^{-1}$ in Wistar rat pups and 314.8 ± 82.4 $ng.ml^{-1}$ in SHR pups; the levels were elevated above the levels in control pups, injected pups as well as pups reared by RF-exposed mothers (p<0.0001 for all groups).

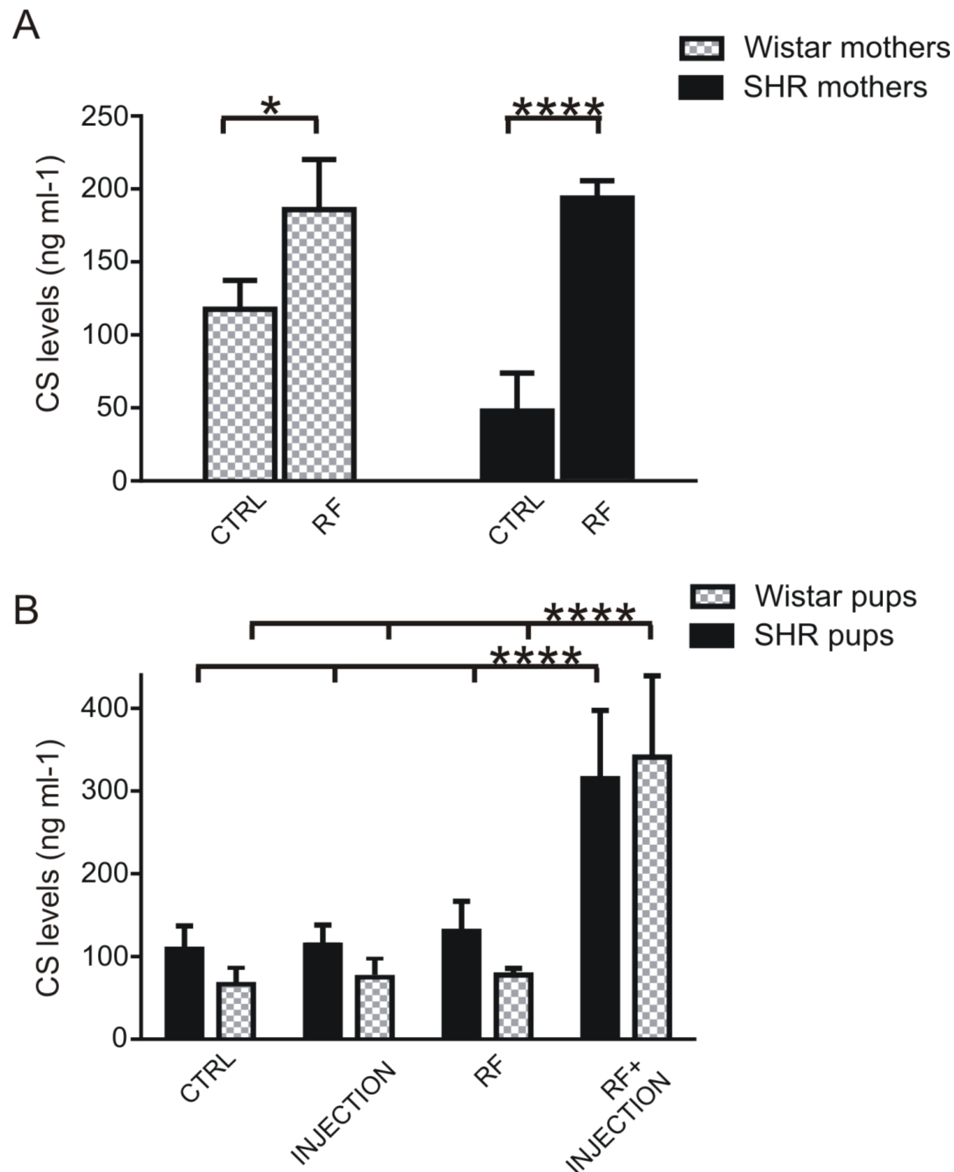


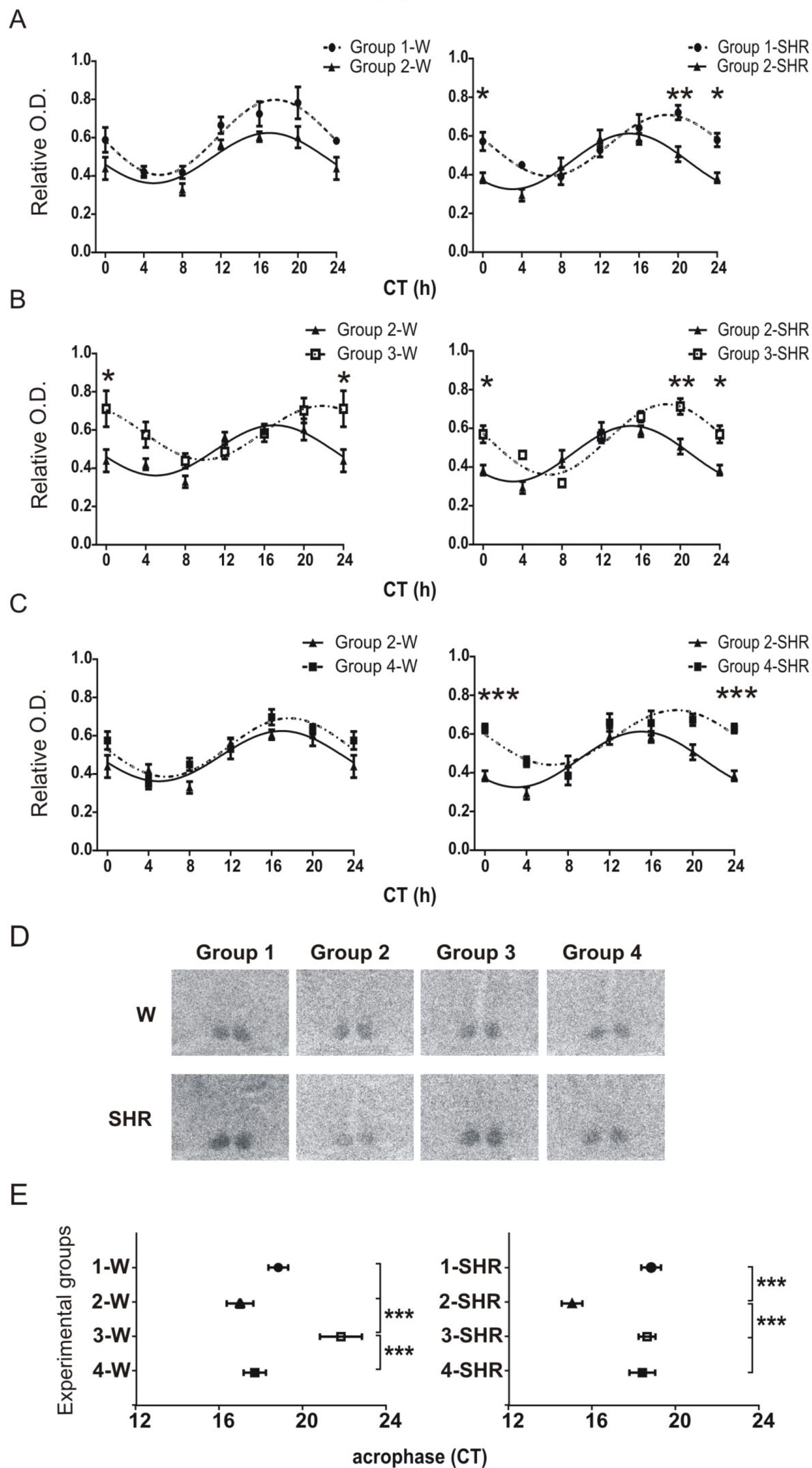
Fig. 18: Plasma corticosterone (CS) levels.
(A) The Wistar rat (shaded columns) and SHR (black columns) mothers were fed ad libitum (CTRL) or exposed to a restricted feeding regimen (RF), and the blood was collected 15 min before the food was provided on RF 4 h after the lights-on of the light/dark cycle (ZT4).
(B) The 4-day-old Wistar rat pups (shaded columns) and SHR pups (black columns) were i) reared by ad libitum fed-mothers and left intact (CTRL), ii) reared by ad libitum fed-mothers and injected s.c. with vehicle (INJECTION), iii) reared by RF-exposed mothers and left intact (RF), iv) reared by RF-exposed mothers and injected with vehicle (RF+INJECTION). The blood was collected 15 min after injections administered before the beginning of the RF, i.e., at ZT4, or at corresponding time in controls. * $p < 0.05$; **** $p < 0.0001$.

5.3.4 Exposure of lactating mothers to RF affects SCN clock in SHR but not Wistar rat pups

To ascertain the effect of GC on the SCN clock of 4-day-old rats, we selected experimental groups based on the results shown in **Fig. 18**; the effect was tested in pups whose GC levels were elevated due to the experimental procedures, i.e., in pups reared by

RF-exposed mothers. Additionally, mifepristone was injected to pups to block GRs. The effect of the experimental procedures was studied on *Per1*, *Per2*, and *Bmal1* expression profiles in the pups' SCN. For the experimental scheme and description of the groups, see **Fig. 4.**

To detect whether the daily exposure of lactating mothers to RF affected the clock in the SCN pups, we compared gene expression profiles in the SCN of pups whose mothers were either fed *ad libitum* and left untreated (group 1-W and group 1-SHR) or exposed to RF (group 2-W and group 2-SHR). For *Bmal1* expression profiles, the two-way ANOVA revealed that the exposure of Wistar rat mothers to RF had no effect on the pups' SCN clock (**Fig. 19A, D**) because no significant differences were detected between the pups reared by *ad libitum* fed mothers (group 1-W) and RF- exposed mothers (group 2-W) at any time point; acrophases (i.e., the peaks of the profiles) were at CT17.7 ± 0.5 and CT17.0 ± 0.6, respectively (p=0.1111). In contrast, the exposure of SHR mothers to RF significantly affected the *Bmal1* expression profile in the SCN of their pups; the expression levels differed between the pups reared by *ad libitum* fed mothers (group 1-SHR) and RF-exposed mothers (group 2-SHR) at CT0 (p=0.0209), CT20 (p=0.0031) and CT24 (p=0.0125), and the group 2-SHR profile was significantly phase advanced compared to the group 1-SHR profile (acrophases at CT15.1 ± 0.5 and CT18.8 ± 0.5, respectively; p<0.0001). For *Per1* and *Per2* gene expression profiles, no significant differences between untreated pups reared by *ad libitum* fed- or RF-exposed mothers (i.e., between groups 1 and groups 2) of both rat strains were detected (**Fig. 20A, D**; **Tables 6** and **7**).



Bmal1
A
Group 1-W
Group 2-W
Group 1-SHR
Group 2-SHR
Relative O.D.
CT (h)
B
Group 2-W
Group 3-W
Group 2-SHR
Group 3-SHR
C
Group 2-W
Group 4-W
Group 2-SHR
Group 4-SHR
D
Group 1
Group 2
Group 3
Group 4
W
SHR
E
Experimental groups
1-W
2-W
3-W
4-W
1-SHR
2-SHR
3-SHR
4-SHR
acrophase (CT)

Fig. 19: Cosine fits of daily profiles of *Bmal1* mRNA levels in the SCN of 4-day-old Wistar rat (left column) and SHR (right column) pups. The profiles of *Bmal1* expression were compared between the groups of pups (**A**) reared by mothers fed ad libitum (group 1-W, group 1-SHR) and those reared by mothers exposed to RF (group 2-W, group2-SHR); pups of both groups were left untreated; (**B**) reared by mothers exposed to RF and either left untreated (group 2-W, group 2-SHR) or injected with vehicle (group 3-W, group 3-SHR); (**C**) reared by mothers exposed to RF and left untreated (group 2-W, group 2-SHR) or injected with mifepristone (group 4-W, group 4-SHR). The CT0 is double plotted as CT24 in groups 2, 3 and 4.
(D) Representative film autoradiographs of the hypothalamic sections containing the SCN of groups 1 - 4 of Wistar rats (W) and SHR processed for in situ hybridization to detect *Bmal1* mRNA levels. The relative optical density (O.D.) of the SCN area (A-C) was measured as described in MATERIALS AND METHODS; the representative sections are from the CT20.
(E) The comparison between acrophases (time of the peak) of the *Bmal1* expression profiles of all experimental groups of Wistar rat (left graph) and SHR (right graph) pups. The data are expressed as means ± SEM. Acrophases were calculated by cosine analysis. The statistical differences between the groups are described in text. For a description of the experimental groups, see Fig. 4. * $p<0.05$; ** $p<0.01$; *** $p<0.001$

		Bmal1		***Per1***		***Per2***	
		F(DFn,DFd)	***p***	**F (DFn, DFd)**	***p***	**F(DFn,DFd)**	***p***
W	**inter.**	F(18,99) = 2.10	0.0109	F(18,99) = 1.14	0.3273	F(18,108) = 1.30	0.2018
	time	F(6,99) = 14.13	< 0.0001	F(6,99) = 92.16	< 0.0001	F(6,108) = 65.90	< 0.0001
	group	F(3,99) = 7.297	0.0002	F(3,99) = 4.62	0.0046	F(3,108) = 11.96	< 0.0001
SHR	**inter.**	F(18,105) = 2.83	0.0005	F(18,109) = 1.45	0.1232	F(18,103) = 2.28	0.0050
	time	F(6,105) = 28.40	< 0.0001	F(6,109) = 38.13	< 0.0001	F(6,103) = 44.35	< 0.0001
	group	F(3,105) = 15.52	< 0.0001	F(3,109) = 2.25	0.0862	F(3,103) = 13.45	< 0.0001

Table 6: Results of two-way ANOVA comparison among the clock gene expression profiles of experimental groups for Wistar rats (group 1-W, group 2-W, group 3-W and group 4-W) and SHR (group 1-SHR, group 2-SHR, group 3-SHR and group 4-SHR); inter.: interaction.

Group	*Per1*		*Per2*		*Bmal1*	
	p	Acro ± SD	*p*	Acro ± SD	*p*	Acro ± SD
W - 1	< 0.0001	6.1 ± 0.4	< 0.0001	9.1 ± 0.4	< 0.0001	17.7 ± 0.5
W - 2	< 0.0001	5.8 ± 0.4	< 0.0001	8.9 ± 0.4	< 0.0001	17.0 ± 0.6
W - 3	< 0.0001	6.4 ± 0.3	< 0.0001	7.6 ± 0.4	0.0018	21.8 ± 1.0
W - 4	< 0.0001	5.6 ± 0.4	< 0.0001	8.9 ± 0.3	< 0.0001	17.7 ± 0.5
SHR - 1	< 0.0001	3.7 ± 0.6	< 0.0001	7.1 ± 0.5	< 0.0001	18.8 ± 0.5
SHR - 2	< 0.0001	3.2 ± 0.6	< 0.0001	6.8 ± 0.5	< 0.0001	15.1 ± 0.5
SHR - 3	< 0.0001	3.2 ± 0.4	< 0.0001	6.6 ± 0.3	< 0.0001	18.7 ± 0.4
SHR - 4	< 0.0001	4.1 ± 0.6	< 0.0001	8.7 ± 0.5	< 0.0001	18.4 ± 0.6

Table 7: Results of cosinor analysis of clock gene expression profiles in the SCN of Wistar and SHR pups at postnatal age P4. W: Wistar rat,; *p*: significance; Acro: Acrophase (peak) ± standard deviation (SD); For description of the groups, see MATERIALS AND METHODS.

5.3.5 Manipulation of pups reared by RF-exposed mothers entrains the SCN clock via GC-dependent pathways

In Wistar rat pups, the *Bmal1* expression profile, which was not affected by maternal RF *per se* (group 2-W) (see **Fig. 19A, D**), was significantly shifted when the pups were injected with vehicle daily from birth until P3 (group 3-W) (**Fig. 19B, D**). The two-way ANOVA revealed significant differences between the non-injected pups (group 2-W) and injected pups (group 3-W) at CT0 and CT24 (p=0.0199 for both), and acrophases of the group 3-W (CT21.8 ± 1.0) and the group 2-W (CT17.0 ± 0.6) profiles were phase shifted (p<0.0001). The injection of mifepristone blocked the effect of the vehicle injection (**Fig. 19C, D**) because acrophases of the mifepristone-treated group 4-W profile (CT17.7 ± 0.5) and the vehicle-treated group 3-W profile were significantly different (p<0.0001), whereas acrophase of the group 4-W was not different compared to that of the non-injected group 2-W (p=0.0944). Therefore, the data demonstrate that postnatal stress imposed via repeated s.c. injections and manipulation on Wistar rat pups reared by RF-exposed mothers produced a phase shift in the *Bmal1* expression profile and the effect was completely blocked by mifepristone (**Fig. 19D**).

In SHR pups reared by RF-exposed mothers, vehicle treatment affected the SCN clock in the same way as in the Wistar rats (**Fig. 19B, D**). The *Bmal1* expression profiles of non-

injected pups (group 2-SHR) and pups repeatedly treated with vehicle (group 3-SHR) were significantly different at CT0 (p=0.0244), CT20 (p=0.0057) and CT24 (p=0.0244). Additionally, acrophase of the group 3-SHR (CT = 18.7 ± 0.4) was shifted compared to that of the group 2-SHR (CT = 15.1 ± 0.5) (p<0.0001). The profile of the non-injected pups (group 2-SHR) was also significantly different compared to the mifepristone-treated pups (group 4-SHR) at CT0 and CT24 (p=0.0003 for both) and acrophases were significantly different (p<0.0001). However, unlike in Wistar rats (**Fig. 19C, D**), there were no differences between the vehicle-treated (group 3-SHR) and mifepristone-treated (group 4-SHR) groups at any time point, nor was there a significant difference between acrophases of these profiles (p=0.5087). Therefore, both mifepristone and vehicle treatments abolished the maternal RF-induced phase advance shift by delaying the profile to the control position (**Fig. 18C, D**); similarly to the vehicle-treated pups (group 3-SHR), the profile of the mifepristone-treated pups (group 4-SHR) differed from the profile of untreated pups (group 2-SHR) significantly at CT0 and CT24 (p=0.0003 for both) and their acrophases also differed significantly (CT18.4 ± 0.6 and 15.1 ± 0.5, respectively; p< 0.0001).

These results demonstrate that the SCN clock of SHR pups, which has already responded by phase advance to maternal RF, may further respond by a phase shift to stress imposed by injecting the pups, but mifepristone is not able to block this double-shift. No significant effects of postnatal stress due to maternal RF in combinations with handling and vehicle or mifepristone treatments were observed on *Per1* and *Per2* gene expression profiles because there were no differences between groups 2, 3 and 4 in any of these rat strains (**Fig. 20B, C, E, F; Tables 6 and 7**).

5.3.6 Spatial distribution of clock genes mRNA in the SCN during peak of their expression is gene-dependent

Because the *Bmal1* and *Per1,2* expressions responded to stress differently, we assessed their spatial localization within the SCN of 4-day-old Wistar rat pups. The area within the SCN where the maximal levels of *Bmal1*, *Per1* and *Per2* mRNA were detected, is shown in the representative film autoradiographs in **Fig. 21**. The images are from the brains sampled at the time points of the maximal binding during the 24 h cycle for each gene, i.e., CT20 for *Bmal1*, CT4 for *Per1* and CT8 for *Per2* (see results in **Figs. 19** and **20**). Matching the histological staining (left side) with the mRNA distribution (right side) of the same SCN section revealed spatial differences between the genes; whereas *Bmal1* mRNA appears to be

distributed uniformly across the entire SCN, *Per1* and *Per2* probes binding is more dense in the dorsomedial part compared to the ventrolateral part of the SCN.

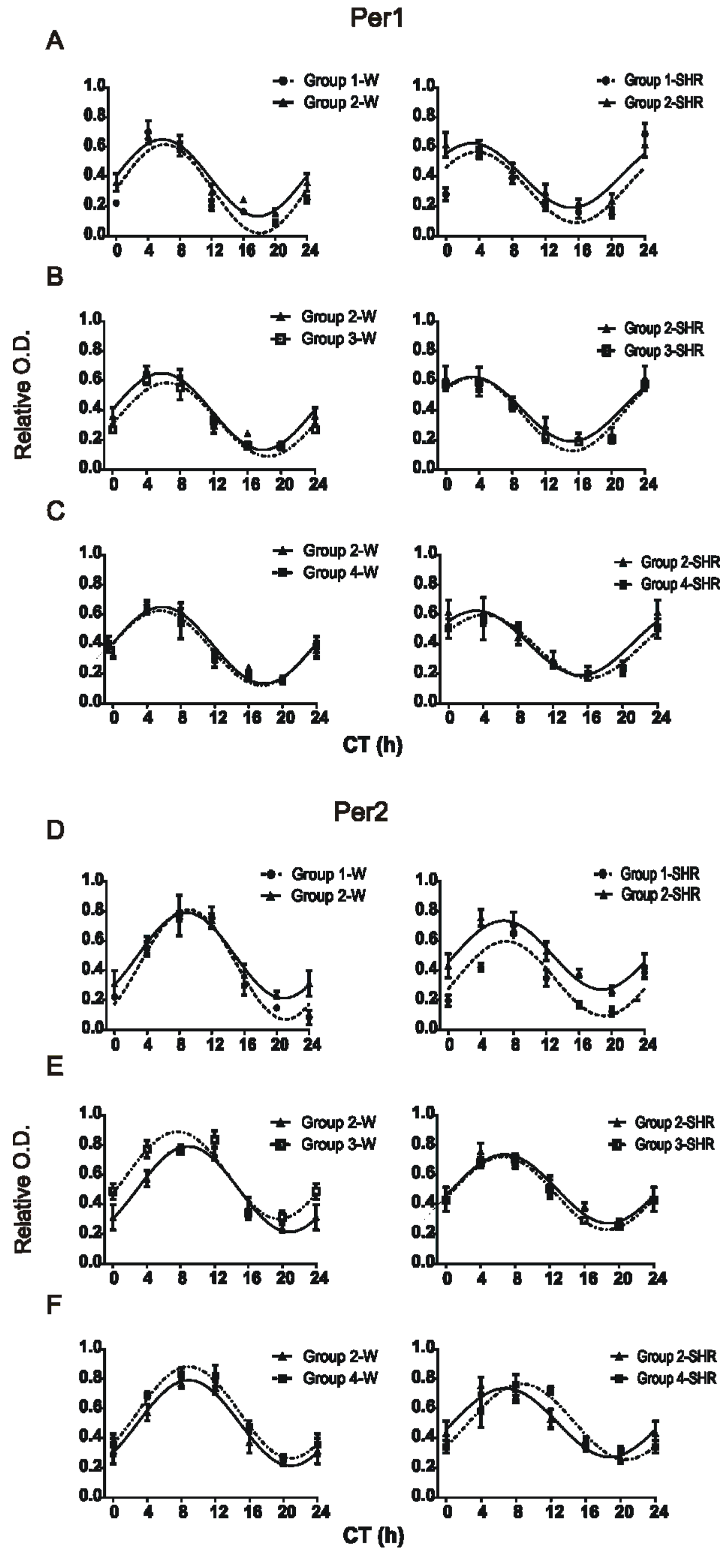


Fig. 20: Cosine fits of daily expression profiles of *Per1* (upper part) and ***Per2*** (lower part) mRNA levels detected by in situ hybridization in the SCN of 4-day-old Wistar rat (left column) and SHR (right column) pups. For a description of experimental groups and other details, see the legend to **Fig. 19**.

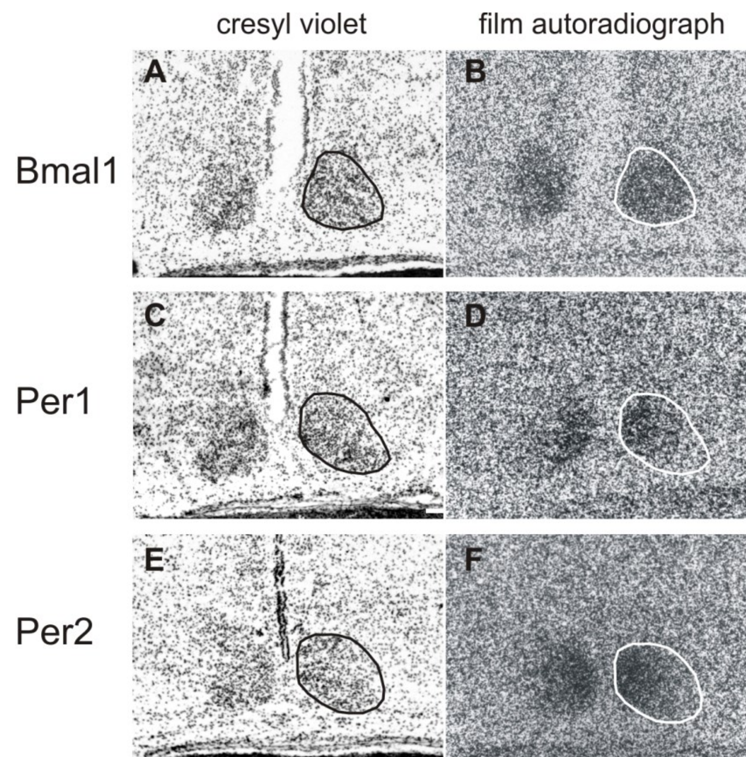


Fig. 21: The gene-specific spatial mRNA distribution within the SCN of 4-day-old Wistar rat pups. The representative sections stained with **cresyl violet (A, C, E)** and their corresponding autoradiographs obtained from **in situ hybridization assay (B, D, F)** to assess *Bmal1* (A, B), *Per1* (C, D) and *Per2* (E, F) mRNA levels are depicted. The sections show localization of the gene expression at the peak time according to results in **Figs**. **19** and **20**, i.e., at CT4 for *Per1*, at CT8 for *Per2* and at CT20 for *Bmal1*. For better clarity, boundaries were precisely delineated for each SCN based on histological staining and the SCN area was copied onto autoradiographic image. Magnification 100x.

5.4 PROJECT 4 – IMPACT OF FOSTER MOTHER ON MOLECULAR CIRCADIAN CLOCK IN RAT PUPS AND ITS LONG-LASTING EFFECT IN ADULTHOOD

The effect of altered maternal care on the circadian clocks in the rat pups was tested in a series of experiments in which the pups were reared by foster mother of the different rat strain (cross-strain fostering) or of the same rat strain (intra-strain fostering) for the entire lactation period (since P1 till P30) or, they were first exposed to the cross-strain fostering and then, at P10, they were returned to their genetic mothers for the rest of the lactation period (**Fig. 5**).

5.4.1 Cross-strain fostering affects circadian clock in the pups' SCN in strain-specific manner

To assess the effect of altered maternal care on the pups' SCN clock, we compared daily profiles of *Per2*, *Rev-erbα* and *Bmal1* expression in the SCN (**Fig. 22**) of 10-day-old and 30-day-old pups which were either reared by their own mothers or were exposed to the cross-strain fostering. The statistical comparison (by two-way ANOVA) between the experimental groups are summarized in **Table 8**, the significant differences in the relative expression levels at the individual time points (results of post-hoc analyses) are depicted in the **Fig. 22A, B**, and the significant differences between acrophases of the cosine fits of the expression profiles are in **Fig. 22C**.

A comparison of acrophases of the clock gene expression profiles (**Fig. 22C**) between the pups of both rat strains reared by their genetic mothers showed that at P30, expressions of all genes were significantly phase advanced in SHR compared to those in Wistar rat pups, but the advances were not significant at P10. These results demonstrate that the positive phase angle of entrainment of the locomotor activity rhythm observed in the adult SHR (Sládek *et al.*, 2012) develops in the pups already by P30. The exposure of 10-day-old Wistar pups (**Fig. 22A**) to the cross-strain fostering significantly modified the clock gene expression profiles of all three tested clock genes, i.e., *Per2*, *Rev-erbα* and *Bmal1*, compared to the control groups (**Table 8** and **Fig. 22A**); acrophase (**Fig. 22C**) in the cross-strain fostered Wistar pups was advanced compared to the pups reared by their own mothers significantly for *Per2* (9.2 ± 0.2 h vs. 8.2 ± 0.3 h) but not significantly for *Rev-erbα* (2.9 ± 0.2 vs. 2.1 ± 0.2 h) and *Bmal1* (18.1 ± 0.4 vs. 16.9 ± 0.4 h) expression profiles. At P30, the cross-strain fostering did not affect the SCN of Wistar rat pups (**Fig. 22B, C**). In contrast to Wistar rat

pups, the cross-strain fostering had no effect on the phase of any of the clock gene expression profiles of SHR pups at P10 (**Fig. 22A, C)** or at P30 (**Fig. 22B, C**). However, at P10, there was a significant effect of the cross-strain fostering on amplitude of the *Bmal1* expression rhythm (**Fig. 22A**), which significantly increased from 0.2335 ± 0.0292 in controls to 0.3618 ± 0.0329 in cross-strain fostered pups (*p*=0.0194); this difference disappeared at P30.

SCN controls vs. cross-strain		***Per2***		***Rev-erbα***		***Bmal1***	
		F(DFn,DFd)	***p***	**F(DFn,DFd)**	***p***	**F(DFn,DFd)**	***p***
Wistar P10	**interaction**	F (6, 52) = 6.002	< 0.0001	F (6, 51) = 5.309	0.0003	F (6, 51) = 2.217	0.0562
	time	F (6, 52) = 110.3	< 0.0001	F (6, 51) = 136.6	< 0.0001	F (6, 51) = 29.92	<0.0001
	group	F (1, 52) = 3.192	0.0798	F (1, 51) = 13.38	0.0006	F (1, 51) = 5.724	0.0205
SHR P10	**interaction**	F (6, 54) = 1.468	0.2068	F (6, 54) = 1.105	0.3718	F (6, 54) = 2.231	0.0538
	time	F (6, 54) = 190.9	< 0.0001	F (6, 54) = 62.02	< 0.0001	F (6, 54) = 33.53	< 0.0001
	group	F (1, 54) = 0.1834	0.6701	F (1, 54) = 9.511	0.0032	F (1, 54) = 9.711	0.0029
Wistar P30	**interaction**	F (6, 45) = 1.821	0.1164	F (6, 53) = 2.128	0.0652	F (6, 51) = 0.9615	0.4605
	time	F (6, 45) = 100.4	< 0.0001	F (6, 53) = 55.36	< 0.0001	F (6, 51) = 32.53	0.0001
	group	F (1, 45) = 3.114	0.0844	F (1, 53) = 12.31	0.0009	F (1, 51) = 5.194	0.0269
SHR P30	**interaction**	F (6, 49) = 2.785	0.0207	F (6, 48) = 1.955	0.0909	F (6, 48) = 2.036	0.0788
	time	F (6, 49) = 116.1	< 0.0001	F (6, 48) = 102.7	< 0.0001	F (6, 48) = 30.21	< 0.0001
	group	F (1, 49) = 2.027	0.1609	F (1, 48) = 3.781	0.0577	F (1, 48) = 0.4667	0.4978

Table 8: Results of two-way ANOVA comparison between the daily profiles of *Per2*, *Rev-erbα* and *Bmal1* expression in the pups SCN. Daily profiles of clock gene expression in the SCN of 10-day-old (P10) and 30-day-old (P30) Wistar rat and SHR pups reared by their own mothers (controls) or exposed to cross-strain fostering (cross-strain).

The results demonstrate that in both rat strains, the effect of cross-strain fostering on the pups' SCN clock was significant already at P10. In Wistar rat pups, the SHR foster mother phase-advanced the clock approximately by 1 h, whereas in SHR pups, the Wistar rat foster mother increased amplitude of *Bmal1* rhythm.

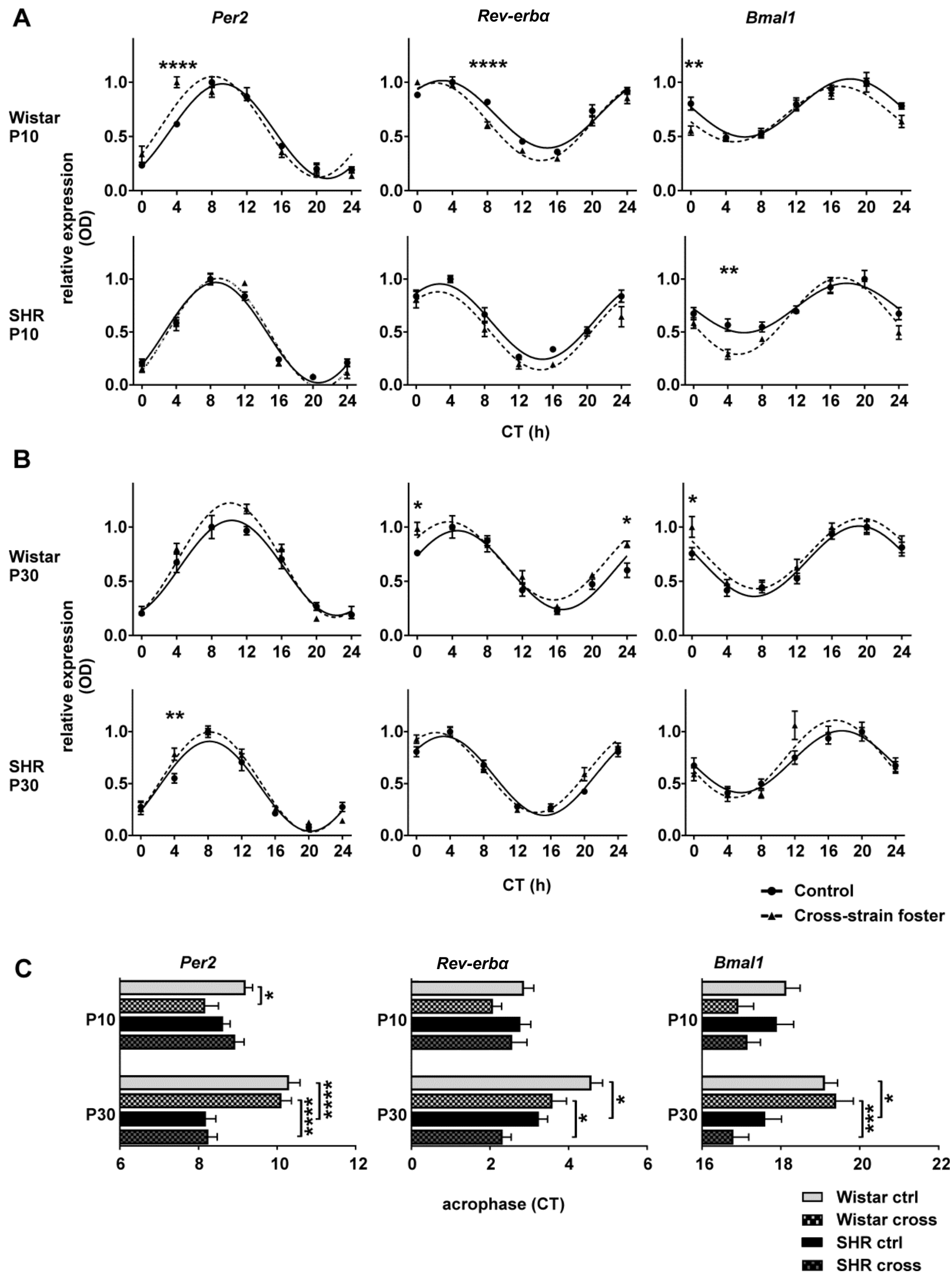


Fig. 22: **The effect of cross-strain fostering on the clock gene expression in the SCN** of pups. Daily *Per2*, *Rev-erbα* and *Bmal1* expression profiles were analysed in **10-day-old (A) and 30-day-old (B)** Wistar rat or SHR pups reared by their genetic mothers as control (full circles, full line) or exposed to cross-strain fostering (full triangles, dashed lines). The pups were sacrificed in 4-h intervals over 24 h. Time is expressed as circadian time (CT); CT0 represents the time of lights on in the previous LD12:12 cycle. The data are expressed as the mean ± SEM, n = 3-5/time point. The results of two-way ANOVA (Table 8) post hoc analyses (Šidák's multiple comparisons) depict differences in the expression levels between the control and cross-strain fostered groups at individual CT. **(C) Comparison of acrophases** (mean ± SEM) of the gene expression profiles shown in A and B by two-way ANOVA with Tukey's multiple comparisons test; the relevant significant differences are depicted. * $p< 0.05$; ** $p<0.01$; *** $p< 0.001$; **** $p< 0.0001$.

5.4.2 Cross-strain fostering affects robustly peripheral circadian clocks of pups in age- and strain-specific manners

To assess the impact of cross-strain fostering on the pups' peripheral clocks, we studied the daily profiles of *Per1*, *Per2*, *Rev-erbα* and *Bmal1* expression in the liver (**Fig. 23**) and colon (**Fig. 24**) of the same pups that were used to detect the effects of cross-strain fostering on the SCN clock. For the results of the statistical comparisons between the groups by two-way ANOVA, see **Tables 9** and **10**; the results of post hoc comparisons between the groups are depicted in the **Figs. 23** and **24**.

In the liver of 10-day-old Wistar pups (**Fig. 23A**), cross-strain fostering significantly down-regulated overall *Per1* and *Per2* expression levels and decreased amplitude of *Rev-erbα* expression rhythm, but the rhythm of *Bmal1* expression was not affected. In a marked contrast, cross-strain fostering had no significant effect on the expression profile of any of the studied genes in the liver of 10-day-old SHR pups (**Fig. 23A**). In 30-day-old pups (**Fig. 23B**), cross-strain fostering affected the clock gene expression profiles in a similar manner in both strains, i.e., amplitudes of the expression rhythms of all studied genes (**Fig. 25A**; cross-strain) and/or their expression levels (**Fig. 23A**) were increased.

Unlike the hepatic clock, the clock in the colon of 10-day-old Wistar pups was not sensitive to cross-strain fostering (**Fig. 24A**), as none of the studied gene expression profiles were significantly affected. In 10-day-old SHR pups, cross-strain fostering selectively dampened amplitude of the *Rev-erbα* expression profile while leaving the expression of the other clock genes unaffected (**Fig. 24A**).

In contrast, at P30, the effect of cross-strain fostering was more pronounced and was similar to the effect seen in the liver, i.e., cross-strain fostering increased amplitudes of expression profiles of all studied clock genes in SHR pups (**Fig. 24B** and **Fig. 25B**; cross-strain), and had a less dramatic effect in Wistar pups, where it significantly increased only amplitude of *Bmal1* expression rhythm (**Fig. 25B**; cross-strain) and also modified the profile of *Per2* (**Fig. 25B**). Because our previous results demonstrated that in adult SHR amplitudes of clock gene expression in the colon were significantly lower compared to Wistar rats (Sládek *et al.*, 2012), we performed additional comparison of amplitudes in the colon of 30-day-old control and cross-strain fostered pups of both strains. The results of this comparison (**Fig. 26**) revealed that already at P30, the rhythm of amplitudes of *Per1* and *Rev-erbα* in control SHR were significantly lower than in Wistar rats (as we found in adults in (Sládek *et al.*, 2012)) and that the cross-strain fostering significantly increased amplitudes for all

genes (with the exception of *Per1* for which amplitude was increased non-significantly). For Wistar rat pups, the comparison confirmed the significant effect of cross-strain fostering only on amplitude of the *Bmal1* expression rhythm (as shown in **Fig. 25B**; cross-strain).

Altogether, these results demonstrate that impact of the cross-strain fostering was dependent on age, peripheral tissue and clock gene. At P10, the procedure significantly suppressed amplitudes of the hepatic clock of Wistar pups but not of SHR pups, and it had almost no effect on the clock in the colon of both strains. Notably, at P30 this procedure had significant impact on amplitudes of the clock in both studied peripheral tissues of pups of both strains. Overall, amplitudes of clock gene expression profiles of cross-strain fostered 30-day-old pups were elevated compared to controls of the same strain, and, in the colon the Wistar rat foster mother improved the lower amplitudes of SHR pups to the same (or higher) levels as were those in Wistar rat pups.

LIVER			*Per1*		*Per2*		*Rev-erbα*		*Bmal1*	
			F(DFn.DFd)	***p***	**F(DFn.DFd)**	***p***	**F(DFn.DFd)**	***p***	**F(DFn.DFd)**	***p***
P10	Wistar controls vs. cross-strain	**interaction**	F (6, 55) = 7.083	< 0.0001	F (6, 54) = 4.636	0.0007	F (6, 54) = 4.012	0.0022	F (6, 55) = 1.115	0.3655
		time	F (6, 55) = 7.846	< 0.0001	F (6, 54) = 3.350	0.0070	F (6, 54) = 9.820	< 0.0001	F (6, 55) = 10.43	< 0.0001
		group	F (1, 55) = 115.9	< 0.0001	F (1, 54) = 274.8	< 0.0001	F (1, 54) = 7.621	0.0079	F (1, 55) = 2.501	0.1195
	SHR controls vs. cross-strain	**interaction**	F (6, 49) = 1.702	0.1405	F (6, 49) = 2.246	0.0541	F (6, 49) = 0.367	0.8962	F (6, 47) = 3.731	0.0041
		time	F (6, 49) = 1.659	0.1513	F (6, 49) = 1.912	0.0975	F (6, 49) = 4.793	0.0006	F (6, 47) = 1.432	0.2227
		group	F (1, 49) = 0.477	0.4931	F (1, 49) = 1.791	0.1869	F (1, 49) = 0.099	0.7533	F (1, 47) = 3.272	0.0769
P30	Wistar controls vs. cross-strain	**interaction**	F (6, 54) = 16.68	< 0.0001	F (6, 54) = 9.325	0.0001	F (6, 54) = 12.07	0.0001	F (6, 54) = 6.609	< 0.0001
		time	F (6, 54) = 25.20	< 0.0001	F (6, 54) = 34.65	0.0001	F (6, 54) = 42.71	< 0.0001	F (6, 54) = 33.10	< 0.0001
		group	F (1, 54) = 35.95	< 0.0001	F (1, 54) = 20.09	< 0.0001	F (1, 54) = 54.16	< 0.0001	F (1, 54) = 101.7	< 0.0001
	Wistar controls vs. cross-strain return	**interaction**	F (6, 56) = 22.80	< 0.0001	F (6, 56) = 4.001	0.0021	F (6, 56) = 13.04	< 0.0001	F (6, 56) = 3.657	0.0039
		time	F (6, 56) = 25.65	< 0.0001	F (6, 56) = 39.05	0.0001	F (6, 56) = 105.0	< 0.0001	F (6, 56) = 31.00	< 0.0001
		group	F (1, 56) = 11.06	0.0016	F (1, 56) = 0.016	0.8994	F (1, 56) = 9.823	0.0027	F (1, 56) = 20.00	< 0.0001
	Wistar controls vs. intra-strain	**interaction**	F (6, 53) = 7.124	< 0.0001	F (6, 55) = 4.832	0.0005	F (6, 54) = 14.26	< 0.0001	F (6, 55) = 1.739	0.1292
		time	F (6, 53) = 8.803	< 0.0001	F (6, 55) = 34.64	0.0001	F (6, 54) = 82.71	< 0.0001	F (6, 55) = 17.11	< 0.0001
		group	F (1, 53) = 0.999	0.3220	F (1, 55) = 1.586	0.2132	F (1, 54) = 39.29	< 0.0001	F (1, 55) = 6.884	0.0112
	SHR controls vs. cross-strain	**interaction**	F (6, 48) = 5.162	0.0004	F (6, 48) = 13.91	< 0.0001	F (6, 48) = 13.02	< 0.0001	F (6, 48) = 11.87	0.0001
		time	F (6, 48) = 16.24	< 0.0001	F (6, 48) = 11.81	< 0.0001	F (6, 48) = 26.93	< 0.0001	F (6, 48) = 23.09	0.0001
		group	F (1, 48) = 14.13	0.0005	F (1, 48) = 49.75	< 0.0001	F (1, 48) = 16.61	0.0002	F (1, 48) = 224.9	0.0001
	SHR controls vs. cross-strain return	**interaction**	F (6, 51) = 3.892	0.0028	F (6, 51) = 10.43	< 0.0001	F (6, 51) = 0.875	0.5200	F (6, 51) = 1.152	0.3465
		time	F (6, 51) = 18.00	< 0.0001	F (6, 51) = 36.87	< 0.0001	F (6, 51) = 15.89	< 0.0001	F (6, 51) = 20.56	< 0.0001
		group	F (1, 51) = 0.496	0.4845	F (1, 51) = 3.921	0.0531	F (1, 51) = 0.549	0.4620	F (1, 51) = 0.08	0.7780
	SHR controls vs. intra-strain	**interaction**	F (6, 50) = 4.443	0.0011	F (6, 47) = 7.096	< 0.0001	F (6, 49) = 4.847	0.0006	F (6, 48) = 6.770	< 0.0001
		time	F (6, 50) = 22.19	0.0001	F (6, 47) = 26.66	< 0.0001	F (6, 49) = 33.60	0.0001	F (6, 48) = 31.03	< 0.0001
		group	F (1, 50) = 13.74	0.0005	F (1, 47) = 13.22	0.0007	F (1, 49) = 0.002	0.9675	F (1, 48) = 17.18	< 0.0001

Table 9: Results of two-way ANOVA comparison between the daily profiles of *Per1*, *Per2*, *Rev-erbα* and *Bmal1* expression in the pups liver. Daily profiles of clock gene expression in the liver of 10-day-old (P10) and 30-day-old (P30) Wistar rat and SHR pups reared by their own mothers (controls), foster mothers of the different strain (cross-strain), foster mothers of the same strain (intra-strain) or they were reared by foster mothers of the different strain since P1 till P10 and then returned to their own mothers (cross-strain return).

COLON			*Per1*		*Per2*		*Rev-erbα*		*Bmal1*	
			F(DFn.DFd)	***p***	**F(DFn.DFd)**	***p***	**F(DFn.DFd)**	***p***	**F(DFn.DFd)**	***p***
P10	Wistar controls vs. cross-strain	**interaction**	F (6, 54) = 0.5541	0.7646	F (6, 54) = 0.4797	0.8205	F (6, 55) = 3,268	0.0081	F (6, 55) = 0.5623	0.7584
		time	F (6, 54) = 1,335	0.2580	F (6, 54) = 1,850	0.1067	F (6, 55) = 0.9945	0.4383	F (6, 55) = 7,519	< 0.0001
		group	F (1, 54) = 2,010	0.1620	F (1, 54) = 8,594	0.0049	F (1, 55) = 0.8589	0.3581	F (1, 55) = 0.6903	0.4097
	SHR controls vs. cross-strain	**interaction**	F (6, 56) = 0.7186	0.6362	F (6, 56) = 1,102	0.3731	F (6, 56) = 2,497	0.0327	F (6, 56) = 0.5723	0.7506
		time	F (6, 56) = 4,969	0.0004	F (6, 56) = 1,096	0.3765	F (6, 56) = 12,40	< 0.0001	F (6, 56) = 3,502	0.0052
		group	F (1, 56) = 1,520	0.2228	F (1, 56) = 4,745	0.0336	F (1, 56) = 20.76	0.0001	F (1, 56) = 13,35	0.0006
P30	Wistar controls vs. cross-strain	**interaction**	F (6, 48) = 0.4782	0.8212	F (6, 48) = 6,481	< 0.0001	F (6, 48) = 0.6245	0.7097	F (6, 48) = 4,806	0.0007
		time	F (6, 48) = 6,314	< 0.0001	F (6, 48) = 8,902	< 0.0001	F (6, 48) = 25,45	< 0.0001	F (6, 48) = 14,99	< 0.0001
		group	F (1, 48) = 11,17	0.0016	F (1, 48) = 11,81	0.0012	F (1, 48) = 1,810	0.1848	F (1, 48) = 59,94	< 0.0001
	Wistar controls vs. cross-strain return	**interaction**	F (6, 50) = 4,866	0.0006	F (6, 50) = 5,697	0.0001	F (6, 51) = 4,123	0.0019	F (6, 50) = 0.7635	0.6020
		time	F (6, 50) = 8,968	< 0.0001	F (6, 50) = 8,416	< 0.0001	F (6, 51) = 16,49	< 0.0001	F (6, 50) = 12,21	< 0.0001
		group	F (1, 50) = 4,989	0.0300	F (1, 50) = 14,00	0.0005	F (1, 51) = 18,23	< 0.0001	F (1, 50) = 0.4523	0.5043
	SHR controls vs. cross-strain	**interaction**	F (6, 48) = 6,239	< 0.0001	F (6, 48) = 2,923	0.0164	F (6, 48) = 7,680	< 0.0001	F (6, 48) = 6,294	< 0.0001
		time	F (6, 48) = 11,09	< 0.0001	F (6, 48) = 7,394	< 0.0001	F (6, 48) = 15,89	< 0.0001	F (6, 48) = 9,065	< 0.0001
		group	F (1, 48) = 80.35	< 0.0001	F (1, 48) = 19,00	< 0.0001	F (1, 48) = 33,66	< 0.0001	F (1, 48) = 131,6	< 0.0001
	SHR controls vs. cross-strain return	**interaction**	F (6, 49) = 3,026	0.0135	F (6, 50) = 1,294	0.2772	F (6, 50) = 2,008	0.0820	F (6, 50) = 0.3778	0.8897
		time	F (6, 49) = 13,30	< 0.0001	F (6, 50) = 22,05	< 0.0001	F (6, 50) = 13,34	< 0.0001	F (6, 50) = 8,787	< 0.0001
		group	F (1, 49) = 1,121	0.2948	F (1, 50) = 48,84	< 0.0001	F (1, 50) = 5,921	0.0186	F (1, 50) = 2,825	0.0990

Table 10: Results of two-way ANOVA comparison between the daily profiles of *Per1*, *Per2*, *Rev-erbα* and *Bmal1* expression in the pups colon. Daily profiles of clock gene expression in the liver of 10-day-old (P10) and 30-day-old (P30) Wistar rat and SHR pups reared by their own mothers (controls), foster mothers of the different strain (cross-strain), or they were reared by foster mothers of the different strain since P1 till P10 and then returned to their own mothers (cross-strain return).

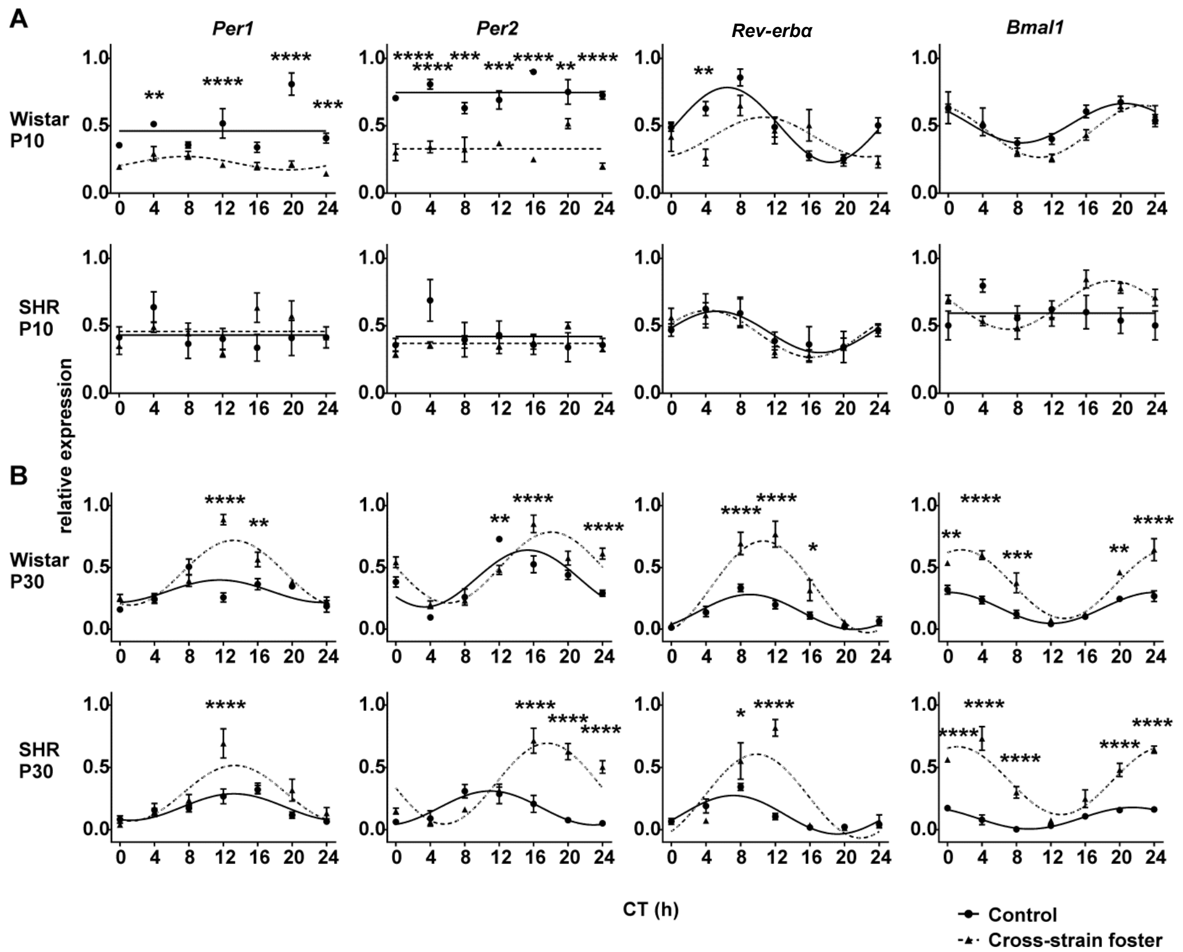


Fig. 23: The effect of cross-strain fostering on clock gene expression in the liver of pups. Daily *Per1*, *Per2*, *Rev-erbα* and *Bmal1* relative expression profiles were detected in 10-day-old **(A)** and 30-day-old **(B)** Wistar rat or SHR pups reared by their genetic mothers as control (full circles, full lines) or exposed to cross-strain fostering (full triangles, dashed lines). The pups were sacrificed in 4-h intervals during 24 h. Time is expressed as circadian time (CT); CT0 represents the time of lights on in the previous LD12:12 cycle. The data are expressed as the mean ± SEM, n = 3-5/time point. The results of two-way ANOVA (Table 9) post hoc analyses (Šidák's multiple comparisons) of differences in the expression levels between the control and cross-strain fostered groups at individual CT are depicted. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

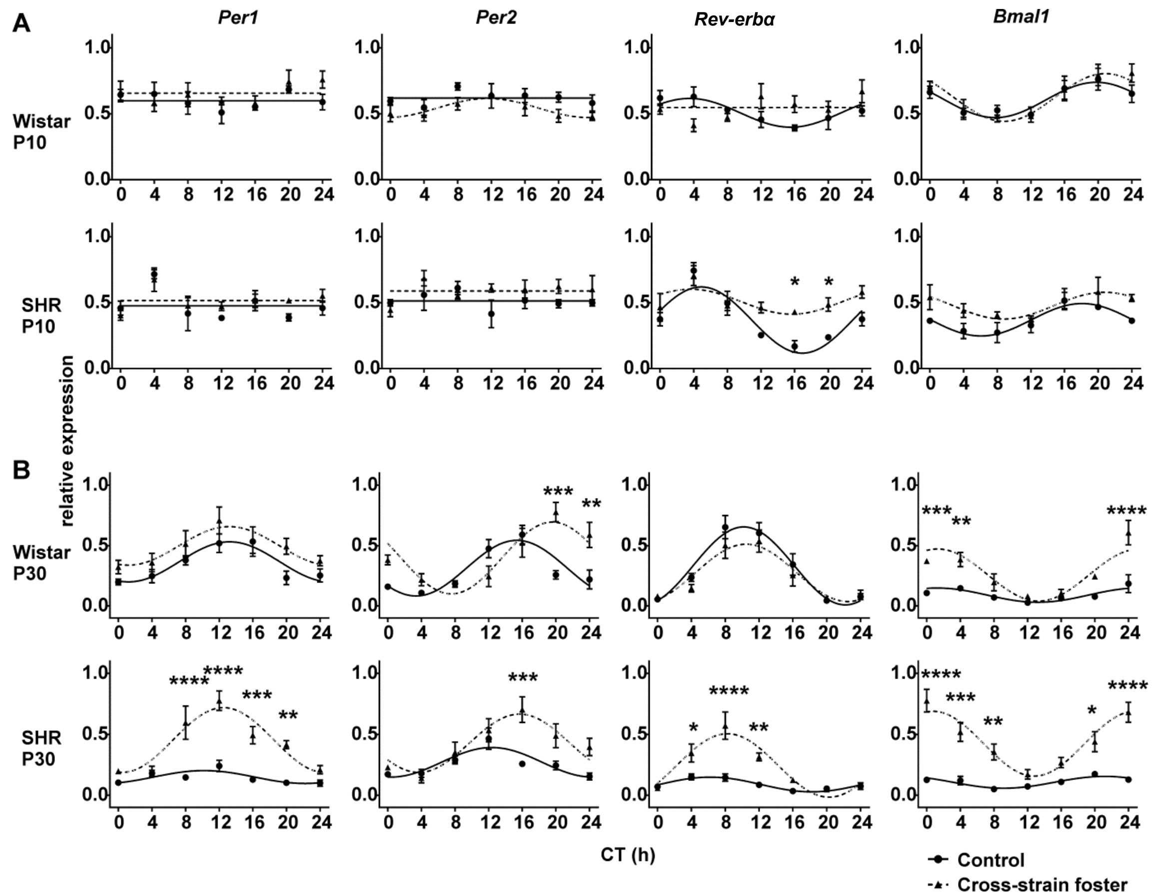


Fig. 24: The effect of cross-strain fostering on the clock gene expression in the colon of pups. Daily *Per1*, *Per2*, *Rev-erbα* and *Bmal1* expression profiles were detected in **10-day-old (A) and 30-day-old (B)** Wistar rat or SHR pups reared by their genetic mothers as control (full circles, full lines) or exposed to cross-strain fostering (full triangles, dashed lines). For more details, see the legend to Fig. 23. For statistical comparisons, see **Table 10.**

5.4.3 Effect of cross-strain fostering on amplitude of peripheral circadian clocks is due to different circadian phenotype of mother

The robust impact of cross-strain fostering on the peripheral clocks in pups at P30 led us to examine whether the effect was a consequence of maternal care provided by a mother of a different strain or due to the presence of a foster mother *per se*. To address the question, we performed two additional experiments (see the scheme in **Fig. 5).** In the first experiment, the pups were exposed to cross-strain fostering at P1, as in the previous experiment, but they were returned to their own genetic mother at P10 and remained with her until P30 (cross-strain return). In the second experiment, the pups were exposed to the fostering procedure at P1, but the foster mother was of the same strain (intra-strain fostering), and the pups remained with the foster mother until they were sampled at P30.

The return of pups exposed to cross-strain fostering to their genetic mothers ameliorated or abolished the effect of cross-strain fostering on the increase in amplitudes of the peripheral clocks in a strain-dependent manner (**Fig. 25**; cross-strain return). In Wistar rat pups, the effect was blocked partially in the liver (only for *Rev-erbα*) (**Fig. 25A**) but almost completely in the colon (**Fig. 25B**). However, in SHR pups, the return to their genetic mother abolished the effect of cross-strain fostering completely both in the liver (**Fig. 25A**) and in the colon (**Fig. 25B**). The fostering procedure per se, i.e., the exchange of a genetic mother with a foster mother of the same rat strain (**Fig. 25A**; intra-strain), did not increase amplitudes of the clock gene expression profiles in the liver of Wistar rats as we observed due to the cross-strain fostering. In the liver of SHR, the intra-strain fostering procedure caused only a small increase in amplitude of *Bmal1*. The effect on intra-strain fostering on the clock in the colon was not examined.

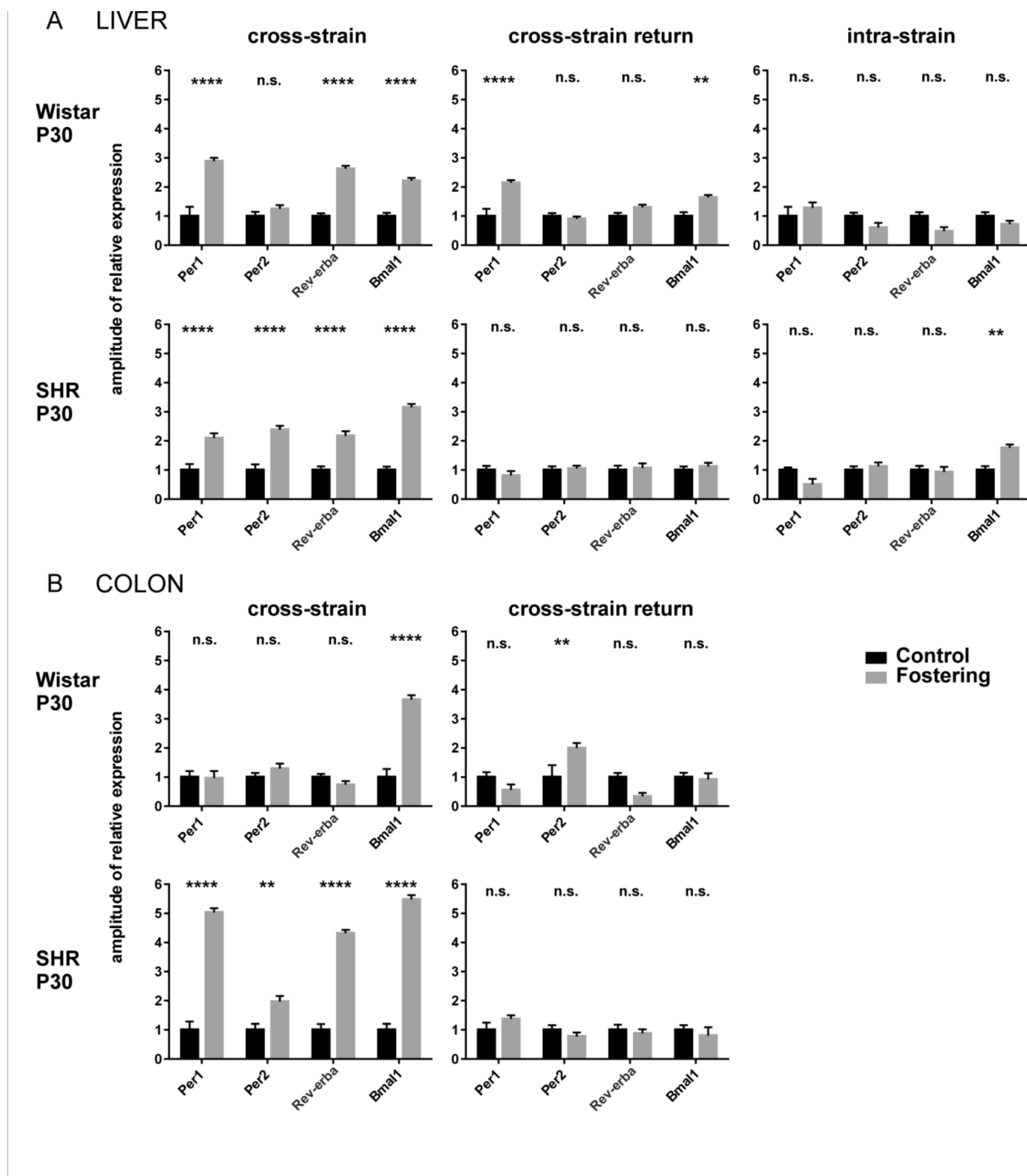


Fig. 25: Amplitudes of the cosine fits of the *Per1*, *Per2*, *Rev-erbα* and *Bmal1* expression profiles in the **liver (A) and colon (B)** of 30-day-old (P30) Wistar rat and SHR pups. For each clock gene, amplitudes from pups reared by their own mother (control) were normalized and compared with profiles from pups reared by foster mothers of the other (cross-strain fostering) or same (intra-strain fostering) strain or with pups who were first exposed to cross-strain fostering and at 10 days of age returned back to their genetic mothers (cross-strain return). The data are expressed as the mean ± SEM. The results of comparisons between groups by two-way ANOVA with Šidák's multiple comparisons test are shown in each graph. ** p<0.01; *** p<0.001; **** p<0.0001.

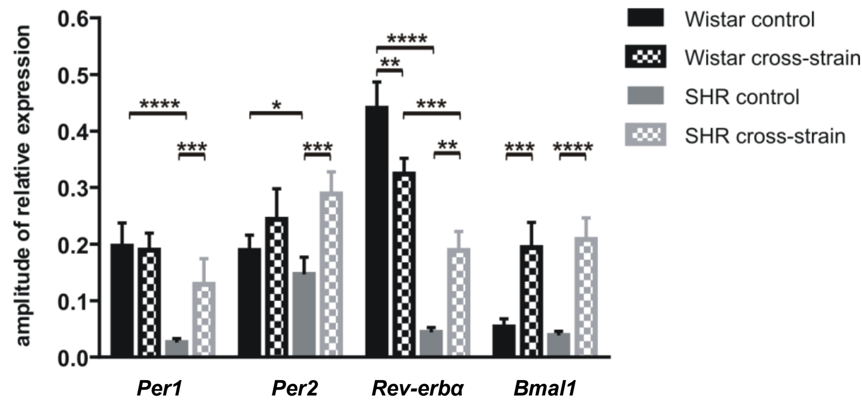


Fig. 26: Comparisons of amplitudes of the cosine fits of the *Per1*, *Per2*, *Rev-erbα* and *Bmal1* expression profiles in the colon between 30-day-old Wistar rat and SHR pups that were reared by their own mothers (control) or by foster mothers of a different strain (cross-strain). The data are expressed as the mean ± SEM. The results of comparisons between groups by two-way ANOVA with Tukey's multiple comparisons test are shown in each graph. * $p<0.05$; ** $p<0.01$; **** $p<0.0001$.

5.4.4 Cross-strain fostering has long lasting impact on circadian behavior

Effect of the cross-strain fostering procedure on the SCN-driven behavioral activity rhythms was assessed in the rats which were monitored shortly after weaning, i.e., since P35 (1 month-old), and again in adulthood (6 months-old) (**Fig. 27**). Results of statistical comparisons among the groups are depicted in the **Fig. 27**. The total activity was not affected by the cross-strain fostering conditions and it also did not change with age under LD12:12 and DD (**Fig. 27A**). Amplitude of the locomotor activity rhythm was measured as the activity/rest ratio (**Fig. 27B**). In LD12:12, amplitude increased significantly with age in control Wistar rats but not in control SHR. As a consequence, amplitude was the not different between 1-month-old in control Wistar rats and SHR, but it in 6-months-old rats it was significantly higher in control Wistar rats than SHR. The cross-strain fostering had no effect on amplitudes of the activity rhythms of Wistar rats at both ages and of 1-month-old SHR. However, it had a significant effect in 6-months-old SHR when it was significantly higher compared to SHR age-matched controls and it reached about the level of control Wistar rats. In rats maintained in DD conditions, amplitude in control SHR was significantly lower than in Wistar rats (similarly as in LD12:12) but the effect of cross-strain fostering was not present. The result suggested that the improvement of the activity/rest ratio due to cross-strain fostering was related to a change in the phase angle of rhythm relative to the LD cycle. Therefore, the phase angle of entrainment of the locomotor activity rhythm was measured as the level of activity during the interval of 3 h before the lights-off on LD12:12 (**Fig. 27C**).

This pre-lights-off activity was significantly increased in SHR compared to Wistar rats at age of 1 month, and even more at age of 6 months. The cross-strain fostering significantly decreased the activity in SHR at age of 6 months when it attained the same level as in Wistar rats. In 1-month-old SHR, the effect was only suggested but not significant. Therefore, the data demonstrate that a small positive phase angle of the entrainment is present already in 1-month-old SHR and it progressively develops later in adulthood. The cross-strain fostering procedure completely blocked development of the positive phase angle of the entrainment in adulthood and improved thus the entrainment of the locomotor activity rhythm of SHR to LD12:12 that attained the same level of the entrainment as in Wistar rats. The period of the free running locomotor activity rhythm measured in 6-months-old rat maintained in DD (**Fig. 27D**) was significantly shorter in SHR compared to Wistar rats and it was not affected by cross-strain fostering. Therefore, in contrast to the phase, the free-running period is a genetic trait that cannot be changed by maternal care.

5.4.5 Cross-strain fostering impacts on cardiovascular function

The effect of cross-strain fostering on HR, MAP, SBP and DBP was studied in 2-month-old rats. The results of statistical comparisons are depicted in **Fig. 28**. Two-way ANOVA revealed control SHR had all four parameters significantly higher compared to age-matched control Wistar. The result confirmed that the cardiovascular pathology had developed with age in our SHR as previously published (Conrad *et al.*, 1995). The maternal care of Wistar rat foster mother significantly decreased the elevated HR of SHR at this age. The maternal care of SHR foster mother had no effect on the parameters in Wistar pups.

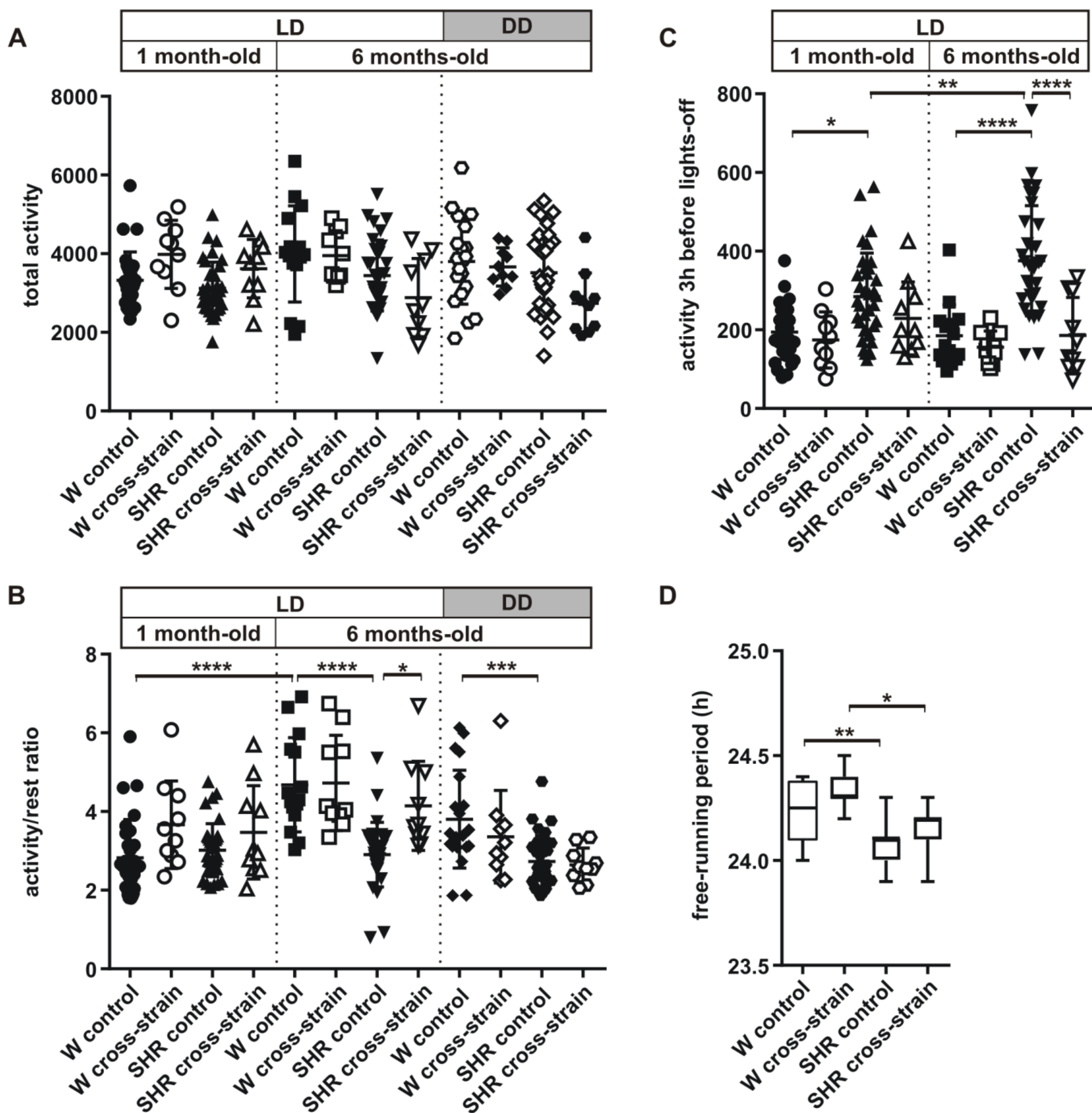


Fig. 27: Analyses of the locomotor activity parameters of 1-month-old and 6-month-old Wistar rats and SHR maintained in light/dark regime (LD) or in constant darkness (DD). **The total activity (A), activity/rest ratio (B), activity in the 3-h interval before lights-off (C) and free-running period (D)** were measured in rats that were reared until weaning by their own mothers (control) and in those that were exposed to cross-strain fostering (cross-strain). The data are expressed as the mean ± SD, n = 10-35/group; whiskers in (D) show min to max values. The results of the statistical comparisons between groups are depicted. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

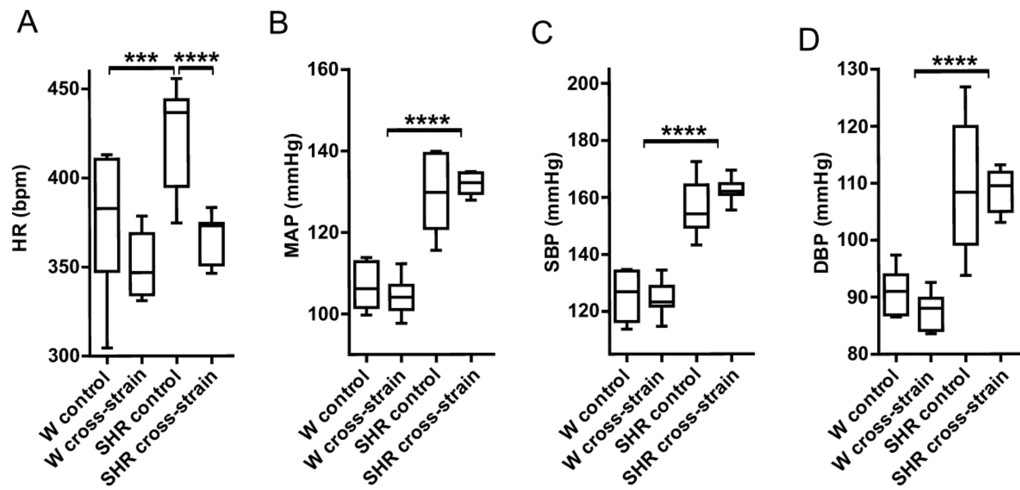


Fig. 28: **Analyses of cardiovascular function parameters** of 2-month-old Wistar rats and SHRs maintained in the light/dark regime. The heart rate (HR) (A), mean arterial pressure (MAP) (B), systolic blood pressure (SBP) (C), and diastolic blood pressure (DBP) (D) were measured in rats that were reared until weaning by their own mothers (control) and those that were exposed to cross-strain fostering (cross-strain). The data are expressed as the mean ± SD, n = 6-8/group; whiskers show min to max values. HR is expressed as beats per minute (bpm) and blood pressure is expressed as millimetre of mercury (mmHg). The results of the statistical comparisons between groups are depicted. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

6 DISCUSSION

6.1 ONTOGENETIC DEVELOPMENT OF CIRCADIAN CLOCK IN COLON OF WISTAR PUPS

In the first experiment we provided a detailed analysis of the development of the rat colonic circadian clock from the prenatal period until the end of weaning. The aim of this study was to detect the developmental period when the *Per1*, *Per2*, *Rev-erbα*, *Cry1*, *Bmal1*, and *Clock* gene expression profiles become rhythmic and when the rhythms become mutually properly phased, ensuring the functional molecular clockwork.

We demonstrated that circadian rhythms in the individual clock gene expression were already detectable in the colons of 20-day-old embryos, but amplitudes of the rhythms were low. The only exception was the *Rev-erbα* expression profile, which exhibited highest amplitude relative to other clock genes studied. This result was in accordance with our earlier study on the development of the circadian clock in the rat liver (Sládek *et al.*, 2007a), in which *Rev-erbα* was also found to be rhythmically expressed at E20. However, in the previous study, we used different criteria for detecting the circadian rhythm and, therefore, the results are not quite comparable. Still, it seems that *Rev-erbα* is regulated in a circadian manner in rat fetal peripheral clocks in the liver and colon. *Per1* and *Per2* were found to be expressed with lower but still reasonably high amplitudes in embryonic colons, whereas the *Cry1* and *Bmal1* expression rhythms exhibited only low amplitudes; *Clock* gene was not expressed rhythmically.

The fact that most of the canonical clock genes at E20 were expressed rhythmically might suggest that a functional circadian clock already works in the fetal colon at this age. However, the peaks of all of the individual clock gene expression rhythms were set to a narrow temporal window at the beginning of the subjective night but the current model of the molecular clockwork predicts that the expression of individual clock genes attains a gene-specific phase, i.e. *Bmal1* and *Rev-erbα* are expressed in an anti-phase (Shearman *et al.*, 2000). Thus, the phasing of all studied clock gene expression rhythms at the same time of a day may reflect a state in which the embryonic clock is driven by a rhythmical signal from a mother. A hypothetical maternal signal would need to be able to induce a signaling pathway that activates the promoters of all of the rhythmic clock genes. According to this hypothesis, the fetal clock would be a slave oscillator driven by rhythmical maternal cues, rather than an autonomous clock. The other explanation can be that the individual cellular clocks in the embryonic colon are functional but mutually desynchronized, which leads to

the detection of low-amplitude rhythms at the cell population level. The latter explanation is not supported by our data because amplitudes of clock gene expression rhythms at E20 were not significantly lower than those at P10 or P20, when a proper mutual phase relationship among the clock gene expression profiles had been established. Altogether, the finding that in the fetal colon, the clock gene expression rhythms are set to the same phase suggests that the rhythms are likely driven by maternal cues.

At P2, only the expression of *Per1*, *Per2*, and *Cry1* was rhythmic. At this age, the pup colon is lacking the rhythmical cues provided from the maternal milieu. Instead, it is exposed to a rhythmic intake of the maternal milk, which may possible entrain, but actually not induce, rhythmicity. This might be the reason why in 2-day-old pups, amplitudes of some of the clock gene expression rhythms vanished for some genes.

In 10 day-old pups, the expression of *Per1*, *Per2*, *Rev-erbα* and *Bmal1* was rhythmic, the rhythm was suggested for *Cry1* and was not present for *Clock*. Notably, at this age, the individual clock gene rhythms peaked at different phases during the 24-h cycle, suggesting that a functional colonic clock may begin to work and/or the maternal breast-feeding regime is able to entrain the clock. Nevertheless, at P10, the timing of acrophases did not completely correspond to the adult-like state, in which the *Rev-erbα* and *Bmal1* are expressed in an anti-phase (Sládek *et al.*, 2007b; Sládek *et al.*, 2012).

At P20, all studied genes with the exception of *Clock* were expressed rhythmically. The phases of *Per2* and *Rev-erbα* were completely phase-reversed compared with P10. At this age, the pups are already sighted and exhibit the nocturnal behavioral activity, which is driven by their SCN (reviewed in Sumova *et al.*, 2012). In addition, the pups begin to consume solid food during the nighttime. The phase reversal of the *Per2* and *Rev-erbα* expression profiles might suggest that their nocturnal feeding becomes a stronger synchronizing cue for the colonic clock than the maternal breast-feeding at this stage.

At P30, *Rev-erbα* and *Bmal1* exhibited high-amplitude rhythms, *Cry1* and *Clock* had low-amplitude rhythms, and the expression of *Per1* and *Per2* was not rhythmic. Importantly, at this developmental stage, the phasing of the clock genes in the colon well corresponded to that in adults (Sládek *et al.*, 2007b; Sládek *et al.*, 2012), reflecting the fact that at this age, the weaning is completed and the colon is exclusively synchronized by the nocturnal feeding regime. 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. These data point to a switch of the strength of the entraining cues for the developing colonic clock, i.e., maternal breast-feeding vs. pups feeding the solid food.

To determine the importance of the rhythmical maternal milieu during the embryonic stage for the development of the colonic clock after birth, the pups were reared by foster mothers, which were entrained to the reversed LD regime compared with their genetic mothers. This allowed us to examine whether prenatal history plays a role in the postnatal phasing of the clock gene expression profiles. The phase reversal of the LD cycle performed prenatally (DL/LD) and postnatally (LD/ DL) had the same effect on the SCN and colonic gene expression profiles in 10-day-old pups. In accordance with the previous data (Sládek *et al.*, 2004; Kováčiková *et al.*, 2006), the SCN at P10 exhibited the significant and high-amplitude rhythms of the clock genes expression, which were already fully entrained by the external LD cycle and their phases corresponded to those in adult animals (Oishi *et al.*, 1998; Sládek *et al.*, 2007b). 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 phasing of the *Per2* and *Rev-erbα* expressions, which were shifted about 4-6 h compared with the non-fostering conditions. Remarkably, for both groups of fostered pups, an anti-phase relationship between *Rev-erbα* and *Bmal1* was already established, which was not yet the case for 10-day-old pups reared by their genetic mothers maintained in the same LD cycle throughout the development. Thus, the data surprisingly suggest that the timing of breast-feeding in nocturnal animals might affect the synchronization of the developing clock. Importantly, from the comparison of the clock gene expression profiles in the colon and SCN of 10-day-old pups, it appeared that these profiles were in the same phase. In contrast, in adults, the phase of the colonic clock significantly lagged behind the SCN (Sládek *et al.*, 2012). The notable finding of 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 SCN, which would otherwise set a phase-delayed rhythm.

In the last experiment, the mothers and their pups were maintained in LL to eliminate the role of the pup SCN in the synchronization of the developing colonic clock. Previous data showed that the prolonged exposure to LL disrupts the synchrony among the SCN oscillatory neurons and the ability of the SCN to generate a coherent circadian signal (Ohta *et al.*, 2005). We have confirmed that the maintenance of pregnant rats on LL during the entire pregnancy leads to a situation in which the maternal SCN clock is incapable to synchronize the embryonic SCN (Nováková *et al.*, 2010). As a consequence, the profiles of clock genes expressions did not exhibit circadian rhythms in the fetal SCN. According to this finding, no rhythms or only faint rhythms in the clock gene expression were detected in

the SCN of pups at P10 and P20, which was, thus, likely unable to synchronize the colonic clock. If mothers maintained in LL were exposed to RF after delivery, the pup SCN profiles were affected only slightly at P10, and no effect was present at P20. The maternal RF during breast-feeding, thus, seemed to be a weak or ineffective entraining cue for the pup SCN. In colons of pups raised by *ad libitum*-fed mothers in LL, the *Rev-erbα* and *Bmal1* expressions retained circadian rhythmicity, but they both peaked at the same phase. Imposing the RF on mothers shifted the *Rev-erbα* and *Bmal1* rhythms in the pup colons, so that they were expressed in an anti-phase, suggesting a functional clock. Thus, whereas the breast-feeding was unable to synchronize the SCN clock, it was a robust synchronizing signal for the colonic clock. The anti-phase relationship was preserved at both ages, despite the fact that the clock gene expression rhythms phase-reversed between P10 and P20, which was similar to the case in which pups were reared by mothers maintained in LD and fed *ad libitum*. Importantly, in this experiment, the age-related phase-reversal between P10 and P20 occurred in pups without the functional SCN due to the prolonged exposure to LL. Thus, the developmental phase shift of the colonic clock observed between P10 and P20 seems to be independent of signaling from the pup SCN.

6.2 DEVELOPMENT OF CIRCADIAN SYSTEM IN SHR PUPS

The results of this study demonstrated that the dynamics of the circadian system development and sensitivity of the system to maternal cues during ontogenesis differ in SHR and Wistar rats. Especially, the dynamics of the *Bmal1* circadian expression development to the adult-like state is delayed in both the SCN and peripheral clocks. Moreover, the data showed that the SHR SCN and peripheral clocks during the fetal stage and the SHR hepatic clock in pups at P10 are sensitive to maternal cues related to changes in the feeding regime.

Analysis of daily profiles of clock gene expression in the SCN of SHR pups revealed that, whereas amplitudes of *Per1*, *Per2* and *Rev-erbα* rhythms increased significantly between P1 and P10, amplitude of the rhythm of *Bmal1*, which was shallow at P1, increased later. In our previous studies on dynamics of the SCN clock ontogenesis of Wistar rat pups we have demonstrated that expression rhythms of all the studied clock genes, including the *Bmal1* rhythm, were fully developed at P10 (Sládek *et al.*, 2004). In the current study, comparison of the clock gene expression profiles in the SCN of 10-day-old SHR and Wistar rat pups confirmed that amplitude of *Bmal1* rhythm was lower in SHR pups. The result suggests that the development of circadian regulation of the *Bmal1* gene expression in the SCN is delayed in SHR. Based on data of this study it could be speculated that the observed higher levels of *Rev-erbα* mRNA in the SCN of SHR may provide explanation of the higher suppression of circadian *Bmal1* expression via binding of REV-ERBA protein to its promoter. During postnatal ontogenesis from P1 until P30, no phase changes of clock gene expression profiles were detected in the SCN of SHR pups, which was in agreement with previously published data in Wistar rats (Kováčiková *et al.*, 2006) and other animal models (Shimomura *et al.*, 2001; Caba *et al.*, 2008; Ansari *et al.*, 2009).

The circadian clocks in the liver and colon of SHR might already operate at P1 because circadian rhythms in expression of all studied clock genes with a proper mutual phasing (*Bmal1* roughly in anti-phase to *Per1*, *Per2* and *Rev-erbα*) were detected. This finding seems in contrast to previously published results in Wistar rats. In the liver and colon of Wistar rats at P1–2, high-amplitude rhythms for *Rev-erbα* and only shallow or no rhythms for other clock genes were detected (Sládek *et al.*, 2007a; Polidarová *et al.*, 2014); however, these clock gene expression rhythms were all in similar phases. During the postnatal development of SHR between P1 and P30, the phases of the clock gene expression in both studied peripheral organs changed in a gene-specific manner. To attain the adult-like pattern in the SHR and Wistar rat liver (Sládek *et al.*, 2012), *Per1* and *Per2* expression rhythms

accomplished two almost complete phase-reversals, the first between P1 and P10 and the second between P10 and P20, whereas the *Rev-erbα* rhythm attained the adult-like state earlier, via only one phase-reversal between P1 and P10 and was thus fully developed as the first of all other gene expression profiles. In contrast to the Wistar rat liver, the phase resetting of *Rev-erbα* rhythm in the SHR liver was accompanied by an apparent dampening of the *Bmal1* expression rhythm during the developmental stages P10–P20 because the rhythm was absent during the transitional interval.

In the SHR colon, the dynamics of the development were different compared with the liver; these rhythms either did not change their phases or shifted only moderately from P1 to P10, however, they all shifted significantly between P10 and P20 when they achieved the adult-like state. The dynamics mostly did not differ to those previously published for the Wistar rat colonic clock (Polidarová *et al.*, 2014).

Together, these results demonstrate that in the liver, development and proper phasing of high-amplitude *Bmal1* rhythm was delayed in SHR compared to Wistar rats, whereas in the SHR colon, the rhythms developed similarly in both rat strains. Notably, the daily *Bmal1* expression levels were significantly suppressed in SHR compared with Wistar rats both in the liver and colon. The results suggest that during ontogenesis, regulation of the *Rev-erbα–Bmal1* loop of the SHR molecular clockwork is different. Above all, these results (together with our previous data on the development of the peripheral clocks in Wistar rats) provide evidence that during ontogenesis, the individual peripheral circadian clocks significantly differ from the adult stage because the individual clock gene expression profiles shift independently of each other during the developmental phase adjustments. We speculate that it might be because the developing clocks in the individual peripheral tissue in vivo may be (i) fully functional on the single cell level but not properly synchronized, (ii) dampened in the individual cells due to confounding signals derived from the maternal and their own developing circadian systems or (iii) responding in a cell-specific manner depending on their function and their maturation stage in the tissue. Functional but desynchronized cellular oscillators would likely lead to absence of the circadian rhythm at the cell population level and high variability in expression levels among the samples. The dampened rhythms in the single cells would manifest as an absence of the rhythm at the tissue level or the individual gene expression would exhibit different phases. Combination of these possibilities is more likely because the peripheral clocks need to be synchronized by various signals from the SCN and/or their local environment to produce coherent output. During the development, these signals might get more complicated than in the adult state because they arrive from the

maternal SCN, the developing pup's SCN, as well as from the maternal care and feeding. These signals may oppose each other and be sensed differently by the individual clock genes; some of the clock genes, e.g. *Rev-erbα*, might be more sensitive to the signals rather than be controlled solely by the transcriptional–translational feedback loop. From our data it appears that high-amplitude *Rev-erbα* rhythm phase leads the adjustment to adult-like stage and represents thus a candidate mechanism for the phase resetting of circadian clocks during the developmental process. We believe that our observation of the apparently incoherent mutual phasing of clock gene expression rhythms during ontogenesis have their biological implications pointing at the specific functional stage of the developing circadian clocks at the tissue level.

The phase shifts of the developing peripheral clocks have been attributed to changes in the maternal-feeding regime during the lactation period (Weinert, 2005; Sumova *et al.*, 2012). The peripheral clocks are dominantly entrained by the feeding regime and the mothers nurse their pups mostly during their resting time which in nocturnal animals occurs during the light phase of the day. The adult-like phases are established during the weaning period when the pups begin to consume solid food during the nighttime. Our data suggest that the SHR mother spends less time in the nest during the nighttime shortly after parturition compared to the Wistar rat mother. While the Wistar rat mother stayed in the nest with her pups almost constantly during the 20-h interval after parturition, the SHR mother left her pups more often during the nighttime. The strain difference was not apparent later than 3 days of the postnatal period, and thereafter, both the SHR and Wistar rat mothers were spending much less time with their litter during the night. Our data are not in accordance with previous findings demonstrating that SHR mothers spend more time nursing and licking their pups and less time away from the litter than normotensive controls (Cierpial *et al.*, 1987; Myers *et al.*, 1989; Gouldsborough *et al.*, 1998). The more frequent nursing was observed in SHR mothers during the daytime (Cierpial *et al.*, 1987). There are many factors that might account for the different outcome of our results and the other studies. In the previous studies, complex maternal behavior in the nest was recorded by personal observation and by monitoring the time spent licking, in arched- and blanket nursing positions, and resting without nursing. In this study, we were not able to reliably distinguish between the maternal behaviors in the nest, i.e. whether the mother was resting and/or feeding the pups, because the mother's body covered the pups to provide the temperature comfort for most time they spent in the nest and obstructed the viewing the pups by video camera. Therefore, the nursing could not be reliably detected by our method. However, in

contrast to previous studies our approach allowed us to monitor animal behavior undisturbed by an observer presence in the animal room. The SHR are generally more sensitive to environmental stress (LeDoux *et al.*, 1982), and therefore, repeated entries into the animal room and the presence of an observer might agitate the SHR mothers more than the controls and, consequently, affect their maternal behavior. Nevertheless, our present data confirm that maternal behavior of SHR differs from that of control rats, and the difference is mainly obvious early after parturition. Our results may suggest that these differences in maternal behavior between both rat strains early after delivery might account for the aforementioned differences in development of the hepatic clocks.

The changes in development of the circadian system in SHR observed in this study might also be caused by difference in their sensitivity to maternal cues. To test this, the mothers maintained on the LD cycle were allowed to consume food only for 6 h during the daytime when they normally sleep. The RF regime was previously shown to provide sufficient caloric intake to SHR and Wistar rats (Polidarová *et al.*, 2013). The RF regime during pregnancy and lactation period imposed a change in the maternal locomotor activity pattern because the dams became active during the food availability, to satisfy their energetic demands, and also during the most of the night because their SCN remained synchronized with the external LD cycle (Nováková *et al.*, 2010; Polidarová *et al.*, 2013). During the time of food availability, the dams were fully occupied by feeding and could not nurse their pups. Consequently, the temporal nursing pattern changed as the RF imposed a distinct rhythm on breast-feeding.

The RF exposure in pregnant rats had an apparent effect already on fetal clocks as evidenced by the phases of clock gene expression rhythms at P1. The fetal SCN clock of SHR was sensitive to maternal RF because it reset the phases of *Per2* and *Rev-erbα* expression rhythms in the SCN of 1-day-old pups. The absence of a detectable shift in *Bmal1* profiles was likely related to the fact that circadian regulation of the gene is not fully developed in SHR at P1, and its expression rhythm is thus only shallow (see above). In contrast, the exposure of pregnant Wistar rats to identical experimental procedure in our previous study did not affect the fetal SCN clock (Nováková *et al.*, 2010). In Wistar rats, the RF had no effect on the fetal SCN clock when the maternal SCN was synchronized with the LD cycle and entrained the fetal clocks accordingly, whereas it was able to synchronize the fetal SCN clocks only when the maternal entraining cues were absent due to the exposure to constant light (Nováková *et al.*, 2010) or surgical removal of the SCN (Weaver and Reppert, 1989). The ability of RF to entrain the fetal SCN clock in SHR mothers entrained to the LD

cycle suggests that either the signals from the maternal SCN are weaker in SHR compared to Wistar rats, or the signal from RF is stronger in SHR than in Wistar rats, or both mechanisms are involved. Both possibilities are plausible because in adult SHR amplitude of the SCN-driven circadian rhythms is lower than in Wistar rats (Lemmer *et al.*, 1993; Cui *et al.*, 2011; Sládek *et al.*, 2012), and the circadian system of SHR is more sensitive to RF than that of Wistar rats (Polidarová *et al.*, 2013). Therefore, the stronger response of SHR mothers to RF could likely provide a stronger entraining cue to their fetuses. However, the nature of the entraining cue remains to be elucidated.

For the fetal peripheral clocks in SHR pups, the maternal RF regime also represented a significant cue. In the liver at P1, all of the rhythmic clock gene expression profiles (*Per1*, *Per2*, *Rev-erbα* and *Bmal1*) were significantly shifted due to maternal RF. In the colon, all expression rhythms, except for *Bmal1*, were also shifted. In this experiment, the *Bmal2* expression rhythm was also detected because the gene was found to be sensitive to RF in the adult SHR liver (Polidarová *et al.*, 2013). In the liver at P1, the *Bmal2* expression rhythm was shifted and suppressed due to the maternal RF but in the colon, *Bmal2* was not expressed rhythmically under either feeding condition similar to adult animals.

Maternal RF also had a significant effect on the developing circadian system of SHR during the later postnatal period. The effect on the SCN clock of 10-day-old pups was disputable because despite statistical significance, the shifts were too small to have any physiological relevance. Similarly, the SCN was not entrained by RF in adult SHR maintained under the LD cycle (Polidarová *et al.*, 2013). At P10, maternal RF shifted *Per1* and *Rev-erbα* rhythms in the liver, and the shift was in the opposite direction compared with that it imposed during the prenatal period. Interestingly, *Bmal2* was expressed rhythmically in the SHR liver at P10 under both feeding conditions, although *Bmal1* was not rhythmic. The results support a possible role of *Bmal2* in regulation of the clock mechanism in the liver of SHR during ontogenesis (results of this study) as well as in adulthood (Polidarová *et al.*, 2013). In the colon, all rhythmically expressed genes were also shifted by maternal RF. Interestingly, while the effect of maternal RF on the hepatic clock persisted until P10 in SHR, this was not the case in Wistar pups, as demonstrated by the phase of *Rev-erbα* (the only gene expressed rhythmically in pups of both strains and under both feeding conditions). Therefore, at P10 the hepatic clock in SHR was likely more sensitive to maternal cues than in Wistar rats.

The differences in ontogenesis of the circadian system and its higher sensitivity to maternal cues in SHR described in this study might arise directly from the SHR genotype

(Woon *et al.*, 2007; Pravenec, Churchill, *et al.*, 2008; Pravenec, Kazdova, *et al.*, 2008). They may be related to a higher overall reactivity of the developing circadian system to changes in the feeding regime as demonstrated previously for adult SHR (Polidarová *et al.*, 2013). They may also be a consequence of an altered maternal behavior, an example of which was observed in this study. Most likely, the effect is due to a combination of these factors. Our ongoing cross-fostering study will answer the questions of whether and how much maternal care contributes to changes in the development of the circadian system in SHR. Understanding the mechanisms is important because disturbances of the circadian system have been attributed to various diseases, including cardiovascular and metabolic diseases, which the animal model examined in this study spontaneously develops. These results underline the importance of the maternal circadian system in the development of the circadian central and peripheral clocks in their offspring.

6.3 COMPARISON OF SENSITIVITY TO MATERNAL STRESS IN WISTAR AND SHR PUPS

The results of this study provide direct evidence that during the early postnatal period, the circadian clock in the SCN contains relevant levels of GRs. We also found that at this developmental stage, the SCN clock is sensitive to stress that may shift its phase via GC-dependent and GC-independent mechanisms. The evidence comes from data on two rat strains with different sensitivities to stress. To distinguish between the GC-dependent and -independent stress mechanisms, we administered the GR antagonist, mifepristone, to pups at the time of the exposure of their mother to stressful stimulus at a dose previously reported in the literature to sufficiently block GRs (Csaba and Inczefi-Gonda, 2000; Csaba and Karabélyos, 2001). The drug is also an efficient antagonist of progesterone receptors (Cadepond F et al. 1997) and the adult SCN contains progesterone receptors (Kato *et al.*, 1993), however, during ontogenesis, these receptors become to be expressed in the hypothalamus only after P8-10 (Kato *et al.*, 1994) and therefore, the participation of this pathway in the observed effects in our 4-day-old pups is unlikely. The experimental approach of repeated injections of mifepristone to newborn pups for a few consecutive days has been validated (Weinstein *et al.*, 1992). The drug reaches maximal plasma levels approximately 1 h after s.c. injection, and effective levels persist until 48 h after a single administration (Lähteenmäki *et al.*, 1987). Therefore, the once-a-day administration for 3 subsequent days used in our study provided a constant blockade of GR throughout the studied interval, from P1 till P4 when the pups were sampled, and did not require pre-treatment with the antagonist before imposing the stress. The participation of GC in the stress response mechanism was assessed via examination of the plasma GC levels in mothers and their pups and exploration the impact of the blockade of GRs on the stress-induced effects in the pups' SCN.

The reactivity of the circadian clock in the SCN to stressful stimuli has been controversial, but most studies have confirmed the resilience of the SCN to stress (Hara *et al.*, 2001; Tahara *et al.*, 2015). This resilience has been explained by the numerous observations that the adult SCN is nearly devoid of GRs (Rosenfeld *et al.*, 1988; Morimoto *et al.*, 1996; Aurelio Balsalobre *et al.*, 2000). Nevertheless, a single study demonstrated the presence of GR-ir in the neonatal SCN of Wistar rats and reported that it declined during the postnatal period (Rosenfeld *et al.*, 1988). Our results extend this report and demonstrate the presence of not only GR-ir but also *Nr3c1* gene expression in the SCN of 4-day-old pups of

two rat strains, namely Wistar rats and SHR, with a trend towards higher *Nr3c1* expression in the SHR. Moreover, our results justified the physiological relevance of the *Nr3c1* mRNA levels in the SCN of both rat pup strains because we detected comparative levels of the mRNA with those in the adult hippocampus, a brain area with high GR levels (Reul and Kloet, 1985). Although previous studies suggested that the exposure to stress might affect the SCN clock in blinded Wistar rat pups (Ohta *et al.*, 2003), evidence of a direct involvement of GC in the stress response has not been provided. Additionally, it was not known whether relatively mild stress might affect the SCN clock in sighted pups maintained in natural conditions of an LD cycle, i.e., when the dominant entraining photic cue was present. Therefore, our study fills the gap and provides evidence for these mechanisms.

To manipulate the GC levels in pups, we used an experimental approach in which stressful stimuli were imposed on neonatal pups indirectly, via manipulating the feeding regimen of their mothers, and directly, via handling/injection of pups. As we expected, the exposure of lactating mothers to RF elevated their plasma GC levels in anticipation of food availability during the daytime. The finding was in accordance with previous reports on the RF-induced changes in GC levels (Krieger, 1974). The RF-induced change in GC levels was significant in mothers of both rat strains; it appeared to be more pronounced in SHR, however, the difference was mainly due to a lower baseline GC level because the RF-increased levels were approximately the same in both rat strains. The higher response of SHR mothers to RF was in accordance with our previous data in male rats showing that compared to Wistar rats maintained on RF, the SHR exhibited earlier and higher food anticipatory behavior and their peripheral clock in the liver was more affected (Polidarová *et al.*, 2013). In the present study, the response of maternal GC levels to RF correlated with the response of the pups' SCN clock, as assessed by the phase of the *Bmal1* expression rhythm; whereas the SCN clock of Wistar rat pups whose mothers exhibited a milder response to RF was not affected, the clock of SHR pups whose mothers exhibited a stronger response to RF was significantly shifted. However, we were not able to detect significant elevation of GC levels in plasma of untreated SHR pups reared by RF-exposed mothers. Therefore, the response of the SHR pups' SCN to maternal stress was most likely mediated by GC-independent mechanism, for example as a response to the maternal RF-induced changes in their feeding regime to which they are more sensitive than Wistar rats, as we discuss below. The differences in maternal behavior shortly after delivery might also participate in the strain-specific effect because we previously found that SHR dams spent less time in the nest with their newborn pups compared to Wistar rat dams (Olejníková *et*

al., 2015). The data are also in accordance with our other previous study showing the impact of RF in pregnant rats on fetal SCN clock; in pregnant Wistar rats the exposure to RF was only a weak signal to fetal SCN clocks that became entrained only when the maternal rhythmic signals were abolished due to the exposure of mothers to constant light (Nováková *et al.*, 2010). However, in pregnant SHR the same manipulation appeared to be a much stronger signal that was also able to entrain the fetal clock when the maternal SCN was functional and synchronized with the LD cycle (Olejníková *et al.*, 2015).

In our in vivo arrangement, a drug to antagonize GR could not be administered to pups via stress-avoiding procedure. Therefore, we tested whether the procedure itself (s.c. injection) affected the GC levels in the pups. We found no significant effect of the injection procedure on plasma GC levels in Wistar rat and SHR pups reared by intact mothers (not exposed to RF); however, the GC levels were significantly elevated in pups treated in the same way but reared by RF-exposed mothers. Because the s.c. injections alone had no effect on the GC levels in pups, this group was not included in the experimental scheme for testing the effect of GC on the SCN clock.

The elevated plasma GC levels in injected/manipulated pups of both rat strains reared by RF-exposed mothers correlated with phase shifts of the clock gene *Bmal1* expression profiles in their SCN. However, there was a difference in mifepristone efficiency to abolish the effect in Wistar rats and SHR. Whereas in Wistar rats the effect was completely blocked by mifepristone and, therefore, was obviously mediated by GC, in SHR the GR antagonist did not block the phase shift and thus involvement of GC in the effect is unlikely. The impact of stress on the pups might thus had been mediated by additional stress mechanisms. The SHR is an animal model with a complex pathophysiology (Pravenec *et al.*, 2004) and apart from the higher sensitivity of the hypothalamo-hypophyseal axis, they also exhibit higher stress-responsiveness in sympathetic tonus (Judy and Farrell, 1979). However, the mechanisms of how these pathways might impact the SCN in pups remain to be uncovered. Nevertheless, based on our data on GC levels in mothers and pups the results provide evidence that the injection/manipulation of the pups per se does not stimulate the pups' stress response but when combined with maternal RF it significantly increases the plasma GC levels in pups which is likely due to combination of maternal milk-derived GC together with sensitization of the pups to maternal stress behavior. Importantly, in Wistar rat pups the elevated GC levels correlated with the phase shifts of their SCN clock that were blocked by GR antagonist mifepristone. The SCN clocks of SHR pups seem to be hypersensitive to changes in the environment; they respond to stress imposed both on their mothers as well as

directly on them likely via GC-independent mechanism. These results justify the suitability of using the SHR as an animal model with stronger RF-related responses.

Importantly, our results provide the first evidence that the rat SCN clock may be shifted in sighted Wistar rat pups maintained under conditions of LD cycle via GC-dependent mechanisms. The *Bmal1* gene represents the positive limb of the transcriptional-translational feedback loop mechanism that is indispensable for the clock function, and its expression is not directly sensitive to light (Bunger *et al.*, 2000). The mechanism by which activated GRs could affect *Bmal1* expression in Wistar rats likely does not involve the classic genomic pathway because a GRE has not been identified in the *Bmal1* promoter. However, previous studies suggested the possibility of an indirect mechanism via non-classical signaling because GR dimers can directly modify the activity of various kinases (Koch *et al.*, 2017) that may potentially affect the clock mechanism. In the immature SCN, the mechanism is clearly distinguished from the light-dependent pathways because as we show here, *Bmal1* is responding to maternal cues, whereas the light-sensitive genes *Per1,2* (Shigeyoshi *et al.*, 1997) did not respond and remained phase-locked to the external LD cycle. The discordance between the *Bmal1* and *Per1, 2* expression profiles due to the stress exposure suggests the presence of subpopulations of cells within the developing pups' SCN that are either entrained with the LD cycle or respond to stress. In support of this speculation, we demonstrate that in the pups SCN, the areas of maximal *Bmal1* and *Per1,2* expression during the 24 h cycle do not entirely overlap. Additionally, recent findings demonstrated different phases of *Per1* and *Bmal1* rhythms that were simultaneously recorded in the same SCN explant in vitro (Ono *et al.*, 2017), further supporting the possibility of their independent regulation. Therefore, our data demonstrate that during the early postnatal period, the SCN clock is able to respond to maternal as well as photic cues. The latter mechanism gradually replaces the maternal entrainment.

6.4 IMPACT OF FOSTER MOTHER ON MOLECULAR CIRCADIAN CLOCK IN RAT PUPS AND ITS LONG-LASTING EFFECT IN ADULTHOOD

Results of our study provide strong evidence for the unexpectedly robust impact of altered maternal care provided by a foster mother on developing circadian clocks and on behavior and cardiovascular function in adulthood. Notably, altered maternal care significantly affected the developing circadian clocks in spite of the fact that the pups were maintained in the light/dark regime that is considered the dominant cue for entraining the circadian clocks, thus demonstrating the relevance of these findings under natural conditions. We found that altered maternal care affected the SCN and peripheral clocks of pups at the molecular level already during the early part of ontogenesis. Moreover, the effect of altered maternal care was exhibited in adulthood at the level of circadian behavior and heart rate. In general, the more intensive maternal care of Wistar rat mothers improved most of the parameters seen with the abnormal SHR circadian phenotype, and during early developmental stages, the less frequent maternal care of SHR mothers worsened these parameters in Wistar rats.

In this study, we found that in Wistar rat pups, the maternal care provided by SHR foster mothers disrupted the proper entrainment of their central SCN clock to the external light/dark cycle because it significantly phase-advanced the clock. The effect imposed on the pup's clock corresponded to the circadian phenotype of the SHR mother. In adult SHR, the phase of the SCN clock is abnormally advanced which causes a positive phase angle of the entrainment of the SCN-driven locomotor activity rhythm relative to the external light/dark cycle. Thus, the adult SHR have already begun to be active before lights off, whereas the Wistar rats become active only at the time of lights off (Sládek *et al.*, 2012). The magnitude of the SCN clock phase advance in 10-day-old Wistar pups reared by SHR foster mothers was approximately 1 h (1.0 h for *Per2*, 0.8 h for *Rev-erbα*, and 1.2 h for *Bmal1*), which is comparable to what we had previously observed in adult SHR (1.4 h for *Per2*, 0.8 h for *Rev-erbα*, and 1.7 h for *Bmal1*). Therefore, the abnormal behavioral phenotype of SHR mothers was completely imprinted on the phase of the SCN clock of 10-day-old pups although the pups were kept in the light/dark cycle that entrains their SCN clock at this age (Matějů *et al.*, 2009). Nevertheless, this detrimental maternal effect on the phase of the SCN clock of pups was not detectable at P30 or after weaning.

In SHR pups reared by their own mothers, the clock gene expression rhythms in the SCN spontaneously phase-advanced between P10 and P30, and in adulthood, their SCN

drove the locomotor activity rhythm that was phase-advanced relative to the light/dark cycle. The maternal care provided to SHR pups by the Wistar rat foster mothers significantly affected this process because it completely abolished the development of the positive phase-angle of the entrainment of the locomotor activity rhythm in adult SHR. Additionally, the maternal care of Wistar rat foster mothers facilitated the development of amplitude of *Bmal1* expression rhythm in the SCN of SHR, which has been found to be delayed in SHR compared to Wistar rats (Olejníková *et al.*, 2015). The cross-strain fostering procedure also completely reversed lower amplitude of the locomotor activity rhythm that was found in adult SHR (Sládek *et al.*, 2012). Therefore, the presumably better (more intensive and properly aligned) maternal care provided by Wistar rat mothers to SHR pups increased the robustness of the SCN clock-driven rhythm and improved its entrainment to the external light/dark cycle in adulthood.

Apart from the altered circadian phenotype and maternal care, nursing regime also differs between Wistar rat and SHR dams. Not only is the presence in the nest more fragmented especially during the first few days after birth (Olejníková *et al.*, 2015), but the milk production is lower in SHR dams as well (Rose and McCarty, 1994). Changes in maternal feeding regime have been shown to have a significant effect on the developing peripheral clocks located in the liver (Sládek *et al.*, 2007a) and colon (Polidarová *et al.*, 2014). Consistent with these findings, we found a significant negative impact of the abnormal maternal care of the SHR mother on the circadian clocks in the liver (but not on the colon) of Wistar rat pups at P10; cross-strain fostering robustly down-regulated the expression of *Per1*, *Per2* and *Rev-erbα* genes. However, the more intensive maternal care provided by the foster Wistar rat mothers to SHR pups had much smaller effect on their clocks in the liver and had almost no effect on the clock in the colon at P10. Unexpectedly, cross-strain fostering had enormous opposite impact on the peripheral clocks of 30-day-old pups of both strains. The expression of most studied clock genes was robustly upregulated during the day and night and/or amplitudes of the rhythmic expression profiles were significantly increased. The general pattern of the effect was very similar in both of the peripheral tissues tested but was more pronounced in the SHR than in the Wistar rat pups. These results led us to investigate whether the effect was caused by the presence of a foster mother of a different circadian phenotype providing altered maternal care or by the fostering procedure per se. We demonstrated that the effect was indeed specific to presence of a foster mother with a different circadian phenotype because when the pups were reared by a foster mother of the same strain, amplitudes of the clock gene expression profiles in the liver and

colon did not change at P30, with the exception of a small increase in amplitude of *Bmal1* of SHR pups. Moreover, returning the pups of both strains exposed to cross-strain fostering to their genetic mothers at P10 prevented the increase in amplitudes of the expression rhythms in the liver and colon of these pups (in Wistar rat pups, the prevention was partial, whereas in SHR pups, it was complete). These results revealed that in contrast to the effect on the SCN clock, the peripheral clocks responded more robustly to cross-strain fostering, and the response was stronger in SHR than Wistar rats. The interval between P10 and P30 represents a developmental period during which pups gain sight and, together with a gradual decline in breast-milk intake, start to consume solid food at night, which affects the phases of their peripheral clocks (Sládek, *et al.*, 2007a; Polidarová *et al.*, 2014). It seems that despite of a gradual weaning of maternal breast-feeding, the presence of a mother whose circadian system is not aligned with the genetically programmed pups' circadian system during this interval still has a significant impact on the pups' peripheral molecular clocks. The greater response of the peripheral clocks of SHR to cross-strain fostering is in agreement with previous findings that this strain exhibits significantly higher sensitivity to environmental cues (Häusler *et al.*, 1983) as well as to changes in feeding regime not only in adulthood (Polidarová *et al.*, 2013) but also during ontogenesis (Olejníková *et al.*, 2015). The generally higher sensitivity of the autonomous nervous system in SHR (Häusler *et al.*, 1983) may play a role in higher sensitivity of their peripheral clocks because activity of the nervous system is considered causal in their cardiovascular pathology, it innervates these peripheral tissues, and the clocks in the liver (Terazono *et al.*, 2003) and in the colon (Malloy *et al.*, 2012) are directly responsive to changes in adrenergic tonus. Apart from sensitivity to feeding regime, SHR also differ in sleep patterns (Carley *et al.*, 1996). The altered sleep pattern of a foster mother may also affect the sleep patterns of her pups, which could account for the observed effects on the circadian clocks. Importantly, the circadian phenotype of the Wistar rat foster mother remedied dampened amplitudes of the colonic clock in SHR pups. The beneficial effect of the care provided by the Wistar rat foster mothers for SHR pups was also confirmed by cardiovascular function measurements. Our data show that cross-strain fostering normalized the increased HR in SHR to the Wistar rat control levels, although it did not affect blood pressure (MAP, SBP and DBP). These results contrast previous studies that demonstrated a reduction in blood pressure due to cross-strain fostering without affecting the heart rate (McCarty *et al.*, 1992; Gouldsborough *et al.*, 1998). The lack of the effect on the blood pressure in our study could be explained by a differences in the age of the animals and the methodology of blood pressure measurements employed in this current study and in

studies from 1990s or by the use of a different strain of normotensive controls. However, in accordance with these previous studies, our results demonstrated that cross-strain fostering had no effect on any of these parameters in normotensive control pups.

In conclusion, while the importance of the maternal circadian system in the entrainment of the rat SCN has been previously suggested (Reppert and Schwartz, 1986; Reppert *et al.*, 1988; Ohta *et al.*, 2002; Varcoe *et al.*, 2013), here we demonstrate that the maternal care of a mother with misaligned circadian clocks may be a strong cue that can positively or negatively affect the circadian clocks in the SCN and the periphery of pups and may have long lasting effects on the circadian regulation of behavior later in adulthood. Altogether, our data provide compelling evidence that the maternal care of a foster mother of a different circadian phenotype may significantly impact the regulatory mechanisms of various physiological parameters even if the pathological symptoms are genetically programmed.

7 CONCLUSION

7.1 Ontogenetic development of circadian clock in colon of Wistar pups

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 for 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. Daily profiles of clock genes *Per1*, *Per2*, *Cry1*, *Cry2*, *Rev-erbα*, *Bmal1*, and *Clock* expression in the colon underwent significant modifications since embryonic day 20 through postnatal days 2, 10, 20, and 30 via changes in the mutual phasing among the individual clock gene expression rhythms, their relative phasing to the light-dark regime, and their amplitudes. An adult-like state was achieved around P20. The fostering study revealed that during the prenatal period, the maternal circadian phase may partially modulate development of the colonic clock. Postnatally, the absence and/or presence of rhythmic maternal care affected the phasing of the clock gene expression profiles in pups at P10 and P20. A reversal in the colonic clock phase between P10 and P20 occurred in the absence of rhythmic signals from the pup SCN. The data demonstrate ontogenetic maturation of the colonic clock and stress the importance of prenatal and postnatal maternal rhythmic signals for its development.

7.2 Development of circadian system in SHR pups

This study was aimed to elucidate how the SHR circadian system develops during ontogenesis and to assess its sensitivity to changes in maternal-feeding regime. Analysis of ontogenesis of clock gene expression rhythms in the suprachiasmatic nuclei, liver and colon revealed significant differences in SHR compare with Wistar rats. In the SCN and liver, the development of high-amplitude expression rhythm selectively for *Bmal1* was delayed compared with Wistar rat. The atypical development of the SHR circadian clocks during postnatal ontogenesis might arise from differences in maternal behavior between SHR and Wistar rats that were detected soon after delivery. It may also arise from higher sensitivity of the circadian clocks in the SHR SCN, liver and colon to maternal behavior related to changes in the feeding regime because in contrast to Wistar rat, the SHR SCN and peripheral clocks during the prenatal period and the hepatic clock during the early postnatal period were phase shifted due to the exposure of mothers to a restricted feeding regime. The maternal

restricted feeding regime shifted the clocks despite the fact that the mothers were maintained under the light/dark cycle. Our findings of the diverse development and higher sensitivity of the developing circadian system of SHR to maternal cues might result from previously demonstrated differences in the SHR circadian genotype and may potentially contribute to cardiovascular and metabolic diseases, which the animal model spontaneously develops.

7.3 Comparison of sensitivity to maternal stress in Wistar and SHR pups

In this study, mothers of two rat strains with different sensitivities to stress, i.e., Wistar rats and SHR, and their pups were exposed to stressful stimuli every day from delivery. The mothers of both strains responded to stressful stimuli with an elevation of plasma glucocorticoid levels, but the response was stronger in SHR mothers. The glucocorticoid receptors were detected at the mRNA and protein levels in the SCN of 4-day-old pups. We found out that in Wistar rat pups, combination of daily maternal stress with the stress caused by their manipulation increased the plasma levels of glucocorticoids and shifted the rhythm of expression of clock gene *Bmal1* in the SCN. This effect was completely blocked by administration of glucocorticoid receptor antagonist. In contrast, in SHR pups, maternal stress on its own was able to shift the phase of the *Bmal1* expression rhythm in the SCN. This effect, however, was mediated probably not only via glucocorticoid-independent mechanism and it would be very interesting topic for further investigation. The expression profiles of *Per1* and *Per2* clock genes in the SCN remained phase-locked to the light/dark cycle in both rat strains. Our results demonstrate that the SCN of rat pups maintained under light/dark conditions expresses glucocorticoid receptors and is sensitive to stressful stimuli early after birth. The impact of stress during early life on health has been a topic of extensive research. This study provides evidence for the existence of a developmental window immediately after birth when stress may affect the function of the circadian clock in the SCN via glucocorticoid-dependent mechanism.

7.4 Impact of foster mother on molecular circadian clock in rat pups and its long-lasting effect in adulthood

The differences in maternal care between Wistar and SHR rats were proved earlier as well as the differences in circadian phenotypes in two studied rat strains. In this study, the impact of altered maternal care on the circadian system was assessed using the cross-strain fostering approach. As normal care we considered the one performed by Wistar mothers and the "abnormal" care was represented by SHR mothers. The SHR mother worsened the

entrainment of the central clock in the SCN to the external light/dark cycle in Wistar rat pups but this negative effect on phasing disappeared after weaning. The presumably better maternal care provided by Wistar rat mother to SHR pups improved amplitude of the SCN clock-driven rhythm and its entrainment to the external light/dark cycle in adulthood. The peripheral clocks in the liver and colon responded more robustly to the cross-strain fostering; importantly, the circadian phenotype of the Wistar rat foster mother improved the dampened amplitudes of the colonic clock in SHR. The beneficial effect of the care provided by the Wistar rat foster mother for SHR was also confirmed by measurement of cardiovascular functions. In general, the more intensive maternal care of the Wistar rat mother improved most of the parameters of the abnormal SHR circadian phenotype and, the less frequent maternal care of the SHR mother worsened these parameters in Wistar rat, but only during early developmental stages. Altogether, our data provide compelling evidence that the maternal care of foster mother of a different circadian phenotype may significantly impact regulatory mechanisms of various physiological parameters even if the pathological symptoms are genetically programmed.

8 SUMMARY

In my PhD thesis, I focused on the ontogenetic development of the circadian system and development of its entrainment in two rat strains – Wistar and SHR.

Our data concerning the development of the colonic circadian clock provide us with the information when the clock in the colon begins to be fully functional. This knowledge may contribute to better understanding of various colonic function-related diseases in newborn children.

SHR is an animal model which is used not only as a model of cardiovascular and metabolic diseases. Based on the previous results of our laboratory, it could also be used as a valuable model of human disorders originating in poor synchrony of the circadian system with external conditions. The insight into the diverse ontogenetic development, altered maternal care and differences in synchronization of circadian clocks in pups of this rat strain may facilitate the understanding of the origin of cardiovascular and metabolic disorders in human. Results of the studies on the development of the circadian system during ontogenesis may help us to understand the connections between its developmental disruptions and various pathologies in adulthood. Our findings underline the importance of regular timing of food intake during development and proper maternal care. They can be utilized as recommendations for maternal care during pregnancy, for care of prematurely born infants and maternal care of full-term born children.

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SHRNUTÍ

Ve své dizertační práci jsem se zabývala ontogenetickým vývojem cirkadiánního systému a vývojem jeho synchronizace u dvou kmenů potkana – Wistar a SHR.

Získaná data o vývoji cirkadiánních hodin v tlustém střevě poskytují informace o tom, kdy začínají být tyto hodiny plně funkční, což může přispět k lepšímu pochopení různých funkčních onemocnění střeva u novorozených dětí.

SHR představuje kmen potkana používaný nejen jako model kardiovaskulárních a metabolických onemocnění, ale na základě předchozích výsledků naší laboratoře může být použit také jako model poruch synchronizace cirkadiánního systému s vnějšími podmínkami. Poznatky o rozdílech v ontogenetickém vývoji, mateřské péči a synchronizaci cirkadiánních hodin u mláďat tohoto kmene mohou usnadnit porozumění vzniku kardiovaskulárních a metabolických poruch u člověka. Výsledky studií o ontogenezi cirkadiánního systému nám mohou pomoci pochopit souvislosti mezi vývojovými poruchami a různými patologickými stavy v dospělosti. Naše zjištění podtrhují význam správného načasování příjmu potravy v průběhu vývoje mláďat a řádné mateřské péče. Výsledky mohou být využity jako doporučení pro těhotné ženy, při péči o předčasně narozené děti i o děti donošené.

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10 ATTACHMENTS

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