Entrainment of the circadian clock within the rat suprachiasmatic nucleus during fetal and early postnatal development

Rehab Essam El-Din El-Hennamy

Supervisor: PharmDr. Alena Sumová, CSc.

Charles University in Prague

Faculty of Sciences

2008

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation and deepest gratitude to PharmDr. Alena Sumová for choosing me to work in her laboratory, her supervision and guidance of the doctoral research and for assistance and encouragement on various phases of this study.

My gratitude is also extended to Prof. Dr. Helena Illnerová for her advice, and valuable lectures on Chronobiology.

I would like to thank all my colleagues in the laboratory, for helping me to do my practical work and their kind support. They include Dr. Zdena Bendová, Kristýna Matějů, Dr..Zuzana Kováčiková, Dr. Martin Sládek, and Dr. Serhiy Sosniyenko. I would like also to thank Eva Suchanová and Jiří Sedlmajer for their excellent technical assistance.

I wish also to express my cordial thanks and gratitude to the staff members of the Institute of Physiology of the Academy of Sciences of the Czech Republic for providing all facilities to achieve this work.

Abstract

All mammals exhibit daily rhythms which persist in non-periodic environment with a period close to 24h. These rhythms are entrained to the 24h day mostly by the light-dark cycle. These circadian rhythms are controlled by a clock (pacemaker) located in the suprachiasmatic nuclei (SCN) of the hypothalamus. In the rat, the circadian clock within the SCN develops gradually from prenatal to postnatal period and is supposed to be synchronized mainly by maternal signals. However, the rat SCN is sensitive to light immediately after birth. **The aim** of the present work was to investigate the mechanism of entrainment of the circadian clock within the rat SCN during fetal and early postnatal development. The specific questions were whether and when the immature fetal and neonatal molecular SCN clocks can be reset by maternal cues, and whether and when the external light-dark cycle (LD) can affect the developing circadian rhythms. The role of light-dark cycle in the development of the photoperiodic entrainment during early postnatal period was also examined. **Experiment no. (1):** Pregnant rats were maintained under a light – dark regime with 12 h of light and 12 h of darkness (LD12:12). At gestational day 20 (E20), the fetuses were sampled throughout the day under either LD12:12 or constant darkness (DD). The daily profile of *c-fos* gene expression was determined by *in situ* hybridization. **Experiment no. (2):** Pregnant rats were maintained under LD 12:12 until the day of delivery, then released into DD and the pups were sampled in DD at postnatal day 3 (P) 3. The daily profiles of clock gene expression within the SCN of pups maintained in DD were compared with those of pups kept under LD12:12 (Sládek, *et al*, 2004). **Experiment (3)**: Pregnant rats were maintained under a long photoperiod (LD 16:8) and their pups were sampled under the same LD regime at P3 and P10. The daily profiles of clock gene expression within the SCN of pups maintained under LD16:8 were compared with those of pups sampled under DD (Kováčiková *et al*, 2005). **Experiment (4):** Pregnant rats were maintained under LD12:12. They were exposed to a 6 h delay of the dark period at different stages of the fetal SCN development and, thereafter, they were released into constant darkness. Adult male rats maintained under the same LD regime were exposed to an identical shifting procedure. Daily rhythms in spontaneous *c-fos, Avp*, *Per1* and *Per2* expression were examined within the adult and newborn rat SCN. **Result (1)**: A clear rhythm of *c-fos* gene expression was detected at E20. **Result (2)**: In pups at P3, significant rhythms in *Per1, Per2*,

Cry1 and *Bmal1* expression were detected under DD. The phase of the rhythms in clock gene expression were not different under LD and DD conditions, however, slight differences in the time of the rise and the decline *of Per1*, *Per2* and *Cry1* mRNA levels between the pups maintained under DD and those maintained in LD were detected. **Result (3**): In 10-day-old pups maintained under LD16:8, presence of the LD cycle induced an advance of the rise in *Per1*, *Per2* and *Bmal1*, expression as compared to pups released into DD. The effect on *Cry1* expression was only suggested. However, the presence of the LD cycle at P3 induced only slight advance of the rise in *Per1* and *Per2* mRNA, but not of *Cry1* and *Bmal1* mRNA. **Result (4)**: Exposure of adult rats to a 6-h phase delay of the dark period (shifting procedure) induced a significant phase delay of locomotor activity within 3 days after the phase shift as well as a delay in the rhythms of *c-fos* and *Avp* expression within three days and *Per1* and *Per2* expression within five days after the shift. Exposure of pregnant rats to the shifting procedure at E18, but not at E20, phase delayed the rhythm in *c-fos* and *Avp* expression in the SCN of newborn pups at P0-1. The shifting procedure at E20 did, however, induce a phase delay of *Per1* and *Per2* expression rhythms at P3 and P6. Hence, five days were necessary for phase-shifting the pups' SCN clock by maternal cues, while only three days were necessary for phase-shifting the maternal SCN by photic cues. From the present studies we can **conclude** that: **(1)** Expression of *c-fos* gene exhibits circadian rhythmicity within the SCN at E20, which indicates a significant rhythm in the neuronal activity during the fetal SCN development. **(2)** Absence of the LD cycle does not affect the phase of the developing rhythms in expression of clock genes within the SCN at P3. Therefore, the phase of the developing circadian clock is primarily set by maternal cues. **(3)** At P10, presence of the LD cycle modulates the photoperiodic entrainment of the rhythm in clock gene expression, namely the *Per1*, *Per2* and *Bmal1* mRNA rhythms in pups maintained under a long photoperiod. At P3, the modulation was only marginal. Thus, presence of the LD cycle facilitates entrainment to the long photoperiod during early postnatal development. **(4)** The SCN clock is capable of significant phase shifts at fetal developmental stages when no or very faint molecular oscillations can be detected. This finding suggests that maternal cues are the most important cues for entrainment of the developing clock during prenatal and early postnatal development.

List of abbreviations

AA-NAT: N-acetyltransferase

AVP: Arginine vasopressin

bHLH: Basic helix loop helix

BIT: Brain immunoglobulin-like molecule

***Bmal1*:** Brain and muscle Arnt-like protein 1

CaMKII: Calcium/calmodulin kinase

***Ck1ε*:** Casein kinase 1ε

CREB: c-AMP response element binding protein

CRH: Corticotrophin releasing-hormone

***Cry*:** Cryptochrome

CSF: Cerebrospinal fluid

DBP: D-element binding protein

DD: constant darkness

DMH: Dorsomedial hypothalamic nucleus

dm-SCN: dorsomedial SCN

dsPVZ: dorsal subparaventricular zone

dSPZ: dorsal subparaventricular zone

E: embryonic day

GABA: γ-aminobutyric acid

GHT: Geniculohypothalamus

GRP: Gastrin releasing peptide

IGL: Intergeniculate leaflet of the thalamus

IEGs: Immediate early genes

LD: light-dark cycle

LDH-A: Lactate dehydrogenase A

LHA: lateral hypothalamus

LP: long photoperiod

MCH: melanin concentrating hormone

MPO: Medial preoptic region

P: postnatal day

PACAP: Pituitary adenylate cyclase-activating peptide

PAS: Period-Arnt-single-minded

***Per*:** Period

PKGII: c-GMP-dependent protein kinase

PVHd: dorsal parvicellular paraventricular nucleus

PVHm: medial parvicellular paraventricular nucleus

RHT: retinohypothalamic tract

SCN: suprachiasmatic nucleus

SP: short photoperiod

sPVZ: Subparaventricular zone

SPZ: Subparaventricular zone

SS: somatostatin

TEF: Transcriptional enhancer factor

TRH: Thyrotropin-releasing hormone

VIP: vasoactive intestinal polypeptide

VLPO: ventrolateral preoptic nucleus

vl-SCN: ventrolateral SCN

VMH: Ventromedial nucleus

vsPVZ: ventral subparaventricular zone

vSPZ: ventral subparaventricular zone

List of Figures and Tables

Contents

1. INTRODUCTION

Most organisms living on the Earth are under the influence of daily and seasonal changes resulting from the planet's rotation around its axis and around the Sun. As a result, many biological functions decrease and increase in cycles that repeat each day, month, or year. This fluctuation in the biological functions is known as a biological rhythm. Biological rhythms are classified into 3 categories according to their period length:

1– Ultradian rhythms are rhythms with a period length shorter than 24 hours, e.g. the rhythm in neuronal firing.

2- Infradian rhythms are rhythms with a period length which is longer than 24 hours, e.g. the menstrual cycle that is completed every 28 days.

3- Circadian rhythms (see bellow).

1. 1. Circadian rhythms

Circadian rhythms are defined as oscillations that display cycles with period length about 24 h, hence the term circadian from the Latin *circa dies*, which means about a day. Many physiological parameters exhibit a circadian rhythm including the sleep-wake cycle, body temperature, immune responses, digestion, susceptibility to anesthesia, or dental pain threshold. Our visual and mental activity also fluctuates during the day. There are daily oscillations in the level of enzymes and hormones that affect the timing of cell function, division, and growth, etc. (Edery, 2000).

Circadian rhythms exhibit 3 characteristics (Pittendrigh and Daan, 1976):

1- They persist (or free-run) with a period of ≈24 h in absence of external time cues (Zeitgebers).

2- They are reset or synchronized by changes in environmental conditions, mostly by the light – dark cycle.
3- Their periods vary only slightly with changes in ambient temperature (temperature compensation).

A circadian rhythm is generated by a molecular clock. The clockwork consists of interlocking transcriptional and translational feedback loops involving at least nine primary clock genes, namely *Per1*, *Per2*, *Per3*, *Cry1*, *Cry2*, *Bmal1*, *Clock*, *Rev-erbα* and casein kinase 1ε (*Ck1ε*) (King and Takahashi, 2000; Lowrey and Takahashi, 2004). The principle molecular clock or the pacemaker is located in the suprachiasmatic nuclei (SCN) of the hypothalamus (Moore, 1991). Beside the SCN pacemaker, nearly every tissue in the body, such as liver, heart, muscle, spleen, etc., contain peripheral clocks that are synchronized by the SCN pacemaker. Peripheral clocks drive local rhythms specific for the tissue function (Schibler *et al*., 2003).

In adult mammals, the circadian clock is mainly entrained by the light-dark cycle that acts via the retinohypothalamic pathway. In altricial rodents, the photic pathways to the fetal SCN are not fully developed. Therefore, non-photic maternal cues seem to be dominant to synchronize fetuses and neonatal pups. The maternal circadian system may also provide the immature mammal with important photoperiodic information (Reppert *et al*., 1985).

2. Review

2.1 Circadian clock

The central circadian clockwork consists of negative and positive feedback loops, or limbs. The positive limb involves CLOCK/BMAL1 heterodimers, two basic helix-loop-helix (bHLH)-PAS (Period-Arnt-Single-minded)-containing transcription factors, (King *et al*., 1997; Gekakis *et al*., 1998; Hogenesch *et al*., 1998; and Bunger *et al*., 2000). CLOCK-BMAL1 heterodimers activate transcription by binding to E box enhancers with highly selective nucleotide sequence CACGTG. Specifically, CLOCK-BMAL1 heterodimers activate transcription of three period genes (m*Per1*-m*Per2*-m*Per3* in the mouse) and two cryptochrome genes (m*Cry1* and m*Cry2).* CRY and PER proteins form oligomers that are transported from the cytoplasm to the nucleus. When located in the nucleus, CRY and PER repress their own transcription by inhibiting CLOCK-BMAL1 (negative limb) (Kume *et al*., 1999; Vitaterna *et al*., 1999; Okamura *et al*., 1999; Shearman *et al*., 2000). CLOCK-BMAL1 also induce expression of the nuclear orphan receptor REV-ERBα (Preitner *et al*, 2002), which in turn represses transcription of *Bmal1* through direct binding to a REV–ERBα/ROR response element in the *Bmal1* promoter (Ueda *et al*., 2002; Preitner *et al*., 2002). mCRY proteins also inhibit *Rev-Erbα* transcription resulting in activation of *Bmal1* transcription (Yu *et al*., 2002, Preitner *et al*., 2002) (Fig. 1).

In addition to transcriptional regulation, the circadian clock is also regulated by posttranslational mechanisms. PER and CRY proteins shuttle between the nucleus and the cytoplasm. In the cytoplasm, *Ck1 ε* (and probably also other kinases) phosphorylates PER and the phospho-PER becomes degraded by the proteasome machinery. Within the nucleus, CRY protein binds PER and prevents it from leaving the nucleus (Yagita *et al*., 2002). Thus, the 24-h periodicity of the circadian clock results from a combination of negative and positive transcriptional feedback loops, nuclear-cytoplasmic shuttling and phosphorylation and degradation of PER.

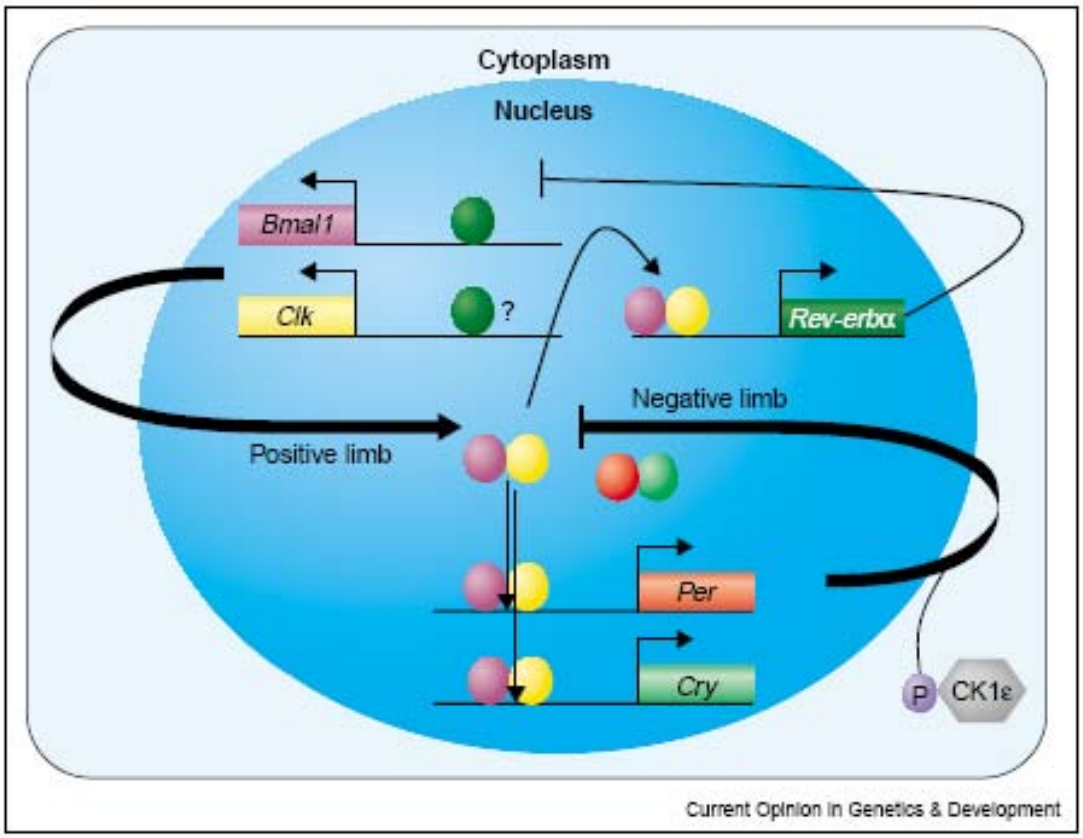


Fig. 1: Diagram of the circadian clock mechanism (after Albrecht and Eichele, 2003).

CLOCK (yellow) and BMAL1 (pink) proteins (positive limb) drive the expression of *Per*, *Cry* and *Rev-erbα* genes in the nucleus. PER (red) and CRY (light green) proteins in the nucleus inhibit CLOCK/BMAL1 action by a yet unknown mechanism and thereby down-regulate their own expression and that of *Rev-erbα* (dark green). When REV-ERBa protein is absent, *Bmal1* (and possibly also Clock) genes are derepressed and hence transcribed to produce new CLOCK/BMAL1 transcription factors that reinitiate a new circadian cycle. Clock proteins are posttranslationally modified; casein kinase I ε (*CKI ε*), for example, phosphorylates PER2. Hyperphosphorylation of PER2 decreases its stability and thus promotes its degradation. A typical circadian cycle would begin with activation of *Per* (and *Cry*) transcription by CLOCK/BMAL1 in the early morning. Transcript levels peak around noon and protein levels in the cytoplasm reach the zenith ~2 hours later. PER shuttles between the cytoplasm and the nucleus. In the cytoplasm, it is degraded following hyperphosphorylation and in the nucleus it is complexed with CRY and thereby blocks CLOCK/ BMAL1 function resulting in termination of *Per* and *Cry* transcription. At some point when much PER is degraded in the cytoplasm, PER concentration in the nucleus is too low to keep up negative feedback and the cycle reinitiates.

2.1.1. Mutations in mammalian clock genes

Loss or mutation of clock genes leads to altered period length (*Cry1, Cry2*, *Per*1*, Per3*, and *CKIε*), deceleration (*Clock*, *Per2*) or immediate loss (*Bmal1*) of circadian rhythmicity under constant conditions (table 1) (Albrecht, 2002). *Cry1*/*Cry2* (van der Horst *et al*., 1999; Vitaterna *et al*., 1999) and *Per1*/*Per2* (Bae *et al*., 2001; Zheng *et al*., 2001) double mutant animals lose circadian rhythmicity immediately under constant conditions, confirming the importance of these genes in the clock mechanism.

Gene Affected	Period Length in Constant Darkness, h	Protein
Bmal1	No rhythm	Probably no functional protein
CkI ε (hamster)	20–22	Arg^{178} to Cys^{178} mutation
Clock	27.26 ± 0.22 (loses rhythm)	Deletion of amino acids 514–564
Cry1	22.51 ± 0.06	No protein
Cry2	24.63 ± 0.06	No protein
Per1	21.6–23.8 22.6 ± 0.3 (loses rhythm)	No protein
Per2	22.1 ± 0.4 (loses rhythm)	Deletion of amino acids 348–434, no protein
Per3	23.27 ± 0.18	No protein

Table 1: Effect of mammalian circadian clock gene mutation on period length and their protein expression (after Albrecht, 2002).

2.1.2 Target genes of the circadian clock

Numerous genes are circadianly regulated in different organs and tissue. These genes are called clock controlled genes and represent the output pathway of the circadian system. They facilitate daily modulation of many physiological processes, such as blood pressure (lowest just after midnight), mental performance (best in the mid afternoon), or hormones (cortisol is highest in the morning, melatonin at night) (Akhtar *et al*., 2001; Panda *et al*., 2002; Storch *et al*., 2002). Some examples of the clock controlled genes that are rhythmically regulated by CLOCK/BMAL1 heterodimers are, e.g., arginine vasopressin gene (AVP) in the SCN (Albrecht and Oster, 2001), as well as transcription factor D-element binding protein (DBP), lactate dehydrogenase A (LDH-A) and transcriptional enhancer factor (TEF) in peripheral tissues (Wuarin and Schibler, 1990, Jin *et al*., 1999, Ripperger *et al*., 2000).

2.2. Suprachiasmatic Nuclei (SCN)

2.2.1. Anatomical background

The SCN of the hypothalamus is the principle circadian pacemaker in mammals responsible for the generation and regulation of rhythms in behavioral state, performance, hormonal secretion, and physiological functions. The SCN also appears to function as a seasonal clock underlying photoperiodic time measurement (Sumova *et al*., 1995b, Schwartz *et al*., 2001). SCN is located bilaterally above the optic chiasm close to the third ventricle in the anterior basal hypothalamus (Meijer and Rietveld., 1989; Klein *et al*., 1991; and Miller *et al*., 1996). Each SCN is 0.9-1.0 mm in length along the rostro-caudal axis and 0.4 mm in width at the central section. The SCN volume of the adult rat is estimated to be 0.13-0.16 mm^3. The SCN has been divided into a dorsomedial shell (dm-SCN) and a ventrolateral core (vl-SCN) according to the innervations pattern and the type of cells (Moore, 1996). Each SCN contain approximately 8000-10000 neurons and a fewer number of glial cells (Van den Pol *et al*., 1992). The SCN neurons are autonomous circadian oscillators (Welsh *et al*., 1995; Herzog *et al*., 1998; and Honma *et al*., 1998). In intact SCN, these neurons usually synchronize each other with defined phase relationships (Herzog *et al*., 1997; Quintero *et al*., 2003; Schaap *et al*., 2003; and Yamaguchi *et al*., 2003). γ-aminobutyric acid (GABA) (Liu and Reppert, 2000),

vasoactive intestinal polypeptide (VIP) and gap-junction communication (Jiang *et al*., 1997; Aton *et al*., 2005; Colwell., 2000; and Long *et al*., 2005) are proposed as mediators maintaining the synchrony between SCN neurons.

2.2.2. Chemical components of the SCN

Most SCN neurons contain an inhibitory transmitter, GABA (Van den Pol, 1986; Okamura *et al*., 1989; More and Speh, 1993). Abundant SCN cells are sensitive to glutamate (Kim and Dudek, 1991; Selim *et al*., 1993). High levels of serotonin (Kiss *et al*., 1984), histamine (Inagaki *et al*., 1988) and moderate levels of norepinephrine are also found in the SCN. Dopamine and acetylcholine are very low (Brownstein and Balkovits, 1984). In addition to the neurotransmitters, the SCN contain several peptides, namely:

a- AVP is localized in the dm-SCN (Okamura *et al*., 1994). AVP concentration exhibit a clear circadian rhythm in SCN and cerebrospinal fluid (CSF) with higher levels during the day time (Earnest and Sladek, 1986, 1987). The rhythm in AVP levels exists under both light-dark cycle (LD) and constant darkness (DD) condition (Söderstein *et al*., 1985; Yamase *et al*., 1991) which suggests that AVP level in the SCN oscillates endogenously (Inouye and Shibata, 1994). AVP has been recognized as a clock controlled gene and, therefore, mediates the output from the SCN pacemaker (Inouye and Shibata, 1994).

b- Somatostatin (SS) is located in the dm-SCN and in the central region along the border with the vl-SCN (Card *et al*., 1988). The SS content displays a circadian rhythm under both LD and DD conditions with higher levels during the day time. SS may regulate circadian rhythm in the SCN (Shinohara *et al*., 1991).

c- VIP is a neuropeptide of the vl-SCN (Card *et al*., 1981). VIP levels in the SCN decrease during the light period of the LD cycle, increase during the dark period of the LD cycle and remain constant under DD condition (Morin *et al*., 1991). Level of VIP could mediate photic information to the pacemaker or act on the input pathway to the pacemaker (Inouye and Shibata, 1994). Recent studies indicate that VIP plays two main roles in the SCN, i.e., a role in sustaining the circadian rhythmicity within a single cell

(Harmar, 2003), and in synchronizing the cells to each other (Aton *et al*., 2005; Maywood *et al*., 2006; Welsh, 2007).

d- Gastrin releasing peptide (GRP) is positioned in the vl-SCN (Okamura *et al*., 1986).

2.2.3. Organization of afferent, efferent and local circuits within the SCN

The core of the SCN receives direct retinal input through the retinohypothalamic tract (RHT), indirect visual input through the geniculohypothalamus tract (GHT) from the intergeniculate leaflet of the thalamus (IGL), and serotonergic input from the raphe nuclei in midbrain. The shell of the SCN possibly receives input from other hypothalamic nuclei and parts of the limbic system. The shell sends efferents to the dorsomedial hypothalamic nucleus (DMH) and medial subparaventricular zone (sPVZ) while the core projects to the lateral sPVZ. The core densely innervates the ipsilateral shell and there is a connection between the two shells and cores through commissural projections (Fig.2) (Leak *et al*., 1999).

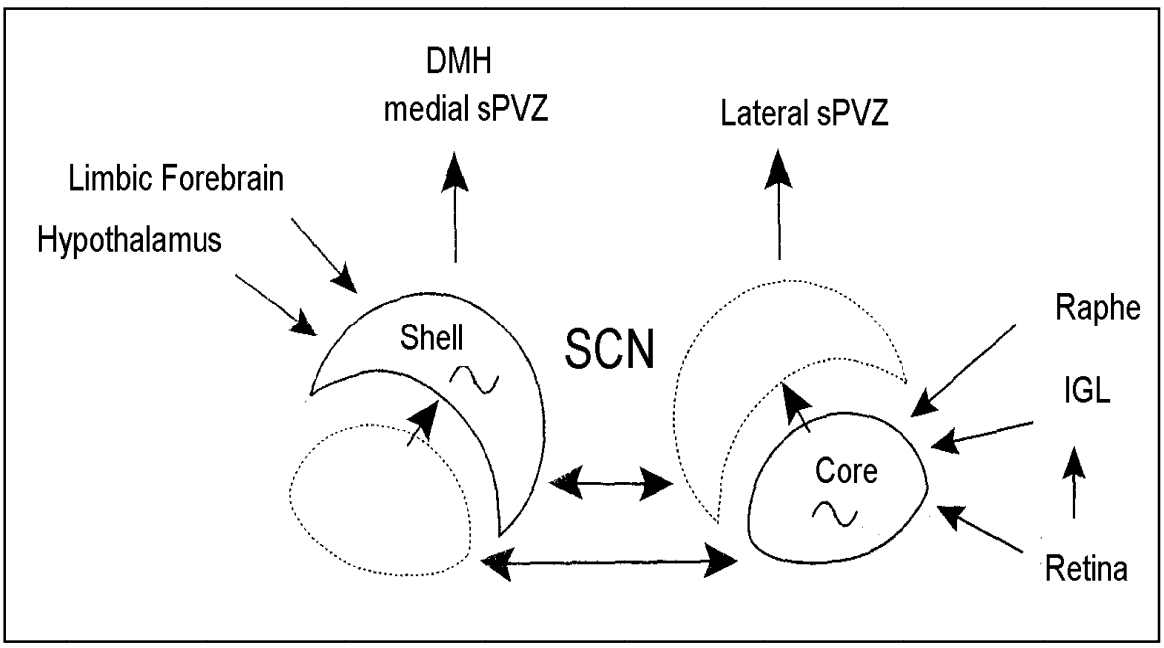


Fig. 2: Organization of afferent, efferent and local circuit within the SCN (after Leak *et al*.,1999).

2.2.4. SCN targets that regulate circadian cycles of specific functions

The SCN sends rostral projection into the preoptic area, caudal projection to the retrochiasmatic area, dorsal pathway to the paraventricular nucleus of the thalamus and to the lateral thalamus, and minor projection to the lateral geniculate nucleus (Watts, 1991). Through the projections, the SCN controls variety of circadian rhythms in different functions. Rhythm in melatonin levels depends on a direct projection to the paraventricular nucleus, whereas direct projection to the dorsal sPVZ (dsPVZ) mediates the body temperature rhythm. However, sleep-wake and locomotor rhythms (and probably feeding and corticosteroid cycles) depend on two relays, one from the SCN to the ventral sPVZ (vsPVZ) and a second from the vsPVZ to the dorsomedial nucleus (Fig.3) (Saber *et al*., 2005).

Apart from neural pathways, the SCN utilizes humoral patways. SCN seems to contain neurons that specifically target the liver, pineal, and adrenal (Kalsbeek, *et al*., 2006) and affects secretion of several hormones, e.g.:

1- Blood corticosterone levels is controlled by SCN via AVP and possibly GABA (Kaneko, *et al*., 1980; Kalsbeek, *et al*., 1996; and Buijs *et al*., 1999). AVP has an inhibitory effect on the release of adrenal corticosterone in nocturnal rodent, while it has a stimulatory effect in diurnal rodents (Kalsbeek, *et al*., 2008).
2- Insulin secretion (Nagai, *et al*., 1996; Kalsbeek and Struble 1998; and La Fleur *et al*., 2001).
3- Melatonin secretion is controlled by the SCN via GABA-mediated inhibition of autonomic PVN neurons (Kalsbeek *et al*., 2000).
4- Gonadal hormones (De La Iglesia *et al*., 1995).

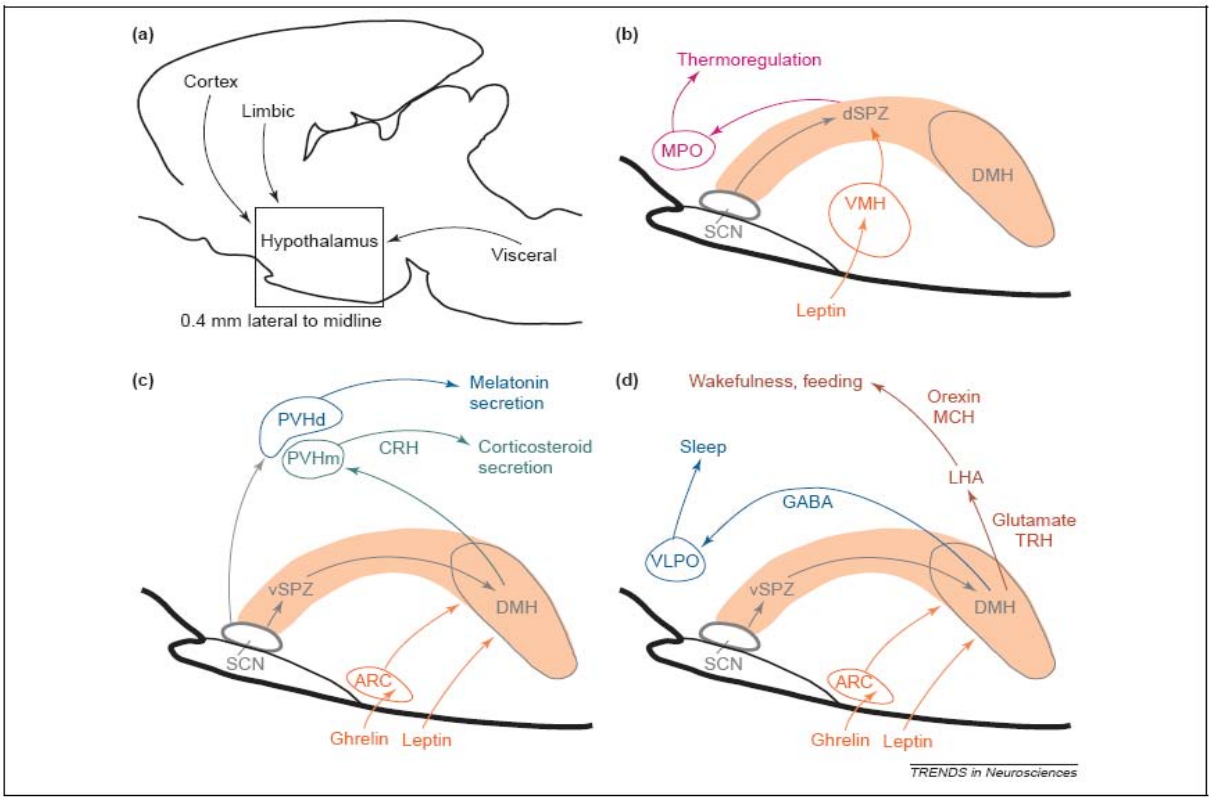


Fig. 3. Summary of the CNS pathways that connect the SCN with other brain areas to drive circadian rhythms of physiology and behavior (after Saber *et al*., 2005).

(a) A sagittal drawing of the rat brain showing the area of the hypothalamus illustrated in panels (b–d). The hypothalamus receives three types of non-photic input that affect the regulation of circadian rhythms: cognitive input from the infralimbic, prelimbic and insular cortex; emotional input from the limbic system, including the hippocampus and amygdala; and visceral input from the nucleus of the solitary tract and parabrachial nucleus. (b–d) The SCN provides its most intense output to the subparaventricular zone (SPZ) and dorsomedial nucleus of the hypothalamus (DMH). Food-related cues from the hormones ghrelin and leptin (orange) are relayed from the arcuate nucleus (ARC) and ventromedial nucleus (VMH), in addition to impinging directly on the DMH and dorsal SPZ (dSPZ). (b) The dSPZ is crucial for controlling circadian rhythms of body temperature, through its projections to the medial preoptic region (MPO), which includes the median preoptic and ventromedial preoptic nuclei. (c) The SCN sends a projection to the dorsal parvicellular paraventricular nucleus (PVHd). PVHd neurons project to sympathetic preganglionic neurons in the spinal cord, which in turn regulate melatonin output by the pineal gland. The ventral SPZ (vSPZ) sends a relay to the DMH, which in turn controls a wide range of circadian responses, including corticosteroid secretion (c), and feeding and sleep–wake cycles (d). Outputs to the medial parvicellular paraventricular nucleus (PVHm) regulate the neurons containing corticotrophin releasing-hormone (CRH), which in turn control pituitary regulation of corticosteroid production. Projections from the DMH to the sleep-promoting ventrolateral preoptic nucleus (VLPO) are GABAergic, whereas outputs to orexin-producing and melanin-concentrating hormone (MCH)-producing neurons in the lateral hypothalamus (LHA) contain glutamate and thyrotropin-releasing hormone (TRH). The orexin-containing and MCH-containing LHA neurons are important for maintaining wakefulness and feeding, consistent with a role for the DMH in driving the arousal components of circadian cycles.

2.2.5. Circadian rhythmicity within the SCN

The SCN neurons themselves exhibit circadian rhythmicity in:

1- Neuronal firing rate (Zlomanczuk and Schwartz 1999).

2- The uptake of 2-deoxyglucose as a marker of metabolic activity (Schwartz 1991).

3- Neuropeptide levels (Inouye and Shibata 1994).

4- Spontaneous as well as light-induced expression of immediate early genes (IEGs), namely *c-fos*, a marker of neural activity (Kornhauser *et al*., 1993, Sumová *et al*., 1998, Guido *et al*., 1999 and Schwartz *et al*., 2000).

2.3. Resetting the circadian clock

2.3.1. Resetting the central clock by light

Environmental light-dark cycle is the most important cue for entraining and resetting the phase of the central clock. Exposing an organism to a light pulse at night induces significant phase shift in circadian rhythms. Pulses administered during late night advance the rhythms while pulses administered during the early part of the night delay them (Hastings and Sweeney, 1958; DeCoursey, 1960). Cycles of light have similar effects as short light pulses. Exposure to constant light disrupts overt rhythms and induces circadian arrhythmicity in mammals (Pittendrigh and Daan, 1976).

In mammals, the circadian photoreceptors responsible for the photic resetting of the circadian clock are localized within the eye and include both visual and non-visual photoreceptors. The visual photoreceptors are classical retinal photoreceptors, rods and

cones. The non-visual photoreceptors are localized within a small subset of retinal ganglion cells containing the opsin-like protein melanopsin that project to the SCN (Beaule *et al*., 2003).

The photic information is transferred from the retina to the SCN via the monosynaptic RHT and polysynaptic GHT tracts. Within the SCN, light induces a cascade involving the release of glutamate from the RHT terminals, stimulation of ionotropic NMDA receptors, influx of Ca^{2+}, activation of nitric oxide synthase and serine/threonine protein kinases, phosphorylation of cAMP response-element binding protein (CREB) and regulation of target gene transcription, e.g., IEGs *c-fos*, *junB*, and clock gene *Per* (Gillette and Tischkau, 1999; Yan *et al*., 1999).

Calcium/calmodulin kinase (CaMKll), which activates the MAPK pathway (Butcher *et al*., 2002), may participate in the phase delay of the clock and in light-dependent induction of *Per1* and *Per2* genes via the CREB/CRE transcriptional pathway (Yokota *et al*., 2001; Nomura *et al*., 2003). In parallel, light induces tyrosine phosphorylation of a transmembrane glycoprotein, BlT (Nakahata *et al*., 2000). Phosphorylation of BlT activates MAPK pathway and resets the clock (Nakahata *et al*., 2003). For phase delay of the clock, photic induction of *Per2* mediated via cGMP-dependent protein kinase ll (PKGll) is required (Oster *et al*., 2003) (Fig.4). Other studies have implicated cGMP-PKG pathway as being critical for the phase advance by light in late night (Gillette and Mitchell, 2002; Tischkau *et al*., 2003).

2.3.2. Resetting the central clock by non-photic stimuli

Several non-photic stimuli are capable of resetting the central clock:

a- Melatonin, a hormone of pineal gland that exhibits a circadian rhythm and its secretion is regulated by the pacemaker (Lynch *et al*., 1975, 1978). In blind individuals, orally administered melatonin synchronized circadian rhythms (Sack *et al*., 1990). In sighted

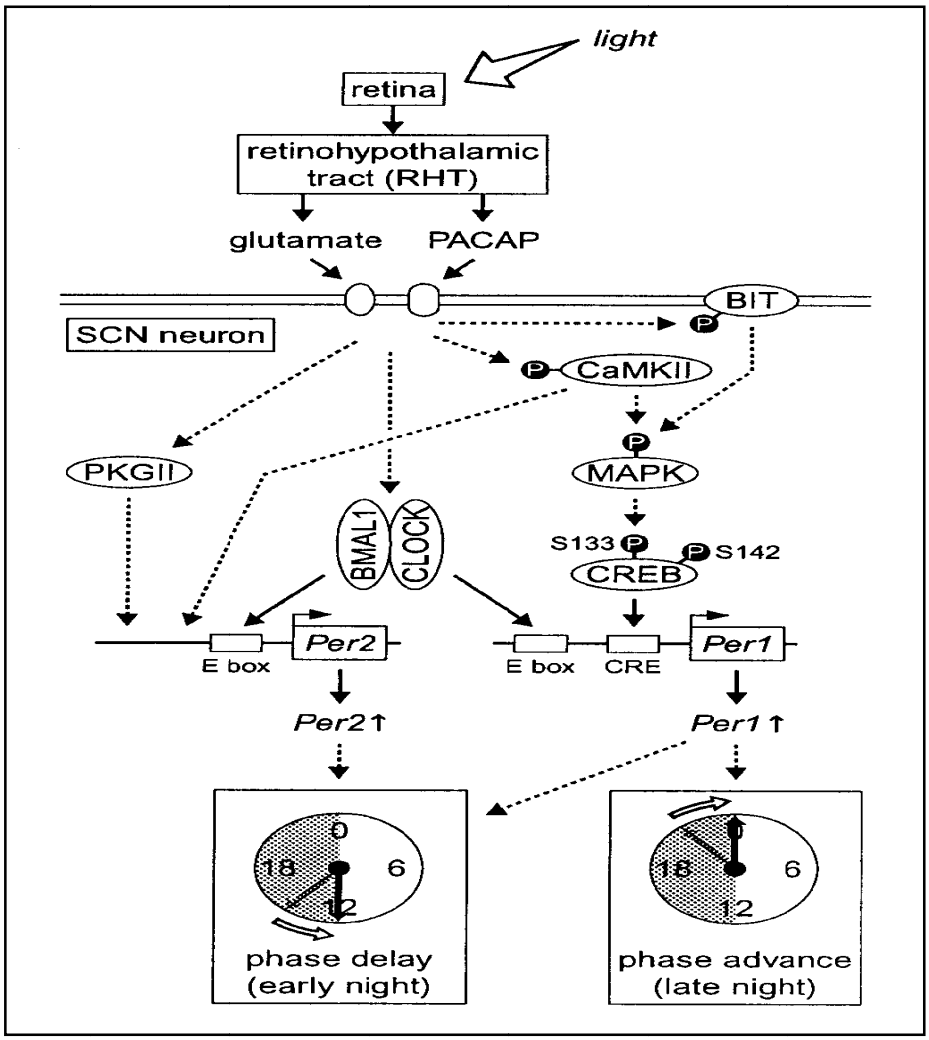


Fig. 4: Photic input signal transduction pathway in the SCN (after Hirota and Fukada, 2004). Solid and dashed lines indicate the direct and indirect pathways, respectively. BIT, brain immunoglobulin-like molecule with tyrosin-based activation motifs; CaMKII, calcium / calmodulin kinase II; CRE, cAMP response element; CREB, CRE-binding protein; PACAP, pituitary adenylate cyclase - activating peptide, PKGII , cGMP - dependent protein kinase II.

individuals, melatonin also induces phase shift; the direction and magnitude of the shift depends on the time of its administration (Lewy, *et.al*. 1990).

b- Arousal and physical activity may modulate circadian rhythms in animals. Some studies demonstrate that periods of physical activity or other forms of arousal, administered in the middle of the rest period, induce phase shift of the circadian rhythm (Pratt and Goldman, 1986; Mrosovsky and Salmon, 1987, Sumova *et al*., 1994).

c- Several chemicals may manipulate circadian rhythms in mammals, including benzodiazepines, a class of hypnotic drugs which include Valium (Ralph and Menaker, 1986), carbachol, phenobarbital, theophylline, and lithium. These chemicals affect specific systems in the brain that are important for neuronal communication and may impinge on the circadian pacemaker (Turek, 1987; Aschoff, 1989; Rusak and Bina, 1990).

2.3.3. Resetting the peripheral clocks

Peripheral clocks are considered to be subordinated oscillators that regulate local rhythms of most tissues. Peripheral clocks are entrained indirectly by light via setting their phase by the SCN clock. SCN may control these clocks by humoral as well as neural pathways. In the liver, glucocorticoids have been proposed to play a role in setting the phase of these clocks (Balsalobre *et al*., 2000). Neural pathways may involve the autonomic nervous system since adrenaline may control gene expression in liver (Terazono *et al*., 2003).

In studies when access to food was restricted to an unusual time of day, the rhythms in clock gene expression in liver, kidney, heart and other tissues were phase shifted relative to that in animals fed *ad libitum*, whereas the phase of the rhythms in clock gene expression within the SCN did not change (Damiola *et al*., 2000). These findings indicate that feeding signals may be dominant time cues for resetting the peripheral clocks.

2.4. Development of the biological clock

The mammalian SCN and its rhythmicity develop through more phases (Moore, 1991). The rat SCN is formed between gestational days 13 and 16 with a peak of mitotic activity occurring on day 15 (Ifft, 1972; Altman and Bayer, 1978). SCN develops from the specialized zone of the ventral diencephalic germinal epithelium as a component of the periventricular cell groups. Immature synapses are first seen within the SCN on gestational day 18. By the 20th day, the SCN neuropil is still very immature and is characterized by a fine lattice-like structure (Koritsanszky, 1981). The vast majority of SCN synapses are formed postnatally (Lenn *et al*., 1977), from postnatal day (P) 4 to P10. Intrinsic SCN rhythmicity is already present in the late embryonic stage: a day-night difference in metabolic activity monitored by 2-deoxyglucose uptake was detected in the fetal rat SCN since embryonic day (E) 19. The fetal rhythm develops from E19 via increase in daytime metabolic activity, with night time activity remaining low (Repperet and Schwartz, 1984). A rhythm of the firing rate was also detected in the SCN neurons at E22 (Shibata and Moore, 1987).

The first significant rhythm in clock gene expression within the rat SCN neurons begins to appear during late prenatal and early postnatal period. At E19, no rhythms in clock genes expression and no clock proteins PER1, PER2, and CRY1 are detectable (Sládek *et al*., 2004). At E20, a formation of rhythm in *Per1* expression is indicated while rhythms of *Per2*, *Cry1*, and *Bmal1* only appear during the first postnatal days (Kováčiková *et al*., 2006). In another study, rhythms in *Per1* and *Per2* expression in the rat SCN have been detected at E20 (Ohta *et al*., 2002 and 2003). The amplitude of the rhythms in *Per1* and *Per2* expression in the SCN cells increases until P10 (Sládek *et al*., 2004) when synaptogenesis is completed and the SCN cells are synchronized with each other. In mice, daily rhythm in m*Per1* mRNA was detected in the fetal SCN at E17 but the rhythm in *Per2* mRNA only at P6 (Shimomura *et al*., 2001).

The rat SCN clock begins to drive output rhythms only around birth as the rhythm in *Avp* heteronuclear RNA is undetectable at E20 but is clearly present at P1 (Kováčiková *et al*., 2006). However, a day-night difference of *Avp* mRNA level was detected already at E21 (Reppert and Uhl, 1987).

2.5. Entrainment of the biological clock during development

The presence of entrained circadian rhythms within the fetal SCN in rodents, sheep, and non-human primates indicate that the fetus is receiving circadian signals. Theoretically, in species in which the RHT innervates the fetal SCN *in utero* (Primates, sheep, precocious rodents) the fetal clock could be entrained directly by the light-dark cycle, provided that enough light reaches the fetus within the uterus. Interestingly, in pregnant sheep, measurements of light inside the uterus show that the fetus is exposed to a miniaturized version of the LD cycle (Parraguez *et al*., 1998). Recent evidence also shows that the circadian system of primate infants is responsive to light at very premature stages and that low-intensity lighting can regulate the developing clock (Rivkees, 2003). In rats, the retinal connections to the SCN develop during early postnatal period. Therefore, non-photic maternal entrainment appears to be dominant in fetuses and during the first week of life (Reppert and Schwartz, 1984). Several studies investigated the nature of maternal entrainment and its importance to fetuses and neonatal pups. In neonates, changes in the timing of maternal care by cross fostering, restricted feeding, or periodic maternal deprivation, were reported to alter the pup circadian rhythms of a pineal enzyme and of certain behaviors (Hiroshige *et al*., 1982a; Hiroshige *et al*., 1982b; Takahashi and Deguchi, 1983; Reppert *et al*., 1984; Sasaki *et al*., 1984; Honma *et al*., 1987a b; Sugishita *et al*., 1993; Duffield and Ebling, 1998; Viswanathan, 1999).

2.6. Photoperiod

Seasonal changes in the environment are caused by the Earth's rotation around the Sun. The most pronounced are the changes in the day-length, i.e., photoperiod. As a result, many species utilize these changes of day length to coordinate seasonal changes of physiology, a phenomenon termed photoperiodism. In different species, timing of reproductive activity varies across the annual cycle dependent on the length of gestation to permit birth in spring, when conditions are optimal for offspring to survive. Prolactin secretion which regulates lactation and fur growth is always maximal during spring-summer (Lincoln, 1999). Many mammals also exhibit annual changes in metabolism, e.g., larger species increase fat stores in preparation for hibernation (Kortner and Geiser, 2000). Because of the importance of photoperiod for annual

physiological rhythms, organism must be able to accurately measure day-length. The hormone melatonin is considered to play an important role in photoperiodic time measurement. The melatonin is secreted by the pineal gland and its syntheses are stimulated by the SCN (Goldman, 2001). The melatonin production is low during the day and high during the night. An inverse relationship between the length of the day and the duration of melatonin secretion is found in many mammals. The duration of melatonin production serves as a photoperiodic message, as the length of the day is encoded in the melatonin signal and decoded in the target tissues of the hormone. During the summer time, when the photoperiod is long, the melatonin signal is short, whereas during the winter time, when the photoperiod is short, the signal is long (Illnerová and Vaněček, 1980; Illnerová, 1991). When animals are transferred from a long to a short photoperiod, duration of elevated activity of arylalkylamine N-acetyltransferase (AA-NAT) which catalyzes formation of the melatonin precursor N-acetylserotonin, as well as duration of high melatonin levels, change gradually (Illnerová *et al*., 1984, 1986; Hastings *et al*., 1987).

2.6.1. Photoperiod regulates SCN physiology

Several evidences indicate that the photoperiod modulates intrinsic rhythmicity of the SCN circadian clock. The first evidence that the SCN is regulated by photoperiod came from studies on its photic sensitivity. Light pulses are able to induce gene expression only during subjective night and, therefore, the light sensitivity is mediated by the circadian clock itself (Kornhauser *et al*., 1993). Sumová *et al*., (1995a) demonstrated that the interval of the rat SCN photic sensitivity depends on photoperiod. The interval was shorter in rats maintained under a long photoperiod (LP) than in those maintained under a short photoperiod (SP) or released into constant darkness. Hamsters maintained under LP also exhibited a greatly compressed duration of photosensitivity (Vuillez and Jacob, 1996). Pinealectomy does not prevent the effect of photoperiod on the SCN photosensitivity and, therefore, the changes in the SCN are not mediated via the pineal melatonin secretion (Sumova *et al*., 1996, Jacob and Vuillez, 1997).

Both parts of the SCN, i.e., the vl- as well as dm- part, are modulated by the photoperiod. Within the rat vl-SCN, the rhythm in *c-fos* photoinduction is photoperiod dependent: the interval

enabling high *c-fos* photoinduction is by 5 h shorter in rats maintained under a long photoperiod than in those maintained under a short photoperiod (Sumová *et al*., 1995b). After a change from a long to a short photoperiod, the interval enabling high *c-fos* photoinduction decompressed gradually within 2 weeks, while the interval was fully compressed within 3 days after a change from a short to a long photoperiod. This indicates a rapid adjustment of the photoperiod dependent-state of the SCN pacemaker to the long days but only a slow one to the short days (Sumová *et al*., 1995a). The dm-SCN rhythmicity is also affected by the photoperiod; interval of high endogenous expression of *c-fos* is longer in long than in short days, mostly due to an earlier rise in this gene expression during a long photoperiod (Sumová *et al*., 2000; Jáč *et al*., 2000a, 2000b).

Electrical activity of the SCN neurons is thought to be a major output of the SCN (Schwartz *et al*., 1987) that carry information about the daytime to other parts of the brain including the pineal gland. The electrical impulse frequency of neuronal populations of the SCN is high during the day and low during the night. Recordings of single cell electrical activity revealed that neurons exhibited phase differences (Quintero *et al*., 2003; Schaap *et al*., 2003; Yamaguchi *et al*., 2003; Brown *et al*., 2005) that may play an important role in the ability of the SCN to encode for day-length (Shaap *et al*., 2003; Rohling *et al*., 2006). Long term recordings of the electrical activity pattern of single SCN cells revealed an increase in phase distribution among oscillating neurons in long days and a decrease in phase distribution in short days (Vander Leest *et al*., 2007).

Within the SCN, photoperiod also modulates the rhythm in *Avp* expression. Under the short photoperiod, the morning rise of *Avp* mRNA within the SCN occurred significantly later than under the long one. However, the levels of *Avp* mRNA were the same in the supraoptic and paraventricular nuclei under both LP and SP. This indicates that photoperiod affects the clock driven *Avp* gene expression especially in the SCN (Jáč *et al*., 2000a).

Indeed, the molecular core clockwork in the SCN is also photoperiod dependent. In rats, the morning and the daytime *Per1* and *Cry1* mRNA rise as well as the morning *Bmal1* mRNA decline were linked to the morning light onset. Consequently, the duration of elevated *Per*

gene expression was longer under the long than under the short photoperiod (Sumová *et al*., 2003). Similarly, *Per1, Per2, Per3* and *Cry2* genes exhibited a longer duration of the peak expression under LP as compared to SP in hamsters (Messager, *et al*.,1999; 2000; Tournier *et al*., 2003), mice (Steinlechner *et al*.,2002) and sheep (Lincoln *et al*.,2002).

2.6.2. How does the photoperiod regulate the circadian clock function?

A theoretical model was postulated to illustrate a mechanism by which the photoperiod regulates the circadian clock. In this model, the circadian clock is comprised of coupled morning (M) and evening (E) oscillators that are synchronized to dawn and dusk respectively. These putative oscillators display differential responses to light; M is accelerated, whereas E is decelerated. Hence, changing photoperiod duration leads to altered phase relationships between these oscillators, providing a representation of external day-length within the endogenous clock (Pittendrigh and Daan, 1976; Daan and Berde, 1978). Daan *et al*., (2001) suggested the morning oscillator consists of *Per1* and *Cry1* and the evening oscillator consists of *Per2* and *Cry2.* Another view supporting the M-E oscillators postulated that the mammalian SCN comprise heterogeneous sub regions and cell types, including neurons that are directly sensitive to photic information, those that exhibit endogenous rhythmicity and others that are apparently arrhythmic (Lee *et al*., 2003). Therefore it is possible that the photoperiodic regulation of SCN gene expression derives from regional variation of SCN function (Johnston, 2005). Studies carried on electrophysiological recordings of multiunit activity in the SCN of mice suggested that the phase distribution of oscillating neurons carries information on the photoperiod's duration (Vander Leest, *et al*., 2007). Thus, it is possible that the actual light-induced adjustment of the pacemaker and its output require interaction between neuronal subpopulations within the SCN rather than being based on the single cell (Meijer, *et al*., 2007).

3. Aim of the work

The aim of the present work was to investigate mechanism of entrainment of the circadian clock within the rat SCN during prenatal and early postnatal development. Both photic and maternal entrainment was studied. We addressed following questions:

3. 1. When and how does the fetal and early postnatal rhythmicity within the SCN develop?

3.1.1. When does a rhythm in neuronal activity within the fetal rat SCN measured by a daily profile of *c-fos* mRNA, develop?

3.1.2. Does a presence of a light-dark cycle before and after birth promote fetal and early postnatal development of rhythms in clock gene expression within the rat SCN?

3. 2. Does a presence of a light-dark cycle facilitate photoperiodic entrainment of the molecular core clock mechanism within the SCN during early postnatal development?

3. 3. Do maternal cues entrain the fetal and early postnatal circadian clock within the SCN?

3.3.1. What is the time interval necessary to accomplish resetting the fetal SCN clock by maternal cues?

4. Results and Discussion

4.1. Development of the circadian clockwork within the rat SCN

4.1.1. Development of the fetal rhythmicity (papers 7.2. and 7.3.)

Several studies demonstrate that the molecular core clock mechanism develops gradually since late prenatal to early postnatal period. In the fetal rat SCN, clock genes were already expressed at E19, but no endogenous rhythm in their expression was detected. Moreover, at E19, clock proteins PER1, PER2 and CRY1 were not detectable at any time during the circadian cycle (Sládek, *et al*., 2004). At E20, *Per1* mRNA exhibited very small daily variations, while *Per2*, *Cry1* and Bma1 expression did not change (Kováčiková *et al*., 2006). However, Ohta and colleagues (2002, 2003) found a rhythm in *Per1* as well as in *Per2* expression within the fetal rat SCN at E20. Rats in Ohta's studies were maintained and sampled under a LD regime while in our studies rats were released into darkness to avoid the masking effect of light. In Syrian hamster, molecular oscillations equivalent to those observed in adults were not detected within the fetal SCN (Li and Davis, 2005) and in mice, a rhythm of *Per1* but not *Per2* expression was detected at E17 (Shimomura *et al*., 2001). Altogether, during the fetal stage, the circadian clock within the rodent SCN does not exhibit robust oscillations in clock gene expression as seen in adult animals.

Our previous studies in rats demonstrated that *Avp* expression within the SCN did not exhibit high amplitude oscillations at E20 (Kováčiková *et al*., 2006). Because *Avp* gene is under the control of BMAL1-CLOCK heterodimer in the SCN, these data support the hypothesis that the molecular core clock mechanism is not operating at this fetal stage. However, levels of *Avp* mRNA exhibited day-night variation at E21 (Reppert and Uhl, 1987).

To provide more insight into the development of the SCN rhythmicity, we measured daily profiles of *c-fos* expression within the SCN of 20-day-old fetuses of mothers that were

maintained under LD12:12 regime or constant darkness. In adult rats, *c-fos* is expressed spontaneously within the SCN and exhibits circadian rhythmicity (Sumova *et al*., 1998). C-fos expression is considered to be a mark of neuronal activity. In the SCN, it is high during the daytime and low during the nighttime (Sumova *et al*. 1998). Previously, a marked rhythm in c-Fos protein immunoreactivity was detected in 3-day-old rat pups (Bendová *et al*., 2004). **In the present study, we found that a significant rhythm in *c-fos* mRNA levels was present already at E20 (Fig. 5) (El-Hennamy, unpublished data).** Pregnant rats were maintained under LD12:12 and at E20, they were either left in LD or released into DD. Fetuses were sampled in 2 h intervals to detect *c-fos* mRNA profiles within the SCN by *in situ* hybridization. A clear rhythm in *c-fos* gene expression was detected under both DD and LD, with a peak during early morning at CT0 and CT2 respectively (Fig.5). Our data thus show that the rhythm in neuronal activity in the rat SCN is present already at the fetal stage when the rhythm in clock gene expression is not detected. This rhythm was not impaired by absence of a light-dark cycle and, therefore, it was likely driven by maternal cues. It is possible that maternal cue-induced *c-fos* expression may provide fetal clock with a daytime signal (Sumová *et al*., 2006a), because *c-fos* expression can be induced within the fetal SCN by activation of dopamine D1 receptors (Weaver *et al*., 1992, Weaver and Reppert, 1995) and activation of the dopaminergic pathways may entrain rodent fetuses (Viswanathan *et al*., 1994). The finding of the significant rhythm in *c-fos* expression at E20 suggests that the maternal cue likely impinges on a large population of the SCN neurons, because the neurons may not be synchronized with each other due to lack of synapses at this developmental stage. Altogether, our finding of spontaneous rhythmicity of *c-fos* expression in the SCN at the developmental stage, when molecular core clockwork seems to be undeveloped, provides further evidence for the hypothesis that the intrinsic rhythmicity in the fetal SCN may be driven by rhythmically appearing maternal cues rather than by the fetal core clockwork.

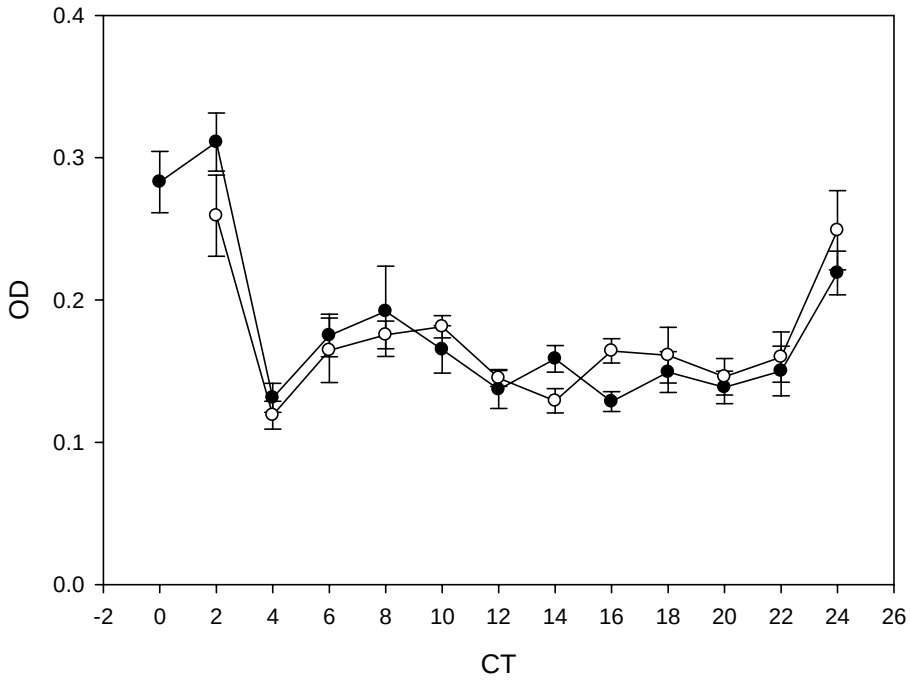


Fig.5: Daily profile of *c-fos* mRNA levels in the rat SCN at E20 under DD (closed circles) and LD (opened circles). mRNA levels are expressed as relative optical density means ± S.E.M. from 4 animals. Time is expressed in circadian time (CT) when CT12 is the beginning of subjective night. (**El-Hennamy *et al*., unpublished data**)

4.1.2. **Development of the circadian rhythmicity during postnatal stage (papers 7.2. and 7.3.)**

After birth, development of the molecular clockwork within the SCN of rat pups matured gradually. At P1, rhythms in *Per1* and *Bmal1* mRNA and a forming rhythm in *Per2*, but no rhythm in *Cry1* mRNA were detected. At P2, the rhythm in *Per1, Per2* and *Bmal1* mRNA became more pronounced and the rhythm in *Cry1* expression began to form (Kováčiková *et al*., 2006). Significant rhythms in *Per1, Per2*, *Cry1*, and *Bmal1* mRNA which resembled those of adults were expressed in the SCN at P3. However, the rhythms continued to mature further as amplitude of *Per1, Per2*, and *Bmal1* mRNA rhythm increased until P10 (Sládek, *et al*., 2004). Interestingly, synaptogenesis within the SCN progresses in a similar fashion. Hence, development of the circadian rhythms in clock gene expression (Sládek *et al*., 2004,

Kováčiková *et al*., 2006) paralleled well the development of the synapses (Moore, 1991). Though development of the circadian core clockwork is genetically predetermined, it is likely controlled by maternal cues: The newly appearing rhythms in clock gene expression developed in synchrony with the maternal clock (Kováčiková *et al*., 2006). In these previous studies, pups were maintained under the LD12:12 regime and released into darkness only on the day of sampling. However, the SCN of newborn rats is able to respond to light as soon as on the day of birth (Speh and Moore, 1993; Leard *et al*., 1994).

To provide evidence that the newly appearing rhythms in clock gene expression are set by maternal cues and not by the LD regime perceived after birth, we studied development of the rhythms in pups reared in DD since birth. Pregnant rats were maintained under the LD12:12 regime and on the day of delivery, light was turned off so that the pups were born into DD and maintained in darkness until they were 3-day old. At P3, they were sampled throughout the circadian cycle and expression of clock gene *Per1, Per2, Cry1* and *Bmal1* was assayed by *in situ* hybridization. All the studied clock genes exhibited significant circadian rhythms in their expression under DD (Fig.6). The profiles of the clock gene expression in DD were compared with those determined previously in pups reared under the LD regime (Sládek *et al*., 2004) by the two-way ANOVA. There was no significant difference between the phases of the clock gene expression profiles of pups reared under LD and DD regime. However, some differences in the time of the rise and the decline *of Per1* (Fig. 6A), *Per2* (Fig. 6B), *Cry1* (Fig. 6C) and *Bmal1* (Fig. 6D) mRNA levels between profiles of the pups maintained under DD and those maintained in LD were detected. The amplitude of the rhythm in *Cry1* (Fig. 6C) mRNA was lower under DD than under LD. Although some differences between the profiles of clock gene expression under the DD and LD condition suggest possible involvement of the external LD regime in entrainment of the rhythm during the first postnatal days, one must take into account the fact that results of two independent studies are compared. Importantly, the phases of the rhythms were the same under the LD and DD conditions. Moreover, the circadian rhythm in m*Per1* expression at P3 was similar in LD and DD conditions in mice (Shimomura *et al*., 2001). These data suggest that the environmental light-dark cycle is not necessary for circadian rhythmicity of m*Per1* mRNA expression in the SCN. However, slight modulation of the profiles by light cannot be excluded. Though previous results demonstrated that the pups begin to be entrained by light since P5-6 (Reppert *et al*., 1984; Sasaki *et al*., 1984; Duncan *et al*., 1986),

our data suggest that transition from maternal to photic entrainment is a gradual process that might begin already around P3 (El-Hennamy *et al*., unpublished data).

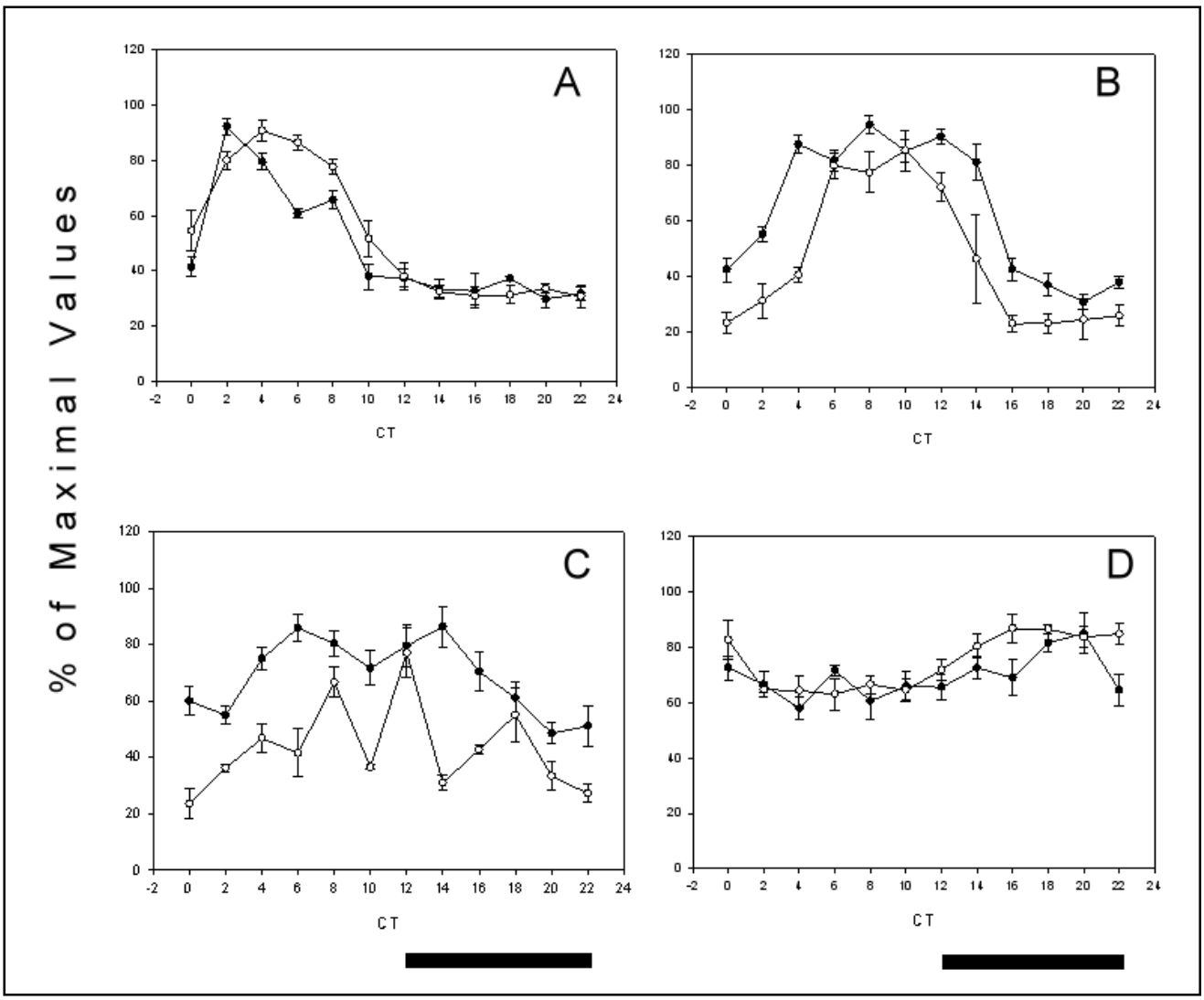


Fig. 6: Daily profiles of *Per1* (A), *Per2* (B), *Cry1* (C), and *Bmal1* (D) mRNA levels at P3 in the SCN of rats maintained under DD from the day of delivery (closed circles, **El-Hennamy *et al.,* unpublished data**) and those maintained under LD 12:12 and released into constant darkness DD at the day of the experiment (open circles, Sládek *et al*., 2004). The mRNA levels are expressed as relative optical density means ±S.E.M. from 4 animals

4.2. Photoperiodic entrainment of the molecular core clock mechanism during development (paper 7.1.)

Development of the photoperiodic entrainment of the rat SCN was first studied using the rhythm in c-Fos immunoreactivity. The spontaneous rhythm of c-Fos immunoreactivity in the dm-SCN was well developed already in 3-day and 10-day-old rats (Bendová, *et al*., 2004) and resembled that in the adults (Sumová, *et al*., 1998). However, photoperiod did not affect the dm-SCN rhythm either at P3 or at P10 (Bendová, *et al*., 2004). A rhythm in light induced c-Fos immunoreactivity within the vl-SCN was present in 10-day- but not in 3-day-old pups. Photoperiod modulated the rhythm in light induced c-Fos immunoreactivity at P10, though the modulation was not yet fully developed as compared to that in adults (Bendová, *et al*., 2004). These data suggest that circadian rhythmicity of the dm-SCN develops earlier than that of the vl-SCN. Once the vl-SCN rhythmicity is structured, it may be entrained by photoperiod (Bendová, *et al*., 2004). Similarly, the pineal rhythm of AA-NAT was fully modulated by the photoperiod in 15-day but not yet in 8-day-old rats (Vaněček and Illnerová, 1985).

Our further studies on photoperiodic entrainment of the molecular core clockwork within the rat SCN demonstrated that the clock gene expression profiles begin to be partially entrained by the photoperiod at P10 (Kováčiková *et al*., 2005; Sumová *et al*., 2006). At P10, but not yet at P3, the photoperiod started to modulate expression of light sensitive genes *Per1* and *Per2*. The morning rise of *Per1* and *Per2* mRNA occurred earlier under the long than under the short photoperiod. Consequently, the interval of elevated *Per1* and *Per2* mRNA levels took longer under the long than under the short photoperiod. However, *Cry1*, the light insensitive gene, was affected by photoperiod only at P20. The light insensitive gene *Bmal1* was not affected by the photoperiod even at P20. These results suggest a dominant role of the light sensitive *Per* genes in the mechanism of photoperiodic entrainment of the SCN molecular core clock. Duration of interval of the elevated levels of *Per1* and *Per2* mRNA at P20 was still shorter than that in adult rats. In adult rats (Sumová, *et al*., 2003), the morning rise of *Per1* mRNA occurred about 4h earlier under the long (LD 16:8) than under the short photoperiod (LD8:16), while in 20-day-old rats the rise occurred only about 2h earlier under LD16:8 than under LD8:16 (Kováčiková, *et al*., 2005). Thus, the photoperiodic entrainment was not fully developed even

around weaning. Altogether, **our data demonstrate that adjustment of the molecular core clock mechanism to the photoperiod proceeds gradually during postnatal development and the complete entrainment is not accomplished before the 20th day of postnatal development** (Kováčiková, *et al*., 2005, **Sumova *et al*., 2006b**).

In the above mentioned experiments, profiles of clock gene expression were assayed under unmasked conditions, i.e., the rat pups were released into DD on the day of sampling. A question remained whether a presence of the LD cycle during the day of experiment may facilitate the photoperiodic entrainment. Therefore, in another experiment, mothers with their pups were kept under a long photoperiod (LD16:8) and the pups were sampled under the LD16:8 regime. The light was on from 0400 to 2000 (Fig7). Expression profiles of *Per1* mRNA (Fig. 7A,B) and *Per2* mRNA (Fig. 7C,D) were significantly different between LD and DD conditions at P10 (Fig. 7B,D) but not at P3 (Fig. 7A,C) (two-way ANOVA). However, at P3, the significant interaction effect suggested some differences in the rise and decline in *Per1* mRNA levels between the LD and DD conditions. The rise in *Per1* (Fig. 7A) and *Per2* (Fig. 7C) mRNA was advanced under LD as compared to DD conditions. Therefore, at P3, a slight effect of the presence of LD on *Per1* and *Per2* mRNA profile was suggested. At P10, the rise in *Per1* (Fig. 7B) and *Per2* (Fig. 7D) mRNA occurred significantly earlier under LD than DD conditions while the decline occurred at about the same time. Consequently, the interval of high *Per1* and *Per2* mRNA levels took longer under the LD than under the DD conditions. The data thus clearly suggest that the presence of light period may facilitate the effect of the long photoperiod on the rhythm in *Per1* and *Per2* expression at P10. The effect of the presence of LD16:8 regime on the profiles of *Cry1* and *Bmal1* was only suggested at P10, but not at P3. **Altogether, these results clearly show that presence of LD cycle may facilitate adjustment of the profiles of *Per1* and *Per2* clock gene expression to a long photoperiod; at P10, the interval of elevated *Per1* and *Per2* mRNAs levels appeared to be longer under LD than that under DD.** At P3, a slight effect of the light-dark cycle on the rhythms of *Per1* and *Per2* mRNA was indicated. **Moreover, the data demonstrate that presence of the LD cycle contributes to development of the photoperiodic entrainment of the molecular clockwork via modulation of the light sensitive *Per1* and *Per2* genes (El-Hennamy *et al*., unpublished data, Sumova *et al*., 2006b)**.

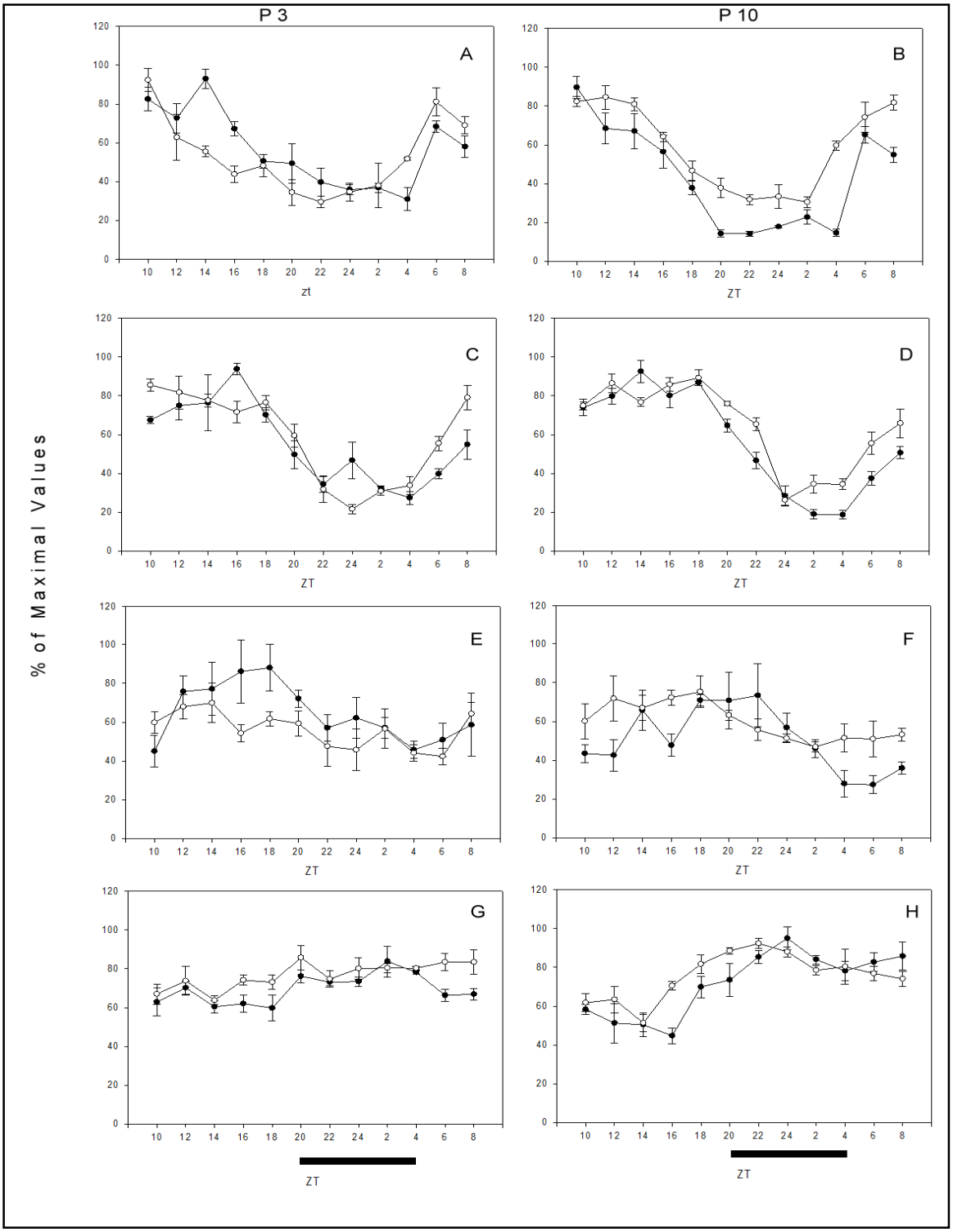


Fig. 7: Daily profiles of *Per1*, *Per2*, *Cry1* , and *Bmal1* mRNA levels at P3 (A, C, E, G) and P10 (B,D,F,H) in the SCN of rats entrained to LD 16:8 and assayed under LD (open circles, **El-Hennamy *et al*., unpublished data**) and under DD (closed circles, **Kováčiková, *et al*.,2005**). The bars indicate the dark period of the cycle. The mRNA levels are expressed as relative optical density means ±S.E.M. from 4 animals.

4.3. Maternal control of the fetal and neonatal rat suprachiasmatic nucleus (paper 7.4.)

During prenatal period, the rhythmically appearing maternal signals may provide an important time cue for setting the phase in the SCN of developing fetuses. For a limited period after birth, stimuli related to maternal care may be more effective in entraining the early postnatal clock than photic stimuli. The aim of our study (**El-Hennamy *et al*., submitted**) was to ascertain whether and how swiftly the immature fetal and neonatal molecular SCN clock can be re-set by maternal cues. Pregnant rats were maintained under LD 12:12 and on gestational day 18 and 20, they were divided into two groups. A control group remained untreated while the other group was exposed to a 6-h phase delay of the dark period, i.e., to prolongation of the light period by 6 hours at gestational days 18 or 20. The next cycle, rats of both groups were released into DD. Pups of both groups were sampled on postnatal day P0-1, P3 and P6. Daily profiles of *c-fos* mRNA and *Avp* hnRNA at P0-1 and of *Per1* and *Per2* mRNA at P3 and P6 were determined by *in-situ* hybridization. Adult male rats were exposed to the same shifting procedure. Daily profiles of *c-fos* and *Avp* expression on the third day and of *Per1* and *Per2* expression on the fifth day after the delay of the dark period in the SCN of control rats and those exposed to the shifting procedure were detected. The interval of three and five days after the phase shift corresponded to the time that elapsed between E20 and P0-1 and between E18 and P0-1. Moreover, a locomotor activity of adult rats before and after the shifting procedure was monitored. At P0-1, we used *c-fos* mRNA and *Avp* hnRNA as a marker of SCN intrinsic rhythmicity. We did not use clock genes *Per1* and *Per2* as a marker at this age because we knew from previous studies (Kováčiková *et al*., 2006) that *Per1* and *Per2* gene expression exhibited only very low amplitude rhythmicity at this age. First, we demonstrated that the shifting procedure affects the maternal SCN. Three days after the shifting procedure, locomotor activity as well as daily profiles of *c-fos* and *Avp* expression in the SCN were phase-delayed. Daily profiles of *Per1* and *Per2* were phase-delayed five days after the shifting procedure. Therefore, the maternal SCN was obviously phase-delayed three days after application of the shifting procedure. Our next aim was to investigate whether exposure of pregnant rats to the shifting procedure on E20 entrains the fetal SCN. To demonstrate whether the phase delay could be accomplished during prenatal or postnatal periods, profiles of *c-fos*

and *Avp* expression were assessed in newborn pups at P0-1 and profiles of *Per1* and *Per2* expression were determined at P3 and P6. We found that at P1, the *c-fos* and *Avp* profiles were not phase-delayed while at P3, the *Per1* and *Per2* profiles were significantly phase delayed. A phase-delay of approximately the same magnitude as that observed at P3 was also confirmed at P6. To elucidate whether the SCN may be entrained by maternal cues during prenatal period, we exposed the pregnant rats to the shifting procedure at E18 rather than at E20 and determined the daily profiles of *c-fos* and *Avp* expression in the SCN at P0-1. This approach allowed the fetal SCN to be exposed to the exclusively prenatal maternal shifting cues for five days instead of three days. We found that both profiles were significantly phase-delayed at P0-1. Therefore, **we provided evidence that the fetal SCN is able to entrain within five days, but not within three days, after exposure of pregnant rats to the shifting procedure (El-Hennamy *et al*., submitted).**

5. Conclusion

5.1. In the present study, we found that a significant rhythm in neuronal activity, as demonstrated by the rhythm in *c-fos* mRNA levels, is fully developed already at E20 (El-Hennamy, unpublished data). Moreover, our data suggest that transition from maternal to photic entrainment is a gradual process that may begin already at P3 (El-Hennamy *et al*., unpublished data).

5.2. The data demonstrate that presence of the light-dark cycle contributes to development of the photoperiodic entrainment of the molecular clockwork (Sumova *et al*., 2006b, El-Hennamy *et al*., unpublished data).

5.3. Our data provide evidence that the fetal SCN is entrained by maternal cues. Moreover, we found that the fetal SCN is able to entrain within five days, but not within three days, after exposure of pregnant rats to the shifting procedure (El-Hennamy *et al*., submitted).

6. References

Akhtar, R. A., Reddy, A. B., Maywood, E. S., Clayton, J. D., King, V. M., Smoth, A. G., Gant, T. W., Hastings, M. H. and Kyriacou, C. P. (2001). Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus. Curr. Biol., 72, 540-450.

Albrecht, U. (2002) Functional genomics of sleep and circadian rhythm nvited review: Regulation of mammalian circadian clock genes, J. Appl. Physiol., 92, 1348-1355.

Albrecht, U., and Eichele, G. (2003) The mammalian circadian clock., Curr. Opi. Genet. & Develp., 13, 271-277.

Albrecht, U., and Oster, H. (2001) The circadian clock and behavior, Behav. Brain Res., 125, 89-91.

Altman, J., and Bayer, S. (1978) Development of the diencephalon in the rat. I. Autoradiographic study of the time of origin and settling patterns of neurons of the hypothalamus., J, Comp, Neurol., 182, 945-972.

Aton, S. J., Colwell, C. S., Harmar, A. J., Weschek, J., and Herzog, E. D. (2005) Vasoactive intestinal polypeptide mediates circadian rhythmicity and synchrony in mammalian clock neurons. Nat. Neurosci., 8, 476-483.

Aschof, J. (1989) Circadian Rhythms in hamster and rats treated with imipramine in drinking water., Chronobiol., 16, 9-20.

Bae, K., Jin, X., Maywood, E. S., Hastings, M. H., Reppert, S. M., and Weaver, D. (2001) Differential unctions of m*Per I*, m*Per 2*, and m*Per3* in the SCN circadian clock, Neuron., 30, 525-536.

Balsalobre, A., Brown, S. A., Marcacci, L., Tronche, F., Kellendonk, C., Reichardt, H. M., Schutz, G. and Schibler, U. (2000) Resetting of circadian time in peripheral tissues by glucocorticoid signaling., Science, 289, 2344-42347,

Beaule, C., Robinson, B., Lamont, E. W., and Amir, S. (2003) Melanopsin in the circadian timing system., J. Mol. Neurosci. 21, 73-89.

Bendová, Y., Sumová, A., and Illnerová, H (2004) Development of circadian rhythmicity and photoperiodic response in subdivisions of the rat suprachiasmatic nucleus., Develop. Brain Res., 148, 105-112.

Brown, T. M., Hughes, A. T., and Piggins, H. D. (2005) Gastrin-releasing peptide promotes suprachiasmatic nuclei cellular rhythmicity in the absence of vasoactive intestinal polypeptide-VPAC2 receptor signaling. J. Neurosci., 25, 11155-11164.

Brownstein, M. J. and Balkovits, M. (1984) Catecholamines, serotonin, acetylcholine, and γ-aminobuteric acid in the rat brain: biochemical studies. In: A. Björklund and T. Hökfelt (eds.) Classical Transmitters in the CNS, part 1, Elsevier, Amsterdam, 23-53.

Buijs, R. M., Wortel, J., Van-Heerikhuize, J. J., Feenstra, M. G., Ter-Horst, G. J., Romijn, H. J., and Kalsbeek, A. (1999) Anatomical and functional demonstration of a multisynaptic suprachiasmatic nucleus adrenal (cortex) pathway. Eur. J. Neurosci., 11, 1535–1544.

Bunger, M. K. *et al.* (2000) Mop3 is an essential component of the master circadian pacemaker in mammals., Cell, 103, 1009-1017.

Butcher, G. Q., Dziema, H., Collamore, M., Burgoon , P. W., and Obrietan, K. (2002) The p24/44 mitogen-activated protein kinase pathway couple photic input to circadian clock entrainment. J Biol. Chem., 277, 29519-29525.

Card, J. P., Brecha, N., Karten, H., and Moore, R. (1981) Immunocytochemical localization of vasoactive intestinal polypeptide-containing cells and processes in the suprachiasmatic nucleus of the rat: light and electron microscope analysis. J. Neurosci., 1, 1289-1303.

Card, J. P., Fitzpatric-McElligot, S., Gozes, I. and Frank-Baldino, J. (1988) Localization of vasopressin-, vasoactive intestinal polypeptide-, peptide histidine isoleucine-, and somatostatin mRNA in rat suprachiasmatic nucleus. Cell Tissue Res.,252, 307-315

Colwell, C. S. (2000) Rhythmic coupling among cells in the suprachiasmatic nucleus., J. Neurobiol., 43, 379-388

Daan, S., and Berde, C., (1978) Two coupled oscillators: simulations of the circadian pacemaker in mammalian activity rhythms. J. Theor. Biol., 70**,** 297-313.

Daan, S., Albrecht, U., van der Host, G.T.J., Illnerova, H, Roennberg, T., Wehr, T. A., and Schwartz, W.J. (2001) Are there M and E oscillators in the genes? J. Biol. Rhythms, 16, 105-116

Damiola, F., Le Minh, N., Preitner, N., Kornmann, B., Fleury-Olela, F., and Schibler, U. (2000) Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus., Genes. Dev. 14, ,2950-2961.

De La Iglesia, H. O., Blaustein, J. D., and Bittman, E. L. (1995) The suprachiasmatic area in the female hamster projects to neurons containing estrogen receptors and GnRH. Neuroreport, 6, 1715–1722.

DeCoursey, P. J., (1960) Daily light sensitivity rhythm in a rodent, Science, 131, 33-35.

Duffield, G. E., and Ebling, F. J. (1998) Maternal entrainment of the developing circadian system in the Siberian hamster (*Phodobus sungorus*). J. Biol. Rhythms, 13, 315-329.

Duncan, M., Banister, M. and Reppert, M. (1986) Developmental appearance of light-dark entrainment in the rat. Brain Res., 369, 326-330.

Earnest, D. J. and Sladek, C. D. (1986) Circadian rhythms of vasopressin release from individual rat suprachiasmatic explants in vitro. Brain Res., 382, 129-133.

Earnest, D. J. and Sladek, C. D. (1987) circadian vasopressin release from perifused rat suprachiasmatic explants in vitro: effects of acute stimulation. Brain Res., 422, 398-402.

Edery, I. (2000) Circadian rhythms in a nutshell. Physiol Genomics, 3, 59-74.

Gekakis. N. *el al*. (1998) Role of the CLOCK protein in the mammalian circadian mechanism. Science, 280, 1564-1569.

Gillette, M. U. and Tischkau, S. A. (1999) Suprachiasmatic nucleus: the brain's circadian clock. Recent Prog. Horm. Res., 54, 33-58.

Gillette, M. U., and Mitchell, J. W. (2002) Signaling in the Suprachiasmatic nucleus: selectively responsive and integrative. Cell Tissue Res., 309, 99-107.

Goldman, B. (2001) Mammalian photoperiodic system:formal properties and neuroendocrine mechanisms of photoperiodic time measurement. J. Biol. Rhythrns, 16, 283-301.

Guido, M., deGuido, L., Goguen, D., Robertson, H., and Rusak, B. (1999) J. Biol. Rhythms, 141, 275-280.

Harmar, A. J. (2003) An essential role for peptidergic signaling in the control of circadian rhythms in the suprachiasmtic nuclei. J. Neuroendocrinol., 15, 335-338.

Hastings, M. H., and Sweeney, B. M. (1958) A persistent diurnal rhythm of luminescene in Gonyaulax polyedra, Biol. Bull., 115, 440-458.

Hastings, M. H., Walker, A., and Herber, T., (1987) Effect of asymmetrical reductions of photoperiod on pineal melatonin, locomotor activity and gonadal condition of male Syrian hamsters. J. Endocrinol.,114, 221-229.

Herzog, E. D., Geusz, M. E., Khalsa, S. B. S., Straume, M., and Block., G. D. (1997) Circadian rhythms in mouse suprachiasmatic nucleus explants on multimicroelectrode plates., Brain Res. 757, 285-290.

Herzog, E. D., Takahashi. J. S., and Block, G. D. (1998) Clock controls circadian period in isolated suprachiasmatic nucleus neurons., Nat. Neurosci., 1, 708-713.

Hiroshige, T., Honma, K. and Watanabe, K. (1982a) Possible zeitgebers for external entrainment of the circadian rhythm of plasma corticosterone in blind infantile rats. J. Physiol. 325, 507-519.

Hiroshige, T., Honma, K. and Watanabe, K.(1982b) Prenatal onset and maternal modifications of the circadian rhythm of plasma corticosterone in blind infantile rats. J. Physiol. (Lond.), 325, 521-532.

Hirota, T. and Fukada, Y. (2004) Resetting mechanism of central and peripheral circadian clocks in mammals. Zoological Science, 21, 359-368.

Hogenesch, I. B., Gu, Y. Z., Jain, S., and Bradfield, C. A. (1998) The basic helix-loop-helix-PAS orphan MOP3 forms transcriptionally active complexes with circadian and hypoxia factors. Proc. Natl. Acad. Sci. U.S.A., 95, 5474-5479

Honma, K., Honma, S., Shirakawa, T. and Hiroshige, T. (1987a) Phase setting of circadian locomotor rhythm of infant rats. Am. J. Physiol., 252, R256-R261.

Honma, S., Honma, K. and Hiroshige, T. (1987b) Restricted daily feeding during nursing period resets circadian locomotor rhythm of infant rats. Am. J. Physiol., 252, 256-261.

Honma. S., Shirakawa, T., Katsuno,Y., Namihira, M., and Honma, K. (1998) Circadian periods of single suprachiasmatic neurons in rats. Neurosci. Lett. 250, 157-160

Ifft, J. D. (1972) An autoradiographic study of the time of final division of neurons in rat hypothalamic nuclei., J. Comp. Neurol. 144, 193-204.

Illnerová, H., and Vaněček, J. (1980) Pineal rhythm in N-acetyltransferase activity in rats under different artificial photoperiods and in natural daylight in the course of a year. Neuroendocrinology, 31, 321-326.

Illnerová, H., Hoffmann, K., and Vaněček, J. (1984) Adjustment of pineal melatonin and N-acetyltransferase rhythms to change from long to short photoperiod in the Djungarian hamster Phodopus sungorus. Neuoendocrinology., 38, 226-231.

Illnerová, H., Hoffman, K., and Vaněček, J. (1986) Adjustment of the rat pineal N-acetyltransferase rhythm to change from long to short photoperiod depends on the direction of the extension of the dark period. Brain Res, 362, 403-408.

Illnerová, H. (1991) The suprachiasmatic nucleus and rhythmic pineal melatonin production. In Suprachiasmatic Nucleus, The Mind's Clock, Klein, D., Moore, R., and Reppert S., eds, 197-216, Oxford University Press, New York.

Inagaki, N., Yamatodani, A., Ando-yYamamoto, M., Tohyama, M., Watanabe, T. and Wada, H. (1988) Organization of histaminergic fibers in the rat brain. J. Comp. Neurol., 273: 283-300.

Inouye, S. T. and Shibata, S. (1994) Neurochemical organization of circadian rhythm in the suprachiasmatic nucleus. Neurosci. Res., 20, 109-130.

Jáč, M., Kiss, A., Sumová, A., Illnerová, H., and Jezová, D., (2000a) Daily profiles of arginine vasopressin mRNA in the suprachiasmatic, supraoptic and paraventricular nuclei of the rat hzpothalamus under various photoperiods., Brain Res., 887, 472-476.

Jáč, M., Sumová, A., and Illnerová, H., (2000b) c-Fos rhythm in subdivisions of the rat suprachiasmatic nucleus under artificial and natural photoperiods., Am, J. Physiol. Regul. Integr. Comp. Physiol., 279, 2270-2276.

Jacob, N., Vuillez, P., and Pevet, P. (1997) Photoperioddoes not act on the suprachiasmatic nucleus photosensitive phase through the endogenous melatonin, in the Syrian hamster. Neurosci. Lett., 229, 117-120.

Jiang, Z. G., Yang, Y. Q., and Allen, C. N. (1997) Tracer and electrical coupling of rat suprachiasmatic nucleus neurons. Neurosci.,77, 1059-1066.

Jin, X., Shearman, L. P., Weaver, D. R., Zylka, M. J., De Vries, G. J., and Reppert, S. M. (1999) A molecular mechanism regulating rhythmic output from the suprachiaslliatic circadian clock, Cell, 96, 57-68.

Johnston, J. D. (2005) Measuring seasonal time within the circadian system: regulation of the suprachiasmatic nuclei by photoperiod. J. Neuroendocrinol., 17, 459-465.

Kalsbeek, A., and Strubbe, J. H. (1998) Circadian control of insulin secretion and glucose homeostasis is independent of the temporal distribution of feeding. Physiol. Behav., 63, 553-560.

Kalsbeek, A., Vanheerikhuize, J. J, Wortel, J., and Buijs, R. M. (1996) A diurnal rhythm of stimulatory input to the hypothalamo-pituitary-adrenal system as revealed by timed intrahypothalamic administration of the vasopressin V-1 antagonist., J. Neurosci., 16, 5555-5565.

Kalsbeek, A., Garidou, M. L., Palm, I. F., Van der Vliet, J., Simonneaux, V., Pevet, P., and Buijs, R. M. (2000) Melatonin sees the light: blocking GABA-ergic transmission in the paraventricular nucleus induces daytime secretion of melatonin. Eur. J. Neurosci., 12, 3146–3154.

Kalsbeek, A., Palm, I. F., La Fleur, S. E., Scheer, F.A., Perreau-Lenz, S., Ruiter, M., Kreier, F., Cailotto, C., and Buijs, R.M. (2006) SCN outputs and the hypothalamic balance of life. J. Biol. Rhythms, 21, 458-469.

Kalsbeek, A., Verhagen, L. A., Schalij, I., Foppen, E., Saboureau, M., Bothorel, B., Buijs, R. M., and Pévet, P. (2008) Opposite actions of hypothalamic vasopressin on circadian corticosterone rhythm in nocturnal versus diurnal species. Eur. J. Neurosci., 27, 818-827.

Kaneko, M., Hiroshige, T., Shinsako, J., and Dallman, M. F. (1980) Diurnal changes in amplification of hormone rhythms in the adrenocortical system. Amer. J. Physiol., 239, 309-316.

Kim, Y. and Dudek, F. (1991) Intracellular electrophysiological study of suprachiasmatic nucleus neurons in rodents: excitatory synaptic mechanism. J. Physiol., 444, 269-287

King, D. *et al*. (1997) Positional cloning of the mouse circadian Clock gene. Cell, 89, 641-653.

King, D. P., and Takahashi, J. S. (2000) Molecular genetics of circadian rhythms in mammals., Annu. Rev. Neurosci., 23, 713-742.

Kiss, J., Leranth, C. S., and Halasz, B. (1984) Serotoninergic endingson VIP-neurons in the suprachiasmatic nucleus and on ACTH-neurons in the arcuate nucleus of the rat hypothalamus. A combination of high resolution autoradiography and electron microscopic immunocytochemistry. Neurosci. Lett., 44, 119-124.

Klein, D.C., Moore,R.Y. and Reppert, S.M. (1991) (eds.) Suprachiasmatic nucleus: The Mind's Clock. Oxford Uni. Press. New York.

Koritsanszky, S. (1981) Fetal and early postnatal cyto- and Synaptogenesis in the suprachiasrnatic nucleus of the rat hypothalamus., Acta Morphol. Acad. Sci Hung, 29,..227-239.

Kornhauser, J.M., Mayo, K.E., and Takahashi, J.S. (1993) Immediate-early gene expression in a mammalian circadian pacemaker: the suprachiasmatic nucleus, in:M.W. Young (eds.), Molecular genetics of Biochemical Rhythms, Dekker, NewYork, pp. 271-307.

Kortner, G., and Geiser, F., (2000) The temporal organization of daily torpor and hibernation: circadian and circannual rhythms., Chronobiol. Inr., 417, 103-128.

Kováčiková, Z., Sládek, M., Laurinová, K., Bendová, Z., Illnerová, H. and Sumová, A. (2005) Ontogenesis of photoperiodic entrainment of the molecular core clockwork in the rat suprachiasmatic nucleus., Brain Res., 1064, 83-89.

Kováčiková, Z., Sládek, M., Bendová, Z., Illnerová, H. and Sumová, A. (2006) Expression of clock and clock-driven genes in the rat suprachiasmatic nucleus during late fetal and early postnatal development. J. Biol. Rhythms, 21, 140-148.

Kume, K. *et al.* (1999) m CRYl and m CRY2 are essential components of the negative limb of the circadian clock feedback loop., Cell, 98, 193-205.

La Fleur, S. E., Kalsbeek, A., Wortel, J., Fekkes, M. L., and Buijs, R. M. (2001) A daily rhythm in glucose tolerance: a role for the suprachiasmatic nucleus., Diabetes, 50, 1237-1243.

Leak, R. K, Card, J. P. and Moore, R. Y. (1999) Suprachiasmatic pacemaker organization analyzed by viral transynaptic transport. Brain Res. 819, 23-32.

Leard, L.E., Macdonald, E.S., Heller, H.C., and Kilduff, T.S. (1994) Ontogeny of photic-induced *c-fos* mRNA expression in rat suprachiasmatic nuclei. Neuroreport, 5, 2683-2687.

Lee, H., Billings, H., and Lehman, M. (2003) The suprachiasmatic nucleus: a clock of multiple components. J. Biol. Rhythms, 18, 435-449.

Lenn, N, J., Beebe, B., and Moore, R. Y. (1977) Postnatal development of the suprachiasmatic nucleus of the rat., Cell Tissue Res., 178, 463-475.

Lewy, A., Sack, R. L., and Latham, J. (1990) Circadian Phase Shifting of Blind and Sighted People With Exogenous Melatonin Administration: Evidence for a Phase Response Curve," paper presented at the Second Annual Conference of the Society for Light Treatment and Biological Rhythms, New York, NY.

Li, X. and Davis, F. C. (2005) Developmental expression of clock genes in the Syrian hamster. Developmental Brain Res., 158, 31-40.

Lincoln, G., (1999) Melatonin modulation of prolactin and gonadotrophin secretion. Systems ancient and modern. Adv. Exp. Med. Biol. 460, 137-153.

Lincoln, G., Messager, S., Andersson, H., and Hazlerigg, D. (2002) Temporal expression of seven clock genes in the suprachiasmatic nucleus and the pars tuberalis of the sheep: Evidence for an internal coincidence timer. Proc. Natl. Acad. Sci. U.S.A., 99,13890-13895.

Liu, C., and Reppert, S. M. (2000) GABA synchronizes clock cells within the suprachiasmatic circadian clock., Neuron, 25, 123-128.

Long, M. A., Jutras, M. J., Connors, B. W., and Burwell, R. D. (2005) Electrical synapses coordinate activity in the suprachiasmatic nucleus., Nat. Neurosci., 8, 61-66.

Lowrey, P., and Takahashi, J., (2004) Mammalian circadian biology: elucidating genome-wide levels of temporal organization. Annu. Rev. Genomics Hum. Genet., 5: 407-441.

Lynch, H. J., Wurtman, R. J., Moskowitz, M. A., *et al.,* (1975) Daily Rhythm in Human Urinary Melatonin, Science, 187, 169-171.

Lynch, H. J., Jimerson, D. C., Ozaki, Y.,*et al*., (1978) Entrainment of Rhythmic Melatonin Secretion From the Human Pineal to a 12-Hour Phase Shift in the Light-Dark Cycle, Life Science, 23, 1557-1564.

Maywood, E. S., Reddy, A. B., Wong, G. K., O'Brien, J. A., McMahon, D. G., Harmar, A. J., Okamura, H., and Hastings, M. H. (2006) Synchronization and maintenance of timekeeping in suprachiasmatic circadian clock cells by neuropeptidergic signaling. Curr. Biol., 16, 599-605.

Meijer, J. H., and Rietveld, W. J. (1989) Neurophysiology of the suprachiasmatic pacemaker in rodents. Physiol. Rev. 69, 671-707.

Meijer, J. H., Michel, S., and Vansteensel, M. J. (2007) Processing of daily and seasonl light information in the mammalian circadian clock. Endocrinology, 152, 159-164.

Messager, S., Ross, A., Barret, P., and Morgan, P. (1999) Decoding photoperiodic time through *Per 1* and ICER gene amplitude., Proc. Natl. Acad. Sci. U.S.A., 96, 9938-9943.

Messager, S., Hazlerigg, D., Mercer, J., and Morgan, P. (2000) Photoperiod differentially regulate the expression of *Per 1* and ICER in the pars tuberalis and suprachiasmatic nucleus of the Siberian hamster. Eur. J. Neurosci., 12, 2865-2870.

Miller, J. D., Morin, L . P., Schwartz, W. J., and Moore, R. Y. (1996) New insights into the mammalian circadian clock. Sleep, 19, 641-667.

Moore, R. Y. (1991) in Suprachiasrnan'c Nucleus: The Mind's Clock, eds. Klein, D. C., Moore, R. Y. and Reppert, S. M.,(Oxford Univ. Press, New York), 197-216.

Moore, R. Y., and Speh, J. C. (1993) GABA is the principal neurotransmitter of the circadian system. Neurosci. Lett., 150, 112.

Moore, R. Y. (1996) Entrainment pathway and the functional organization of the circadian system. Prog. Brain Res. 111, 103-119.

Morin, A. J., Denoroy, L., and Jouvet, M. (1991) Daily variation in concentration of vasoactive intestinal polypeptide immunoreactivity in discrete brain areas of the rat. Brain Res., 538, 136-140.

Mrosovsky, N., and Salmon, R. A. (I987) A behavioral method for accelerating reentrainment of rhythms to new light-dark cycles, Nature, 330, 372-373.

Nagai, K., Nagai, N., Shimizu, K., Chun, S., Nakagawa, H., and Niijima, A. (1996) SCN output drives the autonomic nervous system: with special reference to the autonomic function related to the regulation of glucose metabolism. Pro. Brain Res., 111, 253–272.

Nakahata, Y., Okumura, N., Shima, T.,Okada, M., and Nagai, K. (2000) Light-induced tyrosine phosphorylation of BIT in the rat suprachiasmatic nucleus. J. Neurochem., 74, 2436-2444.

Nakahata, Y., Okumura, N., Otani, H., Hamada, J., Numakawa, T., Sano, S., and Nagai, K. (2003) Stimulation of BIT induces a circadian phase shift of locomotor activity in rats. Brain Res., 976, 194-201.

Nomura, K., Takeuchi, Y., Yamaguchi, S., Okamura, H., and Fukunaga, K. (2003) Involvement of calcium/calmodulin-dependent protein Kinase ll in the induction of m*Per1*. J. Neurosci. Res., 72, 384-392.

Ohta, H., Honma, S., Abe, H. and Honma, K. (2002) Effects of nursing mothers on r*Per 1* and r*Per 2* circadian expression in the neonatal rat suprachiasmatic nuclei vary with developmental stage. European Journal of neuroscience, 15, 1953-1960.

Ohta, H., Honma, S., Abe, H. and Honma, K. (2003) Periodic absence of nursing mothers phase-shifts circadian rhythms of clock genes in the suprachiasmatic nucleus of rat pups., Eur. J. Neurosci., 17, 1628-1634.

Okamura, H., Murakami, S.,Uda, K., Sugano, T., Takahashi, Y., Yanaihara, C., Yanaihara, N., and Ibata, Y. (1986) Coexistence of vasoactive intestinal peptide (VIP)-,Peptide histidine isoleucine amide (PHI)-, and gastrin releasing peptide (GRP)-like immunoreactivity in neurons of the rat suprachiasmatic nucleus. Biomed. Res., 7, 295-299.

Okamura, H., Berod, A., Julien, J., Geffard, M., Kitahama, K., Mallet, J. and Bobillier, P. (1989) Demonstration of GABAergic cell bodies in the suprachiasmatic nucleus: in situ hybridization of glutamic acid decaboxylase (GAD) mRNA and immunocytochemistry of GAD and GABA. Neurosci. Lett., 102, 131-136.

Okamura, H., Inatomi, T., Kanemasa, K., Nagatsu, I., Inouye, S., and Ibata, Y. (1994) Topography of chemically characterized neurons in the suprachiasmatic nucleus. BioSystems.

Okamura, H. *et al*. (1999) Photic induction of m*Per I* and m*Per 2* in cry-deficient mice lacking a biological clock., Science, 286, 2531-2534.

Oster, H., Werner, C., Magnone, M. C., Mayser, H., Feil, R., Seeliger, M. W., Hofman, F., and Albrecht, U. (2003) cGMP-dependent protein kinase ll modulates m*Per1* and m*Per2* gene induction and influences phase shifts of the circadian clock. Curr. Biol., 13, 725-733.

Panda, S., Antoch, M. P., Millar. B. H., Su, A. I., Schook, A. B, Straume, M., Schultz, P. G., Kay, S. A, Takahashi, J.S. and Hogenesch. J. B. (2002). Coordinated transcription of key pathways in the mouse by the circadian clock., Cell, 109, 307-320.

Parraguez, V. H., Sales, F., Valenzuela, G. J., Vergara, M., Catalán, L. and Serón-Ferré, M. (1998) Diurnal changes in light intensity inside the pregnant uterus in sheep. Anim. Reprod. Sci., 52, 123-130.

Pittendrigh, C., and Daan, S. (1976) A functional analysis of circadian pacemakers in nocturnal rodents. J. Comp. Physiol,. 106, 333-355.

Pratt, B. L., and Goldman, B. D. (1986) Environmental Influences on Circadian Periodicity of Syrian Hamsters, Physiol. Behav., 36, 91-95.

Preitner, N. *et al*. (2002) The orphan nuclear receptor REV-ERBa controls circadian transcription within the positive limb of the mammalian circadian oscillator., Cell, 110, 251-260.

Quintero, J. E., Kuhlman, S. J., and McMahon, D. G. (2003) The biological-clock nucleus: a multiphasic oscillator network regulated by light., J. Neurosci., 23, 8070-8076.

Ralph, M. R., and Menaker, M. (1986) Effects of Diazepam on Circadian Phase Advances and Delays, Brain Res., 372, 405-408.

Ripperger, J. A., Shearman, L. P., Reppert, S. M., and Schibler, U. (2000) Clock, an essential pacemaker component, controls expression of the circadian transcription factor DBP. Gemes. Dev., 14, 679-689.

Reppert, S. M. and Schwartz, W. J. (1984) The suprachiasmatic nuclei of the fetal rat: characterization of a functional circadian clock using 14C-labelled deoxyglucose. J. Neurosci. 4, 1677-1682.

Reppert, S. M., Coleman, R. J., Heath, H. W. and Swedlow, J. R. (1984) Pineal N-acetyltransferase activity in 10-day-old rats: a paradigm for studying the developing circadian system. Endocrinology, 115, 918-925.

Reppert, S. M., Duncan, M. J. and Goldman, B. D. (1985) Photic influences on the developing mammal. Ciba Found Symp. 117, 116-128.

Reppert, S. M. and Uhl, G. R. (1987) Vasopressin messenger ribonucleic acid in supraoptic and suprachiasmatic nuclei: appearance and circadian regulation during development. Endocrionology, 120, 2483-2487.

Rivkees, S. (2003) Developing circadian rhythmicity in infants. Pediatrics, 112, 373-381.

Rohling, J., Wolters, L., and Meijer, J. H. (2006) Simulation o f day-length encoding in the SCN: from single-cell to tissue-level organization. J. Biol. Rhythms, 21, 301-313.

Rusak, B., and Bina, K. G. (1990) Neurotransmitters in the Mammalian Circadian System, Annual Rev. Neurosci., 13, 387-401.

Sack, R. L., Stevenson, J., and Lewy, A. J., (1990) Entrainment of a previously free-running blind human with melatonin administration, Sleep Res., 19, 404.

Saber, C.B., Lu, J., Chou, T.C. and Gooley, J. (2005) The hypothalamic integrator for circadian rhythms. Trends in Neuroscinces. 28, 152-157.

Sasaki, Y., Murakami, N. and Takahashi, K. (1984) Critical period for the entrainment of the circadian rhythm in blinded pups by dams. Physiol. Behave., 33, 105-109.

Schaap, J., Albus, H., VanderLeest, H. T., Eilers, P. H., Détári, L., and Meiier, J. H. (2003) Heterogeneity of rhythmic suprachiasmatic nucleus neurons: Implications for circadian waveform and photoperiodic encoding., Proc. Natl. Acad. Sci. U.S.A., 100, 15994-15999.

Schibler, U., Ripperger, J., and Brown, S. A. (2003) Peripheral circadian oscillators in mammals: time and food., J. Biol. Rhythms, 18, 250-260.

Schwartz, W. J. (1991) in Suprachiasmalic Nucleus: The Mind's Clock, eds. Klein, D., Moore, R., and Reppert, S., Oxford Univ Press, New York, 144-156.

Schwatz, W. J., Caprio, A., De La Iglesia, H., Baler, R., Klein, D., Nakabeppu,, Y., and Aronin, A. (2000) Neuroscience 98, 535-547.

Schwartz, W. J., Gross, R. A., and Morton, M. T. (1987) The suprachiasmatic nuclei contain a tetrodotoxin-resistant circadian pacemaker. Proc. Natl. Acad. Sci. U.S.A., 84, 1694-1698.

Schwartz, W. J., de la Iglesia, H. O., Zlomanczuk, P., and Illenrová, H. (2001) Encoding Le Quattro Stagioni within the mammalian brain:photoperiodic orchestration through the suprachiasmatic nucleus. J Biol. Rhythms 16, 302-311.

Selim, M., Glass, J. D., Hauser, U. E. and Rea, M. A. (1993) Serotonergic inhibition of light-induced fos protein expression and extracellular glutamate in the suprachiasmatic nuclei. Brain Res., 621, 181-188.

Shearman, L. P. *et al.* (2000) .Interacting molecular loops in the mammalian circadian clock, Science, 288, 1013-1019.

Shibata, S., and Moore, R. (1987) Brain Res., 431, 311-315.

Shimomura, H., Moriya, T., Sudo, M., Wakamatsu, H., Akiyama, M., Miyake, Y., and Shibata, S. (2001) Differential daily expression of *Per 1* and *Per 2* mRNA in the suprachiasmatic nucleus of fetal and early postnatal mice., Eur. J. Neurosci., 13, 687-693.

Shinohara, K., Isobe, Y., Takeuchi, J. and Inouye, S. (1991) Circadian rhythms of somatostatin-immunoreactivity in the suprachiasmatic nucleus of the rat. Neurosci. Lett., 129, 59-62.

Sládek, M., Sumová, A., Kováčková, Z., Bendová, Z., Laurinová, K., and Illnerová H. (2004) Insight into molecular core clock mechanism of embryonic and early postnatal rat suprachiasmatic nucleus., Natl. Acad. Sci. U.S.A., 101, 6231-6236.

Söderstein, P., Devries, G. J., Buijs, R. M. and Melin, P. (1985) A daily rhythm in behavioral vasopressin sensitivity and brain vasopressin concentrations. Neurosci. Lett., 58, 37-41.

Speh, J. C., and Moore, R. Y. (1993) Retinohypothalamic tract development on the hamster and rats. Brain Res. Dev. Brain Res., 76, 171-181.

Steinlechner, S., Jacobmeier, B., Scherbarth, F., Dernbach, H., Kruse, F., and Albrecht, U. (2002) Robust circadian rhythmicity of *Per 1* and *Per 2* mutant mice in constant light and dynamics of *Per 1* and *Per 2* gene expression under long and short photoperiods. J. Biol. Rythmus., 17, 202-209.

Storch, K. F., Lipan, O., Leykln, I., Viswanathan, N., Davis, F. C., Wong, W. H. and Weltz, C. J . (2002) Extensive and divergent circadian gene expression in liver and heart., Nature, 417, 78-83.

Sugishita, M., Takashima, M., Takeuchi, Y., Kato, Y., Yamauchi, T. and Takahashi, K. (1993) Periodic mother deprivation during the light period reversed the phase of serotonin N-acetyltransferase activity rhythm of the pineal gland of rat pups. Pharmacol. Biochem. Behav., 46, 609-615.

Sumová, A., Ebling, F. J., Maywood, E. S., Herbert, J., and Hastings, M. H. (1994) Non photic circadian entrainment in the Syrian hamster is not associated with phosphorylation of the transcriptional regulator CREB within the suprachiasmatic nucleus, but is associated with adrenocortical activation. Neuroendocrinology, 59, 579-589.

Sumová, A., Trávníčková, Z., and Illnerová, H. (1995a) Memory on long but not on short day is stored in the rat suprachiasmatic nucleus., Neurosci. Lett., 200, 191-194.

Sumová, A., Trávníčková, Z., Peters, R., Schwartz, W., and Illnerová, H. (1995b) the rat suprachiasmatic nucleus is a clock for all seasons., Proc. Nat. Acad. Sci., U.S.A., 92, 7754-7758.

Sumová, A., and Illnerová, H. (1996) Endogenous melatonin signal does not mediate the effect of photoperiod on the rat suprachiasmatic nucleus. Brain Res., 725, 281-283.

Sumová, A., Trávníčková, Z., Mikkelsen, H., and Illnerová, H. (1998) Spontaneous rhythm in c-Fos immunoreactivity in the dorsomedial part of the rat suprachiasmatic nucleous, Brain Res., 801, 254-258.

Sumová, A., Trávníčková, Z., and Illnerová, H. (2000) Spontaneous c-Fos rhythm in the rat suprachiasmatic nucleus: location and effect of photoperiod., Am. J. Physiol. Regul. Integr. Comp. Physiol., 179, 2262-2269.

Sumová, A., Jáč, M., Sládek M., Šauman, I., and Illnerová H. (2003) Clock gene daily profiles and their phase relationship in the rat suprachiasmatic nucleus are affected by photoperiod. J. Biol. Rhythms. 18, 134-144.

Sumová, A., Bendová, Z., Sládek, M., El-Hennamy, R., Laurinová, K., Jindráková, Z. and Illnerová, H. (2006a) Setting the biological time in central and peripheral clocks during ontogenesis. FEBS Letters, 580, 2836-2842.

Sumová, A., Bendová, Z., Sládek, M., Kováčiková, Z., El-Hennamy, R., Laurinová, K., and Illnerová, H. (2006b) The rat circadian clockwork and its photoperiodic entrainment during development, Chronobiol. Intl., 23, 237-243.

Takahashi, K. and Deguchi, T. (1983) Entrainment of the circadian rhythms of blinded infant rats by nursing mothers. Physiol. Behav., 31, 373-378.

Terazono, H., Mutoh, T., Yamaguchi, S., Kobayashi, M., Akiyama, M., Udo, R., Ohdo, S., Okamura. H. and Shibata, S. (2003) Adrenergic regulation of clock gene expression in mouse liver., Proc. Natl. Acad. Sci. U.S.A., 100, 6795-6800.

Tischkau, S. A., Weber, E. T., Abbott, S. M., Mitchell, J. W., and Gillette, M. U. (2003) Circadian clock-controlled regulation of cGMP-protein kinase G in the nocturnal domain. J. Neurosci., 23, 7543-7550.

Tournier, B. B., Menet, J. S., Dardente, H., Poirel, V. J., Malan, A., Masson-Pévet, M.,Pévet, P., and Vuillez, P. (2003) Photoperiod differentially regulates clock genes' expression in the suprachiasmatic nucleus of Syrian hamster. Neurosci. 118, 317-322.

Turek, F. W. (1987) Pharmacological Probes of the Mammalian Circadian Clock: Use of the Phase Response Curve Approach, Trends in Pharm. Sci., 8, 212-217.

Ueda, H. R., *et al.* (2002) A transcription factor response element for gene expression during circadian night., Nature, 418, 534-539.

Van den Pol, A. N., (1986) Gamma-aminobutyrate, gastrin releasing peptide, serotonin, somatostatin, and vasopressin: ultastructural immunocytochemical localization in presynaptic axons in the suprachiasmatic nucleus. Neuroscience, 17, 643-659.

Van den Pol, A. N., Finkbeiner, S. M. and Cornell-Bell, A. H. (1992) Calcium excitability and oscillations in suprachiasmatic nucleus neurons and glia in vitro. J. Neurosci.,12, 2648-2664.

Van der Horst, G. T. J., Muijtjens, M., Kobayashi, K., Takano, R., Kanno, S., Takao, M., de Wit J., Verkerk, A., Eker, A. P. M., van Leenen, D., Buijs, R., Bootsma, D., Hoeijmakers, J. H. J., and Yasui A. (1999) Mammalian *Cry1* and *Cry2* are essential for maintenance of circadian rhythms, Nature, 398, 627-630.

Vander Leest, H. T., Houben, T., Michel, S., Deboer, T., Albus, H., Vansteensel, M. J., Block, G. D., and Meijer, J. H. (2007) Seasonal encoding by the circadian pacemaker of the SCN., Curr. Biol., 17, 468-473.

Vaněček, J., and Illnerová, H. (1985) Effect of short and long photoperiods on pineal N-acetyltransferase rhythm and on growth of testes and brown adipose tissue in developing rats., Neuroendocrinlology, 41, 186-191.

Viswanathan, N., Weaver, D. R., Reppert, S.M. and Davis, F.C. (1994) Entrainment of the fetal hamster circadian pacemaker by prenatal injections of the dopamine agonist SKF 38393., J. Neurosci., 14, 5393-5398.

Viswanathan, N. (1999) Maternal entrainment in the circadian activity rhythm of laboratory mouse (C57BL/6J). Physiol. Behav., 68, 157-162.

Vitaterna, M. H., Selby, C. P, Todo, T., Niwa, H., Thomuson, C., Fruechte, E. M., Hitomi, K., Thresher, R. J., Ishikiwa, T, Miyazaki, J., Takahashi, J. S., and Sancar, A.,. (1999) Differential regulation of mammalian period genes and circadian rhythmicity by crypto chromes 1 and 2., Proc. Natl. Acad. Sci. U.S.A., 96, 12114-12119.

Vuillez, P., Jacob, N., Teclemariam-Mesbah, R., and Pevet, P. (1996) In Syrian and European hamsters, the duration of sensitive phase to light of the suprachiasmatic nuclei depends on the photoperiod., Neurosci. Lett., 208**,** 37-40.

Watts, A.G. (1991) The efferent projections of the suprachiasmatic nucleus:anatomical insights into the control of circadian rhythms. In: D.C. Klein, R.Y. Moore and S.M. Reppert (eds.) Suprachiasmatic nucleus. The Mind's Clock, Oxford University Press, New York, p. 77-106.

Weaver, D. R., Rivkees, S. A. and Reppert, S. M. (1992) Dl-dopamine receptors activate *c-fos* expression in the fetal suprachiasmatic nuclei. Proc. Natl. Acad. Sci. U.S.A., 89, 9201-9204.

Weaver, D. R. and Reppert, S. M. (1995) Definition of the developmental transition from dopaminergic to photic regulation of c-Fos expression in the rat suprachiasmatic nucleus., Mol. Brain. Res., 33, 136-148.

Welsh, D. K., Logothetis, D. E., Meister, M., and Reppert, S. M. (1995) Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms., Neuron, 14, 697-706.

Welsh, D.K. (2007) VIP activates and couples clock cells. Focus on"Disrupted neuronal activity rhythms in the suprachiasmatic Nucleus of vasoactive intestinal polypeptide-Deficient mice" J. Neurophysiol., 97, 1885-1886.

Wuarin, J. and Schibler, U. (1990) Expression of the liver-enriched transcriptional activator protein DBP follows a stringent circadian rhythm, Cell, 63, 1257-1266.

Yagita, K., Tamanini, F., Yasuda, M., Hoeijmakers J. H., van der Horst, G. T., and Okamura, H. (2002) Nucleocytoplasmic shuttling and mCRY- dependent inhibition of ubiquitylation of the mPER2 clock protein., EMBO, J., 21, 1301-1314.

Yamaguchi, S.,Isejima, H., Matsuo, T., Okura, R., Yagita, K., Kobayashi, M., and Okamura, H. (2003) Synchronization of cellular clocks in the suprachiasmatic nucleus., Science, 302, 1408-1412.

Yamase, K., Takahashi, S., Nomura, K., Haruta, K. and Kawashima, S. (1991) Circadian changes in arginine vasopressin level in the suprachiasmatic nuclei in the rat. Neurosci. Lett., 130, 255-258.

Yan, L., Takekida, S., Shigeyoshi, Y. and Okamura, H. (1999) *Per 1*and *Per 2* gene expression in the rat suprachiasmatic nucleus: circadian profile and the compartment-specific response to light. Neuroscience, 94, 141-150.

Yokota, S., Yamamoto, M., Moriya, T., Akiyama, M., Fukunaga, K., Miyamoto, E., and Shibata, S. (2001) Involvement of calcium-calmodulin protein kinase but not mitogen-activated protein kinase in light-induced phase delays and *Per* gene expression in the suprachiasmatic nucleus of the hamster. J. Neurochem., 77, 618-627.

Yu, W., Nomura, M., and Ikea, M. (2002) Interacting feedback loops within the mammalian clock: BMALl is negatively autoregulated and upregulated by CRY1. CRYZ, and PER2. Biochem. Biophys. Rer. Comm., 290, 933-942.

Zheng B., Albrecht, U., Kaasik, K., Sage, M., Lu, W., Vaishnav, S., Li, Q., Sun, Z. S., Eichele, G, Bradley, A., and Lee C. C. (2001) Nonredundant roles of the m*Per I* and m*Per 2* genes in the mamlnalian circadian clock, Cell, 105, 683-694.

Zlomanczuk, P. and Schwartz, W. J. (1999) Cellular and molecular mechanism of circadian rhythmsin mammals. In: Regulation of Sleep and Circadian Rhythms, edited by Turek, F.W. and Zee, P.C. New York: Dekker, 309-342.

7. Publications

Chronobiology International, 23(1&2): 237–243, (2006)

ISSN 0742-0528 print/1525-6073 online
DOI: 10.1080/07420520500522523

Taylor & Francis
Taylor & Francis Group

THE RAT CIRCADIAN CLOCKWORK AND ITS PHOTOPERIODIC ENTRAINMENT DURING DEVELOPMENT

Alena Sumová, Zdenka Bendová, Martin Sládek, Zuzana Kováčiková, Rehab El-Hennamy, Kristýna Laurinová, and Helena Illnerová

Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic

The mammalian circadian pacemaker is located in the suprachiasmatic nucleus (SCN), which is composed of dorsomedial (dm) and ventrolateral (vl) regions. The molecular clockwork responsible for the SCN rhythmicity consists of clock genes and their transcriptional-translational feedback loops. The rat SCN rhythmicity and clockwork are affected by the photoperiod. The aim of this study was to elucidate development of the rat SCN rhythmicity, namely of the rhythmicity of the dm- and vl-SCN and of expression of clock genes and to ascertain when the photoperiod starts to affect the SCN rhythmicity. Rhythmicity of the dm-SCN, measured as the rhythm in spontaneous c-FOS production, developed earlier than that of the vl-SCN, which was measured as the rhythm in c-FOS photoinduction. However, photoperiodic affection of the rhythmicity occurred earlier in the vl-SCN than in the dm-SCN. From the 4 clock genes (*Per1*, *Per2*, *Cry1* and *Bmal1*) studied, the expression of *Bmal1* and *Per1* was rhythmic already in 1-day-old rats; at this age, the *Per2* mRNA rhythm just started to form and no rhythm in *Cry1* expression was detected. After the second postnatal day, all 4 genes were expressed in a rhythmic manner. Thereafter, the rhythms matured gradually via increasing amplitude. *Per1* and *Per2* mRNA rhythms started to be affected by the photoperiod at the 10th postnatal day. The data suggest that the rhythms in clock genes expression in the rat SCN develop mostly postnatally. The molecular clockwork may start to be photoperiod-dependent around the 10th postnatal day.

Keywords Suprachiasmatic nucleus, Clock genes, Photoperiodic entrainment, Development, Circadian rhythm

INTRODUCTION

The rat, like all other mammals, exhibits circadian rhythms from the molecular to the behavioral level. The rhythms are controlled by a clock located in the two suprachiasmatic nuclei (SCN) of the hypothalamus

X. Congress of the EPBRS in Frankfurt am Main, Germany, September 1–5, 2005.
Address correspondence to Dr. Alena Sumová, Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská, 1083, 142 20, Prague 4, Czech Republic. E-mail: sumova@biomed.cas.cz

(Klein et al., 1991). The SCN is composed of a ventrolateral (vl) part, also called a core, and of a dorsomedial (dm) part, called a shell (Moore et al., 2002). The SCN, itself, exhibits rhythms, *e.g.*, in spontaneous (Sumová et al., 1998) as well as light-induced (Schwartz et al., 1995) expression of immediate early genes, namely *c-fos* and in the production of arginine vasopressin (AVP) (Klein et al., 1991). Some of the rhythms are specific for the vl-SCN, *e.g.*, the rhythm in *c-fos* photoinduction (Schwartz et al., 1995), whereas others may be specific for the dm-SCN, *e.g.*, rhythms in spontaneous *c-fos* and *AVP* gene expression (Sumová et al., 1998; Jáč et al., 2000). The SCN rhythmicity is due to the SCN molecular clockwork (reviewed in Fu and Lee, 2003). The 9 mammalian clock genes cloned so far, namely 3 period genes (*Per1, -2,* and *-3*), 2 cryptochrome genes (*Cry1* and *-2*), *Clock*, *Bmal1*, *Rev-erbα*, and casein kinase 1ε (*CK1ε*), are thought to be involved in the clockwork by forming interacting transcriptional-translational feedback loops.

Overt circadian rhythms, namely the rhythm in the pineal melatonin production (Klein et al., 1991) as well as the SCN rhythmicity, itself, *e.g.*, the rhythm in *c-fos* photoinduction (Sumová et al., 1995) or in c-FOS (Sumová et al., 2000) and AVP (Jáč et al., 2000) spontaneous expression, are modulated by day-length, *i.e.*, by the photoperiod. The rat SCN clockwork is affected by the photoperiod as well (Sumová et al., 2003). The rat SCN is formed during the fetal stage. Synaptogenesis in the SCN progresses slowly in the late and early postnatal periods and then markedly increases from postnatal (P4) to postnatal day 10 (P10) (Klein et al., 1991). Intrinsic SCN rhythmicity may be already present in the fetal rat SCN.

The aim of the present work was to elucidate development of the SCN rhythmicity and of its photoperiodic entrainment. The following questions were addressed: 1) Does the rhythmicity of the dm-SCN and of the vl-SCN develop in parallel, and when does it start to be affected by the photoperiod (Bendová et al., 2004)? 2) When and how does the rhythmicity of clock gene expression develop (Sládek et al., 2004; Kováčiková et al., in press)? 3) At what developmental stage does the molecular clockwork respond to the photoperiod (Kováčiková et al., 2005)?

MATERIALS AND METHODS

Animals

Female Wistar rats were maintained under a light-dark (LD) cycle with 12 h of light and 12 h of darkness per day (LD 12 : 12) or under LD 16 : 8 or LD 8 : 16. The day of delivery was designated the postnatal day 0 (P0). On average, 4 pups per each time point were decapitated every 2 h. All experiments were conducted under License No. A5228-01 with the U.S.

National Institutes of Health and in accordance with the Animal Protection Law of the Czech Republic (License No. 1020/491/A/00) and the standards of the Journal (Touitou et al., 2004).

Rhythmicity of the dm- and vl-SCN

Daily profiles of the spontaneous c-FOS immunoreactivity (ir) and of light-induced c-FOS-ir were studied as models of a dm- and a vl- SCN rhythm, respectively. At P3 and P10, in order to avoid the masking effect of the light, either the evening light onset was advanced by 4 h when the afternoon and early night-time points were followed, or the morning light was not turned on, when the late night and daytime points were studied. To analyze the spontaneous c-FOS-ir, pups were killed in darkness. To determine the photoinduced c-FOS-ir, pups were exposed to a 30 min light pulse with an intensity of 200 to 300 lx, then returned to darkness and killed 30 min later. Brains were immediately removed and processed for immunocytochemical demonstration of c-FOS in the SCN as described elsewhere (Bendová et al., 2004).

Rhythms of Clock Gene Expression

At P1, P2, P3, and P10, mothers with their pups were released into constant darkness (DD), *i.e.*, the morning light was not turned on. Pups were sampled throughout the whole circadian cycle and their brains were processed for *in situ* hybridization to determine levels of *Per1*, *Per2*, *Cry1* and *Bmal1* gene mRNAs, as described elsewhere (Sládek et al., 2004).

RESULTS

Development of the dm- and vl-Rhythmicity and its Entrainment by the Photoperiod

In 3- and 10-day-old rats, the dm-SCN rhythm in spontaneous c-FOS-ir was already well expressed, but a response to a photoperiod similar to that in adult rats was not yet been developed (Bendová et al., 2004). The vl-SCN gate for insensitivity of c-FOS production to light at certain times was detected in 10-day-old, but not yet in 3-day-old rats; in the latter, light exposure at any day time induced high c-FOS-ir. In 10-day-old pups, as in adult rats, the gate was shorter under LD 8 : 16 than under LD 16 : 8, but the difference between the short and the long photoperiod did not yet reach that of adult animals (Bendová et al., 2004).

Development of Rhythms in Clock Genes Expression and of Their Photoperiodic Control

At P1, the *Per1* expression was already rhythmic, and after P2, the rhythm was well pronounced (Figure 1A) (Kováčiková et al., in press). The rhythm amplitude continued to increase between P3 and P10 (Sládek et al., 2004). The photoperiod did not yet affect the *Per1* mRNA profile of 3-day-old rats (Figure 2A) (Kováčiková et al., 2005). However, at P10 the morning *Per1* mRNA rise occurred already by about 2 h later under LD 8 : 16 than under LD 16 : 8. The *Per2* mRNA (Figure 1B) rhythm just began to form at P1 (Kováčiková et al., in press). After P2, however, the profile was highly rhythmic (Kováčiková et al., in press), and the rhythm amplitude continued to increase between P3 and P10 (Sládek et al., 2004). The photoperiod did not affect significantly the rhythmic *Per2* expression at P3, but at P10 the duration of low *Per2* expression lasted 2 to 4 h longer under the short

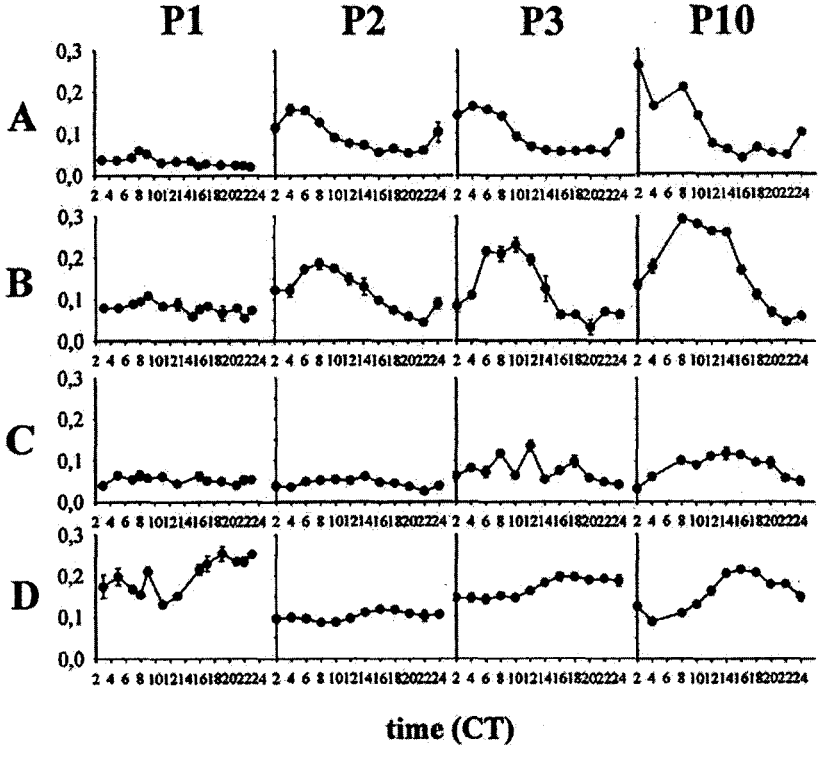


FIGURE 1 Daily profiles of *Per1* (*A*), *Per2* (*B*), *Cry1* (*C*), and *Bmal1* (*D*) mRNA levels at P1, P2, P3, and P10 in the SCN of rats maintained under LD 12 : 12 and released into darkness at the time of the expected light-dark transition. The mRNA levels are expressed as relative optical density means ± S.E.M. from 4 animals. The data are taken from Sládek and colleagues (2004) and Kováčiková and coworkers (in press).

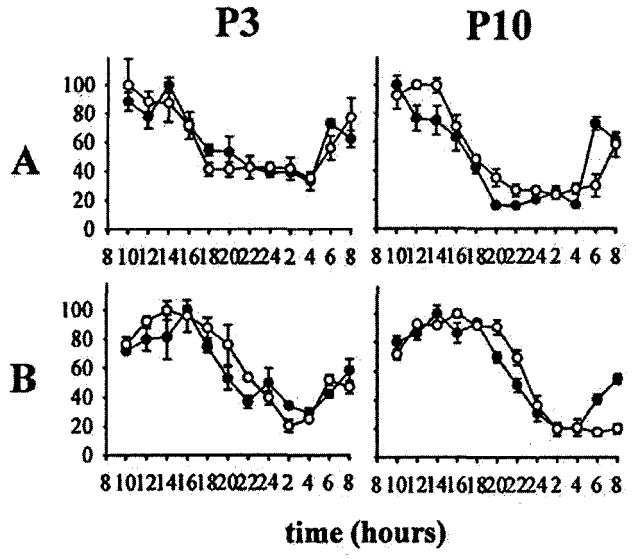


FIGURE 2 Daily profiles of *Per1* (*A*) and *Per2* (*B*) mRNA levels at P3 and P10 in the SCN of rats entrained to LD 16:8 with light on from 04:00 until 20:00 h (*dark circles*) or LD 8:16 with light on from 08:00 until 16:00 h (*open circles*) and released into darkness at the time of expected light-dark transition. The mRNA levels are expressed as percent of maximal relative optical density means ± S.E.M. from 4 animals. The data are taken from Kováčiková et al. (2005).

than under the long photoperiod (Figure 2B) (Kováčiková et al., 2005). *Cry1* mRNA rhythm (Figure 1C) started to form at P2, but there was no rhythm at P1 (Kováčiková et al., in press). Gradually, the rhythm amplitude increased (Sládek et al., 2004). At P3 as well as at P10, the profile of *Cry1* mRNA expression under LD 16:8 did not differ significantly from that under LD 8:16 (Kováčiková et al., in press). *Bmal1* mRNA (Figure 1D) exhibited a highly significant rhythm already at P1 (Kováčiková et al., in press). Initially, the phase of the rhythm was opposite to that of *Per1* expression as is the case in the later postnatal ages; however, its shape differed from that at later ages. Neither at P3 nor at P10 was the rhythmic *Bmal1* expression affected by the photoperiod in a manner similar to that in adult rats (Kováčiková et al., in press).

DISCUSSION

The data suggest that the circadian rhythmicity of the vl-SCN develops later than that of the dm-SCN. We make this suggestion with caution since only the rhythm in c-FOS photoinduction was analyzed in the vl-SCN.

The molecular clockwork develops only gradually. At P1, the rhythm in *Bmal1* and a slight rhythm in *Per1* expression were already present; whereas, the rhythm in *Per2* expression just started and no rhythm in *Cry1* expression was observed. After P2, all studied genes were expressed

in a rhythmic manner and matured mostly via increasing the amplitude. Altogether, the data suggest that rhythms in clock genes expression develop mostly postnatally and not necessarily in parallel.

The photoperiodic control of the SCN circadian rhythmicity may also develop only gradually. At P10, the rhythm of c-FOS photoinduction in the vl-SCN, *i.e.*, the rhythm in photosensitivity, but not yet the spontaneous rhythm in c-FOS production in the dm-SCN, started to be affected by the previously experienced photoperiod. From the studied clock genes, the rhythmic expression of *Per1* and *Per2*, but not that of *Cry1* and *Bmal1*, also started to be affected by the photoperiod at P10, though not yet to the same extent as in adult rats (Sumová et al., 2003). The data suggest a dominant role of light-sensitive *Per1* and *Per2* genes in the SCN photoperiodic entrainment (Hastings, 2001) during development. It was somewhat surprising that the SCN rhythmicity was not affected by the photoperiod at P3, *i.e.*, shortly after birth, as transduction of photoperiodic information from mother to fetus has been demonstrated in several species, *e.g.*, in the Djungarian hamster (Stetson et al., 1986). Thus, the photoperiodic information is not transduced via an undeveloped fetal SCN, or the SCN memory on the fetal photoperiod is only short-term. The postnatal photoperiod may start to affect the circadian system around P10.

ACKNOWLEDGMENTS

The work was supported by the Grant Agency of the Czech Republic, Grant No. 309050350, and by Research Projects Nos. LC 554 and AV0Z 50110509.

REFERENCES

Bendová, Z., Sumová, A., Illnerová, H. (2004). Development of circadian rhythmicity and photoperiodic response in subdivisions of the rat suprachiasmatic nucleus. *Brain Res.* 148:105–112.

Fu, L., Lee, C.C. (2003). The circadian clock: pacemaker and tumor suppressor. *Nature Rev. Cancer* 3: 350–361.

Hastings, M. (2001). Modeling the molecular calendar. *J. Biol. Rhythms* 16:117–123.

Jáč, M., Kiss, A., Sumová, A., Illnerová, H., Ježová, D. (2000). Daily profiles of arginine vasopressin mRNA in the suprachiasmatic, supraoptic and paraventricular nuclei of the rat hypothalamus under various photoperiods. *Brain Res.* 887:472–476.

Klein, D.C., Moore, R.Y., Reppert, S.M. (1991). *Suprachiasmatic Nucleus: The Mind's Clock.* New York: Oxford University Press, p. 467.

Kováčiková, Z., Sládek, M., Bendová, Z., Illnerová, H., Sumová, A. (2005). Expression of clock and clock-driven genes in the rat suprachiasmatic nucleus during fetal and early postnatal age. *J. Biol. Rhythms.* In press.

Kováčiková, Z., Sládek, M., Laurinová, K., Bendová, Z., Illnerová, H., Sumová, A. (2006). Ontogenesis of photoperiodic entrainment of the molecular core clockwork in the rat suprachiasmatic nucleus. *Brain Res.* 1064:83–89.

Moore, R.Y., Speh, J.C., Leak, R.H. (2002). Suprachiasmatic nucleus organization. *Cell Tissue Res.* 309: 89–98.

Schwartz, W.J., Aronin, N., Takeuchi, J., Bennet, M.R., Peters, R.J. (1995). Towards a molecular biology of the suprachiasmatic nucleus: photic and temporal regulation of *c-fos* gene expression. *Semin. Neurol.* 7:53–60.

Sládek, M., Sumová, A., Kováčiková, Z., Bendová, Z., Laurinová, K., Illnerová, H. (2004). Insight into molecular core clock mechanism of embryonic and early postnatal rat suprachiasmatic nucleus. *Proc. Natl. Acad. Sci. USA* 101:6231–6236.

Stetson, M.H., Elliot, J.A., Goldman, B.D. (1986). Maternal transfer of photoperiodic information influences the photoperiodic response of prepubertal Djungarian hamsters (Phodopus sungorus sungorus). *Biol. Reprod.* 36:664–669.

Sumová, A., Trávníčková, Z., Peters, R., Schwartz, W.J., Illnerová, H. (1995). The rat suprachiasmatic nucleus is a clock for all seasons. *Proc. Natl. Acad. Sci. USA* 92:7754–7758.

Sumová, A., Trávníčková, Z., Mikkelsen, J.D., Illnerová, H. (1998). Spontaneous rhythm in c-Fos immunoreactivity in the dorsomedial part of the rat suprachiasmatic nucleus. *Brain Res.* 801: 254–258.

Sumová, A., Trávníčková, Z., Illnerová, H. (2000). Spontaneous c-Fos rhythm in the rat suprachiasmatic nucleus: location and effect of photoperiod. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 279:R2262–R2269.

Sumová, A., Jáč, M., Sládek, M., Šauman, I., Illnerová, H. (2003). Clock gene daily profiles and their phase relationship in the rat suprachiasmatic nucleus are affected by photoperiod. *J. Biol. Rhythms* 18:134–144.

Touitou, Y., Portaluppi, F., Smolensky, M.H., Rensing, L. (2004). Ethical principles and standards for the conduct of human and animal biological rhythm research. *Chronobiol. Int.* 21:161–170.

FEBS Letters 580 (2006) 2836–2842

Minireview

Setting the biological time in central and peripheral clocks during ontogenesis

Sumová Alena*, Bendová Zdenka, Sládek Martin, El-Hennamy Rehab, Laurinová Kristýna, Jindráková Zuzana, Illnerová Helena

Department of Neurohumoral Regulations, Institute of Physiology, Academy of Sciences of the Czech Republic, Videnska 1083, 142 20 Prague 4, Czech Republic

Received 28 February 2006; accepted 6 March 2006

Available online 20 March 2006

Edited by Horst Feldmann

Abstract In mammals, the principal circadian clock within the suprachiasmatic nucleus (SCN) entrains the phase of clocks in numerous peripheral tissues and controls the rhythmicity in various body functions. During ontogenesis, the molecular mechanism responsible for generating circadian rhythmicity develops gradually from the prenatal to the postnatal period. In the beginning, the maternal signals set the phase of the newly developing fetal and early postnatal clocks, whereas the external light–dark cycle starts to entrain the clocks only later. This minireview discusses the complexity of signaling pathways from mothers and the outside world to the fetal and newborn animals' circadian clocks.

Keywords: Circadian system; Suprachiasmatic nucleus; Peripheral circadian clocks; Ontogenesis

1. Introduction

Organisms are exposed to environmental changes that recur mostly in 24-h cycles as a consequence of the Earth's rotation. The most prominent changes are cycles in light and darkness. In response to such changes, organisms evolved an endogenous clock, i.e., a mechanism that enables them to anticipate rhythmically occurring events. Even under constant environmental conditions, the clock generates rhythmic signals in about 24-h cycles and is, therefore, called circadian (from Latin circa diem). Under natural conditions, the circadian clock is entrained to the 24-h day by external cyclically occurring events, mainly by the light period of the day. Due to the entrainment, periods of rest and activity and of many other physiological functions are restricted to a certain time of the day to ensure the best strategy for obtaining food, exposure to optimal outside temperature, protection against predators and excess of sun light, etc.

In mammals, the principal circadian clock resides in cells grouped in two suprachiasmatic nuclei (SCN) of the hypothalamus [1]. In rodents, the paired nuclei are composed of about 20 000 neurons. These neurons are themselves circadian oscillators and are mutually synchronized [2]. Morphologically and functionally, the rodent SCN is divided into at least two parts, namely the ventrolateral (VL) part called the core and the dorsomedial (DM) part called the shell. The VL part receives the photic information from the retina (see below) and expresses mostly light dependent rhythms, e.g., in photoinduction of the immediate early genes (IEGs) *c-fos* and *junB* [3]. The DM part exhibits spontaneous oscillations of many rhythmic variables, like expression of the *arginine vasopressin* and *c-fos* genes [4,5]. Apart from the SCN, nearly every tissue of the body, e.g., liver, kidneys, heart, muscle, spleen, etc., contains a peripheral clock driving local rhythms specific for the tissue function [for review see 6]. Under entrained conditions, the phase of the peripheral clocks is set by the SCN program. However, the peripheral clocks rhythmicity persists even in tissue culture and may not depend on the SCN [7].

The basic molecular core clock mechanism responsible for generation of the rhythmicity within the SCN and peripheral rhythmic cells is formed by interactive transcriptional–translational feedback loops between the clock genes, namely two *Per* (*Per1,2*), two *Cry* (*Cry1,2*), *Clock*, *Bmal1*, *Rev-erbα* and *casein kinase 1 epsilon* (*CK1ε*), and their protein products PER1,2, CRY1,2, CLOCK, BMAL1, REV-ERBα, CK1ε [for review see 8]. Briefly, CLOCK and BMAL1 as a heterodimer positively activates the rhythmic expression of *Per*, *Cry* and *Rev-erbα* genes. In the cytoplasm, the PER and CRY proteins form a complex important for nuclear translocation of both proteins. After shuttling into the nucleus, the PER:CRY complex directly interacts with the CLOCK:BMAL1 heterodimer and inhibits CLOCK:BMAL1 mediated transcription. Regulation of *Bmal1* transcription is mediated mostly by REV-ERBα. The SCN and peripheral clocks operate with similar components and share a similar molecular core clock mechanism. However, some tissue-dependent differences may exist [9]. Also, phasing of clock gene expression differs between the SCN and various peripheral tissues. Peripheral clocks may be phase delayed relative to the SCN by 3–9 h. Although the molecular basis of the circadian clock has been partially defined, the molecular clock outputs that ultimately control circadian rhythms at cellular, organ and system-level are still poorly understood. Components of the core clock mechanism within the SCN and peripheral tissues may serve as down-

*Corresponding author. Fax: +420 241062488.
E-mail address: sumova@biomed.cas.cz (A. Sumová).

0014-5793/$32.00
doi:10.1016/j.febslet.2006.03.023

stream transcription factors. At a certain time of day, they switch on transcription of a great array of tissue specific clock controlled genes that are relevant to distinct functions of these organs [10,11]. For example, about 10% of the liver transcriptome is under circadian control [12,11]. In the SCN, the arginine vasopressin (AVP) gene is one of the best-recognized clock controlled genes: it is expressed in a circadian manner and appears to augment SCN excitability [13].

2. Setting the biological clocks

2.1. Entrainment of the central clock

The phase of the central clock is set mostly by photic stimuli: exposure of animals to light in the first part of the subjective night phase-delays and in the second half phase-advances the clock [14]. The photic information is transferred from the retina to the SCN via the monosynaptic retinohypothalamic (RHT) and polysynaptic geniculohypothalamic (GHT) tracts. Besides the classical retinal photoreceptors cones and rods, a small subset of retinal ganglion cells containing the opsin-like protein melanopsin is also photosensitive and projects to the SCN [for review see 15]. The RHT and GHT terminate on a subset of retino-recipient cells in the VL SCN. The spontaneously rhythmic cells in the DM SCN receive photic information only through the VL part. In the late day, the signal of darkness may be neuropeptide Y, the main neurotransmitter of the GHT [16]. During the night, release of the RHT neurotransmitter glutamate signals "light" to the clock [for review see 17]. Light-induced clock resetting may involve sequential activation of glutamatergic NMDA and non-NMDA receptors. Depending on the time when light impinges on the retina at night, the SCN signals downstream of glutamate may diverge. In the early night, signal transduction leads to activation of ryanodine receptors and release of Ca^{2+}. In the late night, the activated cGMP-dependent pathway downstream of glutamate involves Ca^{2+} influx, nitric oxide synthetase and intracellular movement of nitric oxide. Nitric oxide can activate soluble guanylylcyclase, which increases cGMP and activates cGMP-dependent protein kinase (PKG) [18]. Activation of the second messenger pathways is followed by activation of transcription factors. The Ca_i^{2+}/cAMP response element binding protein (CREB) is phosphorylated [19] and IEGs, namely *c-fos* and *jun-B* [3] and clock genes *Per1* and *Per2* [20] are transcriptionally activated, mostly in the VL SCN. Light induced P-CREB may directly regulate transcription of *Per* genes via a CRE element in the 5′-flanking regions of their promoters [21]. Importantly, light may induce CREB phosphorylation and transcription of IEGs and clock genes only during the interval when light entrains the clock, i.e., during the subjective night [19,3]. While the role of IEGs in photic entrainment has not yet been solved, induction of *Per1* and *Per2* genes is believed to be involved in resetting the core clock molecular mechanism. Via the above-mentioned pathways, the clock may attain a new phase in response to a photic stimulus experienced at night. Also, a long day length, i.e., a long photoperiod, such as during summer days, may modulate the SCN rhythmicity as well as its molecular clockwork [for review see 17,22].

Non-photic stimuli, like enforced locomotor activity, arousal, serotonergic drugs, melatonin, dark pulses, etc., are also supposed to reset the central clock when administered at a critical time of the day, e.g., in the late day [for review see 23]. Due to the complexity of the stimuli, their resetting pathways may vary. These may, however, converge at the same endpoint since it has been demonstrated that several non-photic cues acutely downregulate the *Per1* and *Per2* genes, i.e., act opposite to light stimuli. Hence, the *Per* genes may represent the molecular target for the modulating effect of non-photic stimuli on light signaling to the clock.

2.2. Entrainment of peripheral clocks

Peripheral clocks are indirectly entrained by light via setting their phase by the light entrainable SCN clock. However, they may also be directly entrained by changes in their local environment. Under normal conditions, the indirect and direct pathways act in concert. The SCN-controlled rhythm in spontaneous feeding represents one of the strongest entraining cues for many peripheral clocks. In nocturnal animals, the feeding rhythm is related to another SCN-controlled rhythm, i.e., to the rhythm in locomotor activity. Both locomotor activity and feeding mostly occur during night. However, under certain circumstances, the local entraining cue might be in conflict with the SCN signaling. This may happen in the case when access to food is restricted to an unusual time of the day, i.e., to the daytime rest period. Under such restricted feeding, the rhythmic gene expression in liver, kidneys, heart, and other tissues is phase-shifted relative to that in animals fed ad libitum, whereas the phase of gene expression within the SCN does not change [24]. Under such conditions, entrainment of the peripheral clock mediated via the nutrition supply may uncouple from the SCN entrainment. Besides the feeding rhythm, the SCN may control peripheral clocks by humoral as well as neural pathways. In the liver, glucocorticoids have been proposed to play a role in setting the phase, as administration of dexamethasone acutely shifts rhythmic gene expression in the liver and induces rhythmic *Per* expression in cell cultures [25]. Neural pathways may involve the autonomic nervous system since adrenaline may control gene expression in the liver [26].

3. Ontogenesis of the biological clocks

3.1. Ontogenesis of the SCN clock

Development of the SCN clock proceeds in more stages from fetal to postnatal periods. In the rat, the SCN is formed as a component of periventricular cell groups during embryonic days (E)14 through E17. Neurogenesis is complete at E18 although morphological maturation proceeds until postnatal day (P)10. During prenatal period, the SCN neurons only form a few synapses [27]. In this respect, the fetal SCN might resemble an in vitro culture of dissociated SCN cells where connections between the individual cells are sparse or do not exist. Synaptogenesis progresses slowly around birth and then markedly increases from P4 to P10 [27].

It seems that appearance of the first significant rhythms in clock genes expression within a population of the rat SCN neurons proceeds in parallel with the SCN development. At E19, no rhythms of clock genes expression and no clock proteins PER1, PER2 and CRY1 are detectable [28]. At E20, formation of a rhythm in *Per1* expression is indicated and rhythms of *Per2*, *Cry1* and *Bmal1* only appear during the first postnatal days [29]. In another study, rhythms in *Per1* and *Per2* expression in the rat SCN have been reported at E20 [30,31]. Impor-

tantly, amplitude of the rhythms in *Per1* and *Per2* expression in rhythmic SCN cells increases with age until P10 [28] as the synaptogenesis progresses. The parallelism points to the importance of mutual communication between individual clock cells for generating a marked rhythmic signal. Interestingly, *Bmal1* is strongly expressed in the fetal SCN of rats [28] as well as of hamsters [32], while *Per1* and *Cry1* are expressed only weakly.

The rat SCN clock starts to drive output rhythms only around birth as the rhythm in *AVP* heteronuclear RNA is undetectable in the rat SCN at E20, i.e., 1–2 days before birth, but is clearly present at P1 [29]. The rhythm in *AVP* mRNA is detectable at E21 [33], whereas the rhythm in firing rate only at E22 [34]. Altogether, these data are in favor of the hypothesis that the rat is born with a rather immature SCN clock that develops further postnatally. It remains to be ascertained whether the day–night difference in the SCN metabolic activity, monitored by a 2-deoxyglucose uptake and detected as soon as at E19 [35], i.e., well before the first appearance of the rhythm in clock genes expression, represents an intrinsic SCN rhythmicity or a maternal cue driven change. Also, it is of utmost importance to reveal whether the lack of rhythmicity in clock genes expression within the fetal SCN is due to a lack of synchronization between single oscillating SCN cells. However, the undetectable levels of clock proteins throughout the circadian cycle at E19 [28] suggest rather a not yet fully developed core clockwork in the fetal SCN.

3.2. Ontogenesis of peripheral clocks

Development of peripheral clocks depends on maturation of the organ housing the clock as well as on maturation of the molecular clockwork. The first appearance of molecular oscillations might be thus highly organ- and species-specific. In the rat heart, rhythmic expression of *Per1* and *Bmal1* genes begins between P2 and P5 whereas that of *Per2* begins at P14 [36]. In the rat liver, rhythms in clock gene expression may start from P2 and develop further through P10 until P20 [28]. In the murine cerebral cortex, daily rhythms of *Per1* and *Per2* mRNA are detected from P14 [37].

4. Entrainment of developing clocks

4.1. Maternal signaling to fetal clocks

The fetal SCN clock is supposed to be entrained exclusively by cues delivered periodically by the mother. Though light under certain circumstances may reach the fetus even in the uterus [38], the photic pathways to the fetal SCN in altricial rodents are not fully developed. Therefore, non-photic maternal entrainment appears to be dominant. There is extensive evidence that primarily the maternal SCN sets the phase of the developing fetal clock. First, the rhythm in the fetal SCN metabolic activity is synchronized by the maternal SCN [39]. Second, the newly forming and appearing rhythms in clock genes expression in the very late fetal and early neonatal stages are, from the beginning, in phase with the maternal clock [29]. Moreover, although the maternal SCN does not generate fetal rhythms per se, it ensures the postnatal within litter synchrony [for review see 40]. In hamsters, the postnatal within litter synchrony is established very early during the fetal development as a maternal SCN lesion at E10 but no more at E12 abolishes

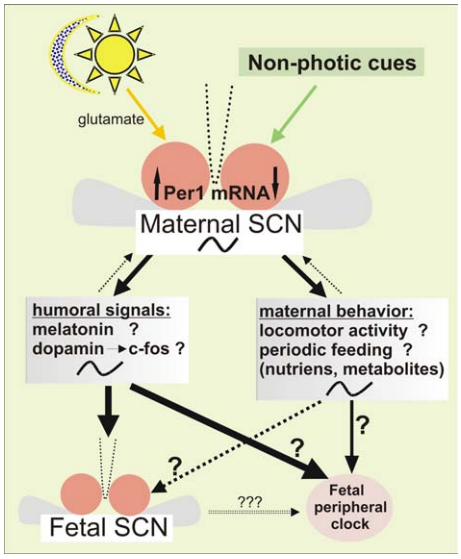


Fig. 1. Signaling to developing fetal clocks. The maternal circadian clock within the suprachiasmatic nucleus (SCN) is entrained mostly by photic and also by non-photic cues with time of the day. The underlying molecular mechanism is symbolized by Per1 mRNA that is upregulated by photic and downregulated by non-photic entraining stimuli. The entrained maternal SCN controls overt humoral and behavioral rhythms that may feedback to the maternal SCN. At the same time, the fetal SCN and perhaps peripheral clocks are entrained via as yet only partially recognized rhythmically delivered maternal stimuli. Although the fetal clocks begin to exhibit intrinsic rhythmicity of the molecular clockwork only around birth and early postnatally, the phase of the newly forming and appearing rhythms in the fetal SCN is set by the maternal SCN early prenatally. Pathways from the maternal to fetal clocks may involve signaling by dopamine via induction of c-fos and/or by melatonin (thick arrow). Also, behavioral maternal rhythms, e.g., locomotor activity and feeding may, hypothetically, entrain the fetal clocks (thin arrows). For more detail see Section 4.1.

the synchrony [41]. In rats, it is suggested that the maternal synchronization of fetal clocks occurs even before the SCN is formed [42]. If this is the case, what is the fetal anatomical substrate that is synchronized by the mother's clock? And as the molecular clockwork in the rat SCN develops mostly postnatally, what is the fetal molecular mechanism that is synchronized by maternal signals? There is also confusion concerning the photoperiodic entrainment of fetuses and newborn rodents. Djungarian hamsters maintain memory of the photoperiod experienced during their fetal stage even postnatally, i.e., the photoperiodic entrainment should be set by their mothers. However, rhythms in clock genes expression or in *c-fos* photoinduction in the neonatal rat SCN are not modulated by the photoperiod experienced by mothers during pregnancy [43,44], though photoperiod modulates the rhythms in the adult rat SCN [for review see 22]. The above-mentioned rhythms, as well as the overt rhythm in the pineal melatonin production, start to be photoperiod dependent only around P10 [for review see 40]. A question arises as to where the memory of the photoperiod experienced during the fetal stage is stored, if not in the neonatal rodent SCN?

Also the entraining signal from mother to fetus is still not completely understood. A designed candidate must exhibit a circadian variation, penetrate the placenta and act at a functional receptor or affect neuronal activity of the fetal SCN. It is difficult to imagine how the fetal clock might become en-

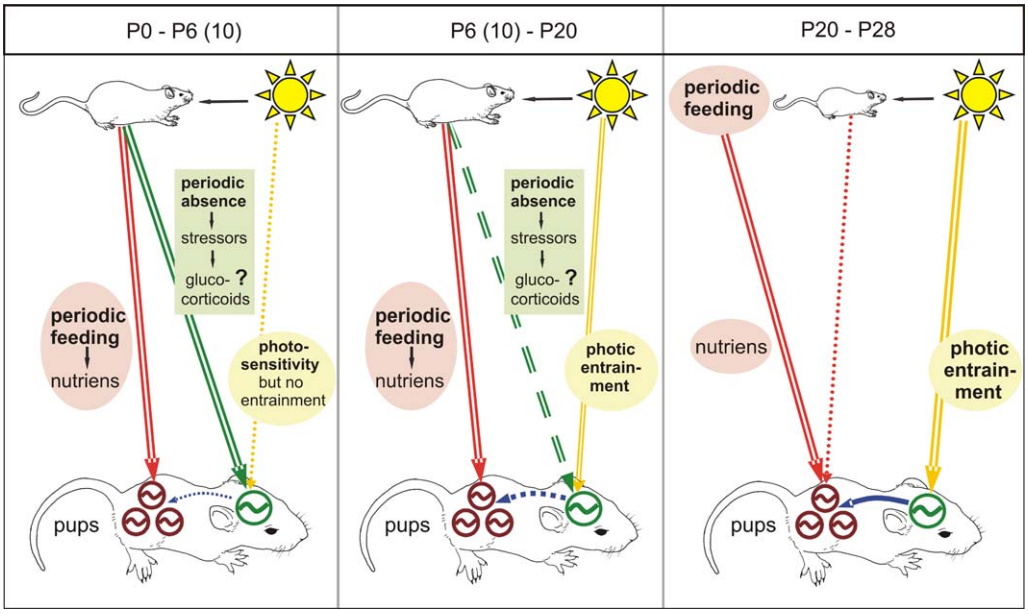


Fig. 2. Signals entraining clocks after birth. Three developmental periods in rodents are depicted: (i) about first week of life, i.e., between the postnatal day 0 and 6–10 (P0–P6(10)); (ii) since the end of the first week until P20 involving the start of weaning (P6(0)–P20); and (iii) between P20 and the end of weaning at P28 (P20–P28). P0–P6(10): During this period, pups are fully dependent on maternal care and maternal entrainment prevails. Periodic absence of the mother might entrain molecular oscillations within the pup's SCN clock via glucocorticoids. Periodic breast feeding and maternal care entrain molecular oscillations in the peripheral clocks. Although the newborn pup's SCN clock is already photosensitive, the photic entrainment does not yet occur. P6(10)–P20: during this period, pups open their eyes and start to be partially independent of their mothers. Significance of maternal absence as an entraining cue of the pup's SCN is losing and the pup's SCN clock begins to be entrained by photic stimuli. At the same time, the pup's SCN may start to control peripheral clocks. Moreover, apart from the maternal day-time feeding, pups begin gradually to forage themselves during the night-time and molecular oscillations of peripheral clocks shift accordingly. P20–P28: during this period, pups become completely independent of their mothers and maternal entrainment is lost. Similarly as in adults, the SCN clock is entrained dominantly by photic cues and peripheral clocks by nocturnal feeding regime. The SCN clock may entrain molecular oscillations in peripheral clocks either directly or rather indirectly via entraining the feeding regime with the external daytime. For more details see Sections 4.2 and 4.3.

trained before it becomes rhythmic itself. Hypothetically, the mechanism might be similar to induction of oscillations in peripheral clock cells in vitro following the addition of serum [45]. At a certain time of day, an entraining maternal cue may trigger a signaling pathway that might impinge onto the yet non-rhythmic clock cells and induce expression of certain genes. Consequently, an imprinting of time awareness might be initiated. Alternatively, a maternal signal might synchronize already existing oscillations in individual cells. Maternal melatonin may fulfill all the criteria of a functional entraining cue and was considered as a first class candidate at least for the photoperiodic entrainment. However, it appears that hormones are not exclusive entraining cues for the fetal clock. Activation of dopaminergic pathways through D1 receptors entrains rodent fetuses as well [46]. Dopamine receptors, as well as melatonin receptors, are present in fetal SCN cells [47]. While melatonin might be considered as the signal of night, dopamine might be the signal of day. The dopaminergic signaling includes activation of the IEG *c-fos* within the fetal SCN [48]. In the adult rat SCN, *c-fos* expression is spontaneously high during the daytime and low during the nighttime [5]. *c-fos* expression is likely related to neuronal activation which is also high during the day and low during the night. Importantly, a marked rhythm in cFos protein immunoreactivity in the neonatal rat SCN is present at P3, i.e., at the earliest time tested [44]. Preliminary data show that the rhythm might be present even at earlier developmental stages (El-Hennamy et al., unpublished results). It is therefore possible that maternal cue-induced *c-fos* expression may provide the fetal clock with a daytime signal and elevation of neuronal activity. It is not yet clear, however, how the suggested signals and pathways may induce rhythmic expression of clock genes. The signaling pathways activating *c-fos* and *Per* genes share a common element, i.e., phosphorylation of CREB. This step might represent the crucial point triggering rhythmic clock gene expression. As more cues share the ability to induce phosphorylation of CREB and expression of *c-fos*, the induction might, hypothetically, represent a common step setting the daytime in the fetal clock (see Fig. 1).

4.2. Signals entraining the central clock postnatally

In rodents, such as rats, mice and hamsters, the newborn neonates are fully dependent on their mothers. In the laboratory, the exquisite maternal entrainment of their rhythmicity becomes less important after the first week of their life when the photic entrainment starts to override the maternal entrainment. In nature, the switch from maternal to photic entrainment may correlate with the ability of pups to leave their underground burrows and get exposed to the environmental light. The mechanism underlying the change in sensitivity of the clock to entraining signals is not fully understood. The phase of the newborn rat SCN clock is set prenatally, synchronously with the mother's clock. Rat pups are born, however, with a low-amplitude oscillation in clock genes expression and the amplitude increases only gradually [28,29]. At the early developmental stage, pups may be partly entrained to the different circadian phase of a foster mother [for review see 40]. This maternal entrainment may be facilitated or even enabled by the low amplitude of pups' clock

oscillations. As the clock rhythmicity strengthens, maternal cues may lose the ability to entrain it and a stronger entraining agent, i.e., light, may take their place.

Maternal cues entraining the pups' clock postnatally may not be the same as those entraining the fetal clock. Many potentially entraining substrates, such as, e.g., melatonin, may be delivered in milk. Recent studies however indicate that this pathway may contribute only little to resetting the pups' clock. When blinded newborn rats are reared by a foster mother on an inverted light–dark regime, the phase of rhythms in *Per1* and *Per2* mRNAs within the pups' SCN is shifted only marginally by about 2 h [30]. Maternal behavior, namely absence of the mother, may, however, strongly entrain the neonatal clock. When newborn pups are deprived of their mothers during the light phase, i.e., at the time when they usually suckle milk, the rhythmic SCN expression of *Per1* and *Per2* genes is completely phase-reversed within six days [31]. Likely, the feeding regime and the periodic partial maternal absence are not the crucial resetting cues for the pups' SCN clock, as they are also reversed under the fostering experiment [30]. The complete absence of the mother at the time when pups are usually fed may be, however, a strong stressor for pups altering expression of stress related genes, such as *corticotropine releasing hormone*, *glucocorticoid receptor* and *AVP* [31]. Hypothetically, the signaling pathway involved in the maternal postnatal entrainment might employ glucocorticoids similarly as with entrainment of peripheral clocks in adults. Sensitivity of the SCN clock to stress diminishes with postnatal age [49].

As innervation of the VL SCN via RHT and GHT develops mostly during the first days after birth, pups become more sensitive to light and gradually the photic entrainment of the SCN clock prevails. The signaling cascade responding to light is functional at least partly immediately after birth: light pulses induce *c-fos* expression in the rat SCN on the day of birth [48] or at P1 [50]. The light induced gene expression is, however, not the only pre-requisite for photic resetting the circadian clock. The photic entrainment may be accomplished mostly due to the fact that light induces the signaling cascade only during a restricted time window that corresponds to the duration of subjective night. During the subjective night, the SCN clock is sensitive to light and photic stimuli may phase delay or phase advance the clock depending upon the time of their administration. The mechanism of how the molecular clockwork gates the response to light is still not understood. The gate for insensitivity to light is not yet developed at P3 since light pulses administered at any time within a 24-h cycle induce high cFos immunoreactivity in the SCN no matter whether it is day or night [44]. In another study [48] a slight gate was indicated at P2. However, the gate for insensitivity to light becomes present only at P10 [44]. This day corresponds well with the developmental stage when photic entrainment begins to override maternal entrainment [for review see 40]. Moreover, at P10 the rat SCN clock starts to be entrained by the photoperiod [43]. In comparison with adult rats [51], the photoperiodic control of the molecular clockwork is only partial and even at P20 it is not yet complete [43]. The data suggest that at least in rodents, the postnatal photic and photoperiodic entrainment develops in dependence on advancement of the mechanism that gates the clockwork insensitivity to light. The development proceeds gradually and may be accomplished at the end of the weaning time (see Fig. 2).

4.3. Signals entraining peripheral clocks postnatally

During postnatal ontogenesis, the circadian expression of clock genes in the rat peripheral clocks might be entrained not only by signals from the developing SCN clock, but also by maternal behavior, namely by the rhythm in breast feeding and care of the newborns. The latter possibility seems to be the case in the first weeks of life. The mother feeds her pups and thus keeps them active mostly during the day. Adult rats, however, are active and consume food mostly at night. During the weaning period, between P14 and P28, the pup's feeding and activity regimes apparently change. In parallel with the changes, the phases of rhythms in genes expression in the heart change as well [36]. First, the phases shift by several hours between P14 and P20. The shifts, though smaller, continue, together with a drastic change of the rhythm's amplitude between P20 and P30, when the matured circadian system seems to have been established. Similarly, during development of the molecular clockwork in the rat liver, rhythms of clock genes expression appear to phase shift during the first weeks of life [28]. Apparently, at this developmental stage, setting peripheral clocks by the feeding regime may prevail upon entrainment by the SCN (see Fig. 2).

5. Concluding comments

This minireview cannot encompass all known data on biological clocks and their entrainment during development. From the data summarized it is, however, obvious how little is known about biochemical signals setting the time in the clocks. Many questions still remain to be answered. What may be the pathways setting the phase of the SCN clock prenatally by the mother at the time when the fetal SCN is not yet formed or at the time when the molecular clockwork is not yet functioning? And what pathways mediate maternal entrainment of the central and peripheral clocks during the first weeks after delivery?

It is of great importance to recognize principles of maternal and photic entrainment of the circadian system during development. This system plays a significant role in controlling many physiological processes and understanding the mechanisms of its entrainment during ontogenesis might facilitate optimization of conditions necessary for its healthy development in animals, as well as human beings.

Acknowledgements: The authors' work is supported by the Grant Agency of the Czech Republic, Grant No. 309050350, by Research Projects Nos. AV0Z 50110509, LC554 and by the 6th Framework Project EUCLOCK No. 018741.

References

[1] Klein, D.C., Moore, R.J. and Reppert, S.M. (1991) in: Suprachiasmatic Nucleus: The Mind's Clock (Klein, D.C., Moore, R.J. and Reppert, S.M., Eds.), pp. 197–216, Oxford University Press, New York.
[2] Welsh, D.K., Logothetis, D.E., Meister, M. and Reppert, S.M. (1995) Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms. Neuron 14, 697–706.
[3] Kornhauser, J.M., Nelson, D.E., Mayo, K.E and Takahashi, J.S. (1992) Regulation of jun-B messenger RNA and AP-1 activity by light and a circadian clock. Science 255, 1581–1584.

[4] Jáč, M., Kiss, A., Sumová, A., Illnerová, H. and Ježová, D. (2000) Daily profiles of arginine vasopressin mRNA in the suprachiasmatic, supraoptic and paraventricular nuclei of the rat hypothalamus under various photoperiods. Brain Res. 887, 472–476.
[5] Sumová, A., Trávníčková, Z., Mikkelsen, J.D. and Illnerová, H. (1998) Spontaneous rhythm in c-Fos immunoreactivity in the dorsomedial part of the rat suprachiasmatic nucleus. Brain Res. 801, 254–258.
[6] Schibler, U., Ripperger, J. and Brown, S.A. (2003) Peripheral circadian oscillators in mammals: time and food. J. Biol. Rhythms 18, 250–260.
[7] Yoo, S.H., Yamazaki, S., Lowrey, P.L., Shimomura, K., Ko, C.H., Buhr, E.D., Siepka, S.M., Hong, H.K., Oh, W.J., Yoo, O.J., Menaker, M. and Takahashi, J.S. (2004) PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. Proc. Natl. Acad. Sci. USA 101, 5339–5346.
[8] Emery, P. and Reppert, S.M. (2004) A rhythmic Ror. Neuron 43, 443–446.
[9] Oishi, K., Fukui, H. and Ishida, N. (2000) Rhythmic expression of BMAL1 mRNA is altered in Clock mutant mice: differential regulation in the suprachiasmatic nucleus and peripheral tissues. Biochem. Biophys. Res. Commun. 268, 164–171.
[10] Jin, X., Shearman, L.P., Weaver, D.R., Zylka, M.J., de Vries, G.J. and Reppert, S.M. (1999) A molecular mechanism regulating rhythmic output from the suprachiasmatic circadian clock. Cell 96, 57–68.
[11] Panda, S., Antoch, M.P., Miller, B.H., Su, A.I., Schook, A.B., Straume, M., Schultz, P.G., Kay, S.A., Takahashi, J.S. and Hogenesch, J.B. (2002) Coordinated transcription of key pathways in the mouse by the circadian clock. Cell 109, 307–320.
[12] Akhtar, R.A., Reddy, A.B., Maywood, E.S., Clayton, J.D., King, V.M., Smith, A.G., Gant, T.W., Hastings, M.H. and Kyriacou, C.P. (2002) Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus. Curr. Biol. 12, 540–550.
[13] Reppert, S.M. and Weaver, D.R. (2001) Molecular analysis of mammalian circadian rhythms. Annu. Rev. Physiol. 63, 647–676.
[14] Aschoff, J., Hoffmann, K., Pohl, H. and Wever, R. (1975) Re-entrainment of circadian rhythms after phase-shifts of the Zeitgeber. Chronobiologia 2, 23–78.
[15] Beaule, C., Robinson, B., Lamont, E.W. and Amir, S. (2003) Melanopsin in the circadian timing system. J. Mol. Neurosci. 21, 73–89.
[16] Gamble, K.L., Novak, C.M. and Albers, H.E. (2004) Neuropeptide Y and *N*-methyl-D-aspartic acid interact within the suprachiasmatic nuclei to alter circadian phase. Neuroscience 126, 559–565.
[17] Meijer, J.H. and Schwartz, W.J. (2003) In search of the pathways for light-induced pacemaker resetting in the suprachiasmatic nucleus. J. Biol. Rhythms 18, 235–249.
[18] Ding, J.M., Buchanan, G.F., Tischkau, S.A., Chen, D., Kuriashkina, L., Faiman, L.E., Alster, J.M., McPherson, P.S., Campbell, K.P. and Gillette, M.U. (1998) A neuronal ryanodine receptor mediates light-induced phase delays of the circadian clock. Nature 394, 381–384.
[19] Ginty, D.D., Kornhauser, J.M., Thompson, M.A., Bading, H., Mayo, K.E., Takahashi, J.S. and Greenberg, M.E. (1993) Regulation of CREB phosphorylation in the suprachiasmatic nucleus by light and a circadian clock. Science 260, 238–241.
[20] Shearman, L.P., Zylka, M.J., Weaver, D.R., Kolakowski Jr., L.F. and Reppert, S.M. (1997) Two period homologs: circadian expression and photic regulation in the suprachiasmatic nuclei. Neuron 19, 1261–1269.
[21] Trávníčková-BCermakian, Z., Cermakian, N., Reppert, S.M. and Sassone-Corsi, P. (2002) Bimodal regulation of mPeriod promoters by CREB-dependent signaling and CLOCK/BMAL1 activity. Proc. Natl. Acad. Sci. USA 99, 7728–7733.
[22] Sumová, A., Bendová, Z., Sládek, M., Kováčiková, Z. and Illnerová, H. (2004) Seasonal molecular timekeeping within the rat circadian clock. Physiol. Res. 53 (Suppl. 1), S167–S176.
[23] Challet, E. and Pevet, P. (2003) Interactions between photic and nonphotic stimuli to synchronize the master circadian clock in mammals. Front. Biosci. 8, 246–257.
[24] Damiola, F., Le Minh, N., Preitner, N., Kornmann, B., Fleury-Olela, F. and Schibler, U. (2000) Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. Genes Dev. 14, 2950–2961.
[25] Balsalobre, A., Brown, S.A., Marcacci, L., Tronche, F., Kellendonk, C., Reichardt, H.M., Schutz, G. and Schibler, U. (2000) Resetting of circadian time in peripheral tissues by glucocorticoid signaling. Science 289, 2344–2347.
[26] Terazono, H., Mutoh, T., Yamaguchi, S., Kobayashi, M., Akiyama, M., Udo, R., Ohdo, S., Okamura, H. and Shibata, S. (2003) Adrenergic regulation of clock gene expression in mouse liver. Proc. Natl. Acad. Sci. USA 100, 6795–6800.
[27] Moore, R.Y. (1991) Development of the suprachiasmatic nucleus in: Suprachiasmatic Nucleus: The Mind's Clock (Klein, D.C., Moore, R.J. and Reppert, S.M., Eds.), pp. 197–216, Oxford University Press, New York.
[28] Sládek, M., Sumová, A., Kováčiková, Z., Bendová, Z., Laurinová, K. and Illnerová, H. (2004) Insight into molecular core clock mechanism of embryonic and early postnatal rat suprachiasmatic nucleus. Proc. Natl. Acad. Sci. USA 101, 6231–6236.
[29] Kováčiková, Z., Sládek, M., Bendová, Z., Illnerová, H. and Sumová, A. (2006) Expression of clock and clock-driven genes in the rat suprachiasmatic nucleus during late fetal and early postnatal development. J. Biol. Rhythms.
[30] Ohta, H., Honma, S., Abe, H. and Honma, K. (2002) Effects of nursing mothers on rPer1 and rPer2 circadian expressions in the neonatal rat suprachiasmatic nuclei vary with developmental stage. Eur. J. Neurosci. 15, 1953–1960.
[31] Ohta, H., Honma, S., Abe, H. and Honma, K. (2003) Periodic absence of nursing mothers phase-shifts circadian rhythms of clock genes in the suprachiasmatic nucleus of rat pups. Eur. J. Neurosci. 17, 1628–1634.
[32] Li, X. and Davis, F.C. (2005) Developmental expression of clock genes in the Syrian hamster. Dev. Brain Res. 158, 31–40.
[33] Reppert, S.M. and Uhl, G.R. (1987) Vasopressin messenger ribonucleic acid in supraoptic and suprachiasmatic nuclei: appearance and circadian regulation during development. Endocrinology 120, 2483–2487.
[34] Shibata, S. and Moore, R.Y. (1987) Development of neuronal activity in the rat suprachiasmatic nucleus. Brain Res. 431, 311–315.
[35] Reppert, S.M. and Schwartz, W.J. (1984) The suprachiasmatic nuclei of the fetal rat: characterization of a functional circadian clock using 14C-labeled deoxyglucose. J. Neurosci. 4, 1677–1682.
[36] Sakamoto, K., Oishi, K., Nagase, T., Miyazaki, K. and Ishida, N. (2002) Circadian expression of clock genes during ontogeny in the rat heart. Neuroreport 13, 1239–1242.
[37] Shimomura, H., Moriya, T., Sudo, M., Wakamatsu, H., Akiyama, M., Miyake, Y. and Shibata, S. (2001) Differential daily expression of Per1 and Per2 mRNA in the suprachiasmatic nucleus of fetal and early postnatal mice. Eur. J. Neurosci. 13, 687–693.
[38] Weaver, D.R. and Reppert, S.M. (1989) Direct in utero perception of light by the mammalian fetus. Dev. Brain Res. 47, 151–155.
[39] Reppert, S.M. and Schwartz, W.J. (1986) Maternal suprachiasmatic nuclei are necessary for maternal coordination of the developing circadian system. J. Neurosci. 6, 2724–2729.
[40] Weinert, D. (2005) Ontogenetic development of the mammalian circadian system. Chronobiol. Int. 22, 179–205.
[41] Davis, F.C. and Gorski, R.A. (1988) Development of hamster circadian rhythms: role of the maternal suprachiasmatic nucleus. J. Comp. Physiol. [A] 162, 601–610.
[42] Honma, S., Honma, K.I., Shirakawa, T. and Hiroshige, T. (1984) Effects of elimination of maternal circadian rhythms during pregnancy on the postnatal development of circadian corticosterone rhythm in blinded infantile rats. Endocrinology 114, 44–50.
[43] Kováčiková, Z., Sládek, M., Laurinová, K., Bendová, Z., Illnerová, H. and Sumová, A. (2005) Ontogenesis of photoperiodic entrainment of the molecular core clockwork in the rat suprachiasmatic nucleus. Brain Res. 1064, 83–89.
[44] Bendová, Z., Sumová, A. and Illnerová, H. (2004) Development of circadian rhythmicity and photoperiodic response in subdivi-

sions of the rat suprachiasmatic nucleus. Dev. Brain Res. 148, 105–112.
[45] Balsalobre, A., Damiola, F. and Schibler, U. (1998) A serum shock induces circadian gene expression in mammalian tissue culture cells. Cell 93, 929–937.
[46] Viswanathan, N., Weaver, D.R., Reppert, S.M. and Davis, F.C. (1994) Entrainment of the fetal hamster circadian pacemaker by prenatal injections of the dopamine agonist SKF 38393. J. Neurosci. 14, 5393–5398.
[47] Weaver, D.R., Rivkees, S.A. and Reppert, S.M. (1992) D1-dopamine receptors activate c-fos expression in the fetal suprachiasmatic nuclei. Proc. Natl. Acad. Sci. USA 89, 9201–9204.
[48] Weaver, D.R. and Reppert, S.M. (1995) Definition of the developmental transition from dopaminergic to photic regulation of c-fos gene expression in the rat suprachiasmatic nucleus. Mol. Brain Res. 33, 136–148.
[49] Takahashi, S., Yokota, S., Hara, R., Kobayashi, T., Akiyama, M., Moriya, T. and Shibata, S. (2001) Physical and inflammatory stressors elevate circadian clock gene mPer1 mRNA levels in the paraventricular nucleus of the mouse. Endocrinology 142, 4910–4917.
[50] Leard, L.E., Macdonald, E.S., Heller, H.C. and Kilduff, T.S. (1994) Ontogeny of photic-induced c-fos mRNA expression in rat suprachiasmatic nuclei. Neuroreport 5, 2683–2687.
[51] Sumová, A., Jáč, M., Sládek, M., Šauman, I. and Illnerová, H. (2003) Clock gene daily profiles and their phase relationship in the rat suprachiasmatic nucleus are affected by photoperiod. J. Biol. Rhythms 18, 134–144.

CIRCADIAN MOLECULAR CLOCKS TICKING ALONG ONTOGENESIS

Sumová Alena, Bendová Zdeňka, Sládek Martin, El-Hennamy Rehab, Matějů Kristýna, Polidarová Lenka, Sosniyenko Serhiy, Illnerová Helena
Institute of Physiology, Academy of Sciences of the Czech Republic, v.v.i., Prague, Czech Republic
Corresponding author:
Alena Sumová
Institute of Physiology
Academy of Sciences of the Czech Republic
Vídeňská 1083
142 20 Praha 4
Czech Republic

Short title

Circadian clocks during ontogenesis

Summary

The circadian system controls the timing of behavioral and physiological functions in most organisms studied. The review addresses the question of when and how the molecular clockwork underlying circadian oscillations within the central circadian clock in the suprachiasmatic nuclei of the hypothalamus (SCN) and the peripheral circadian clocks develops during ontogenesis. The current model of the molecular clockwork is summarized. The central SCN clock is viewed as a complex structure composed of a web of mutually synchronized individual oscillators. The importance of development of both the intracellular molecular clockwork as well as intercellular coupling for development of the formal properties of the circadian SCN clock is also highlighted. Recently, data has accumulated demonstrate that synchronized molecular oscillations in the central and peripheral clocks develop gradually during ontogenesis and development extends into postnatal period. Synchronized molecular oscillations develop earlier in the SCN than in the peripheral clocks. A hypothesis is suggested that the immature clocks might be first driven by external entraining cues, and therefore, serve as "slave" oscillators. During ontogenesis, the clocks may gradually develop a complete set of molecular interlocked oscillations, i.e., the molecular clockwork, and become self-sustained clocks.

Key words

Circadian clock, ontogenesis, suprachiasmatic nucleus, peripheral clock, clock gene

Introduction

The circadian system has evolved as an adaptation to cyclic changes in light and darkness due to the Earth's rotation that occurs within a period of solar day, i.e., 24 hours. It ensures the proper timing of vital processes in most organisms studied thus far. In mammals, the circadian system consists of a central clock in the brain and numerous peripheral clocks that are subordinate to the central clock. Via the molecular clockwork in cells, the clocks generate circadian rhythmicity, which controls bodily functions through rhythmic regulation of gene transcription. The rhythmicity is thus manifested at the behavioral as well as at the physiological levels. The mammalian central clock is strategically located in the suprachiasmatic nucleus (SCN) of the ventral hypothalamus just above the optic chiasm. It receives photic input from the retina, which synchronizes it with the 24-h day. The SCN is a paired organ, and each of the two nuclei is composed of about 10 000 mutually interconnected cells (for review see Moore *et al.* 2002, Lee *et al.* 2003). The peripheral clocks in mammals have lost their photosensitivity during evolution. They are synchronized with the 24-h day mostly via outputs from the central SCN clock, as well as by their local environment (Hastings *et al.* 2003, Yoo *et al.* 2004). The ontogenetic development of the mammalian circadian system has already been the subject of an extensive review (Weinert 2005). This mini-review mainly summarizes recent data that addresses the crucial question of when and how the mammalian circadian molecular clockwork develops during ontogenesis. Are the central and peripheral clocks functional at the time of birth? Do they develop even earlier, i.e., during the fetal stage? Data has recently accumulated to suggest that, although mammalian circadian clocks are genetically equipped to generate rhythmicity well before birth, they undergo a gradual postnatal development in order to function adequately.

The molecular clock within a single cell

Cells in the mammalian body are equipped with a set of genes that are indispensable for circadian clock function. The principles of circadian rhythmicity generation have been partly ascertained at the molecular level and are similar for the central and peripheral clocks. The basic components of the clockwork are the clock genes that encode clock proteins. Malfunction or absence of the clock components render severe abnormalities in circadian rhythmicity (Bae *et al.* 2001, van der Horst *et al.* 1999 Zheng *et al.* 2001). The abnormalities may range from lengthening or shortening of the circadian period to complete arrhythmicity. A contemporary model of the molecular core clockwork presumes that rhythmical expression of clock genes and their proteins drives the circadian clock in a cell-autonomic fashion (for review see Fu and Lee 2003, Ko and Takahashi 2006, Reppert and Weaver 2001). In principle, clock proteins CLOCK and BMAL1 serve as transcriptional activators that switch on the transcription of genes that contain E-box response elements (CACGTG) in their promoters. Both of these proteins contain a basic helix-loop-helix DNA-binding domain and two PAS (Per-Arnt-Sim) protein interaction domains. E-boxes are present in the promoters of the clock genes *Per1,2* and *Cry1,2* and two orphan nuclear receptors *Rev-erbα* and *Rora*, as well as in the promoters of the clock-controlled genes, i.e., the genes that are not part of the core clockwork, but are controlled by it and thus transmit the rhythmical signal outside of the clock. After the CLOCK:BMAL1 heteodimer switches on transcription of *Per1,2*, *Cry1,2* clock genes, the proteins corresponding to these genes are formed with a clock proteinspecific delay. The PER1,2 and CRY1,2 proteins accumulate in the cytoplasm and form homo- and heterodimers via their PAS domains. The dynamics of this checkpoint are controlled by post-translational modifications of the clock proteins, mainly by phosphorylation and subsequent proteasomal degradation. PER protein phosphorylation by CASEIN KINASE 1ε (CKIε) and CKIδ facilitates PER1 and PER2 ubiquitinylation and degradation and masks their nuclear localization signals. Consequently, the entry of the PER:CRY heterodimer into the nucleus is delayed (Lee *et al.* 2001, Lowrey *et al.* 2000, Akashi *et al.* 2002). This may be a crucial step for maintaining the circadian period of the molecular clock. After entering the nucleus, the PER:CRY heterodimers inhibit CLOCK:BMAL1 mediated transcription, most likely by mechanisms involving directed histone deacetylation and other chromatin modification (Etchegaray *et al.* 2003). Later on, PER:CRY repression is relieved by degradation of PERs and CRYs. The rhythmical transcription of the CLOCK:BMAL1 transcription activator complex is controlled via circadian oscillations in the transcription of clock gene *Bmal1*. REV-ERBα and RORA compete to bind to ROR-response elements in the *Bmal1* promoter, and repress or activate its transcription, respectively (Shearman *et al.* 2000, Preitner *et al.* 2002, Sato *et al.* 2004). These interlocked positive and negative transcriptional – translational feedback loops repeat with a circadian period and thus form the basis for a self-sustained circadian clock.

However, recent findings suggest that the core clockwork mechanism might be much more complex and the current model may represent only a part of the complete system. It is plausible that not only more genes, but even additonal mechanisms not considered in the current feedback loop model, may be involved. For example, miRNA, namely miR-219, has recently been implicated in the regulation of the central circadian clock (Cheng *et al.* 2007). miRNAs are small molecules that act as potent silencers of gene expression via translational repression of mRNA

degradation. miR-219 is a target of the CLOCK:BMAL1 complex and exhibits robust circadian rhythms of expression as a clock-regulated gene. Moreover, in-vivo knock-down of miR-219 lengthens the circadian period in a manner similar to a knock-out of the clock gene *Clock*. Translation control via miRNAs may, therefore, represent a novel regulatory level of the circadian clock. Another novel regulatory mechanism might be based on the finding that bZIP transcription factor *E4BP4* is a key negative component of the circadian clock (Ohno *et al.* 2007). Moreover, three recent reports confirmed the importance of targeted protein degradation as a key feature of the circadian clock (Siepka *et al.* 2007, Busino *et al.* 2007, Godinho *et al.* 2007). They revealed that the clock protein CRY is targeted for degradation by a member of F box family of ubiquitin E3 ligases, FBXL3. *Overtime* (Siepka *et al.* 2007) and *after hours* (Godinho *et al.* 2007) mutations both lie in the region of FBXL3 that binds to CRY. Due to these mutations, binding of FBXL3 to CRY is disrupted, CRY degradation is prevented and the duration of its repressive function on clock gene activation is prolonged. Therefore, mice with a targeted mutation of FBXL3 have a longer circadian period than wild-type mice. Although the mechanisms of the degradation pathways have not been fully ascertained, targeted degradation is likely to control not only the rate at which clock protein complexes accumulate in the cytoplasm, but also the rate of their degradation.

Although the basic principles of the core clockwork seem to be conserved across the central and peripheral clocks, they might not be absolutely identical. In contrast to peripheral clocks, the central clock within the SCN is formed of a web of inter-connected cellautonomous oscillators (see bellow). Recent data have demonstrated that the inter-cellular clock mechanisms may significantly contribute to the robustness of the clock system (Liu *et al.* 2007). The oscillator network interactions in the SCN can partly compensate for *Per1* or *Cry1* deficiency and preserve sustained rhythmicity in behavior and *in vitro* in the SCN slices of *Per1* or *Cry1* mutant mice. In contrast, *Per1* and *Cry1* genes are implicitly required for sustained rhythms in peripheral tissues, cells and dissociated SCN neurons (Liu *et al.* 2007). Therefore, a new model that is specific for the central SCN clock is needed to incorporate the ability of inter-cellular coupling among the SCN neurons to confer the robustness of molecular oscillations.

SCN clock: a single cell oscillator or a web of coupled oscillators?

To function properly as a master clock, the SCN must not only be able to generate circadian oscillations, but must also entrain the oscillations at single cell level to cyclically occurring cues and transmit the synchronized rhythmic information to the rest of the body. These tasks are highly dependent upon inter-neuronal coupling within the SCN. In the adult SCN, information regarding photic entraining cues is first processed by a set of neurons located in the retinorecipient zone of the SCN. In rodents, this zone is called the ventrolateral (VL) part or the core of the SCN. Thereafter, the information is sent via intra-SCN coupling pathways to the non-photosensitive cells located mostly within the dorsomedial (DM) part or the shell of the SCN (Yamaguchi *et al.* 2003, Yan and Okamura 2002, Yan and Silver 2004, Yan *et al.* 1999). Inter-cellular communication between clusters of SCN cells has recently been considered to be important for entrainment of the central clock to a change in day length, i.e., in the photoperiod (Inagaki *et al.* 2007, VanderLeest *et al.* 2007). The mechanism by which the coupling is accomplished is not well understood. Several mechanisms underlying the intercellular synchrony have been considered, namely electrical coupling (Aton and Herzog 2005) and coupling by

neurotransmitters, such as vasoactive intestinal polypeptide (VIP) (Harmar *et al.* 2002, Aton *et al.* 2005, Maywood *et al.* 2006) and gamma aminobutyric acid (GABA) (Albus *et al.* 2005, Aton *et al.* 2006).

Recent findings support the idea that inter-cellular coupling is crucial not only for entrainment and transmission of the synchronized output signals out of the clock, but also for the time-keeping mechanism *per se*. In particular, the VIP signaling through the VIPR2 receptor has been shown not only to contribute to synchrony between cells, but also to help maintain a robust rhythmicity in individual SCN neurons. In *Vip*$^{-/-}$ and *Vipr2*$^{-/-}$ mice, the circadian firing rhythm was abolished in about half of all SCN neurons (Aton *et al.* 2005). Similarly, the rhythmicity was disrupted in cells within the SCN organotypic slices from *Vipr2*$^{-/-}$ mice carrying *Per1::luciferase* and *Per1::GFP* reporter transgenes as reporters of activity within the core circadian feedback loop (Maywood *et al.* 2006). Recently, it has been shown that coupling between single cell SCN oscillators may amplify and stabilize unstable component oscillators, and, therefore, establish a more reliable rhythmicity at the SCN and behavioral level (Liu *et al.* 2007, To *et al.* 2007). Thus, the coupling between individual rhythmic cells is likely to contribute the autonomous time-keeping mechanism and ensure stability of the central clock.

Development of the central SCN clock

From the data summarized above, it is obvious that the adult central clock is not only a simple sum of self-oscillating neurons, but is rather a well organized entity. The multi-level organization includes coupling between individual neurons as well as coupling between the defined sub-divisions of the nucleus. Therefore, development of the central clock within the SCN obviously does not depend only on the presence of individual components of the molecular core clockwork and the ability of single cells to oscillate, but also on development of a hierarchical organization of the nucleus. Only maturation of the complex clock enables the development of synchronized oscillatory signaling from the central clock to the rest of the body. In this context, data regarding the morphological development of the mammalian SCN might be highly relevant.

Morphologically, the rodent SCN develops gradually (Moore 1991). Gestational periods among different rodent species differ and therefore, for simplification, most of the further discussion relates to development of the rat SCN. In the rat, the prenatal period lasts about 22 days. Neurogenesis of the SCN begins on embryonic day (E) 14 and continues through E17 from a specialized zone of the ventral diencephalic germinal epithelium as a component of periventricular cell groups. Neurons of the VL SCN are generated at E15 - E16 and those of the DM SCN at E16 - E17. The neurogenesis is completed at E18, but the morphological maturation of the SCN neurons gradually proceeds until postnatal day (P) 10. Synaptogenesis in the SCN is a slower process; at E19, only very sparse synapses may be observed. It begins to progress only in the late prenatal and early postnatal periods, and then increases noticeably from P4 to P10 (Weinert 2005). Therefore, during the prenatal period, the SCN neurons are present but the multi-level inter-cellular coupling may not yet be functional. The coupling strengthens during the first postnatal week and, the rat SCN is fully developed to its full complexity only at P10.

Intrinsic rhythms in the SCN may appear as early as the late embryonic stage. A daynight variation in metabolic activity monitored by a 2-deoxyglucose uptake was detected in the fetal

rat SCN from E19 through E21 (Reppert and Schwartz 1984), in the *Avp* mRNA level at E21 (Reppert and Uhl 1987) and in the firing rate of the SCN neurons at E22 (Shibata and Moore 1987). All these rhythms are supposed to be driven by the SCN clock in adults, and the rodent fetal clock has therefore been considered to be functionally developed well before birth. However, direct evidence that the above mentioned fetal SCN rhythms are indeed driven by the molecular core clockwork is lacking. Alternatively, the observed rhythmicity might arise from cyclically appearing maternal cues, which impinge on fetal SCN neurons and driving the oscillations in a "slave" oscillator-like fashion. Such maternal "zeitgebers" might trigger the rhythm in neuronal activity, as reflected in the rhythms in firing rate and metabolic activity, as well as in gene transcription, as is the case with the observed rhythm in *Avp* mRNA levels. It is relevant to note that transcription of *Avp* might be regulated not only by the clockwork via activation of the E-box sequence in its promoter (Jin *et al.* 1999), but also via activation of CRE (Iwasaki *et al.* 1997, Burbach *et al.* 2001) and AP1 (Burbach *et al.* 2001) elements by a non-clock-related mechanism. Moreover, recent data using detection of heteronuclear RNA as a nascent transcript, which is a more reliable marker of transcriptional rate than detection of mRNA, did not reveal any circadian rhythmicity in transcription of the *Avp* gene in the rat SCN at E20. However, the expression was rhythmic at P1 (Kováčiková *et al.* 2006).

A solution to the question of whether the central clock is functional before birth might come from studies on the development of the molecular core clockwork mechanism. According to the current model described above, the circadian rhythms in the levels of the clock gene transcripts and protein products are essential for the molecular timekeeping mechanism. Therefore, several groups of researchers have measured the daily profiles of clock gene expression in the rat SCN by *in situ* hybridization, but outcome of these studies was ambiguous. Ohta et al. reported high amplitude rhythms of *Per1* and *Per2* mRNA in the fetal rat SCN at E20 (Ohta *et al.* 2002, 2003). Other authors studied the daily profiles of *Per1*, *Per2*, *Cry1* and *Bmal1* mRNA at E19, i.e., at the embryonic day when the fetal rat SCN is already formed (Moore 1991) and the rhythm in metabolic activity present (Reppert and Schwartz 1984). However, none of the above-mentioned clock genes were expressed rhythmically at that embryonic stage (Sládek *et al.* 2004). Moreover, levels of clock gene proteins PER1, PER2 and CRY1 not only did not exhibit any circadian variation, but were in fact undetectable at E19 (Sládek *et al.* 2004). These data suggest that at this stage of fetal development, the SCN circadian clock might not be able to generate synchronized oscillations. The same authors performed a detailed developmental study and found that at E20, some of the rhythms were just about beginning to form, but the amplitude of rhythmicity was very low or did not reach a significant level (Kováčiková *et al.* 2006). Rhythms in clock gene expression developed gradually during the postnatal period, and adult-stage-like amplitudes were achieved only at P10 (Kováčiková *et al.* 2006) (Fig 1). Similarly, molecular oscillations equivalent to those observed in adults were not detected in the fetal hamster SCN (Li and Davis 2005). In mice, Shimomura et al. found a significant oscillation in *Per1* but not in *Per2* mRNA in the SCN at E17, and the amplitude of the oscillations increased progressively with postnatal age (Shimomura *et al.* 2001).

Using this approach, it was possible to study the development of synchronized rhythmicity, but not the development of single cell rhythmicity. Low amplitude rhythms in clock gene expression might already be present in individual SCN neurons, but they may not yet be mutually synchronized due to insufficient synapses in the embryonic SCN (Moore 1991). The

increase in the amplitude of the rhythms in clock gene expression correlated well with synaptogenesis within the SCN. Therefore, it is plausible that mutual synchronization of the SCN neurons due to developing synapses may account for the gradual rise in the amplitude of clock gene oscillations. Theoretically, development of the synapses might also be conditional for the oscillations. Daily profiles of clock gene expression in the rat SCN at E19 seem to support the idea of undeveloped molecular oscillations in individual neurons rather than the idea of fully developed but desynchronized oscillations, since levels of constitutively expressed *Per1*, *Cry1* and *Bmal1* genes at E19 corresponded either to the minimum or maximum, but not to the mean of their P3 values. Moreover, at E19, not only rhythms in clock gene mRNA levels, but also protein products PER1, PER2 and CRY1 were undetectable. In fact, no PER1, PER2 and CRY1immunoreactive cells were detected in the fetal SCN at any circadian time (Sládek *et al.* 2004). The absence of the basic components of the molecular core clockwork is rather in favor of the hypothesis that the mechanism enabling the rhythmic expression of clock genes may not yet be mature at E19. However, the possibility cannot be ruled out that only a very small proportion of the SCN cells is rhythmic during the fetal stage, and that the number of rhythmic cells increases due to development of synaptic communication between these rhythmic cells and the non-rhythmic ones. The methodological approach used in the above-mentioned studies would not detect a very low oscillating signal, which could potentially arise from a few SCN cells.

The use of newly introduced experimental tools, such as transgenic animals, will be necessary for addressing these issues in the future. In SCN slices explanted from transgenic animals, it is possible to detect rhythms in clock gene expression with a single cell resolution. However, even in the case of detection of a significant rhythm in clock gene expression at a single cell level during the fetal stage, the question still remains as to whether these rhythms are indeed reliable markers of a functional circadian clock. If so, individual cellular oscillators without any coupling must be able to drive synchronized rhythmicity. However, such characteristics have not been observed in *in vitro* cell lines that are devoid of inter-cellular coupling like the fetal clock. Without entraining cues, these cells are desynchronized or arrhythmic. As soon as the cultured cells are subjected to a “zeitgeber”, e.g., to serum shock, the cells become synchronized and exhibit synchronized rhythmicity (Balsalobre *et al.* 1998). Therefore, the question of whether the fetal SCN cells are able to maintain oscillations, or whether the oscillations would soon be dampened in the isolated SCN may still remain. The possibility that other mechanisms besides the molecular clockwork might drive the SCN rhythmicity during late embryonic development should be considered. For example, maternal cues, such as dopamine or melatonin, might directly trigger the fetal SCN rhythm in metabolic activity (Davis and Mannion 1988, Weaver *et al.* 1995). Complete lesions of the maternal SCN at E7 disrupt rhythms in SCN glucose utilization in rat fetuses (Reppert and Schwartz 1986). This disruption might be due to desynchronization among the fetuses, but also might be due to the lack of a rhythmical input to the slave fetal clock. Strikingly, a periodic feeding cue delivered to SCN-lesioned pregnant rats is sufficient to entrain the fetal SCN clock (Weaver and Reppert 1989). The fetal clock is therefore sensitive to feeding cues in a way similar to adult peripheral clocks, but not the adult SCN clock. These observations suggest that multiple and more complex pathways mediate rhythmic information to the fetal SCN clock as compared to the adult SCN clock. They also indicate that formal properties of the fetal and of the postnatal SCN clock may differ. Therefore, the possibility cannot be excluded that during ontogenesis, the SCN clock

develops spontaneously from a slave oscillator at the prenatal stage to a master clock at the postnatal stage.

More strikingly, the restructuring of the slave oscillator to the master clock may occur spontaneously without entraining cues driven by the maternal SCN. Surgical ablation of the maternal SCN did not prevent development of the clock during the postnatal period (Reppert and Schwartz 1986, Davis and Gorski 1988). Moreover, genetic ablation of functional central as well as peripheral maternal clocks did not prevent spontaneous development of the clocks, since heterozygous off-spring of m*Per1*^{Brdm1}/*Per2*^{Brdm1} and *mPer2*^{Brdm1}/*Cry1*$^{-/-}$ double mutant arrhythmic females crossed with wild-type males developed circadian rhythm in locomotor activity. However, within a litter, pups were less synchronized than pups born to wild-type controls (Jud and Albrecht 2006). Also, transplantation of fetal SCN tissue to arrhythmic SCN-lesioned animals leads to a recovery in the circadian rhythm of locomotor activity (Ralph *et al.* 1990). Therefore, development of the circadian clock appears to be genetically predetermined.

Peripheral clocks during ontogenesis

Studies on the development of peripheral clocks have only recently commenced after the finding that rhythms in clock gene expression are detectable in cells of the peripheral organs, and are therefore not unique to the central SCN clock (Abe *et al.* 2001, Balsalobre 2002, Schibler and Sassone-Corsi 2002). Two methodological approaches for these studies were used. In the first approach, daily profiles of clock gene expression within a peripheral tissue sampled throughout the circadian cycle were examined. In the rat heart, circadian rhythms in the expression of clock genes *Per1, Per2* and *Bmal1* and a clock-controlled gene *Dbp* were not detected by Northern blot analysis on P2 (Sakamoto *et al.* 2002). Expression of *Per1, Bmal1* and *Dbp* began to be rhythmic between P2 and P5, but expression of *Per2* did not exhibit any rhythmicity until P14. Similarly, in the rat liver, clock gene expression as determined by RT-PCR developed gradually during postnatal ontogenesis (Sládek *et al.* 2004) (Fig.1). At E20, only *Rev-erbα* mRNA exhibited a significant, high amplitude circadian oscillation, but the expression of *Per1*, *Per2*, *Cry1*, *Bmal1* and *Clock* mRNA did not. Even at P2, *Rev-erbα* was still the only gene expressed rhythmically with high amplitude. At P10 *Per1* mRNA and at P20 *Per2* and *Bmal1* also began to be expressed in a circadian way. Only as late as at P30, all of the studied clock genes were expressed rhythmically in an adult-like pattern (Sládek *et al.* 2004). Development of the molecular oscillations in the liver was therefore similar to that in the heart. Apparently, rhythms in synchronized clock gene expression develop earlier in the central SCN clock (see above) than in peripheral oscillators. The stable detection of the high-amplitude rhythm in *Rev-erbα* expression throughout ontogenesis rules out the possibility that the lack of rhythmicity in the early development is due to desynchronization of oscillating cells in the liver. Unlike the SCN clock cells, the peripheral oscillating cells are not mutually interconnected via synapses and are likely to be synchronized by rhythmic humoral or neuronal cues impinging upon individual cells. The significant rhythm in *Rev-erbα* expression in the absence of rhythms in other clock genes during ontogenesis may give us clues regarding the mechanism that underlies peripheral clock development. During an early developmental stage, rhythmic expression of *Rev-erbα* might be triggered by mechanisms other than E-box mediated induction. Apart from the E-box, the *Rev-erbα* promoter contains other response elements that may be responsible for switching on/off gene transcription, namely Rev-DR2/RORE, DBPE/D-box etc. (Adelmant *et al.* 1996, Raspe *et*

al. 2002, Yamamoto *et al.* 2004). The rhythmically appearing mediators may activate transcription of *Rev-erbα* by stimulation of some of these elements independently of the core clockwork. Moreover, it is tempting to speculate that the rhythmic expression of *Rev-erbα* might trigger the newly appearing rhythms in clock gene expression, since a constant phase relationship between rhythms in the expression of *Rev-erbα* and other clock genes is maintained during different developmental stages (Sládek *et al.* 2004) (Fig.1). Therefore, theoretically, a peripheral clock may function as a slave oscillator during early ontogenesis, and may only later, with the development of clock gene oscillations, become a self-autonomous clock.

Importantly, phases of the rhythms in clock gene expression in the liver (Sládek *et al.* 2007) as well as in the heart (Sakamoto *et al.* 2002) change during development. The acrophase of these rhythms shifts in a coordinated manner so that the expressions of individual clock genes keep stable phase relationships throughout development (Fig.1). Feeding regimes accompanied by behavioral activity may account for these phase changes. Mothers feed their pups mostly during the daytime; therefore, during the period of maternal breast feeding, pups are diurnal rather than nocturnal in their food consumption (Weinert 2005). The nocturnal feeding pattern develops during the weaning period, but it is preceded by a period when pups still suckle some maternal milk during the daytime and consume solid food during the nighttime. These changes in feeding behavior appear to be mirrored in changing phases of the rhythms in clock gene expression.

Recently, another approach was used for studying the ontogenesis of oscillations in clock gene expression (Saxena *et al.* 2007). *In vivo* rhythms in bioluminiscence were monitored *in utero* in the fetuses of transgenic rats carrying *Per1:: luciferase* transgene throughout the whole gestational period (Saxena *et al.* 2007). The bioluminiscence increased dramatically at E10 and continued to increase progressively until birth. Diurnal fluctuations in *Per1* expression in the whole body were already suggested prior to birth. From this study, it is not apparent which parts of the fetal body might account for the whole-fetal bioluminescence recorded in vivo or for the suggested day-night differences observed. It is possible that some peripheral clocks may start to exhibit circadian rhythms in *Per1* expression before birth. Tissue-specific differences in the development of molecular oscillations in peripheral clocks are thus suggested.

Conclusion

The data summarized in this mini-review cannot definitively answer the question of exactly when the central and peripheral clocks develop during the mammalian ontogenesis. Depending on the methods used, different results have been produced; therefore, more studies are still needed. However, most results support the hypothesis that synchronized oscillations in clock gene expression develop gradually during ontogenesis, and development extends well into the postnatal period. It is feasible that the ability to function as a self-sustained clock may develop gradually, and that the immature clock may function first as a “slave” oscillator. Only later, with the development of a complete set of molecular oscillations, may it become a selfsustaining clock. Such development occurs earlier in the central SCN clock than in peripheral clocks.

Acknowledgement

The authors are supported by the Grant Agency of the Czech Republic, grant No. 309050350; Grant Agency of the Academy of Sciences of the Czech Republic, grant No. IAA500110605; by Research Projects AV0Z 50110509, and LC554; and by the 6th Framework Project EUCLOCK 018741.

References

ABE H, HONMA S, NAMIHIRA M, MASUBUCHI S, IKEDA M, EBIHARA S, HONMA K: Clock gene expressions in the suprachiasmatic nucleus and other areas of the brain during rhythm splitting in cs mice. *Mol Brain Res* **87**: 92-99., 2001.

ADELMANT G, BEGUE A, STEHELIN D, LAUDET V: A functional rev-erb alpha responsive element located in the human rev-erb alpha promoter mediates a repressing activity. *Proc Natl Acad Sci U S A* **93**: 3553-3558, 1996.

AKASHI M, TSUCHIYA Y, YOSHINO T, NISHIDA E: Control of intracellular dynamics of mammalian period proteins by casein kinase i epsilon (ckiepsilon) and ckidelta in cultured cells. *Mol Cell Biol* **22**: 1693-1703, 2002.

ALBUS H, VANSTEENSEL MJ, MICHEL S, BLOCK GD, MEIJER JH: A gabaergic mechanism is necessary for coupling dissociable ventral and dorsal regional oscillators within the circadian clock. *Curr Biol* **15**: 886-893, 2005.

ATON SJ, COLWELL CS, HARMAR AJ, WASCHEK J, HERZOG ED: Vasoactive intestinal polypeptide mediates circadian rhythmicity and synchrony in mammalian clock neurons. *Nat Neurosci* **8**: 476-483, 2005.

ATON SJ, HERZOG ED: Come together, right now: Synchronization of rhythms in a mammalian circadian clock. *Neuron* **48**: 531-534, 2005.

ATON SJ, HUETTNER JE, STRAUME M, HERZOG ED: Gaba and gi/o differentially control circadian rhythms and synchrony in clock neurons. *Proc Natl Acad Sci U S A* **103**: 19188-19193, 2006.

BAE K, JIN X, MAYWOOD ES, HASTINGS MH, REPPERT SM, WEAVER DR: Differential functions of mper1, mper2, and mper3 in the SCN circadian clock. *Neuron* **30**: 525-536, 2001.

BALSALOBRE A: Clock genes in mammalian peripheral tissues. *Cell Tissue Res* **309**: 193-199, 2002.

BALSALOBRE A, DAMIOLA F, SCHIBLER U: A serum shock induces circadian gene expression in mammalian tissue culture cells. *Cell* **93**: 929-937, 1998.

BURBACH JP, LUCKMAN SM, MURPHY D, GAINER H: Gene regulation in the magnocellular hypothalamo-neurohypophysial system. *Physiol Rev* **81**: 1197-1267, 2001.

BUSINO L, BASSERMANN F, MAIOLICA A, LEE C, NOLAN PM, GODINHO SI, DRAETTA GF, PAGANO M: Scffbxl3 controls the oscillation of the circadian clock by directing the degradation of cryptochrome proteins. *Science* **316**: 900-904, 2007.

DAVIS FC, GORSKI RA: Development of hamster circadian rhythms: Role of the maternal suprachiasmatic nucleus. *J Comp Physiol [A]* **162**: 601-610, 1988.

DAVIS FC, MANNION J: Entrainment of hamster pup circadian rhythms by prenatal melatonin injections to the mother. *Am J Physiol* **255**: R439-448, 1988.

ETCHEGARAY JP, LEE C, WADE PA, REPPERT SM: Rhythmic histone acetylation underlies transcription in the mammalian circadian clock. *Nature* **421**: 177-182, 2003.

FU L, LEE CC: The circadain clock: Pacemaker and tumour suppressor. *Nature Reviewes Cancer* **3**: 350-361, 2003.

GODINHO SI, MAYWOOD ES, SHAW L, TUCCI V, BARNARD AR, BUSINO L, PAGANO M, KENDALL R, QUWAILID MM, ROMERO MR, O'NEILL J, CHESHAM JE, BROOKER D, LALANNE Z, HASTINGS MH, NOLAN PM: The after-hours mutant reveals a role for fbxl3 in determining mammalian circadian period. *Science* **316**: 897-900, 2007.

HARMAR AJ, MARSTON HM, SHEN S, SPRATT C, WEST KM, SHEWARD WJ, MORRISON CF, DORIN JR, PIGGINS HD, REUBI JC, KELLY JS, MAYWOOD ES, HASTINGS MH: The vpac(2) receptor is essential for circadian function in the mouse suprachiasmatic nuclei. *Cell* **109**: 497-508, 2002.

HASTINGS MH, REDDY AB, MAYWOOD ES: A clockwork web: Circadian timing in brain and periphery, in health and disease. *Nat Rev Neurosci* **4**: 649-661, 2003.

CHENG HY, PAPP JW, VARLAMOVA O, DZIEMA H, RUSSELL B, CURFMAN JP, NAKAZAWA T, SHIMIZU K, OKAMURA H, IMPEY S, OBRIETAN K: MicroRNA modulation of circadian-clock period and entrainment. *Neuron* **54**: 813-829, 2007.

INAGAKI N, HONMA S, ONO D, TANAHASHI Y, HONMA K: Separate oscillating cell groups in mouse suprachiasmatic nucleus couple photoperiodically to the onset and end of daily activity. *Proc Natl Acad Sci U S A* **104**: 7664-7669, 2007.

IWASAKI Y, OISO Y, SAITO H, MAJZOUB JA: Positive and negative regulation of the rat vasopressin gene promoter. *Endocrinology* **138**: 5266-5274, 1997.

JIN X, SHEARMAN LP, WEAVER DR, ZYLKA MJ, DE VRIES GJ, REPPERT SM: A molecular mechanism regulating rhythmic output from the suprachiasmatic circadian clock. *Cell* **96**: 57-68., 1999.

JUD C, ALBRECHT U: Circadian rhythms in murine pups develop in absence of a functional maternal circadian clock. *J Biol Rhythms* **21**: 149-154, 2006.

KO CH, TAKAHASHI JS: Molecular components of the mammalian circadian clock. *Hum Mol Genet* 15 Spec No **2**: R271-277, 2006.

KOVÁČIKOVÁ Z, SLÁDEK M, BENDOVÁ Z, ILLNEROVÁ H, SUMOVÁ A: Expression of clock and clock-driven genes in the rat suprachiasmatic nucleus during late fetal and early postnatal development. *J Biol Rhythms* **21**: 140-148, 2006.

LEE C, ETCHEGARAY JP, CAGAMPANG FR, LOUDON AS, REPPERT SM: Posttranslational mechanisms regulate the mammalian circadian clock. *Cell* **107**: 855-867, 2001.

LEE HS, BILLINGS HJ, LEHMAN MN: The suprachiasmatic nucleus: A clock of multiple components. *J Biol Rhythms* **18**: 435-449, 2003.

LI X, DAVIS FC: Developmental expression of clock genes in the syrian hamster. *Dev Brain Res* **158**: 31-40, 2005.

LIU AC, WELSH DK, KO CH, TRAN HG, ZHANG EE, PRIEST AA, BUHR ED, SINGER O, MEEKER K, VERMA IM, DOYLE FJ, 3RD, TAKAHASHI JS, KAY SA: Intercellular coupling confers robustness against mutations in the scn circadian clock network. *Cell* **129**: 605-616, 2007.

LOWREY PL, SHIMOMURA K, ANTOCH MP, YAMAZAKI S, ZEMENIDES PD, RALPH MR, MENAKER M, TAKAHASHI JS: Positional syntenic cloning and functional characterization of the mammalian circadian mutation tau. *Science* **288**: 483-492., 2000.

MAYWOOD ES, REDDY AB, WONG GK, O'NEILL JS, O'BRIEN JA, MCMAHON DG, HARMAR AJ, OKAMURA H, HASTINGS MH: Synchronization and maintenance of timekeeping in suprachiasmatic circadian clock cells by neuropeptidergic signaling. *Curr Biol* **16**: 599-605, 2006.

MOORE RY: Development of the suprachiasmatic nucleus. In: *Suprachiasmatic nucleus: The mind's clock.* DC Klein, RY Moore, SM Reppert (eds), Oxford Univ. Press, New York, 1991, pp 197-216.

MOORE RY, SPEH JC, LEAK RK: Suprachiasmatic nucleus organization. *Cell Tissue Res* **309**: 89-98, 2002.

OHNO T, ONISHI Y, ISHIDA N: The negative transcription factor e4bp4 is associated with circadian clock protein period2. *Biochem Biophys Res Commun* **354**: 1010-1015, 2007.

OHTA H, HONMA S, ABE H, HONMA K: Effects of nursing mothers on rper1 and rper2 circadian expressions in the neonatal rat suprachiasmatic nuclei vary with developmental stage. *Eur J Neurosci* **15**: 1953-1960, 2002.

OHTA H, HONMA S, ABE H, HONMA K: Periodic absence of nursing mothers phase-shifts circadian rhythms of clock genes in the suprachiasmatic nucleus of rat pups. *Eur J Neurosci* **17**: 1628-1634, 2003.

PREITNER N, DAMIOLA F, LOPEZ-MOLINA L, ZAKANY J, DUBOULE D, ALBRECHT U, SCHIBLER U: The orphan nuclear receptor rev-erbalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell* **110**: 251- 260, 2002.

RALPH MR, FOSTER RG, DAVIS FC, MENAKER M: Transplanted suprachiasmatic nucleus determines circadian period. *Science* **247**: 975-978, 1990.

RASPE E, MAUTINO G, DUVAL C, FONTAINE C, DUEZ H, BARBIER O, MONTE D, FRUCHART J, FRUCHART JC, STAELS B: Transcriptional regulation of human reverbalpha gene expression by the orphan nuclear receptor retinoic acid-related orphan receptor alpha. *J Biol Chem* **277**: 49275-49281, 2002.

REPPERT SM, SCHWARTZ WJ: Maternal suprachiasmatic nuclei are necessary for maternal coordination of the developing circadian system. *J Neurosci* **6**: 2724-2729, 1986.

REPPERT SM, SCHWARTZ WJ: The suprachiasmatic nuclei of the fetal rat: Characterization of a functional circadian clock using 14c-labeled deoxyglucose. *J Neurosci* **4**: 1677-1682, 1984.

REPPERT SM, UHL GR: Vasopressin messenger ribonucleic acid in supraoptic and suprachiasmatic nuclei: Appearance and circadian regulation during development. *Endocrinology* **120**: 2483-2487., 1987.

REPPERT SM, WEAVER DR: Molecular analysis of mammalian circadian rhythms. *Annu Rev Physiol* **63**: 647-676, 2001.

SAKAMOTO K, OISHI K, NAGASE T, MIYAZAKI K, ISHIDA N: Circadian expression of clock genes during ontogeny in the rat heart. *Neuroreport* **13**: 1239-1242, 2002.

SATO TK, PANDA S, MIRAGLIA LJ, REYES TM, RUDIC RD, MCNAMARA P, NAIK

KA, FITZGERALD GA, KAY SA, HOGENESCH JB: A functional genomics strategy reveals rora as a component of the mammalian circadian clock. *Neuron* **43**: 527-537, 2004.

SAXENA MT, ATON SJ, HILDEBOLT C, PRIOR JL, ABRAHAM U, PIWNICA-WORMS D, HERZOG ED: Bioluminescence imaging of period1 gene expression in utero. *Mol Imaging* **6**: 68-72, 2007.

SHEARMAN LP, SRIRAM S, WEAVER DR, MAYWOOD ES, CHAVES I, ZHENG B, KUME K, LEE CC, VAN DER HORST GT, HASTINGS MH, REPPERT SM: Interacting molecular loops in the mammalian circadian clock. *Science* **288**: 1013-1019, 2000.

SHIBATA S, MOORE RY: Development of neuronal activity in the rat suprachiasmatic nucleus. *Brain Res* **431**: 311-315, 1987.

SHIMOMURA H, MORIYA T, SUDO M, WAKAMATSU H, AKIYAMA M, MIYAKE Y, SHIBATA S: Differential daily expression of per1 and per2 mrna in the suprachiasmatic nucleus of fetal and early postnatal mice. *Eur J Neurosci* **13**: 687-693., 2001.

SCHIBLER U, SASSONE-CORSI P: A web of circadian pacemakers. *Cell* **111**: 919-922, 2002.

SIEPKA SM, YOO SH, PARK J, SONG W, KUMAR V, HU Y, LEE C, TAKAHASHI JS: Circadian mutant overtime reveals f-box protein fbxl3 regulation of cryptochrome and period gene expression. *Cell* **129**: 1011-1023, 2007.

SLÁDEK M, JINDRÁKOVÁ Z, BENDOVÁ Z, SUMOVÁ A: Postnatal ontogenesis of the circadian clock within the rat liver. *Am J Physiol Regul Integr Comp Physiol* **292**: R1224-1229, 2007.

SLÁDEK M, SUMOVÁ A, KOVÁČIKOVÁ Z, BENDOVÁ Z, LAURINOVÁ K, ILLNEROVÁ H: Insight into molecular core clock mechanism of embryonic and early postnatal rat suprachiasmatic nucleus. *Proc Natl Acad Sci U S A* **101**: 6231-6236, 2004.

TO TL, HENSON MA, HERZOG ED, DOYLE FJ, 3RD: A molecular model for intercellular synchronization in the mammalian circadian clock. *Biophys J* **92**: 3792-3803, 2007.

VAN DER HORST GT, MUIJTJENS M, KOBAYASHI K, TAKANO R, KANNO S, TAKAO M, DE WIT J, VERKERK A, EKER AP, VAN LEENEN D, BUIJS R, BOOTSMA D, HOEIJMAKERS JH, YASUI A: Mammalian cry1 and cry2 are essential for maintenance of circadian rhythms. *Nature* **398**: 627-630, 1999.

VANDERLEEST HT, HOUBEN T, MICHEL S, DEBOER T, ALBUS H, VANSTEENSEL MJ, BLOCK GD, MEIJER JH: Seasonal encoding by the circadian pacemaker of the SCN. *Curr Biol* **17**: 468-473, 2007.

WEAVER DR, REPPERT SM: Periodic feeding of scn-lesioned pregnant rats entrains the fetal biological clock. *Dev Brain Res* **46**: 291-296., 1989.

WEAVER DR, ROCA AL, REPPERT SM: C-fos and jun-b mrnas are transiently expressed in fetal rodent suprachiasmatic nucleus following dopaminergic stimulation. *Dev Brain Res* **85**: 293-297, 1995.

WEINERT D: Ontogenetic development of the mammalian circadian system. *Chronobiol Int* **22**: 179-205, 2005.

YAMAGUCHI S, ISEJIMA H, MATSUO T, OKURA R, YAGITA K, KOBAYASHI M, OKAMURA H: Synchronization of cellular clocks in the suprachiasmatic nucleus. *Science* **302**: 1408-1412, 2003.

YAMAMOTO T, NAKAHATA Y, SOMA H, AKASHI M, MAMINE T, TAKUMI T: Transcriptional oscillation of canonical clock genes in mouse peripheral tissues. *BMC Mol Biol* **5**: 18, 2004.

YAN L, OKAMURA H: Gradients in the circadian expression of per1 and per2 genes in the rat suprachiasmatic nucleus. *Eur J Neurosci* **15**: 1153-1162, 2002.

YAN L, SILVER R: Resetting the brain clock: Time course and localization of mper1 and mper2 protein expression in suprachiasmatic nuclei during phase shifts. *Eur J Neurosci* **19**: 1105-1109, 2004.

YAN L, TAKEKIDA S, SHIGEYOSHI Y, OKAMURA H: Per1 and per2 gene expression in the rat suprachiasmatic nucleus: Circadian profile and the compartment-specific response to light. *Neuroscience* **94**: 141-150, 1999.

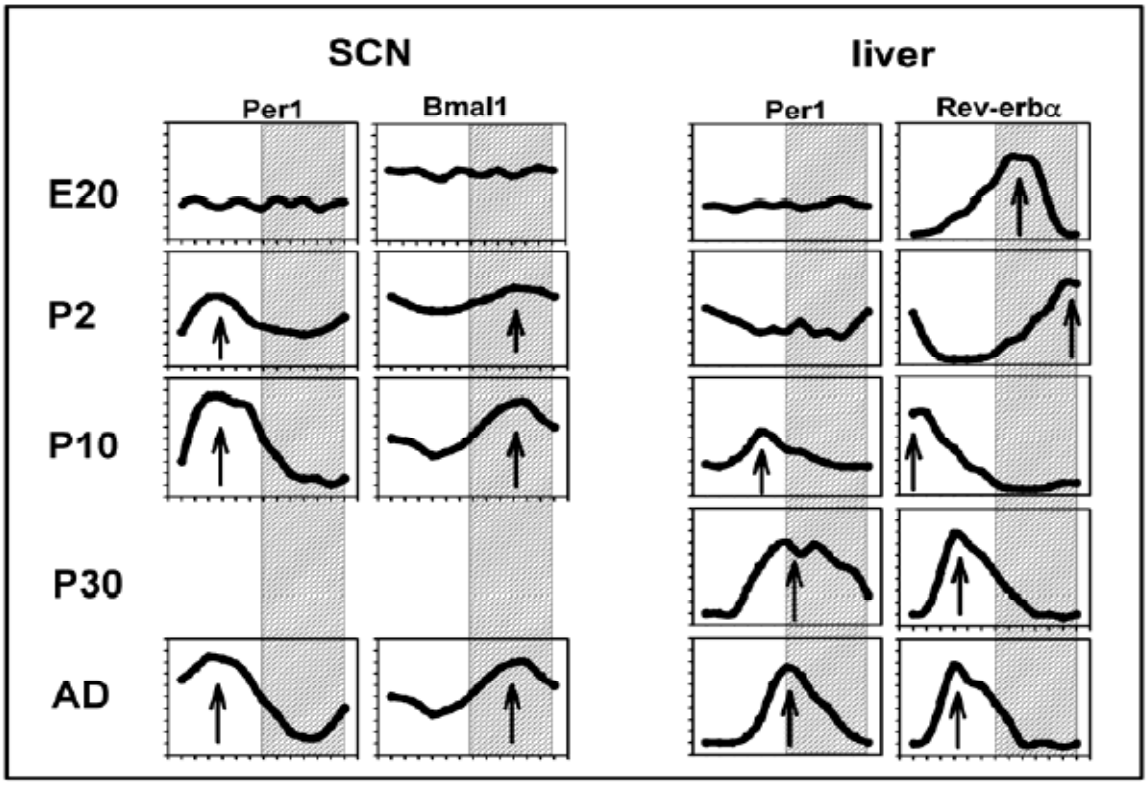
YOO SH, YAMAZAKI S, LOWREY PL, SHIMOMURA K, KO CH, BUHR ED, SIEPKA SM, HONG HK, OH WJ, YOO OJ, MENAKER M, TAKAHASHI JS: Period2:Luciferase real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. *Proc Natl Acad Sci U S A* **101**: 5339-5346, 2004.

ZHENG B, ALBRECHT U, KAASIK K, SAGE M, LU W, VAISHNAV S, LI Q, SUN ZS, EICHELE G, BRADLEY A, LEE CC: Nonredundant roles of the mper1 and mper2 genes inthe mammalian circadian clock. *Cell* **105**: 683-694, 2001.

Legend to figures

Fig. 1. Schematic drawings of development of the circadian clocks in the rat. Daily profiles of clock gene *Per1* and *Bmal1* mRNA in the SCN and *Per1* and *Rev-erbα* in the liver are depicted in 20-day-old embryos (E20), in pups at postnatal day 2 (P2), P10, P30 and in adult rats. X axis represents day time with the shaded area defining night hours. Y axis represents relative mRNA levels. Drawings are based on results published previously in Sládek et al., 2004, Kováčiková et al., 2006 and Sládek et al., 2007.

Fig.1



Maternal control of the fetal and neonatal rat suprachiasmatic nucleus

Rehab El-Hennamy, Kristýna Matějů, Zdena Bendová, Serhiy Sosniyenko, Alena Sumová

Institute of Physiology, Academy of Sciences of the Czech Republic, v.v.i., Prague, Czech Republic

Running title: Maternal entrainment of fetal SCN

Corresponding author:
Alena Sumová, PhD
Institute of Physiology, v.v.i.
Academy of Sciences of the Czech Republic
Vídeňská 1083
142 20 Praha 4
Czech Republic
Phone number: +420241062528
Fax number: +420241062488
Email: sumova@biomed.cas.cz

Number of pages: 24
Number of figures: 5

Abstract

The molecular clockwork underlying the generation of circadian rhythmicity within the suprachiasmatic nucleus (SCN) develops gradually during ontogenesis. The authors' previous work has shown that rhythms in clock gene expression in the rat SCN are not detectable at embryonic day (E)19, start to form at E20 and develop further via increasing amplitude until postnatal day (P)10. The aim of the present work was to elucidate whether and how swiftly the immature fetal and neonatal molecular SCN clocks can be reset by maternal cues. Pregnant rats maintained under a light-dark (LD) regime with 12h of light and 12h of darkness were exposed to a 6 h delay of the dark period and released into constant darkness at different stages of the fetal SCN development. Adult male rats maintained under the same LD regime were exposed to an identical shifting procedure. Daily rhythms in spontaneous *c-fos*, *Avp*, *Per1* and *Per2* expression were examined within the adult and newborn SCN by *in situ* hybridization. Exposure of adult rats to the shifting procedure induced a significant phase delay of locomotor activity within 3 days after the phase shift as well as a delay in the rhythms of *c-fos* and *Avp* expression within three days and *Per1* and *Per2* expression within 5 days. Exposure of pregnant rats to the shifting procedure at E18, but not at E20, delayed the rhythm in *c-fos* and *Avp* expression in the SCN of newborn pups at P0-1. The shifting procedure at E20 did, however, induce a phase delay of *Per1* and *Per2* expression rhythms at P3 and P6. Hence, five days were necessary for phase-shifting the pups' SCN clock by maternal cues, be it the interval between E18 and P0-1 or the interval between E20 and P3, while only three days were necessary for phase-shifting the maternal SCN by photic cues. These results demonstrate that the SCN clock is capable of significant phase shifts at fetal developmental stages when no or very faint molecular oscillations can be detected. This finding suggests that maternal cues may drive rather than entrain the immature fetal rat SCN clock.
Key words: circadian system, suprachiasmatic nucleus, ontogenesis, maternal entrainment, rat

INTRODUCTION

The intrauterine period is important for postnatal development of mammals. During this period, a developing organism is mostly protected from the influence of the external environment, but it is continuously exposed to the internal maternal milieu. The majority of maternal signals that are delivered to the fetus trans-placentally exhibit circadian rhythms. Maternal circadian rhythms are driven by an endogenous clock located within the suprachiasmatic nuclei (SCN) of the hypothalamus (Klein et al., 1991). The clock is dominantly entrained by the light-dark regime of the solar day, mainly by its light period (Pittendrigh, 1981). Therefore, timing of maternal cues may provide fetuses with information about the external time. However, it is still not known whether and how maternal cues impinge on the fetal SCN clock.

In the rat, the prenatal period lasts for about 22 days. Neurogenesis of the SCN begins on embryonic day (E) 14 and is completed at E18, but the morphological maturation of the SCN neurons proceeds gradually until postnatal day (P) 10 (Moore, 1991). Synaptogenesis within the SCN appears to be a slower process: at E19, only sparse synapses can be observed. The process begins to progress only in the late prenatal and early postnatal periods, and then increases noticeably between P4 and P10 (Weinert, 2005). Thus, during the prenatal period, although SCN neurons are present, the multi-level intercellular coupling may not yet be functional. The coupling strengthens during the first postnatal week, and the rat SCN is developed to its full complexity only at P10. Therefore, morphological development of the

SCN extends well into the postnatal period. The development is genetically determined and occurs without input from the external environment (Jud and Albrecht, 2006). A question remains as to whether the morphologically immature SCN clock serves as a self-sustaining clock or whether it functions first as an hourglass oscillator and only later, it becomes an autonomous clock (Sumová et.al, in press). During the late prenatal period, the SCN exhibits day-night variation in metabolic activity (Reppert and Schwartz, 1984), in *Avp* mRNA levels (Reppert and Uhl, 1987) and in the firing rate of its neurons (Shibata and Moore, 1987). In adults, all of these rhythms are supposed to be driven by the SCN clock. However, within the fetal SCN, the rhythms might arise also from cyclically-appearing maternal cues that impinge on fetal SCN neurons. Such maternal "zeitgebers" might trigger rhythms in neuronal and metabolic activity as well as in *Avp* mRNA levels. Indeed, it has been demonstrated that transcription of *Avp* might be regulated by a non-clock-related mechanism (Iwasaki et al., 1997; Burbach et al., 2001). Recent data using a more reliable marker of transcription rate than detection of mRNA, i.e., detection of heteronuclear RNA as a nascent transcript, revealed circadian rhythmicity in transcription of the *Avp* gene in the rat SCN only at P1, but not at E20 (Kováčiková et al., 2006).

A self-sustained clock generates circadian rhythmicity through molecular clockwork composed of interactive transcriptional–translational feedback loops. A contemporary model of the molecular core clockwork presumes that rhythmic expression of clock genes, namely *Per1*, *Per2*, *Cry1*, *Cry2*, *Rev-erbα*, *Bmal1*, as well as their proteins, drives the circadian clock in a cell-autonomous fashion (for review see Fu and Lee, 2003; Ko and Takahashi, 2006; Reppert and Weaver, 2001). The molecular core clockwork develops gradually during ontogenesis. Ohta et al. reported clear daily rhythms of *Per1* and *Per2* mRNA in the rat SCN at E20 (Ohta et al., 2002, 2003). However, other authors did not detect significant rhythms in *Per1*, *Per2*, *Cry1* and *Bmal1* mRNA in the rat SCN at E19 (Sládek et al., 2004), when the fetal rat SCN is already formed (Moore, 1991) and the rhythm in metabolic activity has become apparent (Reppert and Schwartz, 1984). Moreover, at that stage, clock gene proteins PER1, PER2 and CRY1 not only did not exhibit any circadian variation, but were in fact undetectable (Sládek et al., 2004). At E20, the rhythm in *Per1* expression began to form, but the amplitude was very low (Kováčiková et al., 2006). Rhythms in clock gene expression developed gradually during the postnatal period, and adult-level amplitudes were achieved only at P10 (Kováčiková et al., 2006). Similarly, molecular oscillations equivalent to those observed in adults were not detected in the fetal hamster SCN (Li and Davis, 2005). In mice, Shimomura et al. reported a significant oscillation in *Per1* but not in *Per2* mRNA in the SCN at E17, and that the amplitude of the oscillations increased progressively with postnatal age (Shimomura et al., 2001). The aforementioned data suggest that during fetal development, the SCN circadian clock is not able to generate high-amplitude synchronized oscillations in clock gene expression and, therefore, may not be able to function as a self-sustained clock.

In the present study, we aimed to determine whether and how swiftly the immature fetal and neonatal molecular SCN clock can be re-set by maternal cues. The maternal SCN clock was shifted by a 6h delay of the dark period at different stages of fetal SCN development, and the phases of the spontaneous oscillations in *c-fos*, *Avp*, *Per1* and *Per2* expression within the newborn SCN were examined. We assumed that a circadian clock that is not capable of generating synchronized molecular oscillations, as is the case for the fetal rat SCN, would not be entrained by maternal cues. However, we found a significant phase-shift of the SCN clock at fetal developmental stages when no or only faint molecular oscillations were detected. This finding may suggest that maternal cues drive rather than entrain the immature fetal rat SCN clock.

MATERIAL AND METHODS

Animals

Male and female Wistar rats (Bio Test s r.o., Konárovice, Czech Republic) were maintained for at least 4 weeks at a temperature of 23 ± 2^0 C and under a light-dark cycle with 12h of light and 12h of darkness (LD 12:12) per day, with lights on from 07:00 to 19:00. The rats had free access to food and water throughout the whole experiment. Light was provided by overhead 40-W fluorescent tubes, and illumination was between 50 and 200 lx depending on cage position in the animal room. Vaginal smears were taken from females to determine the day of estrus; on the same day, females were mated with males. The day when female rats were found to be sperm-positive was designated embryonic day 0 (E0); the day of delivery, which occurred at about E22, was designated postnatal day 0 (P0).

All experiments were conducted under license no. A5228-01 with the U. S. National Institutes of Health and in accordance with Animal Protection Law of the Czech Republic (license no. 42084/2003-1020).

Experimental protocol

On gestational days 18 and 20, pregnant rats were divided into two groups. A control group remained untreated under the previous LD regime. The other group was exposed to a 6h delay of one dark period, so that the light was switched off and on by 6h later than before, i.e., at 01:00 and 13:00, respectively (Fig.1). At the next cycle, rats of both groups were released into constant darkness (DD) at 19:00 and were kept in darkness until pups were born and sampled at 2h intervals at P0-P1, P3 and P6. The time of the release into DD was designated as circadian time (CT)0. Simultaneously, adult male rats were subjected to treatment identical with that experienced by control and phase-shifted groups of pregnant rats, in order to provide evidence for the efficiency of the procedure for phase-delaying circadian rhythmicity within the adult SCN. Adult rats were monitored for locomotor activity and/or sacrificed under anesthesia at 2h intervals throughout the 24h cycle, three or five days after the 6h delay of the dark period. Pups were killed by rapid decapitation at 2h intervals of the 24h cycle at (P) 0-1, P3 and P6. Brains were removed, immediately frozen on dry ice and stored at -80^oC. They were sectioned into five series of 12μm thick slices in alternating order throughout the whole rostro-caudal extent of the SCN. Sections were further processed for *in situ* hybridization in order to determine profiles of *c-fos* mRNA and *Avp* hnRNA at P0-P1 and in adults, and of *Per1* and *Per2* mRNA at P3, P6 and in adults.

Locomotor activity monitoring

Adult female rats were maintained individually in cages equipped with infrared movement detectors attached above the centre of the cage top, enabling detection of locomotor activity across the whole cage. Activity was measured every minute using a circadian activity monitoring system (Dr. H.M. Cooper, INSERM, France) and was analyzed by Actiview Biological Rhythms Analysis software (Mini Mitter, Oregon, USA). Double-plotted actograms were generated for visualization of data. The activity onset and offset was determined by two independent observers by fitting lines connecting at least five successive activity onsets or offsets by eye before and after the shift of the light-dark cycle.

In situ hybridization

The cDNA fragments of rat *c-fos* (1160 bp; corresponds to nucleotides 141-1300 of the sequence in GenBank accession number X06769), *Avp* (506 bp; identical to nucleotides 796-1302 of the intronic sequence in GenBank accession no. X01637), *Per1* (980 bp; corresponds to nucleotides 581-1561 of the sequence in GenBank accession no. AB002108) and *Per2* (1512 bp; corresponds to nucleotides 369-1881 of the sequence in Genbank with accession no. NM031678) were used as templates for *in vitro* transcription of complementary RNA probes. The *Per1* and *Per2* fragment-containing vectors were generously donated by Professor H. Okamura (Kobe University School of Medicine, Japan) and the *c-fos* fragment-containing-vector was generously donated by Dr. Tom Curran (Children's Hospital of Philadelphia, Pennsylvania, USA). The *Avp* cDNA was cloned in our laboratory (Kovacikova et al., 2006). Probes were labeled using ^{35}S-UTP, and the *in situ* hybridizations were performed as described previously (Kováčiková et al., 2006; Shearman et al., 2000; Sládek et al., 2004). The sections were hybridized for 20h at 60 ^{0}C. Following a post-hybridization wash, the sections were dehydrated in ethanol and dried. Finally, the slides were exposed to the film BIOMAX MR (Kodak) for 10 -14 days and developed using the ADEFO-MIX-S developer and ADEFOFIX fixer (ADEFO-CHEMIE Gmbh, Germany). Brain sections from control and phase-shifted animals were processed simultaneously under identical conditions.

Autoradiographs of sections were analyzed using an image analysis system (Image Pro, Olympus, New Hyde Park, NY) to detect relative optical density (OD) of the specific hybridization signal. In each animal, mRNA or hnRNA was quantified bilaterally, always at the midcaudal SCN section containing the strongest hybridization signal. Each measurement was corrected for nonspecific background by subtracting OD values from the same adjacent area in the hypothalamus. The background signal of that area served as an internal standard and it was consistently low and did not exhibit marked changes with time of the day. Finally, slides were counterstained with cresyl violet to check the presence and the midcaudal position of the SCN in each section. For each time point, 3-4 rats were sacrificed. The OD for each animal was calculated as a mean of values for the left and right SCN.

Data analysis

Data were analyzed by 2-way ANOVA for the group and time differences. Subsequently, the Student-Newman-Keuls multiple range test was used, with a significance level of $p<0.05$.

RESULTS

Effect of a 6 h phase delay of the dark period on the locomotor activity and profiles of c-fos, Avp, Per1, and Per2 expression in the SCN of adult rats

We first aimed to determine how the shifting procedure affects the maternal SCN. Dynamics of the SCN entrainment were assessed from the locomotor activity recordings before and after the 6h shift of the dark period. A representative locomotor activity actogram is depicted in Fig.2. The phase-delay of locomotor activity was fully completed by the third day after the shift of the dark phase.

To provide further evidence that the maternal SCN had fully phase-shifted, we determined daily profiles of *c-fos* and *Avp* expression on the third day and of *Per1* and *Per2*

expression on the fifth day after the delay of the dark period in the SCN of control rats and those exposed to the shifting procedure (shifted group).

For *c-fos* mRNA levels (Fig.3A), the two-way ANOVA revealed a significant effect of time ($F=35.9$, $p<0.001$). Although the effect of group was not significant, the highly significant interaction effect ($F=10.2$, $p<0.001$) suggested differences between the *c-fos* mRNA profiles in rats of the control and shifted group. *c-fos* mRNA levels increased at CT24 from a low level at CT20 ($p<0.05$) in controls, but only at CT2 from a low level at CT24 ($p<0.001$) in the shifted group. The elevated levels declined at CT14 in controls (CT10 vs. CT12, $p<0.01$), but not until CT16 in the shifted group (CT16 vs. CT14, $p<0.01$). Moreover, levels at CT24 and CT2 were significantly higher ($p<0.001$) and levels at CT12 and CT6 were significantly lower ($p<0.001$) in controls than in the shifted group. The entire daily profile of *c-fos* expression was thus phase-delayed by about 2 h in animals exposed to the shifting procedure as compared to the profile of control animals.

For *Avp* hnRNA levels (Fig.3B), the two-way ANOVA revealed a significant effect of time ($F=189.5$, $p<0.001$), of group ($F=30.8$, $p<0.001$) as well as a significant interaction effect ($F=14.2$, $p<0.001$). In control rats, *Avp* hnRNA levels at CT20 and CT22 were significantly higher than the levels measured at CT16 ($p<0.05$ and $p<0.001$, respectively), but the increase was not seen until CT24 in the shifted group (from a low level at CT22, $p<0.001$). The decline occurred at the same time in both groups, i.e, at CT16 (from high levels at CT14, $p<0.001$). Moreover, the *Avp* hnRNA levels were significantly higher at CT22, CT24 and CT2 ($p<0.001$) and significantly lower at CT12 ($p<0.05$) in controls compared to the shifted group. Apparently, the rise but not the decline in *Avp* expression was phase-delayed in animals exposed to the shifting procedure compared to the control animals.

For *Per1* mRNA levels (Fig.3C), the two-way ANOVA revealed significant effects of time ($F=167.4$, $p<0.001$), group ($F=6.2$, $p<0.05$) and interaction ($F=33.3$, $p<0.001$). In controls, the *Per1* mRNA levels began to increase at CT24 (vs. a low level at CT22, $p<0.001$) and the rise continued until CT4 whereas in the shifted group, the levels started to rise only at CT4 (vs. a low levels at CT2, $p<0.001$) and continued to increase until CT6. The first significant decline of *Per1* mRNA occurred at CT12 in controls (vs. CT10, $p<0.001$), but not until CT14 in the shifted group (vs. CT12, $p<0.001$). Furthermore, levels were significantly higher at CT2 and CT4 ($p<0.001$) and lower at CT12, CT14 ($p<0.001$) and CT16 ($p<0.05$) in the control than in the shifted group. Thus, the entire daily profile of *Per1* expression was phase-delayed in animals exposed to the shifting procedure as compared to the controls.

For *Per2* mRNA levels (Fig.3D), the two-way ANOVA revealed a significant effect of time ($F=58.7$, $p<0.01$). Although the effect of group was not significant, the highly significant interaction effect ($F=7.7$, $p<0.001$) suggested the presence of differences between the Per2 expression profile for controls and the shifted group. *Per2* mRNA levels began to rise significantly at CT2 in controls (vs. CT22, $p<0.001$), but only at CT6 in the shifted group (vs. CT4, $p<0.001$). The levels declined at CT18 in controls (vs. elevated level at CT16, $p<0.001$), but did not decrease until CT20 in the shifted group (vs. high level at CT18, $p<0.001$). Moreover, *Per2* mRNA levels were significantly higher at CT2 and CT4 ($p<0.001$) and significantly lower at CT18 ($p<0.001$) in controls than in the shifted group. The entire daily profile of *Per2* expression was thus phase delayed in animals exposed to the shifting procedure as compared with that of the control animals.

Effect of the maternal phase shift at E20 on profiles of c-fos and Avp expression in the SCN at P0-1 and of Per1 and Per2 expression at P3 and P6

Our second aim was to investigate whether exposure of pregnant rats to the shifting procedure on gestational day 20 (E20) entrains the fetal SCN. To determine whether the shift could be accomplished during prenatal or postnatal periods, the profiles of *c-fos* and *Avp* expression were assessed in newborn pups at P0-1 and the profiles of *Per1* and *Per2* expression were determined at P3 and P6.

For levels of *c-fos* mRNA at P0-1 (Fig.4C), the two-way ANOVA revealed a significant effect of time ($F=28.2$, $p<0.001$), of group ($F=10.6$, $p<0.01$) as well as a significant interaction effect ($F=3.6$, $p<0.001$). The levels at CT16, CT20, CT2 ($p<0.05$) and CT6 ($p<0.001$) were significantly lower and at CT22 ($p<0.05$) significantly higher in the control than in the shifted group. A significant rise in *c-fos* mRNA levels occurred at CT2 in controls (vs. CT20, $p<0.01$) and at CT24 in the shifted group (vs. CT22, $p<0.001$). The elevated levels declined at CT16 in the control (vs. CT10, $p<0.05$) and at CT18 in the shifted group (vs. at CT12, $p<0.05$). Altogether, at P0-1, the profile of *c-fos* expression was not phase-shifted in pups of mothers exposed to the shifting procedure at E20 compared to the profile of pups born to control mothers.

For levels of *Avp* hnRNA at P0-1 (Fig.4D), the two-way ANOVA revealed a significant effect of time ($F=22.1$, $p<0.001$) and group ($F=5.3$, $p<0.05$), but not for the interaction. The daily profile of *Avp* expression in pups of mothers exposed to the shifting procedure during E20 was not phase-shifted relative to the profile of pups born to control mothers.

For levels of *Per1* mRNA at P3 (Fig.5A), the two-way ANOVA revealed a significant effect of time ($F=39.7$, $p<0.001$). Although the effect of group was not significant, the highly significant interaction effect ($F=6.3$, $p<0.001$) suggested the presence of differences between groups. *Per1* mRNA levels first increased at CT24 in controls (vs. CT22, $p<0.01$), but only at CT2 in the shifted group (vs. CT24, $p<0.05$). In control animals, the elevated levels started to decline at CT8 (vs. CT6, $p<0.001$) and continued falling until CT12 (vs. CT10, $p<0.05$). In the shifted group, the decline was not apparent until CT10 (vs. CT8, $p<0.05$) and was completed only at CT16 (vs. CT12, $p<0.05$). Moreover, *Per1* mRNA levels at CT24, CT2 and CT4 were significantly higher ($p<0.001$) and at CT8, CT10 and CT12 significantly lower ($p<0.001$, 0.05 and 0.001, respectively) in controls as compared to the shifted group. Altogether, at P3, the entire daily profile of *Per1* expression was phase-delayed in pups born to mothers exposed to the shifting procedure at E20 relative to the profile of pups born to control mothers.

Regarding levels of *Per2* mRNA at P3 (Fig.5B), the two-way ANOVA revealed a significant effect of time ($F=50.9$, $p<0.001$), group ($F=12.9$, $p<0.001$) as well as a significant interaction effect ($F=11.2$, $p<0.001$). *Per2* mRNA levels increased at CT24 in controls (vs. CT22, $p<0.001$), but this rise was delayed until CT2 in the shifted group (vs. CT24, $p<0.001$). Similarly, the decline from elevated levels occurred earlier in the control than in the shifted group, i.e., at CT14 (vs. CT12, $p<0.001$) and at CT18 (vs. CT14, $p<0.01$), respectively. Moreover, *Per2* mRNA levels at CT14 ($p<0.001$), CT16 and CT18 ($p<0.01$) were significantly lower and at CT24 and CT8 ($p<0.001$) were significantly higher in controls than in the shifted group. Therefore, at P3, the daily profile of *Per2* expression was phase-delayed in pups born to mothers exposed to the shifting procedure at E20 relative to the profile of pups born to control mothers.

For levels of *Per1* mRNA at P6 (Fig.5C), the two-way ANOVA revealed a significant effect of time ($F=24.1$, $p<0.001$) though not of group. However, the highly significant interaction effect ($F=6.2$, $p<0.001$) suggested that there may indeed be differences between the control and the shifted groups. *Per1* mRNA levels increased significantly at CT2 in controls (vs. CT20, $p<0.01$), but only at CT4 in the shifted group (vs. CT2, $p<0.05$). Decline from elevated levels occurred at CT14 in controls (vs. CT12, $p<0.01$), but only at CT18 in the shifted group (vs. CT14, $p<0.01$). *Per1* mRNA levels were significantly higher at CT24 ($p<0.05$), CT2 and CT4 ($p<0.001$) and lower at CT8, CT10 ($p<0.01$) and CT14 ($p<0.001$) in the control group as compared to the shifted group. Thus, at P6, the entire daily profile of *Per1* expression in pups of mothers exposed to the shifting procedure at E20 was phase delayed relative to the profile of pups born to control mothers.

Finally, for levels of *Per2* mRNA at P6 (Fig.5D), the two-way ANOVA revealed a significant effect of time ($F=89.1$, $p<0.001$). Although the effect of group was not significant, a highly significant interaction effect ($F=8.5$, $p<0.001$) again suggests that the profiles of the different groups were not identical. *Per2* mRNA levels first increased at CT2 (vs. CT24, $p<0.001$) in controls, but the increase was delayed until CT6 (vs. CT4, $p<0.001$) in the shifted group. Decline from elevated levels occurred at CT18 in both controls and the shifted group (vs. CT16, $p<0.001$ and $p<0.05$, respectively), but the decline continued through CT18 to CT20 in the latter group ($p<0.001$). *Per2* mRNA levels were significantly higher at CT2 and CT4 ($p<0.001$) and lower at CT18 ($p<0.001$) in controls compared to the shifted group. In summary, at P6, the entire daily profile of *Per2* expression was phase-delayed in pups born to mothers exposed to the shifting procedure at E20 relative to the profile of pups born to control mothers.

The data indicate that the fetal SCN did not entrain in parallel with the maternal SCN, i.e., within three days after the shifting procedure, but rather later during the postnatal period at P3, i.e., within five days after the shift. A phase delay of approximately the same magnitude as that observed at P3 was also confirmed also at P6.

Effect of the maternal shift at E18 on profiles of c-fos and Avp expression in the SCN at P0-1

To elucidate whether the prenatal SCN can be entrained by maternal cues, pregnant rats were exposed to the shifting procedure at E18 rather than E20, and profiles of *c-fos* and *Avp* gene expression were determined in the newborn pups at P0-1. This experimental design allowed the fetal SCN to be exposed to the shifting maternal entraining cues for five days instead of three days, i.e., for the same time interval as the period between a shift at E20 and sampling of pups at P3 (see above). This arrangement permitted exclusively prenatal maternal cues to impinge on the pup's SCN.

For levels of *c-fos* mRNA at P0-1 (Fig.4A), the two-way ANOVA revealed a significant effect of time ($F=47.6$, $p<0.001$). The effect of group was not significant, but a highly significant interaction effect ($F=8.0$, $p<0.001$) implied differences between the profiles. In the control group, *c-fos* mRNA levels rose at CT2 (vs. CT24, $p<0.001$), but only at CT4 in the shifted group (vs. CT2, $p<0.001$). A significant decline from the elevated levels was apparent at CT10 in both groups. However, the decline began after CT6 in controls ($p<0.001$) and after CT8 in the shifted group ($p<0.001$). Moreover, *c-fos* mRNA level at CT2 was significantly higher ($p<0.001$) and at CT4 and CT8 lower ($p<0.001$) in the control compared to the shifted group. It appears that at P0-1, the daily profile of *c-fos* expression in pups born

to mothers exposed to the shifting procedure at E18 was phase-delayed relative to the profile of pups born to control mothers.

For levels of *Avp* hnRNA at P0-1 (Fig.4B), the two-way ANOVA revealed a significant effect of time ($F=39.7$, $p<0.001$). The effect of group was not significant, but again, the highly significant interaction effect ($F=9.2$, $p<0.001$) indicates the presence of differences between the profiles. *Avp* hnRNA levels began to rise at CT24 (vs. CT20, $p<0.01$) and further increased until CT2 (vs. CT24, $p<0.001$) in the controls, but the levels increased first only at CT2 in the shifted group (vs. CT22, $p<0.01$) and the rise concluded at CT4 (vs. CT2, $p<0.001$). A significant decline from elevated levels occurred at CT8 in controls, but was delayed until CT10 in the shifted group (vs. CT6 and CT8, respectively, $p<0.001$). Moreover, *Avp* hnRNA levels at CT24 and CT2 were significantly higher ($p<0.01$ and $p<0.001$, respectively) and at CT8, CT10 and CT12 lower ($p<0.001$, $p<0.05$ and $p<0.01$, respectively) in the control compared to the shifted group. Therefore, at P0-1, the entire daily profile in *Avp* expression in pups born to mothers exposed to the shifting procedure at E18 was phase delayed relative to the profile of pups born to control mothers.

These data indicate that the fetal SCN did entrain within five days after exposure of pregnant rats to the shifting procedure at E18.

DISCUSSION

Our data demonstrate that exposure of pregnant rats to a 6 h delay in the dark period induced a significant phase delay in the profiles of *c-fos* and *Avp* expression within the newborn pup's SCN at P0-1 if the shifting procedure was performed at E18, but not at E20. The shifting procedure at E20 was, however, able to phase-shift the profile of *Per1* and *Per2* expression in pups at P3 and P6.

The efficiency of the procedure for phase-shifting the maternal SCN clock was proven by a noted phase delay of locomotor activity as well as of profiles of *c-fos*, *Avp*, *Per1* and *Per2* gene expression within the SCN of adult rats. Locomotor activity recordings revealed that entrainment to the shifting procedure in rats released thereafter into DD was accomplished within three days post-shift. On the third and fifth day after the shifting procedure, profiles of *c-fos* and *Avp*, and *Per1* and *Per2* gene expression, respectively, within the SCN of adult rats released into DD were significantly phase-delayed compared to the profiles of control rats. Similarly, another study showed that profiles of *Per1*, *Per2* and *Cry1* expression in the mouse SCN were phase-delayed within two cycles after the mice were exposed to a 6 h delay of the LD cycle and kept in a new LD regime (Reddy et al., 2002). The magnitude of phase delays was, however, larger in mice held in LD than in rats kept under DD conditions. Nevertheless, our data clearly demonstrate that after the shifting procedure and subsequent release of rats into DD, the maternal SCN was significantly phase-delayed. The delay was clearly detectable on the third day after the shift both at the locomotor activity as well as the gene expression profile levels.

In newborn rat pups at P0-1, significant circadian rhythms in *c-fos* and *Avp* expression were detected (this study, Kováčiková et al., 2006; Leard et al., 1994). Therefore, we used profiles of *c-fos* mRNA and *Avp* hnRNA as phase-markers of circadian rhythmicity within the newborn SCN. At P3 and P6, we used *Per1* and Per2 mRNA profiles as phase markers of the rhythmicity, as our recent studies have shown that those rhythms exhibit already significant amplitude at these developmental stages. In previous studies, maternal entrainment during fetal stages was studied by monitoring various overt rhythms postnatally, e.g., the pineal

arylalkylamine N-acetyltranferase in rats after P10 (Reppert and Schwartz, 1986b; Duncan et al., 1986), the running wheel activity and drinking behavior rhythm in rats and hamsters following weaning (Davis and Gorski, 1985a;1985b; Viswanathan et al., 1994; Weaver and Reppert, 1989; Bellavia et al., 2006) or the corticosterone rhythm after P28 (Honma et al., 1984). The general outcome of the studies was that the fetal SCN was entrained by maternal cues. However, in these reports, a relatively long time elapsed between manipulation of pregnant rats and the recording period and therefore, postnatal entraining cues might have also interfered. In the fetal rat SCN, a rhythm in metabolic activity detected by 2-deoxyglucose uptake was used to study maternal entrainment (Reppert and Schwartz, 1984, 1986a). Usually, one day-time and one night-time point were determined and significance of difference between the two time-points increased between E19 and E21 (Reppert and Schwartz, 1984). After complete surgical removal of the maternal SCN, the day-night difference was abolished (Reppert and Schwartz, 1986b). It was concluded that this resulted from a de-synchronization of the rhythms between individual fetuses rather than to loss of the fetal SCN rhythmicity. The maternal SCN was thus recognized as an important component of maternal entrainment during fetal development.

In the current study, we examined the impact of a phase-shift of an intact maternal SCN on the phase of the newborn rat SCN. Exposure of mothers to a 6 h delay of the dark period at E18 induced a significant phase delay in the rhythms of *c-fos* and *Avp* expression in the SCN of newborn pups at P0-1. As pregnant rats were released into DD immediately after the manipulation and pups were born in darkness, the observed phase shift in both gene expression profiles was accomplished solely by non-photic maternal cues during the fetal development. This phase-delay was of approximately the same magnitude as the phase shift observed in their mothers. However, when the same shifting procedure was applied to pregnant rats at E20, profiles of *c-fos* and *Avp* expression at P0-1 were not phase-shifted. This result could not be attributed to the possibility that the phase-shifting of the maternal SCN had not completed within three days (see above), nor to the possibility that the fetal SCN was not sensitive to the shift, because a significant delay in the *Per1* and *Per2* expression profiles was detected at P3 and at P6. Rather, it seems that the interval elapsing between the maternal manipulation and detection of the phase shift within the pup's SCN is important: five days were necessary for phase-shifting the pup's SCN clock by maternal cues, be they the interval between E18 and P0-1 or the interval between E20 and P3. In contrast, only three days were needed to completely phase-shift the maternal SCN by photic cues. Our data thus support the hypothesis that the maternal SCN is necessary for entrainment of the fetal SCN clock. If entrainment of the fetal SCN were mediated independently of the maternal SCN, e.g., via a direct effect of a cue upon the fetal SCN, the maternal and fetal SCN would shift at the same rate.

Our results show for the first time a maternal entrainment of rhythms in clock gene expression at such early developmental stages. In previous studies, only about 2 h phase shifts of *Per1* and *Per2* rhythms were demonstrated at P6 in the SCN of blinded pups, induced by nursing of the pups by foster mothers synchronized to a reverse LD cycle. Between P6 and P13, maternal cues were ineffective (Ohta et al., 2002). The ability of maternal cues to entrain the pup's molecular clockwork thus decreases during postnatal development. Maternal entrainment is gradually replaced by developing photic entrainment, which may be capable of overriding the maternal cues after P6 (Duncan et al., 1986).

The most intriguing question still remains as of what is the substrate that mediates the maternal entrainment of the fetal SCN clock. In the rat SCN, synchronized spontaneous rhythms in the SCN clock gene expression had only begun to develop and the rhythm in clock

controlled *Avp* gene expression was not detectable by late fetal stages (Kováčiková et al., 2006; Sládek et al., 2004). This might result from a lack of inter-cellular communication between individual SCN neurons at fetal stages when the rate of synaptogenesis was still very low. Indeed, the increase in amplitude of clock gene expression rhythms nicely paralleled synaptogenesis; the amplitude achieved the adult like magnitude only at P10 when synaptogenesis was completed. The data suggest that only a small population of the SCN cells may be spontaneously rhythmic during late fetal stage. As synapses gradually maturate during early postnatal development, more and more SCN cells may become synchronized and rhythmic. Alternatively, a minority of the SCN cells may become rhythmic because they are selectively sensitive to the cyclically-appearing maternal SCN-derived cues. These cues may in turn directly drive the activity of these cells during fetal stages. Later on, as synaptogenesis progresses, previously insensitive cells may also become rhythmic, allowing development of the autonomous SCN clock to proceed spontaneously. With the gradual development of the autonomous clock, the original mechanism that drove the rhythmicity becomes redundant. The developmental decline in sensitivity of the SCN clock to maternal cues may favor the latter hypothesis. To achieve more insight into the underlying mechanisms, future studies should investigate the nature of the maternal entraining cues and the phenotype of the rhythmic fetal SCN cells.

ACKNOWLEDGEMENTS

The authors wish to thank Prof. Helena Illnerová for her heplful comments on the manuscript and Eva Suchanova and Jiri Sedlmajer for their excellent technical assistance. The authors' work is supported by the Grant Agency of the Czech Republic, Grants No. 309050350, 309080503 and 30908H079, by Research Project Nos. AV0Z50110509, LC554 and by the 6th Framework Project EUCLOCK No. 018741.

REFERENCES

Bellavia SL, Carpentieri AR, Vaque AM, Macchione AF, and Vermouth NT (2006) Pup circadian rhythm entrainment - effect of maternal ganglionectomy or pinealectomy. Physiol Behav 89:342-349.

Burbach JP, Luckman SM, Murphy D, and Gainer H (2001) Gene regulation in the magnocellular hypothalamo-neurohypophysial system. Physiol Rev 81:1197-1267.

Davis FC, and Gorski RA (1985a) Development of hamster circadian rhythms. I. Within-litter synchrony of mother and pup activity rhythms at weaning. Biol Reprod 33:353-362.

Davis FC, and Gorski RA (1985b) Development of hamster circadian rhythms: Prenatal entrainment of the pacemaker. J Biol Rhythms 1:77-89.

Duncan MJ, Banister MJ, and Reppert SM (1986) Developmental appearance of light-dark entrainment in the rat. Brain Res 369:326-330.

Fu L, and Lee CC (2003) The circadian clock: Pacemaker and tumor suppressor. Nature Reviews Cancer 3:350-361.

Honma S, Honma KI, Shirakawa T, and Hiroshige T (1984) Maternal phase setting of fetal circadian oscillation underlying the plasma corticosterone rhythm in rats. Endocrinology 114:1791-1796.

Iwasaki Y, Oiso Y, Saito H, and Majzoub JA (1997) Positive and negative regulation of the rat vasopressin gene promoter. Endocrinology 138:5266-5274.

Jud C, and Albrecht U (2006) Circadian rhythms in murine pups develop in absence of a functional maternal circadian clock. J Biol Rhythms 21:149-154.

Klein DC, Moore RY, and Reppert SM, eds (1991) In *Suprachiasmatic Nucleus: The Mind's*

Clock, New York, Oxford University Press.
Ko CH, and Takahashi JS (2006) Molecular components of the mammalian circadian clock. Hum Mol Genet 15 Spec No 2:R271-277.
Kováčiková Z, Sládek M, Bendová Z, Illnerová H, and Sumová A (2006) Expression of clock and clock-driven genes in the rat suprachiasmatic nucleus during late fetal and early postnatal development. J Biol Rhythms 21:140-148.
Leard LE, Macdonald ES, Heller HC, and Kilduff TS (1994) Ontogeny of photic-induced c-fos mRNA expression in rat suprachiasmatic nuclei. Neuroreport 5:2683-2687.
Li X, and Davis FC (2005) Developmental expression of clock genes in the Syrian hamster. Dev Brain Res 158:31-40.
Moore RY (1991) Development of the suprachiasmatic nucleus. In *Suprachiasmatic nucleus: The mind's clock*, DC Klein, RY Moore and SM Repperts, eds, pp 197-216, Oxford Univ. Press, New York.
Ohta H, Honma S, Abe H, and Honma K (2002) Effects of nursing mothers on rPer1 and rPer2 circadian expressions in the neonatal rat suprachiasmatic nuclei vary with developmental stage. Eur J Neurosci 15:1953-1960.
Ohta H, Honma S, Abe H, and Honma K (2003) Periodic absence of nursing mothers phase-shifts circadian rhythms of clock genes in the suprachiasmatic nucleus of rat pups. Eur J Neurosci 17:1628-1634.
Pittendrigh CL (1981) Circadian systems: Entrainment. In *Handb Behav Neurol*, Vol. 4, J Aschoff, ed, pp 95-124, Plenum, New York.
Reddy AB, Field MD, Maywood ES, and Hastings MH (2002) Differential resynchronisation of circadian clock gene expression within the suprachiasmatic nuclei of mice subjected to experimental jet lag. J Neurosci 22:7326-7330.
Reppert SM, and Schwartz WJ (1984) The suprachiasmatic nuclei of the fetal rat: Characterization of a functional circadian clock using 14C-labeled deoxyglucose. J Neurosci 4:1677-1682.
Reppert SM, and Schwartz WJ (1986a) Maternal endocrine extirpations do not abolish maternal coordination of the fetal circadian clock. Endocrinology 119:1763-1767.
Reppert SM, and Schwartz WJ (1986b) Maternal suprachiasmatic nuclei are necessary for maternal coordination of the developing circadian system. J Neurosci 6:2724-2729.
Reppert SM, and Uhl GR (1987) Vasopressin messenger ribonucleic acid in supraoptic and suprachiasmatic nuclei: Appearance and circadian regulation during development. Endocrinology 120:2483-2487.
Reppert SM, and Weaver DR (2001) Molecular analysis of mammalian circadian rhythms. Annu Rev Physiol 63:647-676.
Shearman LP, Sriram S, Weaver DR, Maywood ES, Chaves I, Zheng B, Kume K, Lee CC, van der Horst GT, Hastings MH, and Reppert SM (2000) Interacting molecular loops in the mammalian circadian clock. Science 288:1013-1019.
Shibata S, and Moore RY (1987) Development of neuronal activity in the rat suprachiasmatic nucleus. Brain Res 431:311-315.
Shimomura H, Moriya T, Sudo M, Wakamatsu H, Akiyama M, Miyake Y, and Shibata S (2001) Differential daily expression of Per1 and Per2 mRNA in the suprachiasmatic nucleus of fetal and early postnatal mice. Eur J Neurosci 13:687-693.
Sládek M, Sumová A, Kováčiková Z, Bendová Z, Laurinová K, and Illnerová H (2004) Insight into molecular core clock mechanism of embryonic and early postnatal rat suprachiasmatic nucleus. Proc Natl Acad Sci U S A 101:6231-6236.
Sumová A, Bendová Z, Sládek M, El-Hennamy R, Matějů K, Polidarová L, Sosniyenko S, Illnerová H (2008) Circadian molecular clocks ticking along ontogenesis. Physiol Res *in press*
Viswanathan N, Weaver DR, Reppert SM, and Davis FC (1994) Entrainment of the fetal

hamster circadian pacemaker by prenatal injections of the dopamine agonist SKF 38393. J Neurosci 14:5393-5398.
Weaver DR, and Reppert SM (1989) Periodic feeding of SCN-lesioned pregnant rats entrains the fetal biological clock. Dev Brain Res 46:291-296.
Weinert D (2005) Ontogenetic development of the mammalian circadian system. Chronobiol Int 22:179-205.

Legends to figures

Figure 1. Experimental protocol for the shifting procedure. Pregnant rats were maintained in a light-dark cycle with 12 h of light and 12 h of darkness per day (LD12:12) with lights on at 07:00 and lights off at 19:00. On gestational day 18 or 20, rats were divided into two groups. For the group exposed to the shifting procedure, the light period was extended so that lights off and on were delayed by 6 h and occurred at 01:00 and 13:00, respectively. Rats in the control group remained under the previous LD regime. Thereafter, the lights were turned off at 19:00 and rats of both groups were maintained in constant darkness (DD) until sampling of their pups. Time of the release into DD was designated as CT0. Pups were sampled at 2 h intervals at postnatal day P0-1, P3 and P6 starting at CT12. Simultaneously, adult male rats were subjected to treatments identical to those of the control and phase-shifted groups of pregnant rats and sampled three and five days after the treatment.

Figure 2. Representative double-plotted actogram of locomotor activity of one female rat subjected to the experimental procedure described in Fig.1. The white and black bars on the top of the actogram represent the light and dark periods of the LD cycle prior to exposure to the shifting procedure. On the left side, the actogram shows raw data without labeling. On the right side, the actogram is depicted with a shaded area that marks time when the rat was maintained in darkness. Activity onset and offset before and after the phase shift was determined by two independent observers by fitting lines connecting at least five successive activity onsets or offsets by eye (the vertical lines on the right side actogram).

Figure 3. Daily profiles of *c-fos* (A), *Avp* (B), *Per1* (C) and *Per2* (D) mRNA within the SCN of adult male rats. Control rats (full circles) were released into constant darkness and sampled three (A,B) and five (C,D) days later throughout the 24 h cycle, starting at CT12. Group of rats exposed to the shifting procedure and released into darkness (open circles) were sampled three (A,B) and five (C,D) days after the treatment starting at CT12. For further details of experimental protocol, see Material and Methods. Data represent means of four animals per time point and S.E.M.

Figure 4. Daily profiles of *c-fos* mRNA (A,C) and *Avp* hnRNA (B,D) within the SCN of pups at P0-1 born either to control mothers (full circles) or to those exposed to the shifting procedure (open circles) at gestational day 18 (A,B) or 20 (C,D). For further details of the experimental protocol, see Material and Methods. Data represent means of four (occasionally three) animals per time point and S.E.M.

Figure 5. Daily profiles of *Per1* (A,C) and *Per2* (B,D) mRNA within the SCN of rat pups at P3 (A,B) and P6 (C,D) born to control mothers (full circles) and those exposed to the shifting procedure (open circles). For further details of the experimental protocol, see Material and Methods. Data represent means of four animals per time point and S.E.M.

Fig.1

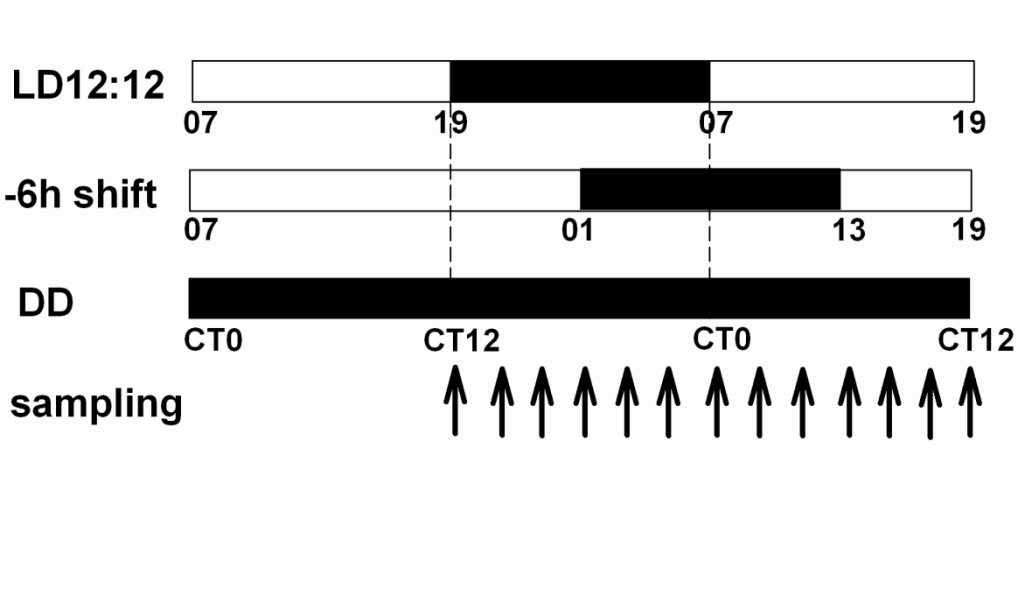

LD12:12
07
19
07
19
-6h shift
07
01
13
19
DD
CT0
CT12
CT0
CT12
sampling

Fig.2

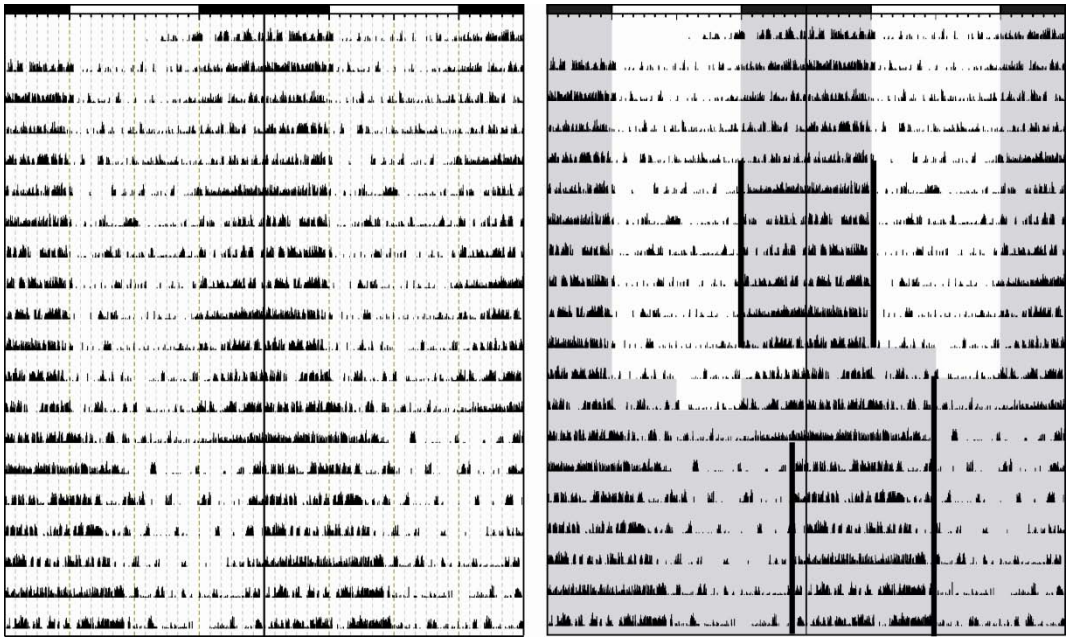


Fig. 3

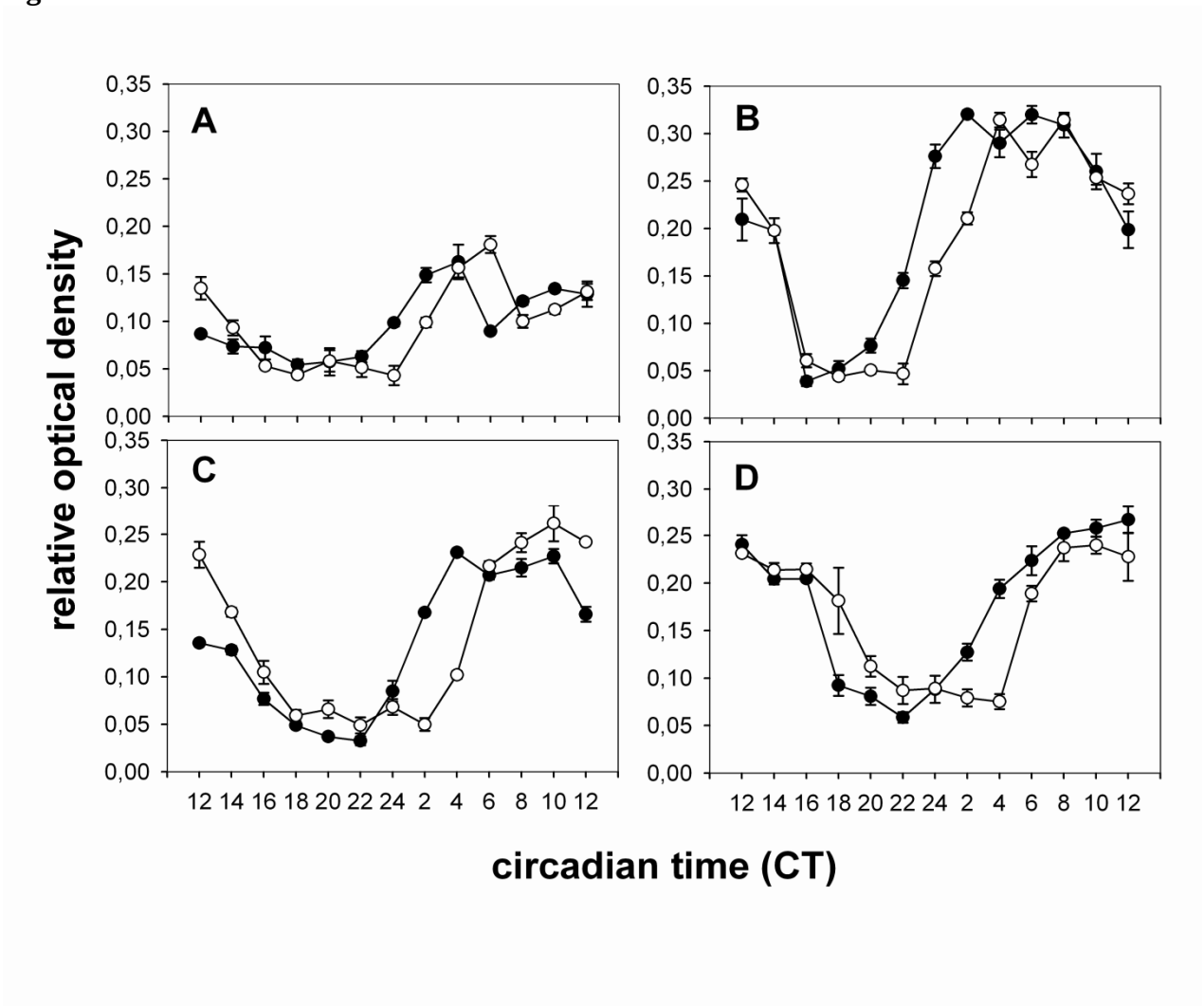

A
B
C
D
relative optical density
0,35
0,30
0,25
0,20
0,15
0,10
0,05
0,00
12 14 16 18 20 22 24 2 4 6 8 10 12
circadian time (CT)

Fig. 4

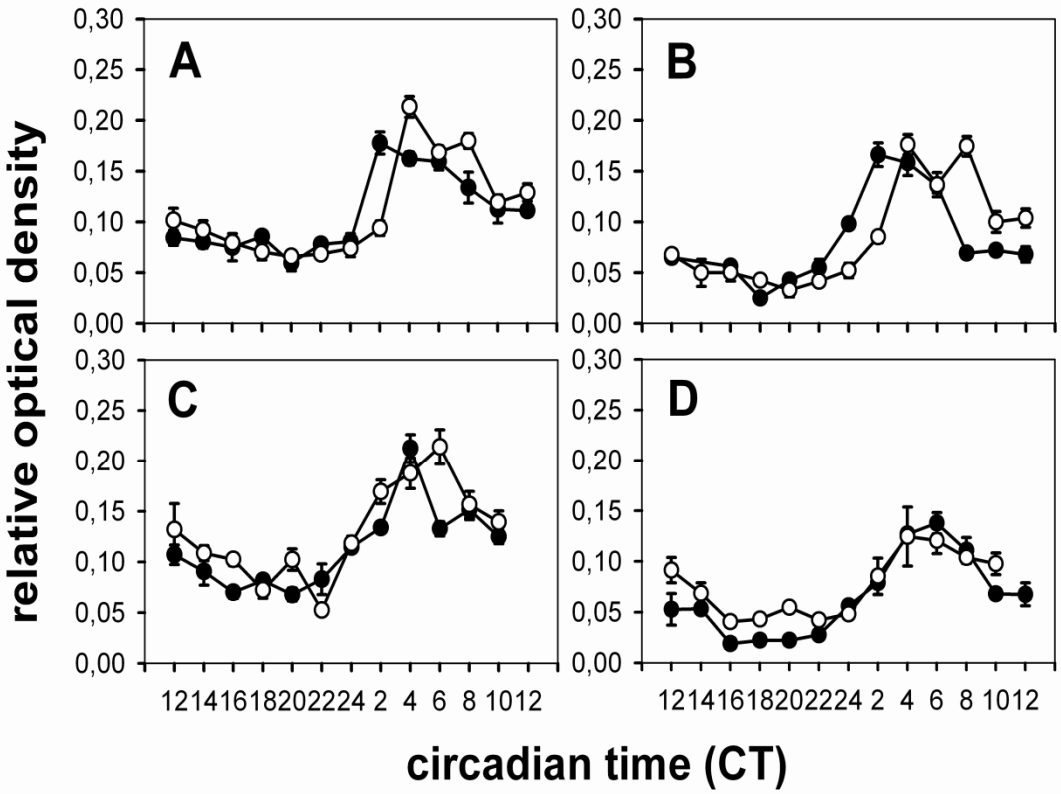
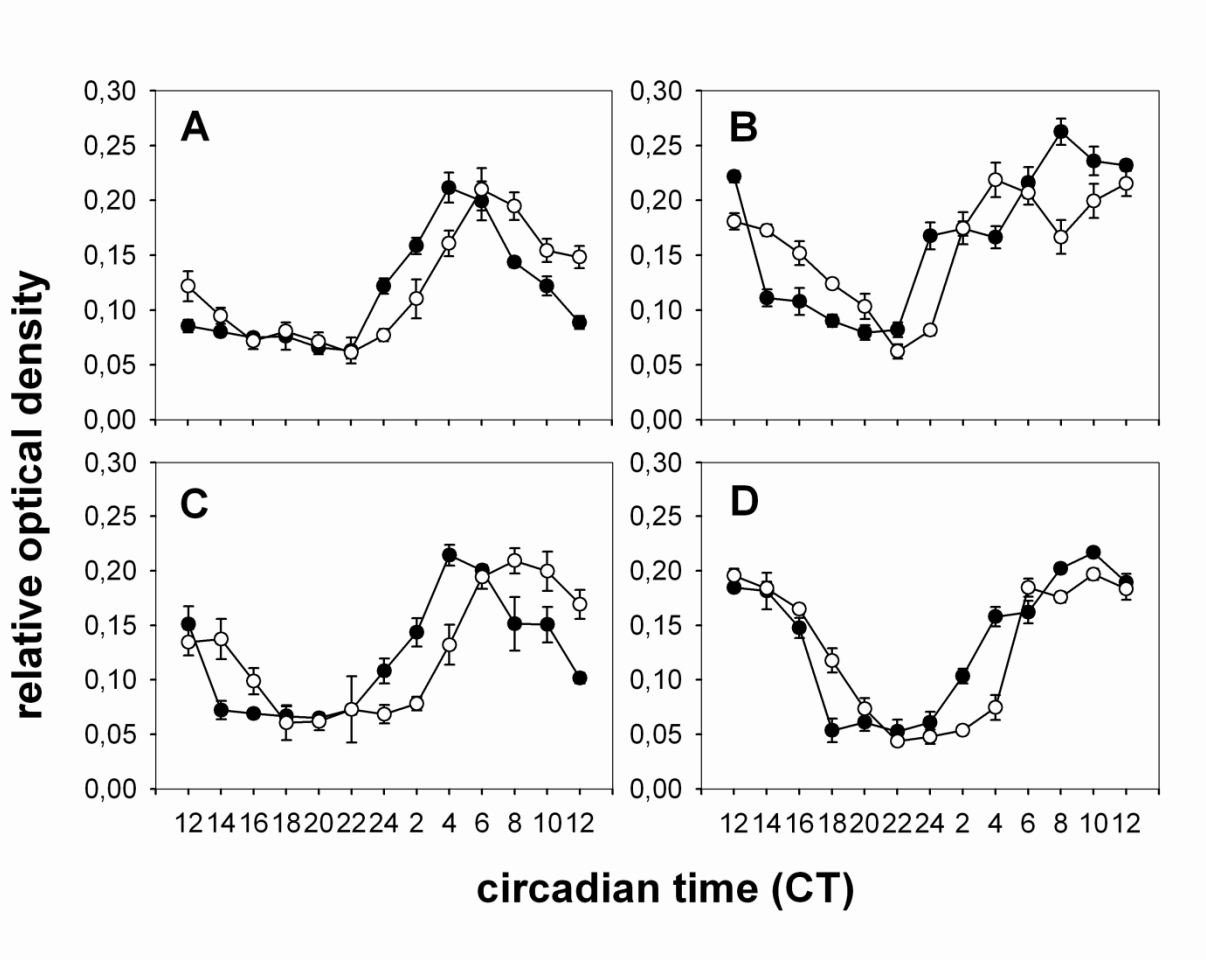

A
B
C
D
relative optical density
0,30
0,25
0,20
0,15
0,10
0,05
0,00
12 14 16 18 20 22 24 2 4 6 8 10 12
circadian time (CT)

Fig. 5


A
B
C
D
relative optical density
0,00
0,05
0,10
0,15
0,20
0,25
0,30
12 14 16 18 20 22 24 2 4 6 8 10 12
circadian time (CT)