Circadian rhythm and fetal programming

Ph.D. Thesis

Dr. Krisztina Mészáros

Doctoral School of Clinical Medicine, Semmelweis University

Program: Prevention of Chronic Diseases in Childhood

Tutor / Supervisor: Prof. Attila J. Szabó, M.D., Ph.D., D.Sc.

1st Department of Pediatrics, Semmelweis University, Budapest, Hungary

Consultant: Prof. Franz Schaefer, M.D.

Center for Pediatrics and Adolescent Medicine, University of Heidelberg, Germany

Budapest, 2017

1. INTRODUCTION

Evidence exists that many physiological processes, e.g. sleep-wake cycle, timing of hormones release as well as several aspects of cardiovascular and renal functions are governed by the circadian clock. The fundamental circadian clockwork generating and sustaining circadian oscillation is based on series of transcriptional-translational feedback loops involving the core clock genes (i.e. *Clock*, *Bmal1*, *Per1/2*, *Cry1/2* and *Rev-erbα*) and tissue specific clock-controlled genes (*CCGs*) at the cellular level. The synchronization between the "single cell clocks" is tightly regulated at both central and peripheral level. The central circadian pacemaker residing in the suprachiasmatic nucleus (SCN) of the hypothalamus requires exogenous input, e.g. rhythmic photic stimuli (entrainment) to keep harmony with the environmental time. Then, through its complex output pathways due to neuronal-, endocrine signals (e.g. timed glucocorticoid and melatonin secretion) as well as the regulation of the behavior driven by the rest-activity cycle synchronizes the peripheral clocks with the day-night rhythm. Additionally, evidence has been accumulated that the long-term misalignment between the daily behavioral pattern (e.g. sleep-wake, or fasting-feeding

cycles) and the internal circadian rhythms influences essential physiological processes that are relevant to human diseases.

In the recent years, it has been recognized that before the light entrainment is established, the fetus is exposed to the maternal rhythms related to e.g. the sleep-wake status, thus melatonin signaling and feeding behavior via hormonal and metabolic cues. Furthermore, adverse effects of the circadian disruption have been linked to e.g. compromised pregnancy. At the same time, there is a growing evidence that suboptimal intrauterine environment leads to “fetal programming” of late-onset diseases. In this content, it is tempting to consider that the disturbance of the circadian rhythm during the prenatal period by altered feeding and sleeping pattern of the mother might influence the circadian system of offspring and have potentially long-lasting effects later in life.

2. PLAN OF THE STUDY

In the present work we investigated whether the maternal circadian disruption during the intrauterine period has direct and/or long-lasting effects on the peripheral circadian organization in the kidney. We hypothesized that the maternal circadian alteration modifying fetal programming has an impact on the intrauterine growth or the kidney development and thus on renal function including blood pressure regulation later in life. We tested our hypothesis using a rat model intrauterine exposed to modified light-dark cycles ("**LL**"- constant light exposure, "**DD**"- constant darkness, "**6:6-LD**"- shortened, ultradian photoperiod and "**3:21-LD**"- prolonged dark phase condition) relative to normal laboratory condition ("**LD**"-12 h:12 h light-dark cycle). Additionally, we investigated the long-term effects of prenatal time-restricted feeding ("**FR-LD**", food was available only in the inactive period) regime altering the maternal circadian rhythms on the offspring.

We divided our study in 3 different experiments: in **experiment-1** we studied the early effects of prenatal maternal circadian disruption on the dam and on the offspring till the birth-time (at

embryonic day 20 = **E20**). While in **experiment-2** we observed the postnatal changes of the renal clockwork (at 1, 4 and 12 weeks of age = **1W**, **4W**, **12W**), and investigated the potential Zeitgebers for the kidney in this particular period. Last, but not least, in **experiment-3** we examined the long-lasting effects of the disturbed prenatal circadian condition on the offspring (at 34-week of age = **34W**) focused on the renal, cardiovascular and metabolic function.

Because the sex differences have not been studied at the level of the molecular clock in the kidney previously, thus we aimed to explore the **gender related differences** in the circadian oscillation of the clock genes at different age (at **1W** and **12W**).

All animals were handled according to written approval from the local authority for animal experiments (Regierungspräsidium Karlsruhe, 35-9185.81/G-29/11).

3. LABORATORY METHODS

Tissue sampling

The kidney tissue of offspring (n = 84/ group, 42 male) at embryonic day 20 (**E20**), as well as at **1W**, **4W** and **12W** from each perinatal photoperiod conditions (**LD, LL, DD, 6-6 LD and 3-21 LD**) and from **FR-LD (**only at E20**)** were sampled every 4 hours to cover one entire circadian cycle (ZT 2-24 h, ZT0 = light on at 06:00 h). Dams' body and placentas' weight and weight of the offspring at all in investigated timepoint were measured.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from homogenized kidney tissue (10–50 mg) of offspring (n = 7/group) using the RNeasy Mini Isolation Kit (Cat.No.74106, Qiagen, Hilden,Germany) according to the manufacturer's protocol. The reverse transcription reaction was performed using 1 µg of total RNA and GeneAmp RNA-PCR Kit. The quantitative PCR reactions, followed by melt curve analysis were run on a StepOnePlus Real-Time PCR System. The number of the specific transcripts in each

sample was calculated using relative standard curves normalized to the ribosomal protein 18S housekeeping gene. Primer sets for the clock genes *Clock*, *Bmal1*, *Rev-erbα, Per1*, *Per2*, *Cry1*, *Cry2* and clock controlled genes *αENaC, SGK1, ENH3, AVPR2* were designed with Primer Express Software v2.0. The specificity of the products was checked in the BLAST database (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi) against reference sequences of mRNA in the Rattus norvegicus genome.

Urinalysis

Urine samples were collected from dams at 4-hour intervals over a 24 h period in metabolic cages at 1., 2. and 3. gestational week (**1.GW**, **2.GW** and **3.GW**) in **LD**, **LL** and **DD** groups (n = 8-12/ 4h over 24 h period in each group) and from offspring at 34. week of age (**34W**) at 4- and 24-hour intervals in all investigated groups (n = 8-12 / 4 h and n = 8-12 / 24 h). Urinary parameters, i.e. sodium, potassium, phosphate, calcium, glucose and albumin excretion were analyzed using standard laboratory methods. Additionally, daily excretion of urinary melatonin (at **2.GW** in **LD** and **DD**) and aldosterone (**1.GW** and **3.GW** in **LD** and **LL**) was determined during the gestation period.

Behavioral/locomotor activity measurements

Mothers with their pups in **LD** were maintained in cages equipped with infrared video camera. Feeding times of the mothers, as well as the locomotor activity, the frequency of food and water intake of the pups at age 4 and 12 weeks and the mothers were evaluated by video analysis.

Telemetric measurements of blood pressure

Dams at embryonic day 2 in group **LD** (n = 4), **LL** (n = 4), **DD** (n = 6) and **3:21-LD** (n = 7) as well as female offspring at **34 weeks** of age (± 2 weeks) in group **LD** (n = 9), **LL** (n = 11), **DD** (n = 8), **6:6-LD** (n = 5) and **3:21-LD** (n = 7) and **FR-LD** (n = 6) underwent an implantation of telemetrically device (model PA-C40; Data Science International) to monitor blood pressure and motor activity. After a 7-14 days recovery, measurements were taken under normal feeding regime (except FR-LD) under ambient temperature. Signals were transmitted (ca. 10.000 readings/rat) in a 10- minute average from 3 consecutive measurements with use of Dataquest system for further analysis.

Echocardiography

At age 34 week in **LD, LL, 6:6-LD, 3:21-LD** and **FR-LD** group the left ventricular function was measured under isoflurane anesthesia. End-diastolic left ventricular internal diameter (EED) and end-systolic left ventricular internal diameter (ESD) were measured. The percentage of fractional shortening (FS%) was calculated ([(EDD-ESD)/EDD) x100], n = 6-10 / group).

Statistical analysis

Data are presented as mean ± SD or SEM. The statistical analysis was performed using SigmaPlot (version 11.0) program. Results were considered significant when $p < 0,05$.

Circadian rhythms were analyzed by the single cosinor procedure including the fit of a cosinus wave to the data by least-squares linear regression. The characteristics of the rhythms were described by the peak of the oscillation wave, i.e. the acrophase φ (hh:mm) and the double amplitude 2A (%). Statistical analyses were performed with R (version 3.0.3) and cosinor functions by Charles W. Berry program.

4. MAIN RESULTS

Experiment-1

The main finding of this study is the evidence for the presence of a functioning molecular clockwork (circadian oscillation in the daily mRNA expression of ***Clock*** [2A = 22 (%), ϕ = 16:32 (ZT), p = 0,008], ***Per2*** [2A = 36 (%), ϕ = 15:05 (ZT), p = 0,014] and ***Rev-erbα*** [2A = 78 (%), ϕ = 15:54 (ZT), p = 0,014]) in the late fetal kidney, earlier than in other tissues (at E20). While rhythms which are directly controlled by the master clock (SCN) do not yet function at birth in rats, we assume that the rhythms observed at E20 reflect intrinsic, autonomous oscillation of renal tissues. Furthermore, we documented a temporary alteration of the intrarenal, molecular circadian clockwork in the fetus (E20) induced by prenatal modified light exposure or restricted maternal feeding regime. However, at this point, it is difficult to demonstrate the actual physiological relevance of the observed alterations in the daily expression level, those might trigger adverse effects later in life.

In contrast to previous animal experiments in which the fetuses presented either growth restriction or elevated birth weight after

disturbance of the light-dark cycle, e.g. chronic phase shift of the mother, we did not find significant different fetal and placental weight at E20 between the different groups.

Experiment-2

This work extends the previous findings of others by providing evidence that oscillating renal clock gene and target gene expression correlates with nutritional cues. We have described that during early postnatal life, the daily pattern of some intrarenal clock gene expression undergoes a phase shift apparently driven by the timing of nutrient uptake processes (i.e. depending on the maternal breastfeeding or nursing behavior). In order to provide further interventional experiment exploring the entrainment of the intrarenal molecular clockwork by maternal feeding behavior, we removed the mothers from their pups during the 4 hours of maximal spontaneous feeding activity (ZT 3 to 7). After 7 days of cyclic absence of the mother the phase of *Clock* and *Bmal1* gene expression was inverted (acrophases: ϕ of Clock 05:12 vs. 19:07, ϕ of Bmal1 06:12 vs. 19:34) whereas circadian rhythmicity of the other investigated genes was completely lost. The relatively rapid changes of the *Clock* and *Bmal1* expression in response to the changes of the feeding regime could temporarily disturb the

rhythm of the other genes. Although it is assumed that feeding regime is a dominant Zeitgeber for peripheral clock entrainment, it should be considered that other daily rhythms e.g. the sleeping pattern or body temperature (which in adults is driven by the SCN) might also contribute in the peripheral regulations.

Experiment-3

With aging (**34W**) we have observed an increasing tendency to obesity gender specific manner. The body weight of females in all investigated group except LL (thus in **DD**, **6:6-LD**, **3:21-LD** and **FR-LD**) was significantly higher compared to the control group (**LD**). While aged male only in **6:6-LD** had higher body-weight compared to the control group.

We found that aged females gestated under ultradian light-dark condition (**6:6-LD**) or exposed to altered maternal feeding behavior during the gestational period (**FR-LD**) exhibited higher systolic daily blood pressure and decrease in the urinary sodium excretion. Along the same line, the evaluation of our long-term study revealed that aged females (but not males) with prenatal

maternal light exposure (**LL**) presented renal phosphate wasting with hypercalciuria and albuminuria.

Taken together, the above findings indicate that both the prenatal light hygiene and the altered feeding regime has a long-lasting impact on the renal function including e.g. blood pressure regulation in adult offspring.

Gender related differences

Gender related differences of the renal clock gene oscillation patterns were observed at **1W** in the control group, which led us to speculate with a gender specific response to the entraining signaling in the early postnatal period. However, we found no differences in the expression pattern of *Bmal1* and *Rev-erbα* at adult age (12W) in the kidney between females and males. Exception for the Clock, which displayed a distinct circadian oscillation in female, but not in male offspring' kidney.

5. CONCLUSIONS

This study showed for the first time that clockwork machinery in the kidney is functioning at the late gestational period and developing postnatally driven by different Zeitgebers. Thus, our findings provide evidence for the renal molecular clockwork as a fundamental physiological mechanism by which intrarenal gene activity can adapt to changing environmental conditions. Whether tubular functions are a more endpoint of circadian homeostatic regulation or whether changes in electrolyte and water homeostasis can feed back on the clockwork machinery, remains to be elucidated.

Furthermore, the results of the present experiments might implicate a profound long-lasting effect of the prenatal maternal circadian disturbance. Adverse intrauterine effects related to maternal feeding regime and circadian cycle seems to be involved in the programming of the late-onset diseases. It is still unclear, however, through which molecular mechanism these changes are programmed.

In our 7/24 society in which there is an increasing tendency of the onset of diseases associated to cardiovascular (hypertension,

myocardial ischemia, stroke and renal failure) and metabolic dysfunction (overweight and diabetes) the prevention should be more emphasized. Through the understanding of the circadian clockwork machinery and the potential effects of its dysfunction, especially in the developing fetus, we could be one step closer to the prevention and therapy of chronic diseases.

6. OWN PUBLICATIONS

Publication related to the dissertation:

Mészáros K, Pruess L, Szabó AJ, Gondan M, Ritz E, Schaefer F. Development of the circadian clockwork in the kidney. Kidney Int. 2014 Nov; 86:915-22.

Other publications:

Pasti K, Szabo AJ, Prokai A, **Meszaros K**, Peko N, Solyom R, Sallay P, Reusz G, Rusai K. Continuous glucose monitoring system (CGMS) in kidney-transplanted children. Pediatr Transplant. 2013 Aug; 17:454-60.

Rusai K, Prokai A, Juanxing C, **Meszaros K**, Szalay B, Pásti K, Müller V, Heemann U, Lutz J, Tulassay T, Szabo AJ. Dexamethasone protects from renal ischemia/reperfusion injury: a possible association with SGK-1. Acta Physiol Hung. 2013 Jun; 100:173-85.

Rusai K, Prókai A, Szebeni B, **Mészáros K**, Fekete A, Szalay B, Vannay Á, Degrell P, Müller V, Tulassay T, Szabó AJ. Gender differences in serum and glucocorticoid regulated kinase-1 (SGK-1) expression during renal ischemia/reperfusion injury. Cell Physiol Biochem. 2011; 27:727-38.