Development of the circadian clockwork in the kidney

Krisztina Mészáros[1,2], Linda Pruess[1], Attila J. Szabó[2], Matthias Gondan[3], Eberhard Ritz[4] and Franz Schaefer[1]

[1]Division of Pediatric Nephrology, Center for Pediatrics and Adolescent Medicine, University of Heidelberg, Heidelberg, Germany; [2]Nephrological Research Laboratory of the Hungarian Academy of Science and the First Department of Pediatrics, Semmelweis University, Semmelweis, Hungary; [3]Department of Psychology, University of Copenhagen, Copenhagen, Denmark and [4]Department of Internal Medicine, University of Heidelberg, Heidelberg, Germany

The circadian molecular clock is an internal time–keeping system composed of centrally synchronized tissue–level pacemakers. Here, we explored the ontogeny of the clock machinery in the developing kidney. Pregnant rats were housed at 12–12 h light–dark cycles. Offsprings were killed at 4-h intervals on embryonic day 20 and at postnatal weeks 1, 4, and 12. Canonical clock gene (Clock, Bmal1, Rev-erbα, Cry1, Cry2, Per1, Per2) and kidney-specific clock-controlled gene (αENaC, SGK1, NHE3, AVPR2) expression was profiled by RT-PCR. To investigate the role of nutritional cues, the feeding pattern was modified postpartum. Clock, Rev-erbα, Per2, αENaC, SGK1, NHE3, and AVPR2 showed circadian expression at the end of intrauterine development. By 1 week, all genes oscillated with a distinct acrophase shift toward the time of peak feeding activity. Daily 4-hour withdrawal of mothers induced a 12-hour phase shift of Clock and Bmal1 expression, while disrupting oscillations of the other genes. After weaning, oscillation phases shifted back toward the adult pattern, which was fully expressed at 12 weeks. Thus, functional circadian molecular clockwork evolves in the late fetal and early postnatal kidney. During the nursing period, oscillations are entrained by nutritional cues. The coupling of the circadian expression of tubular regulators of fluid and electrolyte excretion to the feeding-entrained clockwork may be important to maintain homeostasis during this critical period.

Kidney International advance online publication, 4 June 2014; doi:10.1038/ki.2014.199

KEYWORDS: circadian rhythm; clock genes; development; food; kidney; rat

Numerous functions of the kidney follow a circadian rhythm driven by an internal time–keeping system.[1,2] Several external cues, including the light–dark cycle and nutrient intake, operate as Zeitgebers capable of synchronizing the endogenous time with the environment. However, even in the absence of Zeitgebers, most processes exhibit diurnal rhythmicity on the basis of the molecular machinery of the clockwork.[3–5] In mammals this system is comprised of central and peripheral components using a set of molecular constituents, i.e., Clock, Bmal1, Cry1/2, Per1/2, and Rev-erbα.[6,7] These clockwork elements form interlocking transcription–translation feedback loops generating circadian oscillations of messenger RNA and proteins.[8–10] In addition, posttranslational modification of clock proteins has an important role in the generation of the circadian rhythm mainly via regulating nuclear import/export as well as protein degradation.[11,12]

Approximately 10% of all genes are known to display circadian rhythms, which are considered to be outputs of the molecular clockwork.[13,14] In the kidney, numerous genes critical for renal function have been identified to be clock controlled. For instance, NHE3, the Na^+/H^+ exchanger in the proximal tubule regulating pH, Na^+, and water balance, has a fundamental role in basic homeostasis. Rhythmic expression of the *NHE3* gene is directly regulated by the Clock:Bmal1 heterodimer and blunted in Cry double-mutant mice.[15,16] Similarly, the expression of arginin-vasopressin receptor 2 (AVPR2), a key regulator of water resorption in the distal nephron and collecting duct, is controlled by clock gene products.[17] In addition to direct control of renal genes by the intrinsic renal molecular clockwork, the circadian regulation of kidney functions is effected by circadian endocrine input. Several tubular channel proteins are regulated by steroid hormones, which themselves are subject to circadian rhythmicity.[18,19] Recently, the clock proteins Cry1, Cry2, and Clock have been demonstrated to have an impact on aldosterone synthesis, and Per1 to regulate the aldosterone-controlled renal epithelial sodium channel (αENaC).[20–23] Furthermore, SGK1 (serum and glucocorticoid-inducible kinase 1), a pro-survival kinase induced by glucocorticoids and other endocrine stimuli, has a significant role in the

Correspondence: *Krisztina Mészáros, Division of Pediatric Nephrology, Center for Pediatrics and Adolescent Medicine, University of Heidelberg, Im Neuenheimer Feld 430, 69120 Heidelberg, Germany. E-mail: Krisztina.Meszaros@med.uni-heidelberg.de*

Received 5 February 2014; revised 3 April 2014; accepted 10 April 2014

regulation of sodium transport (e.g., αENaC, NHE3).[24–26] These mechanisms contribute to long-term blood pressure control.[27,28]

The perinatal development of rhythms underlying the expression of clock genes has been analyzed for the central circadian clock, the suprachiasmatic nucleus (SCN).[29,30] In addition, prenatal and early postnatal circadian gene expression patterns have been studied in the liver and heart.[31,32] These circadian oscillations develop gradually during the late embryonic and early postnatal stages. With respect to the kidney, however, such information is not yet available. This prompted us to explore the ontogeny of circadian gene expression in the kidney. Here, we describe the development of the rhythmic expression of clock (Clock, Bmal1, Rev-erbα, Cry1, Cry2, Per1, Per2) and clock-controlled genes (*αENaC*, *SGK1*, *NHE3*, *AVPR2*) in the rat kidney, and the role of external Zeitgebers in their regulation.

RESULTS

Time definition

Zeitgeber time (ZT) is a standardized way to express time on the basis of the period of normal light–dark cycles (12–12 h light–dark) in animal laboratories. Light onset defines Zeitgeber time ZT0, thus ZT0-12 represents the light and ZT12-24 the dark period. We chose 0600 hours clock time as ZT0.

Circadian gene variation at birth

At embryonic day 20 (E20), the clock genes *Clock* ($P = 0.008$), *Per2* ($P = 0.014$), and *Rev-erbα* ($P = 0.014$) as well as the clock-controlled genes *αENaC* ($P < 0.001$), *SGK1* ($P = 0.017$), *NHE3* ($P < 0.001$), and *AVPR2* ($P = 0.003$) showed significant circadian oscillation of expression (Figures 1a and 2a; Supplementary Figure S1a online and Supplementary Table S2 online). The peak of the oscillations was observed during the dark period (ZT15-19). The amplitude of the rhythms was smaller at E20 (mean $2A = 53 \pm 22\%$) than later in life (Figure 3).The expression of Bmal1, Cry1, Cry2, and Per1 showed no significant variation with time.

Postnatal changes in circadian gene variation

At 1-week postpartum, significant circadian oscillations were observed for all genes (*Clock* ($P = 0.009$), *Bmal1* ($P = 0.021$), *Rev-erbα* ($P = 0.006$), *Per1* ($P = 0.038$), *Per2* ($P = 0.012$), *Cry1* ($P = 0.008$), *Cry2* ($P = 0.013$), *αENaC* ($P < 0.001$), *SGK1* ($P = 0.001$), *NHE3* ($P < 0.001$), see Figures 1b and 2b; Supplementary Figure S1b online; Supplementary Table S2 online). The amplitudes of rhythmic expression more than doubled from birth (mean $2A = 117 \pm 25\%$), and a phase shift from nighttime to early daytime was observed for all genes except Rev-erbα, which peaked at nighttime (Figure 3).

At 4 weeks, the circadian component of Clock, Cry2, and AVPR2 was lost. The rhythm of *Bmal1* ($P = 0.012$), *Rev-erbα* ($P = 0.014$), *Per1* ($P = 0.039$), *Per2* ($P = 0.044$), *Cry1* ($P < 0.001$), *αENaC* ($P = 0.010$), *SGK1* ($P < 0.001$), and *NHE3* ($P = 0.01$) showed significant variation with time (Figures 1c and 2c; Supplementary Figure S1c online; Supplementary Table S2 online). The oscillation phase shifted back from daytime to nighttime (ZT14-19) for Per1, Per2, Cry1, αENaC, and SGK1, whereas the phase of Rev-erbα was inverted from nighttime to daytime (ZT7). Bmal1 peaked at the beginning of the light phase (ZT0, Figure 3a). The amplitudes of the circadian expression of Rev-erbα, Bmal1, and Cry1 increased further (mean $2A = 167 \pm 71\%$), whereas the amplitudes of the rhythmic oscillations of Per1, Per2, αENaC, SGK1, and NHE3 were reduced (mean $2A = 76 \pm 18\%$, Figure 3b).

In adult rats 12 weeks after birth, profound rhythmic expression was observed for Bmal1 ($P < 0.001$), Rev-erbα ($P < 0.001$), Cry1 ($P < 0.001$), Cry2 ($P < 0.001$), Per1 ($P < 0.001$), Per2 ($P < 0.001$), and SGK1 ($P = 0.004$) (Figures 1d and 2d; Supplementary Figure S1d online; Supplementary Table S2 online). Compared with those at age 4 weeks, the amplitudes of most investigated genes increased (Figure 3b). The phase was unchanged for Rev-erbα and Cry1 (acrophase differences < 30 min), but delayed by 1–2 h for Cry2, Per2, and SGK1, and advanced by 1–3 h for Bmal1 and Per1 (Figure 3a).

Postnatal behavioral changes

The relationship of feeding time to activity level and oscillatory gene expression is depicted in Figure 4.

The 1-week-old offsprings were fed mostly during daytime by breastfeeding during the resting period of the mother animals. The oscillation peaks of all studied genes (except *Rev-erbα*) were observed during this time (ZT2-6). The mothers showed nocturnal activity and consumed food (peak time: ZT18) and water (peak time: ZT16) mostly during the dark period. Maternal activity peaked twice, at ZT14 and ZT18. The acrophase of Rev-erbα expression in the pup kidneys coincided with the maximum of maternal food consumption (ZT18).

At completion of weaning (age 4 weeks), the pups were active and consumed solid food mostly at night. The activity was simultaneous to feeding time and water consumption (with two peaks at ZT14 and ZT22). Most genes, except *Rev-erbα* and *NHE3*, peaked during the dark period (ZT12-24).

Feeding behavior remained constant from 4 to 12 weeks of age, whereas the maximum activity advanced from the second half to the beginning of the dark period. The expression of Rev-erbα showed stable oscillation (peaking at ZT7), whereas the other genes closely followed the activity pattern. The expression of Per1, Per2, and Cry2 peaked at the beginning (ZT12-15), whereas that of Bmal1 and AVPR2 at the end (ZT23-24) of the dark period. The acrophase of Cry1 and SGK1 oscillation was observed between these two peaks (ZT19).

Periodic maternal withdrawal

The daily separation of the pups from their mothers during the feeding period (ZT3-7) for 7-day-long postpartum disturbed the rhythmicity of the daily feeding. The mothers fed and nursed their pups predominantly during the dark period (Supplementary Figure S2 online). The intervention