Postnatal ontogenesis of the circadian clock within the rat liver

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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–R1229, 2007. First published November 9, 2006; doi:10.1152/ajpregu.00184.2006.—In mammals, the circadian oscillator within the suprachiasmatic nuclei (SCN) entrains circadian clocks in numerous peripheral tissues. Central and peripheral clocks share a molecular core clock mechanism governing daily time measurement. In the rat SCN, the molecular clockwork develops gradually during postnatal ontogenesis. The aim of the present work was to elucidate when during ontogenesis the expression of clock genes in the rat liver starts to be rhythmic. Daily profiles of mRNA expression of clock genes Per1, Per2, Cry1, Clock, Rev-Erb, and Bmal1 were analyzed in the liver of fetuses at embryonic day 20 (E20) or pups at postnatal age 2 (P2), P10, P20, P30, and in adults by real-time RT-PCR. At E20, only a high-amplitude rhythm in Rev-Erb and a low-amplitude variation in Cry1 but no clear circadian rhythms in expression of other clock genes were detectable. At P2, a high-amplitude rhythm in Rev-Erb and a low-amplitude variation in Bmal1 but no rhythms in expression of other genes were detected. At P10, significant rhythms only in Per1 and Rev-Erb expression were present. At P20, clear circadian rhythms in the expression of Per1, Per2, Rev-Erb, and Bmal1, but not yet of Cry1 and Clock, were detected. At P30, all clock genes were expressed rhythmically. The phase of the rhythms shifted between all studied developmental periods until the adult stage was achieved. The data indicate that the development of the molecular clockwork in the rat liver proceeds gradually and is roughly completed by 30 days after birth.

IN MAMMALS, MOST PHYSIOLOGICAL and behavioral processes exhibit daily cycles, which persist even in constant conditions with a period close to 24 h. These circadian rhythms are controlled by a circadian clock located within the suprachiasmatic nuclei of the hypothalamus (SCN). The SCN clock is entrained to the 24-h day by environmental stimuli, such as the light-dark (LD) cycle. Apart from the SCN, numerous endogenous circadian clocks operate in neuronal and nonneuronal peripheral tissues like liver, spleen, lung, heart, muscle, etc. The SCN synchronizes the peripheral clocks via incompletely understood humoral and neuronal signals so that they run in phase with the external LD cycle. In SCN-lesioned mice, the peripheral clocks still oscillate, but their mutual synchronization, as well as the synchronization with the LD cycle, is lost (35). Besides synchronization by the SCN, many peripheral clocks may also be entrained by daily feeding cycles. Under natural conditions, the SCN clock controls circadian rhythms in activity-rest and food intake. However, when feeding of nocturnal rodents is restricted to the light part of the day when they usually rest, the phase of peripheral clocks becomes uncoupled from that of the SCN, which is set predominantly by the LD cycle (4). Physiological relevance of peripheral clocks is highlighted by the high number of rhythmically regulated genes responsible for tissue-specific functions. For example, in the liver, many rhythmically controlled genes encode enzymes involved in food processing (e.g., phosphoenolpyruvate carboxykinase, glycogen synthase), heme synthesis (aminolevulinate synthase), detoxification (components of cytochrome P450), bile acids synthesis (cholesterol 7α-hydroxylase), etc. (2, 10, 16).

The molecular mechanism of the circadian clockwork is evolutionary conserved across diverse species, such as the fruit fly and mouse (23), and is considered to be composed of interlocked autoregulatory feedback loops of gene expression and translation (for review, see Ref. 24). Several mammalian clock genes have been cloned so far, and it is likely that more will be discovered in the near future. Transcription factors named CLOCK and BMAL1 form heterodimers, bind to E-box-containing sequences of Per and Cry genes and drive their expression. PER and CRY proteins form complexes and translocate to the nucleus where they interact with CLOCK/BMAL1 and inhibit their own expression, probably due to directed histone deacetylation and methylation (5, 25). Other important genes regulated by CLOCK/BMAL1 are Rev-Erb, which encodes an orphan nuclear receptor that periodically represses Bmal1 transcription (18), and retinoic acid receptor-related orphan receptor-α, which competes with Rev-Erb and activates Bmal1 transcription (27). In addition, phosphorylation of clock proteins by various kinases, regulation of nuclear import/export, and protein ubiquitination also play important roles in fine-tuning the oscillations (11). Output from the core oscillator is mediated by CLOCK/BMAL1-regulated transcription of clock-controlled genes containing E-boxes in their enhancer sequences. The molecular mechanism generating circadian rhythmicity is shared by the SCN and peripheral oscillators, although some tissue-specific differences also exist (12, 15, 20).

We recently reported a gradual development of the molecular core clock mechanism within the rat SCN (8, 9, 29). Morphologically, the rat SCN develops between the 14th day of embryonic development (E14) through E17. Synaptogenesis proceeds predominantly postnatally and accelerates around postnatal day 4 (P4); the adult level of synaptic density is achieved around P10. Intrinsic rhythmicity in the SCN can already be detected prenatally (21, 22, 28). Also, the oscillations in clock genes expression might be present prenatally (13, 14), although they gradually develop mostly postnatally (9, 29). Little is known, however, about development of clock gene expression in peripheral tissues. The aim of the present study was to partly fill this gap in our knowledge by describing daily profiles of clock gene expression in the rat liver during...
development. The liver was chosen because it is currently the most studied model of peripheral clock. Development of rhythms in clock gene expression was studied by embryonic day 20 and postnatal days 2, 10, 20, and 30. These days represent important stages in rat ontogenesis: from a prenatal stage at E20 and a newborn completely dependent on maternal care at P2, through a stage when light starts to entrain the SCN clock at P10 and the time of weaning at P20, until P30 when pups gradually become completely independent of maternal care. Profiles of Per1, Per2, Rev-erbα, Bmal1, Cry1, and Clock mRNAs were determined as these genes are expressed with high-amplitude oscillations in adult liver.

MATERIALS AND METHODS

Animals and tissue sampling. Male and female Wistar rats (BioTest, Konarowice, Czech Republic) were housed at a temperature of 23 ± 2°C with free access to food and water. They were maintained under a 12:12-h light-dark cycle (LD 12:12) for at least 2 mo. Illumination was provided by overhead 40 W fluorescent tubes (50–200 lux at the level of cages). Day 0 of gestation, when the rats were found to be sperm-positive, was designated the embryonic day 0 (E0); day of delivery was designated the postnatal day 0 (P0). Pregnant rats at gestation day E20 and newborn pups with their mothers at P2, P10, P20, and P30, as well as adult male rats were released into darkness at the time of the usual light onset (circadian time 0 (CT0)) and three fetuses, pups, and adult animals, respectively, per time point were sampled in 4-h intervals throughout the circadian cycle (CT4–24). Roughly 4-mm thick pieces of liver tissue were dissected, quickly placed into RNAlater stabilization reagent (Qiagen, Valencia, CA), and subsequently stored at −20°C until RNA isolation and subsequent real-time RT-PCR. All experiments were conducted under license A5228-07 with the U. S. National Institutes of Health and in accordance with the Animal Protection Law of the Czech Republic (license 10201491A100).

RNA isolation and RT-PCR. Total RNA was isolated from 20–50 mg of homogenized liver tissue by means of Trizol-based reagent (RNA-Blue; Top-Bio, Czech Republic) according to the manufacturer’s instructions. RNA concentrations were determined by spectrophotometry at 260 nm. RNA quality was assessed by electrophoresis on 1.5% agarose gel. Moreover, the integrity of total RNA was tested by Agilent 2100 Bioanalyzer. Total RNA (1 μg) was reverse transcribed to cDNA using the ImProm-II RT kit (Promega, Madison, WI) with random hexamer primers according to the manufacturer’s instructions. The cDNA reaction (20 μl) was diluted 1:2 in DEPC-treated water to eliminate inhibition of Taq polymerase. Diluted cDNA (2 μl) was amplified in a 20 μl PCR reaction containing commercial SYBR Green and Hot Start Taq polymerase mix (QuantiTect SYBR Green PCR kit, Qiagen) with random hexamer primers according to the manufacturer’s instructions. The cDNA reaction (20 μl) was diluted 1:2 in DEPC-treated water to eliminate inhibition of Taq polymerase. Diluted cDNA (2 μl) was amplified in a 20 μl PCR reaction containing commercial SYBR Green and Hot Start Taq polymerase mix (QuantiTect SYBR Green PCR kit, Qiagen) with random hexamer primers according to the manufacturer’s instructions. The cDNA reaction (20 μl) was diluted 1:2 in DEPC-treated water to eliminate inhibition of Taq polymerase. Diluted cDNA (2 μl) was amplified in a 20 μl PCR reaction containing commercial SYBR Green and Hot Start Taq polymerase mix (QuantiTect SYBR Green PCR kit, Qiagen) with random hexamer primers according to the manufacturer’s instructions. The cDNA reaction (20 μl) was diluted 1:2 in DEPC-treated water to eliminate inhibition of Taq polymerase.

Statistical analysis. Daily profiles of clock genes are expressed as means of three animals ± SE per time point. Data were analyzed by one-way ANOVA for time differences and subsequently by Student-Newman-Keuls multiple range test, with P < 0.05 required for significance.

RESULTS

Fig. 1 shows daily profiles of Per1, Per2, Rev-Erbα, Cry1, Bmal1, and Clock expression in the liver of 20-day-old fetuses and 2-, 10-, 20-, and 30-day-old and adult rats.

At E20, the one-way ANOVA revealed a significant effect of time on expression of Rev-Erbα (P < 0.01) and Cry1 (P < 0.05), but not on expression of Per1, Per2, Bmal1, and Clock. Rev-Erbα expression at CT16 was significantly higher than that at CT04, 08, 20, and 24 (P < 0.01) but not that at CT12. Thus, the levels in Rev-Erbα mRNA started to rise between CT08 and 16 and to decline between CT16 and 20. Cry1 expression was elevated at CT24 when compared with CT04–16 (P < 0.05). Hence, the levels in Cry1 mRNA increased between CT16 and 24 and declined between CT24 and 04. The amplitude of the oscillations was, however, lower than that during later developmental stages (see data for P30 and adults).

At P2, the one-way ANOVA revealed a significant effect of time on expression of Rev-Erbα (P < 0.001) and Bmal1 (P < 0.01), but not on expression of Per1, Per2, Cry1, and Clock. Rev-Erbα mRNA levels at CT20 and 24 were significantly higher than those at CT04–16 (P < 0.001). Moreover, the levels at CT16 and 04 were higher than that at CT08 (P < 0.05). Rev-Erbα mRNA rose slowly between CT08 and 16 and faster between CT16 and declined between CT04 and 08. For Bmal1 expression, the level at CT16 was significantly higher than that at CT04, 08, 24 (P < 0.01), and 20 (P < 0.05) but not that at CT12. At CT16, the level was significantly elevated above that at CT04 (P < 0.05). Bmal1 mRNA apparently started to rise between CT20 and 12 and to decline between CT16 and 20. The amplitude of the rhythm was low

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compared with those at later developmental stages (at P20, P30, and in adults).

At P10, the one-way ANOVA revealed a significant effect of time only on expression of Per1 and Rev-Erbα (P < 0.05) but not on the expression of Per2, Cry1, Bmal1, and Clock. Per1 expression at CT8 was significantly higher than that at CT12–24 (P < 0.05). Per1 mRNA levels increased between CT24 and 08 and declined between CT08 and 12. Expression of Rev-Erbα at CT4 was significantly elevated above that at all other time points (P < 0.05). Rev-Erbα mRNA rose significantly between CT24 and 04 and declined between CT04 and 08.

At P20, the one-way ANOVA revealed a significant effect of time on expression of Per1, Rev-Erbα, Bmal1 (P < 0.01), and Per2 (P < 0.05) but not yet for Cry1 and Clock expression. Per1 mRNA levels peaked at CT16; at that time it was significantly higher than at any other CT (P < 0.01). However, the level at CT12 was already higher than that at CT04 (P < 0.05). Per1 mRNA thus rose slowly between CT04 and 12 and faster between CT12 and 16, and declined between CT16 and 20. Per2 expression at CT12 and 20 was significantly higher than that at CT4 (P < 0.05); values at CT12–24 did not differ significantly. Per2 mRNA levels increased between CT4 and 12 and declined between CT20 and 04. Rev-Erbα mRNA at CT12 was higher than that at CT04, 20, and 24 (P < 0.01) and CT08 and 16 (P < 0.05). Moreover, levels at CT08 and 16 were higher than those at CT04 and 20 (P < 0.05). Rev-Erbα mRNA thus started to rise between CT04 and 08, reached its maximum at CT12, and then started to decline between CT12 and 16 to reach the minimal level only at CT20. Bmal1 mRNA at CT24 was higher than at all other CTs (P < 0.01). It rose between CT20 and 24 and declined between CT24 and 04. At that developmental stage, the rhythm in Bmal1 expression was already roughly in opposite phase to those in Per1 and Rev-Erbα expression but not yet to the rhythm in Per2 expression.

At P30, the one-way ANOVA revealed a highly significant effect of time on expression of all studied clock genes (P < 0.01). Per1 expression at CT12 was significantly higher than that at CT04, 08, and 24 (P < 0.01) but not than that at CT16 and 20. At CT20, the expression was still higher than at CT24 (P < 0.05). Per1 mRNA thus rose between CT08 and 12 and declined significantly between CT20 and 24. Per2 expression at CT20 was significantly higher than at CT04–12 and at CT16, the expression was higher than at CT04 and 08 (P < 0.01). Per2 mRNA thus started to rise between CT08 and 16 and to decline between CT20 and 04. Rev-Erbα expression at CT08 and 12 was significantly higher than that at all other CTs (P < 0.01). Cry1 expression at CT20 and 24 was significantly higher than at CT04–12 and at CT16, the expression was higher than at CT04 and 08 (P < 0.01). Cry1 mRNA thus started to rise between CT08 and 16 and to decline between CT20 and 04. Rev-Erbα mRNA increased between CT04 and 08 and declined between CT12 and 16. Cry1 expression at CT20 and 24 was significantly higher than expression at all other CTs (P < 0.001), and at CT16 it was higher than at CT08 and 12 (P < 0.01 and P < 0.05, respectively). Cry1 mRNA levels thus started to rise slowly between CT08 and 16, reached maximal level at CT20, started to decline between CT20 and 24 and reached minimal level at CT08. Expression of Bmal1 at CT20 and 24 was significantly higher than at CT08–16 (P < 0.001) and at CT04 (P < 0.05). Also, at CT04 the expression was higher than at CT08–16 (P < 0.001). Bmal1 mRNA thus rose between CT16 and 20 and declined after CT24 to reach
minimum level at CT08. Clock expression at CT20 was significantly higher than that at CT08–16 (P < 0.05). Clock mRNA thus increased between CT16 and 20 and declined between CT20 and 08. Both Clock and Bmal1 rhythms were roughly in opposite phase to those in Per1 and Rev-Erbβ but not to the rhythms in Per2 and Cry1 expression.

In adult rats, the one-way ANOVA revealed a highly significant effect of time on expression of all the studied genes (P < 0.001). Expression of all genes exhibited circadian rhythms, in agreement with previously reported data (3, 7, 31, 34). The peak in Per1 expression at CT12 was significantly higher than values at other CTs (P < 0.01). Per1 mRNA thus rose between CT08 and 12 and declined between CT12 and 16. Per2 expression at CT12–16 was significantly higher than that at other CTs (P < 0.01). Per2 mRNA increased between CT08 and 12 and declined between CT16 and 20. Rev-Erbβ expression at CT8–12 was significantly higher than at other CTs (P < 0.01). Rev-Erbβ mRNA thus rose between CT04 and 08 and declined between CT12 and 16. Cry1 expression at CT16–24 was higher than at other CTs (P < 0.05); it started to rise between CT16 and 12 and declined between CT24 and 04. Bmal1 expression at CT4 and again at CT24 was significantly higher than that at CT8–16 (P < 0.01); at CT20 the level was also higher than that at CT08–16 (P < 0.05). Thus, Bmal1 mRNA started to rise between CT16 and 20, reached the maximal level at CT24, and declined between CT04 and 08. Clock expression at CT24 was significantly higher than at other CTs (P < 0.05); in addition, it was higher at CT4 than at CT12 and 16 (P < 0.05). Clock mRNA thus rose between CT20 and 24 and started to decline between CT24 and 04, reaching the minimal level at CT12. Both Clock and Bmal1 rhythms were roughly in opposite phase to those in Per1, Per2, and Rev-Erbβ but not to the rhythm in Cry1 expression.

DISCUSSION

The data demonstrate that the postnatal development of the molecular clockwork in the rat liver proceeds gradually. While the expression of Rev-Erbβ exhibited a significant rhythm throughout all the studied developmental stages (i.e., from E20 till adulthood), high-amplitude oscillations in expression of all other clock genes were detectable only at P10 and later.

At E20, only expression of Rev-Erbβ exhibited a circadian rhythm with high amplitude. Expression of Cry1 varied also significantly across time, but the amplitude was lower compared with that of adult rats. Moreover, the variation ceased to be significant at later developmental stages until P30. At P2, again only Rev-Erbβ exhibited a significant high-amplitude rhythm in its expression. Variation in Bmal1 expression, although significant, exhibited only a low amplitude and the rhythm was not detectable at P10. At P10, expression of Per1 and Rev-Erbβ, but not of other clock genes, exhibited a significant circadian rhythm. At P20, Per1, Per2, Rev-Erbβ, and Bmal1, but not yet Cry1 and Clock, exhibited a clear rhythm. As late as at P30, expression of all the studied clock genes became rhythmic. It seems that the entire molecular clockwork in the rat liver was not yet fully developed at the age of 20 days and attained a mature state only around P30.

Recently, a gradual postnatal development of the molecular oscillations within the rat SCN was reported (9, 29). Similarly as in the liver, the rat SCN clock also does not exhibit high-amplitude oscillations in Per1, Per2, Cry1, Bmal1, and Clock mRNA during embryonic stages. However, in contrast to the liver, at P2 it already operates with rhythmically expressed clock genes, although amplitudes of the rhythms at that developmental stage are lower than in adult animals and increase progressively with age. Unfortunately, data on development of rhythm in Rev-Erbβ expression in the rat SCN are not available. In another peripheral oscillator located in the rat heart, the circadian expression of clock genes Per1 and Bmal1 starts between P2 and P5, while expression of Per2 does not exhibit any rhythmicity until P14 (26). Apparently, rhythms in clock gene expression develop earlier in the central SCN clock than in the peripheral oscillator, be it in the liver or the heart. Tissue specific differences in programming of the development of molecular oscillations are also suggested. Such differences might help to elucidate specific roles of individual components of the molecular core clockwork in the SCN as well as in peripheral organs during development.

While in the SCN the phase of the newly appearing rhythms in clock gene expression is roughly in phase with that of adult rats since the beginning (29), this apparently does not hold true for the liver. The phase changed between all the developmental intervals studied, i.e., between the intervals E20–P2, P2–P10, P10–P20, and P20–P30. Rev-Erbβ expression peaked in the subjective night at E20, at the onset of the subjective day at P2, in the first half of the day at P10, at the end of the day at P20 and in the second half of the day at P30. Per1 peaked in the second half of the subjective day at P10, during the night at P20 and at the end of the subjective day at P30. It seems that during the development of the rhythms in expression of at least these two clock genes in the rat liver, the phase first delayed, and then between P20 and P30 slightly advanced. At P30, rhythms of all the studied clock genes were roughly in phase with those in adult rats. Apparently, the liver rhythms at the beginning are entrained prevalently by cues independent of the SCN. Food intake represents a potent synchronizing cue for liver oscillations in adult animals. When restricted to an unusual daytime, food intake may rapidly synchronize liver clock gene expression independent of the SCN (4, 6, 30). Restricted feeding and wheel running can even induce circadian rhythms in the liver of ultradian common voles that display no circadian clock gene expression in the liver during ad libitum feeding (32). After delivery, the rat mother nurses her newborns periodically, predominantly in her rest time, i.e., during the day, and leaves them so that she may feed herself during the night. The feeding regimen of the pups is thus mostly in opposite phase to those of adults. The partly restricted feeding of the suckling pups to the daytime might directly entrain the liver clock. Since the second week of life, the rat pups open their eyes and start to change their feeding habits, i.e., to consume solid food (in addition to milk) during the night hours. After the end of weaning around P28, the SCN controls rhythmic ad libitum feeding that mostly takes place during the nighttime. Changes in the feeding regimen during development may also be reflected in the daily rhythm of liver glycogen concentration, which peaks in newborn pups in the middle of the dark phase, 8 h later than in adult rats (33). Apparently, changing the feeding regimen during postnatal development might affect the phase of more liver rhythms, and in the first place the phase of rhythms in clock gene expression. Similarly, the phase of rhythms in clock gene expression in the rat heart changes...
between P14 and P20 and further between P20 and P30 when mature rhythms are established (26). However, if the feeding regimen were the sole entraining agent of the developing liver clock, it would be difficult to explain why the rhythm in Rev-Erbα expression at E20 is in opposite phase to that in the adult liver.

Absence of the rhythms in clock gene expression in the liver during early developmental stages might reflect lack of synchronization between otherwise functional individual oscillators in the liver cells. Actually, mouse embryonic fibroblasts that harbor peripheral circadian clock, can be synchronized in vitro (17). However, the high-amplitude rhythm in Rev-Erbα expression at E20 is not in agreement with such a possibility. Rather, the Rev-Erbα mRNA profile reflects a rhythmic expression of a well-synchronized population of liver cells at E20. Even at P2, Rev-Erbα is the only clock gene expressed in a rhythmic way. This finding raises a question and that is what cue may drive the rhythm in Rev-Erbα if not the positive and negative feedback elements of the clockwork? Apart from an E-box, promoter of Rev-Erbα contains other response elements that might be responsible for switching on/off the gene transcription (Rev-DR2/RORE, DBPE/D-box) (1, 19, 34). It is also 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 the rhythms in Rev-Erbα and other clock genes is maintained during all the developmental stages studied in this work.

In conclusion, our data demonstrate that in the rat liver, only expression of Rev-Erbα exhibits a significant circadian rhythm at E20 but not the expression of other clock genes studied. Rhythmic expression of clock genes develops gradually and the entire molecular clockwork in the liver is not yet fully developed at P20. The adult-like state of clock gene expression is attained as late as at P30. The phase of the rhythmic clock gene expression changes during postnatal development, and this is most likely due to changes in the feeding regimen.

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