Circadian rhythm and fetal programming

Ph.D. Thesis

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Budapest
2017

"Nothing is as powerful as an idea whose time has come."

(Victor Hugo)

CONTENTS

ABBREVIATIONS

AAD	aromatic amino acid decarboxylase
AANAT	arylalkylamine N-acetyltransferase
ACTH	adrenocorticotrop hormon
AMPK	AMP (adenosine monophosphate) activated protein kinase
ARNT	Arylhydrocarbon Receptor Nuclear Translocator
AVPR2	arginine vasopressin type 2 receptor
bHLH	basic-helix-loop-helix protein structural motif
BMAL	brain and muscle Arnt-like protein, also known as ARNT-Like Protein
cAMP	cyclic adenosine monophosphate
Circadian	from Latin: *circa* = approximately, *diem* = day
CCGs	clock controlled genes
CKI	casein kinase 1
CLOCK	Circadian Locomotor Output Cycles Kaput, its paralog NPAS2
CRE	cAMP response element
CRY	Cryptochrome (Cry1/2)
DMH	dorsomedial hypothalamus
DRN	dorsal raphe nucleus
E-box	enhancer box
ELISA	enzyme-linked immunosorbent assay
ENaC	epithelial sodium channel (also known as amiloride-sensitive sodium channel)
E20	embryonic day 20
GHT	geniculohypothalamic tract
GC	Glucocorticoid
HIOMT	hydroxyindole-O-methyl transferase enzyme

HPA hypothalamic–pituitary–adrenal axis.

IGL intergeniculate leaflet

LD light-dark cycle

LT long-term

miRNA small non-coding RNA molecule (ca. 22 nucleotides)

MPO medial preoptic region/area (abbreviation also known as mPOA)

MRN median raphe nucleus

MT melatonin receptor (MT1 and MT2 subtypes)

NHE3 sodium-hydrogen antiporter 3 protein encoded by the scl9a3 genes.

NPAS2 Neuronal PAS domain containing protein 2

NPY neuropeptide Y

PAS Per/Arnt/Sim protein domain

PER period gene (per1 and its homologes per2 and per3)

PVN paraventricular nucleus of the thalamus

RHT retinohypothalamic tract

ROR/RZR retinoic acid receptor-related orphan nuclear receptor subfamily

Rev-erbα encoded on the opposite strand of the alpha-thyroid hormone receptor (*c-erbα*); member of nuclear receptor subfamily

SCN paired suprachiasmatic nuclei

Sgk1 serum- and glucocorticoid-inducible kinase

TRPH tryptophan hydroxylase

VIP vasoactive intestinal peptide

Zeitgeber from German, means “time giver”

3-ß-HSD 3β-hydroxysteroid dehydrogenase

6-SMT 6-sulfatoxymelatonin

1. INTRODUCTION

1.1. Circadian rhythm in mammals: an endogenous, self-sustained system

The rhythmical environmental cues related to e.g. the Earth rotation forced life forms to synchronize their physiology via "predictive" rather than "reactive" mechanisms.[1,2] The temporal organization of behavior into circadian cycles of rest- and activity periods is a fundamental feature of the organisms' adaptation. Most organisms including mammals operate with a self-sustaining, endogenous, time-keeping cellular machinery, called the circadian clock, that allows them to facilitate their anticipatory adaptation to the rhythmic environmental changes, e.g. to the light-dark cycles.[3-7] Because of its molecular regulation, the outputs of the circadian clock machinery, the circadian rhythms persist even in constant conditions (i.e. deprived solar or social contacts) with an endogenous (i.e. free running) period close to 24 h.[8-11] Evidence exists that many physiological processes, e.g. sleep-wake cycle, the body temperature fluctuations, timing of hormones release as well as several aspects of cardiovascular and renal functions are governed by the circadian clock.[12,13]

Although the internal circadian clock is not the most accurate system: its free running period is only approximate that of the environment (24 h).[14] However, under natural conditions the phase and the period of the circadian rhythms are being entrained (synchronized /adjusted /reset) by time cues (Zeitgebers) to maintain harmony with the external, environmental light-dark cycle.[15] It is well established that the solar light is the major Zeitgeber, but other cyclic cues e.g. rhythmic feeding regime, behavioral activity are also dominant Zeitgebers entraining the clock.[16]

So far, three essential components of the circadian timing system have been described and intensively investigated: input pathways that mediating entrainment (i.e. Zeitgebers), the circadian oscillators (i.e. cellular circadian clock machinery) and the output pathways (i.e. circadian rhythms) that express the circadian function.[4]

1.1.1. Dysfunction of the system: what happens when the clock goes wrong?

In the recent years, it has been recognized that the misalignment between the behavioral pattern driven even by social cues (social jet lag) and the endogenous rhythms causes surprisingly broad unwanted effects. Ignoring the internal circadian clock in our "24/7" society (e.g. due to rotating shift work, jet lag) leads to reduced sleep efficiency which is associated with cognitive impairment and impaired social functions caused by mood disturbance.[17] Above that, the long-term misalignment between the daily behavioral pattern (e.g. sleep-wake, or fasting-feeding cycles) and the internal circadian rhythms is known to influence essential physiological processes that are relevant to human diseases.[18-20] For example, the disruption of the temporal endocrine regulation, as results of physiologic maladaptation to sleeping and eating at abnormal circadian time chronically, has a severe impact on metabolic health.[20,21] For example, recent study from Harvard showed that a rearrangement of the day-night rhythm in healthy volunteers can cause insulin resistance.[18]

Furthermore, adverse effects of the circadian disruption have been linked to e.g. cardiovascular dysfunctions and compromised pregnancy.[22-25] An increased mortality from coronary heart disease among shift workers has been demonstrated in a historical cohort of Swedish pulp and paper industry workers.[23] A correlation between shift work and prematurity or intrauterine growth retardation has been confirmed in large cohort study from China.[26] Another study from Denmark also confirmed the adverse effect of the circadian disturbance by shift work on the fetal development and birth weight.[25]

More focused involvement of the circadian dysfunction has been observed in tumor development and progression.[27] The disturbed internal clock has been linked to increased risk of e.g. breast cancer in women who work irregular shift patterns.[28] Animal experiments have shown conclusively that the circadian disturbance accelerates diverse tumor development and play a significant role in the progression.[29,30]

1.2. Molecular network of the circadian rhythms: what is ticking?

Understanding how the circadian system is controlled, first we need to understand the molecular-genetic processes at the cellular level. While, current evidence suggests that the circadian system itself is composed of as many tiny autonomously ticking clocks as there are cells of the body.[31-34] On the molecular level, the fundamental circadian network which is essential for generating and sustaining circadian oscillation is based on series of transcriptional-translational feedback loops.[19,35-42] To our knowledge, the mammalian genome encodes 4 core circadian genes directly involved in the regulation of the core feedback loop: *Clock* (its paralog protein: *Npas2*), *Bmal1* (also known *Arntl-2*), *Period* (*Per* homologs 1, 2 and 3) and *Cryptochrome* (*Cry* homologs 1 and 2). See Figure 1.

It should be noted that this scheme is oversimplified, but to put it briefly: the transcription factors that positively regulate the feedback loop are the CLOCK and BMAL1. These proteins have basic-helix-loop-helix (bHLH) PAS domains, so they can form heterodimer and bind to E-box elements in the promoter of their target genes, thus induce the expression of other clock components including *Period 1-3* and *Cryptochrome 1-2*.[38,43-45] The activation of the target gene expression is associated with chromatin remodeling by histone acetylation.[46-49] The CLOCK protein itself has an intrinsic histone acetyltransferase activity.[46]

Then, closing the negative limb of the feedback loop, the homologues of PER and CRY proteins form complexes, accumulate rhythmically in the nucleus and once these complexes reached a critical concentration, inhibit their own transcription interacting with the BMAL1-CLOCK heterodimers.[35,40,50,51] CLOCK-BMAL1 also drives the expression of nuclear hormone receptors i.e. orphan nuclear receptors: *Rev-erbα* and *RORα* which are also involved in the negative feedback loop. REV-ERBα represses, while RORα activates the *Bmal1* expression.[35,50,52-54] Thus, the mRNA expression and protein level of the core genes (except *Clock* in the many tissues) oscillate with a period close to 24h.[52,55]

Diverse ranges of transcripts (involved in multiple physiological functions) have been identified as targets, directly controlled by the core circadian component, e.g. CLOCK-BMAL1 heterodimer. These target genes called clock-controlled genes (*CCGs*) exhibit

rhythmic expression.[44,56,57] Current estimations indicate that the clock-controlled genes constitute circa 10% of the expressed transcripts.[43]

Evidence suggests that the direct post-transcriptional mechanisms might have potent role in the circadian control.[58,59] Recent studies have implicated that the micro-RNAs (miRNAs) - which are small non-coding RNAs regulate stability and/or translation of their target-mRNA - are involved in the precise regulation of the circadian network.[60-62]

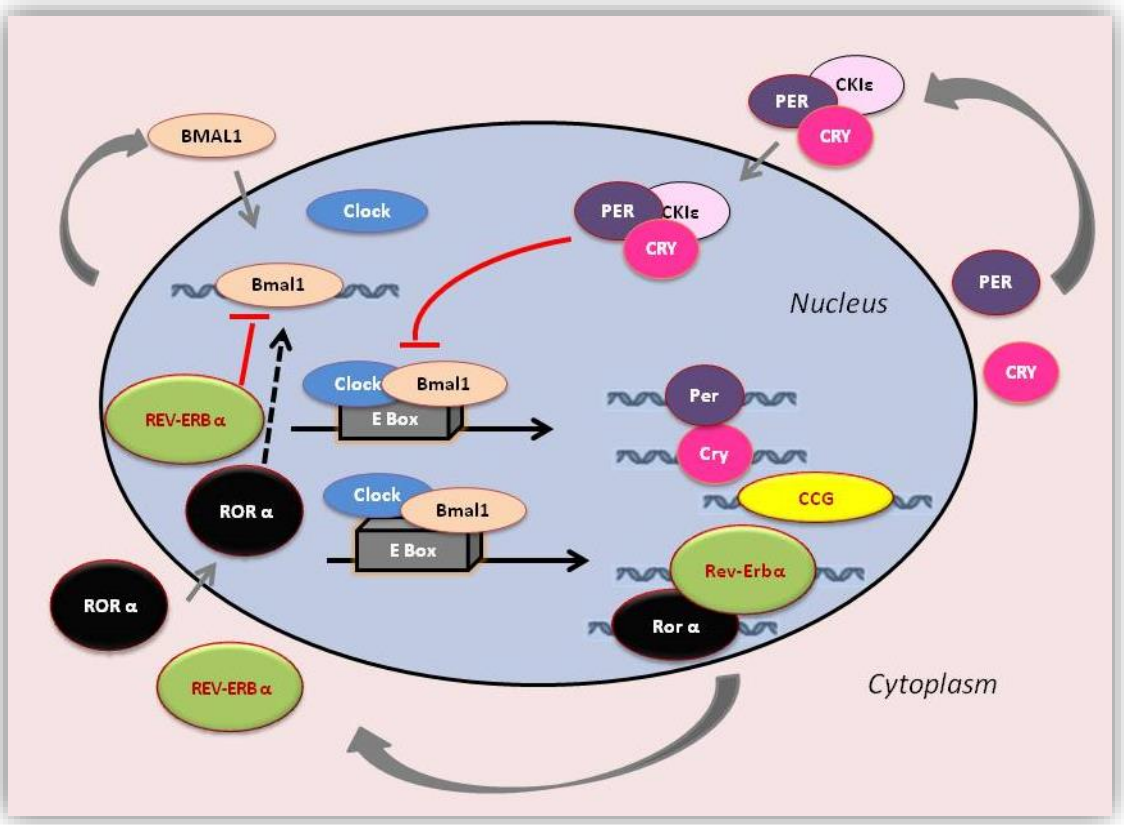


Figure 1. The mammalian circadian clockwork. The circadian clock network involves transcription-translation feedback loops comprised of set core clock genes, i.e. *Clock*, *Bmal1*, *Period (Per1*, *Per2* and *Per3)*, *Cryptochrome (Cry1* and *Cry2).* CLOCK and BMAL1 circadian proteins form heterodimer complexes and activate the transcription of the *Per* and *Cry* genes. PER and CRY proteins heterodimerize, translocate back to the nucleus and interact with the CLOCK–BMAL1 complex to inhibit their own transcription. Retinoic acid-related orphan receptors, RORα and REV-ERBα which are also targets of the CLOCK–BMAL1 complex activates or represses *Bmal1* transcription respectively, functioning as a secondary feedback loop. In addition, as post-translational modification of the clock proteins by phosphorylation i.e. casein kinases (CKIε, CKIδ) are crucial for the circadian control. Clock-controlled genes (*CCGs*) directly driven by the CLOCK–BMAL1 complex show circadian expression.

Post-translational modifications of the clock proteins are also involved in the fine control by e.g. phosphorylation modulating the stability, heterodimer formation, subcellular localization (nuclear entry) and the degradation of the circadian clock proteins.[51,63,64] The key kinases, CKIε, CKIδ, which are involved in the PER CRY and BMAL1 proteins phosphorylation for accelerated degradation and clearance from the nuclei are essential component of the clock machinery network.[65,66] The mutations of these kinases could modify the stability of the regulated proteins, thus the period length or the amplitude of the circadian cycle.[67,68] Interestingly, the first mammalian circadian mutation was documented in "tau mutant" hamster characterized by an abnormally short free running period (20 h) in behavior and the secretion pattern of melatonin as well as glucocorticoids in temporal isolation.[69] It took ca. 10 years till Takahashi and colleagues revealed that the ground of abnormal circadian "phenotype" was a point mutation in the *CK1ε*.[67] In humans, the same mutation leads to advance sleep phase disorders with a ca. 4-hour advance of the sleep onset in individuals, who are also called "morning larks".[68]

1.3. Circadian organization: the central and peripheral circadian clocks

The mammalian endogenous timing system is divided into a central pacemaker which generate and synchronize the endogenous circadian rhythms and peripheral circadian oscillators sharing the same molecular clockwork at the cellular level. (Figure 1) In the current model of its regulation the central pacemaker is described as a conductor in an orchestra, in which the peripheral clocks can play music autonomously.[70]

1.3.1. The master clock: SCN

The central pacemaker of the circadian clock is located in the paired suprachiasmatic nuclei (SCN) of the anterior hypothalamus.[35,71,72] The SCN cells (ca. 2x 10.000) generate and maintain self-sustained and cell-autonomous, strongly coupled (possibly mediated by GABA and VIP) circadian oscillations of the firing rhythms, metabolism as well as the expression of clock genes and their proteins at the tissue level.[73,74] Recent studies have shown that SCN neurons are not identical in intrinsic properties and

function: the dorsal “shell” region (characterized by vasopressin expression) has been appeared to be the primary pacemaker, while the ventral “core” region receiving direct innervations from the retina (i.e. input pathways) is responsible for the (re)entrainment of the intrinsic oscillations.[19] The circadian rhythm of the entire SCN is tightly synchronized, but the phase between the two regions could be different.[74]

The role of the SCN as the master clock in the circadian control has been extensively investigated. Accumulation of evidence includes lesion studies which are demonstrating the loss of circadian rhythmicity of the behavior pattern following ablation of the SCN in animal experiments. More importantly, the transplantation of an intact SCN grafts is restoring the rhythmicity.[72 75]

1.3.2. Input to the SCN

In order to keep harmony with the environmental time, SCN requires rhythmic exogenous input. The SCN receives photic and nonphotic information. The most important are the light cues detected by a subset of melanopsin expressing non-image-forming retinal ganglion cells which are directly projected to the SCN via the retinohypothalamic tract (RHT).[70,76,77] Glutamatergic terminals from the retina activate multiple pathways in the “core” region of the SCN leading to light dependent phase advances or delays of the daily oscillation in clock gene expression.[55] This stimulus drives a trans-synaptic activation of the “shell” region.[19] In that way, continuous light could desynchronize the molecular rhythms of the SCN.[78]

On the other hand, RHT transfers photic information indirectly to the SCN via the geniculohypothalamic tract (GHT) pathway associated with the reward system by releasing NPY and GABA on the SCN neuron. However, its role remains controversial, serotonergic (5-HT) tracts end directly on the SCN mediating non-photic signals from the dorsal and median raphe nuclei.[70,79-81] (Figure 2) These connections are made during the early postnatal period in rats and probably in the late gestation period in humans.[82]

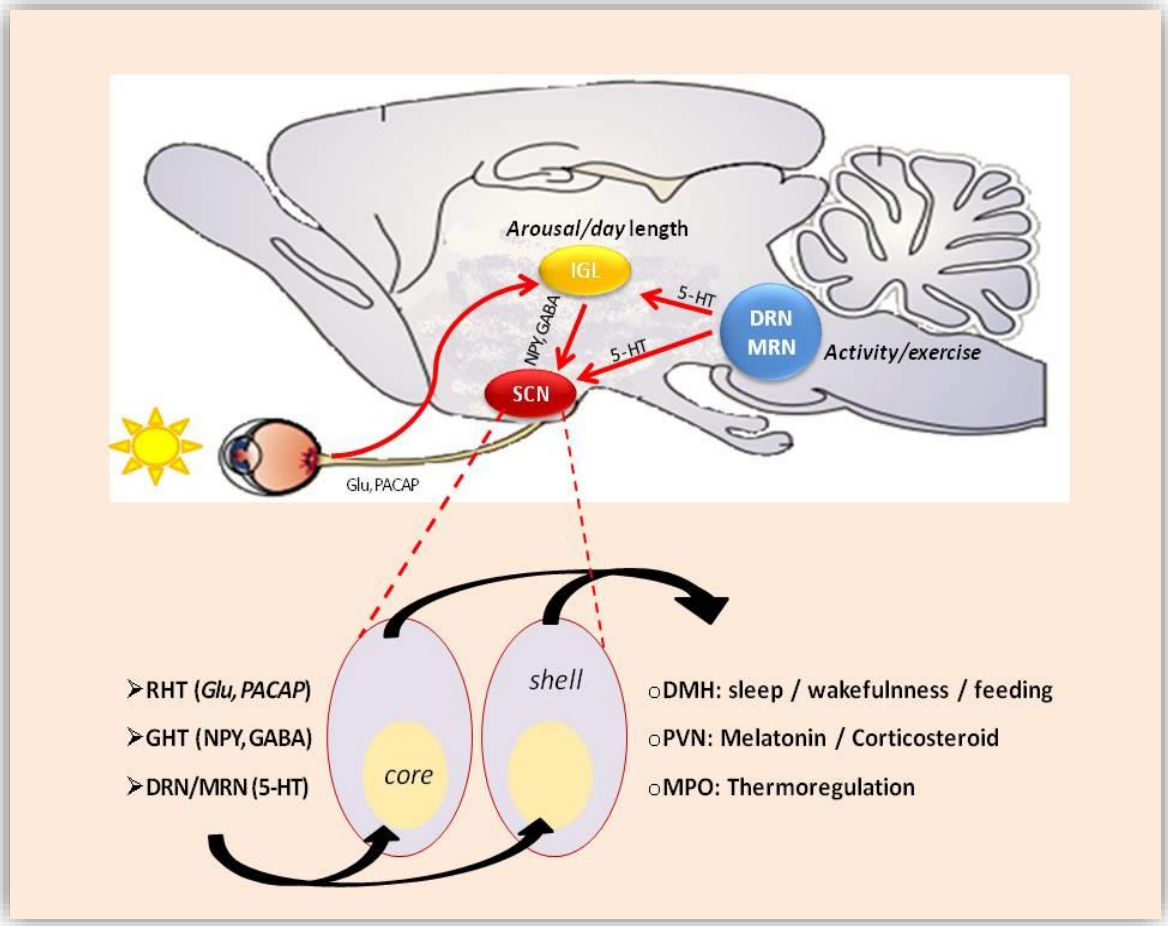


Figure 2. Main input and output pathways of the SCN in rat. Photic input to the SCN through the retinohypothalamic tract (RHT) via glutaminergic pathway (Glu), non-photic input from the dorsal and medial raphe nucleus (DRN and MRN) via serotoninerg pathway (5-HT) and from the intergeniculate leaflet (IGL) as indirect photic input through geniculohypothalamic tract (GHT) via GABA and neuropeptide Y (NPY) pathways.

1.3.3. Output from the SCN

SCN neurons innervate diverse target regions. The main output pathway ends on the dorsomedial hypothalamus (DMH) contributing to the circadian regulation of the orexinergic system, thus to the timing of sleep and wakefulness.[11,83] Through their neuronal connections SCN projects to the paraventricular nucleus of the thalamus (PVN) influencing behavior related to feeding and reward processing and to the medial preoptic region (MPO) controlling proper timing of diverse physiological functions.[3]

(Figure 2) Furthermore, SCN modulates the neuroendocrine and autonomic nervous system setting the sympathetic/parasympathetic balance.[84]

Beyond the sleep dependence hormonal secretion, e.g. growth hormone and prolactin, the glucocorticoid synthesis and release, as well as the nocturnal secretion of melatonin are controlled by SCN.[11,85,86] Thus, the master clock, the SCN is able to synchronize circadian clocks on the periphery with the day-night rhythm due to its neuronal-, endocrine signals and the regulation of the behavior driven by the rest-activity cycle, as well as the body temperature rhythms.[9,77]

1.3.4. Other ins and outs: feeding the clock

The synchronization of the peripheral clocks by the SCN depends on the SCN's control governing the rest-activity, thus the fasting-feeding cycles via autonomic and hormonal pathways.[87,88] E.g. hormones which are driven by feeding or fasting might be able to adjust the phase of circadian oscillators in the periphery.[89,90] However, the central pacemaker, the SCN itself has been shown to be unaffected by feeding-depend signaling pathways.[91-93]

The feeding time appears to be a potent Zeitgeber in some peripheral tissue i.e. in the liver, kidney and heart. [91,94,95] Under certain conditions, e.g. sudden disruption of the regular feeding regime, the peripheral oscillators can be uncoupled from the SCN's synchronization. When the food ability is experimentally restricted to the inactive phase of nocturnal rodents (to the light period) the circadian oscillations in the periphery have been shown to be phase shifted accordingly.[91,92,96,97] Furthermore, for example in the liver, the restricted feeding regime can entrain the phase of the clock gene expression in SCN-ablated mice which are otherwise completely arrhythmic.[98]

In mammals, most of the gene-expression patterns which are involved in the metabolic regulation show daily variation govern by the circadian clock.[43,99,100] The circadian rhythms at the cellular level result from the rhythmic expression of 2 types of transcript groups: the central core circadian genes and the clock controlled genes (*CCG*), which are the downstream output driven by the core component. In the absence of feeding

cues, however, there is only a small part of transcripts including the core clock genes which maintain circadian oscillation in the peripheral tissues.[101,102]

Otherwise, in liver specific clock gene mutation experiments, in which the mice sustained rhythmic feeding behavior, revealed a subset of hepatic circadian clock controlled genes which maintain oscillation.[103]

It is becoming clear that the temporal metabolic status of a tissue might affect the circadian clock machinery of individual cells.[64,104] It has been reported for example that the ratio of NADH to NAD^+ is affecting the dimerization of Bmal1/Clock and their binding to their DNA sequences (E-boxes). Higher ratio of the reduced forms, NADH (NADPH) enhance, while higher ratio of the oxidized forms, NAD^+, ($NADP^+$) diminish the transcriptional activation by BMAL1/CLOCK heterodimer.[64]

Furthermore, it has been proposed that the adenosine monophosphate activated protein kinase (AMPK) acting as a food sensor directly phosphorylates the Cry1 protein, thereby marking it for degradation.[105] Thus, Cry clock protein is functioning as a sensor of cellular energy rate and might have a pivotal role in the synchronization of the metabolism, e.g. glucose homeostasis at cellular level.[106] Thus, signals related to food intake have major roles in the regulation of the daily expression pattern of clock- and clock controlled genes at the cellular level.[107,108] In addition to the time of feeding, food content e.g. high salt diet can alter the circadian gene expression in the liver and kidney as well.[109,110]

1.3.5. Other ins and outs: glucocorticoids as potential synchronizers

A group of steroid hormones involved in diverse functions including stress response, anti- inflammations, cardiovascular and metabolic functions are synthesized and secreted in circadian manner.[86,111-113] Son et al. have reported that the rhythmic release of glucocorticoids (GCs) from the adrenal cortex is controlled by the peripheral clock in the adrenal gland.[114,115] Otherwise it is known that the synthesis is controlled by the SCN via direct neuronal pathways.[85,116] (Figure 4) Data are suggesting that the sensitivity of adrenal gland to ACTH depends on the daytime and the daily GCs peak is driven by the SCN.[83,116] The ablation of SCN leads to loss of rhythmic secretion of

glucocorticoids in rats.[71] Intracellular glucocorticoid receptors are expressed in almost all cells in the body (except the SCN, it has no GC receptors) which suggests their pivotal role in the synchronization of the peripheral clocks with the central pacemaker.[89,108,117,118] Interestingly, the intracellular GC receptors, as well as numerous cytochrome P450 enzymes that are important in the steroids metabolism show robust circadian oscillation at the transcript level.[119-122]

The generally accepted mechanism for GC's effects is the transcriptional activations of selective target genes, however, nongenomic mechanisms of the GC are also intensively studied.[123,124] Indeed, a direct interaction between CRY1/2 and glucocorticoids has been recently reported.[44,106] Furthermore, because of the CRE sequences of Period (1/2), it is strongly affected by glucocorticoid via cAMP/CRE signaling pathways.[125] In animal studies, the synthetic glucocorticoid, dexamethasone can induce a phase shift of circadian rhythmicity in different peripheral tissue, while the SCN remains unaffected.[89,126-128] However, in adrenalectomized rats most of the clock genes except Per1/2 remain rhythmic in the periphery.[129] Furthermore, the circadian rhythm of GC in rodents might influence the daily rhythm of serotonin synthesis in the raphe nuclei.[130] In adrenalectomized rats, serotonin is expressed at constant level which might indicate the circadian control of the behavioral and emotional function by the SCN through the GC rhythms.[130]

1.3.6. Other ins and outs: melatonin, the night manager

It has become evident for decades that the mammalian pineal gland (has been called as a "third eye") is involved in the synchronization of the circadian clock of the body to the external light-dark cycles.[131-134] Melatonin is synthesized by the pinealocytes from the essential amino acid tryptophan with circadian manner (Figure 3).

Its synthesis is driven by the SCN via multi-synaptic neural pathway and inhibited by light exposure.[135,136] During the light period serotonin, as an intermediate metabolite tends to be stored in the pinealocytes. With the onset of darkness begins the final step mediated by arylalkylamine N-acetyl-transferase (AANAT) of the melatonin production which is driven by the SCN via noradrenergic sympathetic fibers.[137-139] The sympathetic

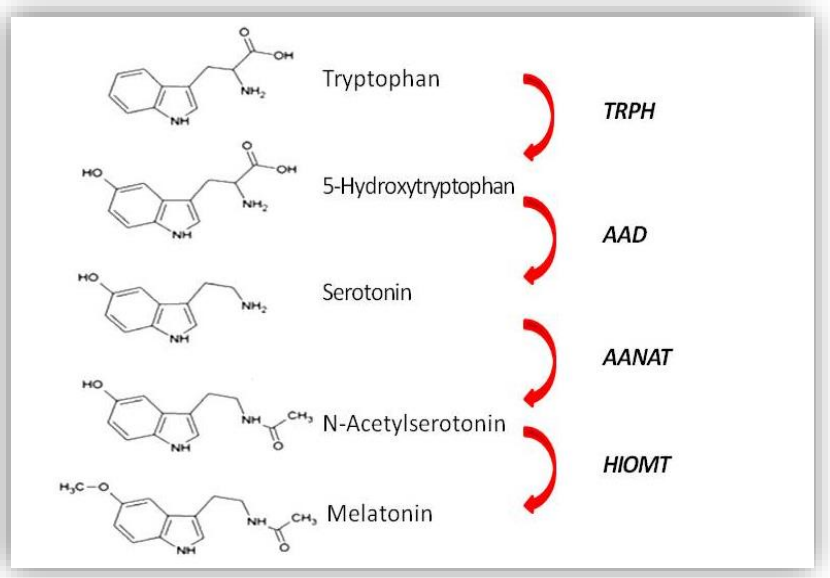


Figure 3. The melatonin synthesis pathway. Melatonin is synthesized from tryptophan. The rate limiting steps mediated by arylalkylamine N-acetyl-transferase (AANAT) and hydroxyindole-O-methyl transferase (HIOMT). TRPH: tryptophan hydroxylase, AAD: aromatic amino acid decarboxylase.

control is necessary in this regulation: it is known that patients with a cervical cord injury have a loss of circadian melatonin synthesis.[140,141] Melatonin is released rapidly into the blood stream and the nocturnal plasma melatonin concentration is ascending more than 10-fold of the daytime level.[141] (Figure 4) The circulated melatonin is then rapidly inactivated in the liver, thus its plasma level precisely reflects its pineal synthesis essentially. However, there is new evidence of its extrapineal synthesis (i.e. gut, lymphocytes and placenta), the extrapineal melatonin production might not contribute significantly to its plasma level concentration.[142-144]

It has been suggested that melatonin has diverse roles in the physiology, the most important to see that diurnal melatonin release synchronizes the entire body with the nighttime.[145,146] It is known that the production of melatonin declines with age. This decline may reflect the progressive age-related calcification of the pineal gland.[147]

However, the exact effects and side effect of the exogenous melatonin are not fully explored, melatonin supplementations have been used for decades as dietary supplements to treat “sleep problems” especially in the older population. Interestingly,

symptoms related to endogen overproduction of melatonin, i.e. Shapiro's syndrome including periodic syncope, hypothermia and hyperhidrosis.[148] On the other hand, the destruction of the functioning pineal gland is associated with reproductive disorder, e.g. precocious puberty.[149]

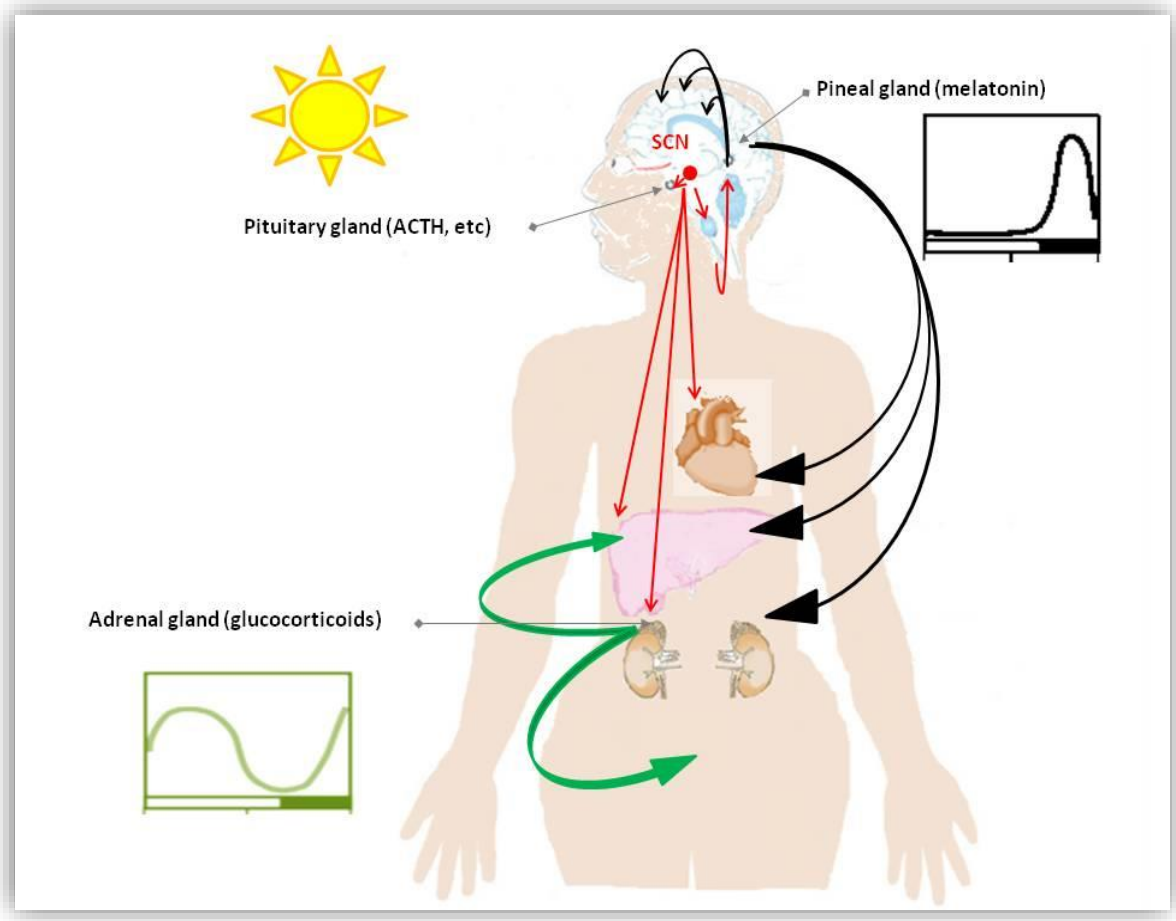


Figure 4. Melatonin, the night manager and glucocorticoid signaling are master clock outputs and internal Zeitgebers. (The figure has been redrawn according to Pevet et al. 2011[132]**)** The master circadian clock is located in the SCN. Numerous peripheral oscillators are found in the brain and peripheral tissues expressing melatonin and glucocorticoidreceptor. Red arrows are presenting the neuronal fibers targets different tissue as outputs of the SCN. Melatonin synthesis is also driven by the SCN via noradrenergic sympathetic fibers. Black arrows are examples of melatonin signaling synthesized by the pineal gland and secreted at night. The daily rhythm of plasma glucocorticoids (signaling pathway marked as green) are synthesized by the adrenal glands and secreted mainly around activity onset is controlled by the SCN.

In mammals, its effects are mediated in part through its G-protein-coupled membrane receptors known as melatonin receptor 1 and 2 (MT1, MT2).[150,151] Furthermore, due to its lipophilic nature, melatonin may mediate intracellular signaling through the nuclear receptors that belong to the RORα/RZR family, or the quinone reductase II enzyme which has been described as intracellular melatonin receptor (MT3).[150]It has been recently reported that the expression of the melatonin receptors in different tissue varies with the time of the day and may vary with the seasons and developmental stage, or age.[152-155] Thus, the effects of the melatonin depend on its plasma concentration and the concentration of its receptors in the target organs i.e. the brain, cardiovascular and immune system, testes, ovary, skin, liver, kidney, adrenal cortex, placenta, breast, retina, pancreas and spleen. The presence of melatonin receptors in the SCN indicates that melatonin has a feedback effect on the central pacemaker.[151] E.g. melatonin signal mediated through the MT1 receptor in the SCN inhibits the firing of the SCN neurons during night.[133]

As an example of its effects in inflammation processes: it has been documented that MT2 receptor inhibits leukocyte rolling while MT3 regulates the adhesion of the leukocytes to the endothelium.[156]

1.4. Circadian rhythms during fetal development

During gestation, the optimal maternofetal milieu ensures adequate intrauterine development and appropriate transition to the extra-uterine life. In humans, prenatal circadian rhythms e.g. daily oscillations of fetal movements and heart rate have been observed.[157,158] The central clock (SCN) in the fetus and the in- and output pathways of the SCN develop gradually and become sensitive to the external light-dark cues during late prenatal and early postnatal period.[159] In rodents the neurogenesis of the SCN starts at embryonic day 13-14, the innervations by the RHT and morphological maturation are completed between the postnatal day 4 and 10 (P4-10).[160,161] In humans, these processes are completed already by midgestation.[162,163] It is considered that the fetal organs involving the fetal SCN are functioning as peripheral oscillators governed by maternal signals during the early and mid-gestation.[164] The core components of the circadian

machinery that generate oscillations are already expressed in several fetal tissues, e.g. the fetal brain, liver, kidney and adrenal gland.[165-167] Interestingly, no circadian variation has been observed in embryonic tissue for BMAL1 and Per2 in vivo, however cultured cells from this tissue have capacity to express active circadian oscillation.[168] A plausible explanation is that the individual cells which have the functioning circadian clockwork might not be synchronized until later development stage.

Before the light entrainment is established, the fetus is exposed to the maternal rhythms e.g. related to the sleep-wake status, thus melatonin signals.[169] (Figure 5) Another potent Zeitgeber during pregnancy is the time of feeding via hormonal and metabolic cues.[170]

The melatonin production by the pineal cells starts postnatal in rats and humans.[171] Thus, during the intrauterine development the maternal melatonin provides the photoperiodic information crossing the placenta and binding to the fetal e.g. SCN neurons, kidney and adrenal gland.[172-176] Interestingly, the expression of the melatonin receptor (MT1) in the fetal SCN shows circadian manner which might reflect the maternal regulation.[177,178]

Studies in rats demonstrated that during the developmental stage the clockwork machinery undergoes developmental changes in tissue specific manner.[163,165-167,179,180] In all studies increase in the amplitude of clock gene mRNA expression and phase shift of the circadian oscillation have been reported. In parallel with that human and animal experiments documented that the amplitude of the maternal nocturnal melatonin peak and the concentration of the maternal glucocorticoid level are elevated in the late

pregnancy.[173] One of the underlying molecular mechanisms is the alteration of the placental expression of 11-ß-hydroxysteroid-dehydrogenase (11ßHSD) enzyme throughout the pregnancy which regulates the passage of maternal glucocorticoids to the fetus.[181]

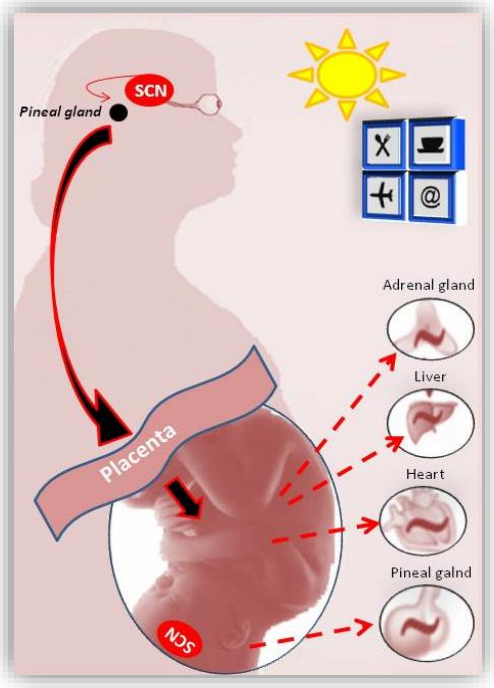


Figure 5. Schematic representations of the proposed entrainment pathways of the fetal SCN and fetal peripheral oscillators (adrenal gland, liver, heart, pineal gland and kidney)

The maternal SCN is entrained by the light-dark cycle, whereas peripheral clocks, thus, the fetal SCN, as well as the other fetal peripheral clocks are entrained by the maternal SCN through humoral and neuronal signals. E.g. maternal melatonin, feeding and metabolic signals that able to cross the placenta might contribute to the entrainment of the peripheral clocks in the fetus.

1.5. Fatal consequences of the fetal programming

It became evident that suboptimal intrauterine environment altering the organogenesis has potential long-lasting effects on diverse organ function.[182-184] However, early postnatal development and adult behavioral factors modify the progression of these diseases; many factors have been described to have deleterious effects on fetal development e.g. inappropriate nutrient supply, high maternal salt intake, alcohol and smoking.[185-190] Epidemiological studies reported that shift work during pregnancy is associated with an increased risk of preterm birth and low birth weight.[24,25] A series of studies reported increased risk of late onset of cardiovascular, renal and metabolic diseases associated to low birth weight.[191-194] Evidence is accumulating that suggests that circadian disruption as a suboptimal condition during pregnancy leads to adverse programming late-onset e.g. cardiovascular and metabolic diseases.[195-197]

The developing central circadian clock, the SCN could be vulnerable to adverse maternal exposure during the neuronal development which might have a long-lasting impact on the circadian timing system later in life.[170,198] It has been reported that

intrauterine malnutrition as well as maternal stress during the last week of pregnancy can alter the sleep-wake cycle in the offspring.[199,200]

Maternal withdrawal in neonatal period can alter the circadian rhythm, basal- and stress-induced corticosterone plasma level and growth rate in the offspring.[201,202] Prenatal overexposures to glucocorticoids, depending on the timing of exposure, has many adverse effects altering fetal development influencing the blood pressure control later in life.[203-207] It is known that growth restricted infants have small kidneys with fewer nephron number.[208] And the congenital nephron deficit caused by adverse intrauterine effects is strongly correlated with increased risk of hypertension and renal diseases in adulthood.[194,209,210] It should be noted that the nephron deficit (e.g. children with only one kidney) do not necessarily lead to hypertension.[211]

1.6. Circadian control of the kidney functions

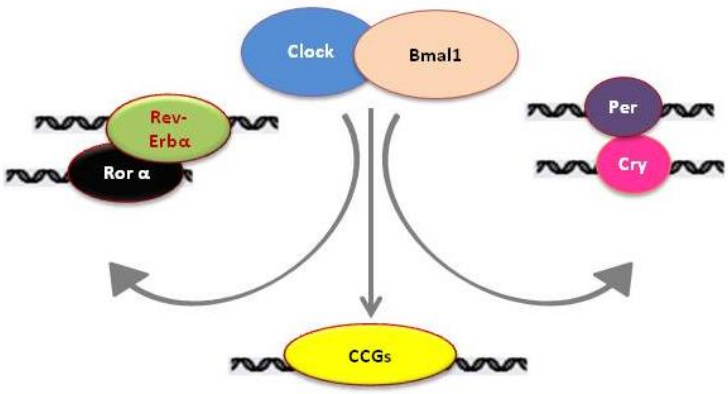
The circadian rhythm of various renal homeostatic functions e.g. daily fluctuations in urine volume, renal blood flow, glomerular filtration rate, sodium and water excretion, as well as the blood pressure has been described for decades and became a well-known phenomenon.[212-215] The molecular clockwork which governs the circadian fluctuations of the renal function has been recently established and since then intensively investigated.[56,216-222] It became evident that the nocturnal dipping of the normal blood pressure (10-20% decrease in nighttime) is regulated by the circadian clock.[12,223,224] Although the underlying mechanisms are not fully understood, sympathetic vascular tone, renal sodium handling and NO signaling are involved in the circadian control.[215,225-227] Hormones which are critical for the blood pressure regulation, e.g. plasma renin, ACE activity, angiotensin II and aldosterone exhibit daily oscillation.[228,229] It is not surprising that the hypothalamic-pituitary-adrenal (HPA) axis has been revealed being under circadian control as well.[230,231] Several reports link aldosterone signaling and the inappropriate sodium transport to the disruption of the circadian pattern of the blood pressure: e.g. patients suffering hyperaldosteronism exhibit the non-dipper pattern.[232] Furthermore, the dietary sodium restriction in subjects with hyperaldosteronism can restore the nocturnal dipping blood pressure pattern.[232]

Individuals with chronic kidney disease frequently loose the night time dipping of the blood pressure which is associating with a faster decline in renal function and increased risk for cardiovascular event.[233,234] And vice versa, the non-dipping pattern itself seems to be a preclinical marker for cardiovascular and renal diseases.[235-238]

Functional studies explored that the local renal circadian clock is involved in renal functions, including e.g. the maintenance of water, calcium, magnesium and acid-base balance. For example, growing number of genes, which encode products including sodium and water transporters along the nephron segments appear to be under the control of the circadian clockwork.[239-245] (See Figure 6.) The gene of the Na^+/H^+ exchanger isoform 3 (*NHE3)* was the first identified clock-controlled gene (*CCG*) directly regulated by the CLOCK/BMAL1 heterodimers in the kidney.[246,247] The circadian expression of the *NHE3* is well documented. Its function is the absorption of NaCl in the proximal tubule and NaHCO3 in both the proximal tubule and the thick ascending limb.[248] It has been observed that the rhythmic expression of mRNA encoding *NHE3* is blunted in *Cry1/Cry2*-null mice.[246]

The epithelial sodium channel (ENaC) plays a crucial role for the sodium reabsorption in the aldosterone-sensitive distal nephron, thus in the long-term blood pressure control. It is regulated by hormones such as aldosterone.[249,250] The channel consists of three subunits, α, β and γ.[251] It has recently been shown that *αENaC* is regulated by the circadian protein PER1, as well on a basal, as on an aldosterone-mediated level.[252,253] The transcription of *αENaC* is induced by the interaction of Per1 with its E-box.[240]

Sgk1, a well-known serin-threonine kinase that activate variety of sodium transporters including ENaC appeared to be also directly regulated by the circadian clock.[56] It is regulated by different hormones such as glucocorticoids on the genomic level. In humans, the overexpression of SGK1 is associated with variety of pathophysiological functions including high blood pressure.[254] Furthermore, microarray studies (using microdissected nephron segments) revealed that many genes express daily variation in the kidney.[239]



Clock controlled target genes in the kidney (CCGs):		
Gene	**Function**	**RNA Source**
Scnn1α (αENaC)	epihtelial sodium channel	aldosterone-sensitive distal nephron
Sgk1	serine-threonine kinase	whole kidney
Slc9a3 (NHE3)	sodium/hydrogen exchange	whole kidney
Avpr2	water channel	cortical collecting duct

Figure 6. The investigated clock controlled target genes in the kidney (*CCGs*)

Arginin-vasopressin which displays a circadian variation has a crucial role in the salt and water handling greatly affecting urine production and storage. However, vasopressin is synthesized in different region of the hypothalamus, significant circadian changes in mRNA and protein level have been only detected in the SCN.[255,256] The SCN-derived vasopressin is probably one of the major rhythmic hormonal outputs of the central pacemaker. Daily expression level of vasopressin type 2 receptor (*AVPR2*) linked to its diuretic function has been shown to follow temporarily synchronized circadian oscillations.[257] Interestingly, the suppression of the *Clock gene* leads to significant changes in the expression levels of this transcript. Furthermore, the phenotype analysis of Clock-deficient mice revealed an impaired capacity of the kidney to concentrate urine, a condition called as partial diabetes insipidus.[216,239]

1.7. Renal phenotypes in clock mutant models

Significant milestones in our understanding the effects of the single core circadian component have reached by knockout models. However, in animal models in which a single clock gene mutation causes behavioral misalignment, e.g. sleeping, feeding activity, it is difficult to evaluate the principal effect of peripheral disruption of the circadian network. Thus, tissue specific deletions of clock components might lead us to better understand the underlying mechanism.[257] It should be considered additionally, that many genes which have no circadian oscillation are up- or down regulated by the core components.[43]

Bmal1 (Brain and muscle arnt like protein-1)

Bmal1 KO models display a wide range of organ pathologies e.g. loss of circadian rhythm of blood pressure, increased endothelial dysfunction, severe arteriosclerotic disease[258,259], development of cardiomyopathy with age[260], modified glucose homeostasis[261-263], altered sleep pattern and infertility.[264,265] Specific deletion of BMAL1 in renin-producing cell leads to e.g. decrease of plasma aldosterone and lower blood pressure.[257] Mice with a specific deletion of BMAL1 in the central nervous system associated with reduced food intake and loss of body weight are not capable for re-entrainment by restricted feeding.[266] In the conventional global BMAL1 KO mice display progressive muscle atrophy, behavior arrhythmic with a markedly reduced mobility, premature aging and shortened life span.[267,268] However, when Bmal1 deletion is induced at an adult age, or at early developmental stages but selectively e.g. in the skeletal muscle, unaltered body weight, muscle mass and life span could be observed.[257,269,270] Thus, the emerging role of the BMAL1 during development is supported by these findings.[271]

Clock (Locomotor Output Cycles Kaput)

Clock KO mice have NPAS2 as paralog within the SCN which can be the partner of Bmal1 driving the circadian rhythmicity of the gene expression.[272] Thus, Clock KO mice sustain the circadian rhythm of behavior, which makes this mutation a suitable

model exploring the peripheral circadian dysfunction.[273,274] However, clock delta19 mutation which compromises its transcriptional activation causes a longer free-running period (ca. 28 h) associated with metabolic syndrome in mice.[261,274] Clock KO mice are hypotensive, displaying mild polyuria due to altered water and sodium handling and altered aldosterone plasma level.[216,239] This mice display a slight degree of nephrogenic diabetes insipidus. This model also displays decreased fertility.[275]

Cryptochrome(s)

It has been observed that *Cry*-null mice exhibit no circadian pattern of the locomotor activity.[276] Furthermore, the circadian oscillation of blood glucocorticoid levels is disturbed with significantly increased plasma aldosterone level which leads to increased kidney damage.[11,277] A fascinating study revealed the molecular background: the *Cry* null mice (*Cry1* and *Cry2*) exhibit enhanced aldosterone production due to marked increase in type VI 3ß-hydroxyl-steroid dehydrogenase (Hsd3b6) mRNA expression and enzyme activity by the adrenal gland.[278] As previously mentioned, in this model the *NHE3* expression seems to be severely blunted.[246]

Period(s)

The reduced level of *Per1* in mice leads to sodium wasting.[279] *Per1* KO mice express significantly lower blood pressure, reduced insulin secretion, higher melatonin level during the active phase and reduced daily expression level of *αENaC*.[242,252,280] Furthermore, *Per1* KO mice exhibit increased level of corticosterone.[281] *Per2* KO mice are losing the circadian rhythmicity of behavioral activity, blood pressure and heart rate under constant darkness.[282-284] Furthermore, they exhibit impaired endothelium-dependent relaxation.[285,286]

Rev-erbα

Rev-erbα KO mice exhibit altered period length and phase shifting in the molecular clock oscillatory properties and altered circadian wheel running behavior.[52] KO mice display disturbed lipid metabolism and increased adiposity.[287]

2. THE AIMS OF THIS STUDY

Based on the presented, largely unexplored background we addressed the role of the prenatal maternal circadian misalignment in the fetal programming of chronic diseases in adulthood. We studied circadian entrainment during intrauterine period. We investigated the long-term effects of the maternal circadian disruption in early postnatal period and in adulthood.

While the kidney is one target organ of specific interest for us, our working hypothesis was the following: a disturbed maternal circadian rhythm due to altering fetal programming potentially addressing clock genes has adverse effects on the renal function of the offspring resulting i.e. hypertension later in life.

The following questions were intended to be answered:

- Whether the maternal circadian disruption during the intrauterine period influences the peripheral circadian clock machinery in the offspring's kidney at the birth (more precisely at embryonic day 20 = E20), or later in life: i.e. in the postnatal period (at 1-week-old = 1W), at the weaning time (at 4-week-old = 4W) and in adult age (at 12-week-old = 12W)?
- Whether the prenatal circadian disruption adversely affects the intrauterine growth and/ or the kidney development?
- Whether the maternal circadian disruption during the intrauterine development period has any impact on the kidney functions and the blood pressure regulation in their offspring later in life?

3. MATERIALS AND METHODS

3.1. Time definition

Zeitgeber time (ZT) is a standard time based on the period of normal light-dark cycles (12:12h light - dark) in animal laboratories. Light onset defines Zeitgeber time ZT0. Thus, ZT0-12 represents the light and ZT12-24 the dark period under normal laboratory condition. We chose 06:00 a.m. as ZT0.

3.2. Animals and experimental protocols

All animals were handled according to written approval from the local authority for animal experiments (Regierungspräsidium Karlsruhe, 35-9185.81/G-29/11). Pregnant Sprague Dawley rats (n = 240, pregnancy rate 93,5 %) were obtained from Charles River Co. (Sulzfeld, Germany) two days after conception. At the second day of gestation (considered the fetuses, in the following mentioned as embryonic day 2, **E2**) rats were randomly allocated to the following light-dark cycles with food and water supplied ad libitum under controlled temperature (21 ± 2°C):

1. 12 h:12 h light-dark (**LD**) cycle, normal photoperiod, ZT0 = 06:00 h
2. constant illumination, 24 h light (**LL**)
3. constant darkness (**DD**)
4. shortened, ultradian, 6 h:6 h light - dark cycle (**6:6-LD),** ZT0 = 06:00 h
5. prolonged dark phase condition, 3 h:21 h light - dark cycle (**3:21-LD**), ZT0 9:00h

Dams were housed (3-5 dams / 1800 cm², in Typ IV cages) under the same photoperiod conditions during the entire pregnancy. At the day before the expected delivery, i.e. at embryonic day 20 (**E20**) dams were housed separately (1 dam / Typ IV cages). Cohorts

of animals at age E20, as well as at 1. (1W), 4. (4W) and 12. (W) and 34. week of age (34W, long term= LT) postnatally were used in the following experiments: Experiment1, 2 and 3. (Figure 7)

3.2.1. Disruption of maternal feeding regime

To alter the normal maternal feeding regime, a subgroup of pregnant rats (n = 16) under normal 12:12 light-dark (LD) cycle were randomly assigned to be fed only during the light phase throughout the entire pregnancy (E2 - E21). Food was restricted to the 06:00-18:00 hour (ZT0-12) interval with free access to water. (**FR-LD**) (Figure 7)

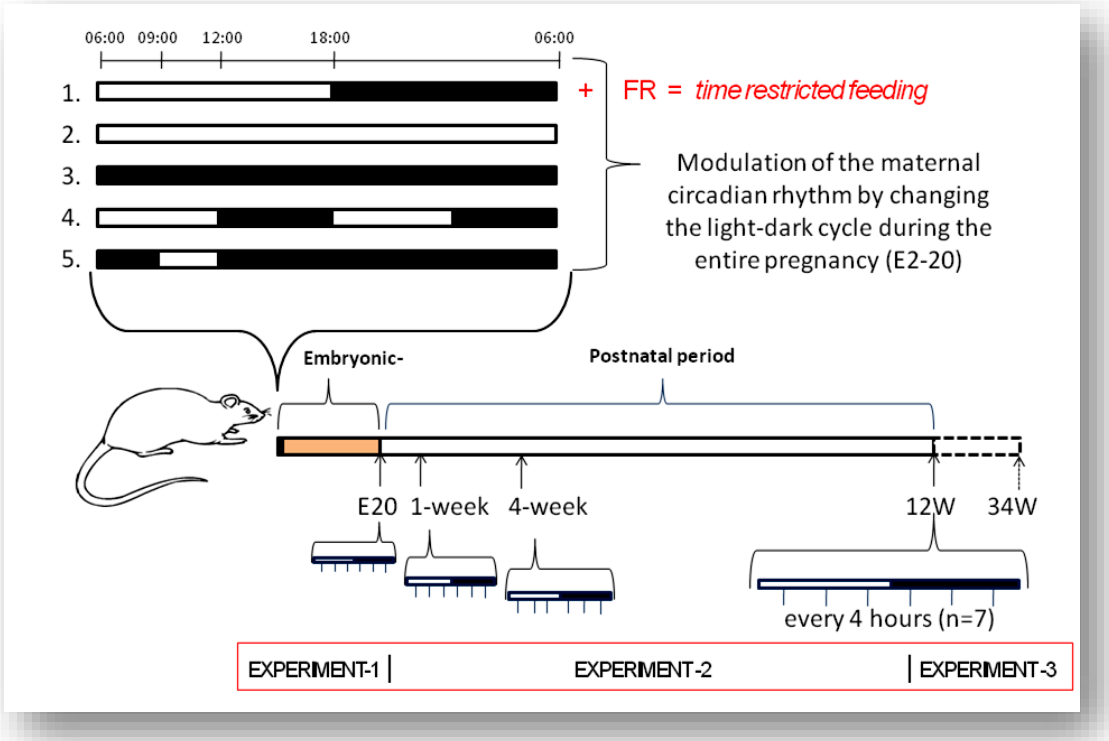


Figure 7. Experimental protocols (1-5) were used to modulate the maternal circadian rhythm during the entire pregnancy (details see in the text). At embryonic day 20 (E20), groups of pregnant rats (n = 6-10 / from each group) were sacrificed with their fetuses (n = 12 ± 2 mothers) every 4 hours to cover one entire circadian period (ZT4-24 h, ZT0 = 06:00 a.m.) Postnatal, at 1, 4 and 12 weeks after birth (1W, 4W, 12W) 14 rats (7 male, 7 female) were sacrificed in 4 hours interval in the following photoperiod conditions: LD, LL, DD, 6:6-LD and 3:21-LD. Offspring (n=20/group) were investigated at long term (LT = 34W) period in each groups.

3.3. EXPERIMENT 1: Early effects of prenatal maternal circadian disruption

At embryonic day 20 (**E20**), groups of pregnant rats (n = 6-10 / group) from each photoperiod conditions (**LD, LL, DD, 6-6 LD and 3-21 LD**) and from **FR-LD** were anesthetized (100 mg/kg ketamine and 3 mg/kg xylazine, which very rapidly passes the placenta) and sacrificed with their fetuses (n = 12 ± 2 mothers) every 4 hours to cover one entire circadian cycle (ZT 2-24 h, ZT0 = 06:00 h). Dams' body and placentas' weight were measured. Fetal tissues (kidney, heart and liver) were dissected and stored.

3.3.1. Effect on the fetal intrarenal clockworks

In the kidney, gene expression patterns of core clock genes (*Clock*, *Bmal1*, *Rev-erbα, Per1*, *Per2*, *Cry1*, *Cry2*) and renal clock controlled genes (*αENaC, Sgk11, ENH3, AVPR2*) were further analyzed in males.

3.3.2. Telemetric measurements of the dams over a 24h period

A subgroup of pregnant rats at embryonic day 2 in group **LD**, **LL**, **DD** and **3:21-LD** (n = 4-6 / group) underwent implantation of telemetrical device (model PA-C40; Data Science International) to monitor BP, HF and motor activity. After a 7-10 days recovery period daily measurement was recorded. The measurements at the third gestational week (3.GW) were further analyzed. A detailed description of the implantation and measurements can be seen below. (Page 37)

3.3.3. Circadian variation in renal excretion function during pregnancy

Urine samples were collected at 4-hour intervals over a 24 h period in metabolic cages (TECNIPLAST S.p.A., Buguggiate, Italy) 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). Immediately after collection urine volumes were measured and fractionated into 2-3 equal parts. Aliquots were stored in a freezer at -20 °C until further analysis. Urinary parameters, i.e. sodium, potassium, phosphate, calcium, glucose and albumin

excretion were analyzed using standard laboratory methods. (Central-labor, University of Heidelberg)

3.3.3.1. Urinary aldosterone excretion over the pregnancy

In group **LD** (n = 8 / 4h over 24 h period) and **LL** (n = 12 / 4h over 24 h period) the daily pattern of urinary aldosterone excretion rate with a 4-hour interval over a 24 h at 1. and 3. gestational week (1.GW and 3.GW) was measured using ELISA Kit. (The measurement was performed at the University of Silesia in Katowice, Poland)

3.3.3.2. Urinary melatonin excretion

At the second week of gestation (2.GW) in **LD** (n = 5 / 4h over 24 h period) and **DD** (n = 7 / 4h over 24 h period) urinary melatonin concentration was measured with a 4-hour period over a 24 h. Urine samples were subjected in duplicate to Melatonin-Sulfat Urine ELISA Kit (IBL, Hamburg, Germany) to determine the immunoreactivity of urinary melatonin metabolite (6-SMT). The absorbance of the immunoreactions was recorded spectrophotometrically at 450 nm in an automated ELISA reader and hormone concentration was calculated using an automated method with standard curve. The intra- and inter-assay coefficients were 5.8–204 ng/ml (5.2–12.2%) and 12.4–220 ng/ml (5.1–14.9%).

3.4. EXPERIMENT 2: postnatal changes

After the delivery, all dams along with their offspring (n = 12 ± 2 / liter) were housed with free access to food and water under constant temperature (21 ± 2°C) exposed to standard 12-12h light-dark cycles (light on at 06:00 h) with illumination ca. 200 lx at the cage level. The offspring were weaned at 4 weeks of age and housed in standard Typ IV cages (2-3 males or 4-5 female per cage). The following studies were performed in offspring from different mothers, in order to avoid litter effect.

At 1, 4 and 12 weeks after birth (**1W, 4W, 12W)** 14 rats (7 male, 7 female) were sacrificed in 4 hours intervals to cover one entire circadian period (ZT4-24 h, ZT0 = 06:00 a.m.) in the following photoperiod conditions: LD, LL, DD, 6:6-LD and 3:21-LD. (Figure 8) Under global anesthesia (100 mg/kg ketamine and 3 mg/kg xylazine) the abdominal aorta was catheterized and retrograde pressure-controlled perfusion fixation was performed using 4% phosphate-buffered formaldehyde for morphological and 0,9% NaCl for molecular investigation, respectively. Body and organ (kidney, heart and liver) weight were recorded.

3.4.1. Circadian gene variation after maternal circadian disruption

The gene expression pattern of core clock (*Clock*, *Bmal1*, *Per1*, *Per2*, *Cry1*, *Cry2*, *Rev-erbα*) genes and clock controlled genes (*αENaC, Sgk11, ENH3, AVPR2*) in the male kidney were studied in LD, LL and DD groups. Other tissue samples were stored for further analysis.

Figure 8. Clockwork of offspring after prenatal circadian disruption were investigated at embryonic day 20 (E20), as well as 1 (1W), 4 (4W) and 12 week (12W) of age.

3.4.2. Postnatal behavioral measurements of offspring

Mothers with their pups were maintained in cages equipped with infrared video camera (Sygonix, CCD-Camera and Digitalrecorder). The Hosttech video capture program was used to record maternal nursing- and feeding behavior, daily activity, feeding and drinking pattern of the offspring at age 4 and 12 weeks. Feeding times of the mothers, as well as the locomotor activity, the frequency of food and water intake of the pups and the mothers were evaluated by video analysis.

3.4.3. Effect of timed maternal melatonin treatment in the early postnatal period

6 dams with their pups were randomly allocated in a subgroup under LD condition (body weight at 3. GW 359,5 ± 23,5 g, 11,8 ± 3,8 pups / mothers). From the day of the delivery dams were treated with intraperitoneal injection of melatonin (**Me**: 4,8 mg/kg ip. at ZT3) or its vehicles (**Vech**: 1,4 g/kg, 6% ethanol in 0.9% NaCl at ZT3) over a week period. Pups (female n = 5-7 per 4 h over 24 h) were anesthetized (100 mg/kg ketamine and 3 mg/kg xylazine) and sacrificed at 4 h intervals for 24h at 1 week of age. As a second control, females from **LD** at 1 week were sampled (n = 7 / 4 h over a 24 h period). Body weights were measured. Fetal tissues (kidney, heart and liver) were dissected, measured and stored. In the kidney, gene expression patterns of core clock genes (*Clock, Bmal1, Rev-erbα, Per1, Per2, Cry1, Cry2)* and clock controlled genes (*αENaC, Sgk11, ENH3, AVPR2*) were further analyzed.

3.4.4. Periodic maternal withdrawal at the first postnatal week period

Pregnant rats (n = 5) exposed to 12-12h light-dark cycles under the above described conditions were randomly allocated in a subgroup. After the delivery (Body weight at 3.GW 376 ± 14 g, 12,2 ± 2,5 pups / mothers), the pups were separated from their mothers for a 4- hour (Maternal withdrawal, **MW**: ZT3-7) period starting on the first postnatal day. The control group remained with their mother all the time. Five pups (male) per time point were anesthetized (100 mg/kg ketamine and 3 mg/kg xylazine) and sacrificed at 4 h intervals for 24h at 1 week of age. Body weights were measured. Fetal tissues (kidney, heart and liver) were dissected, measured and stored. In the

kidney, gene expression patterns of core clock genes (*Clock*, *Bmal1*, *Rev-erbα, Per1*, *Per2*, *Cry1*, *Cry2*) and clock controlled genes (*αENaC, Sgk11, ENH3, AVPR2*) were further analyzed.

3.5. EXPERIMENT 3: long lasting effects

From each photoperiod group (LD, LL, DD, 6:6-LD, 3:21-LD) and FR-LD mothers were returned to LD condition after delivery. Offspring (n = 8-12 / group) weaned at 4-weeks-old were kept for longer period (34 ± 2 Week) under standard condition with free access to food and water under constant temperature (21 ± 2°C) exposed to standard 12-12h light-dark cycles (ZT0 at 06:00 a.m.) with illumination between 50 and 200 lx at the cage level.

3.5.1. Renal function

Urine was sampled in 24-hour intervals in metabolic cages at 34. week of age in all investigated groups (n = 8-12 / 4 h interval over a 24 period in each group). Urinary parameters, i.e. sodium, potassium, phosphate and calcium excretion, as well as albumin- and glucosuria were analyzed using standard laboratory methods. (Figure 9)

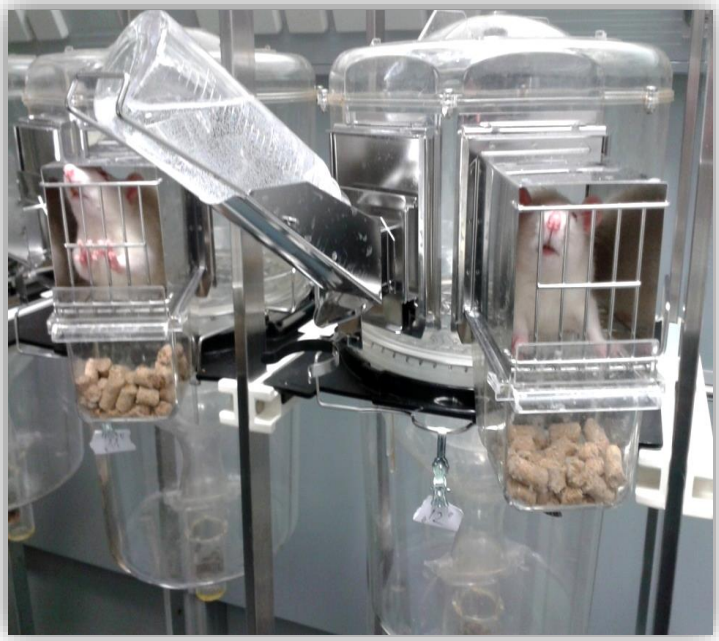


Figure 9. Metabolic cages (TECNIPLAST)

3.5.2. Telemetric measurement

A subgroup of female rats at 34 weeks of age in group **LD**, **LL**, **DD**, **6:6-LD**, **3:21-LD** and **FR-LD** (n = 5-11 / group) underwent implantation of telemetrical device (model PA-C40; Data Science International) to monitor BP, HF and motor activity. After a 7-10 days recovery period daily measurement was recorded. See below detailed description of the implantation and measurements. (Page 37)

3.5.3. 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, after the anterior chest was shaved with transthoracic echocardiography. A parasternal short-axis view was performed for M-mode imaging at the papillary muscle level (See Figure 10.) using a 13-MHz linear transducer (GE 12L-RS, GE Healthcare), connected to an echocardiographic imaging unit (Vivid i, GE Healthcare). 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)

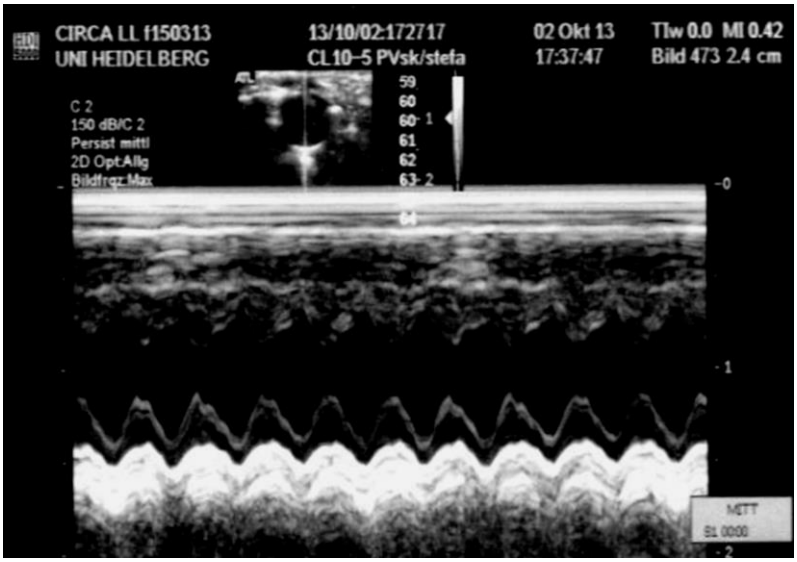


Figure 10. A representative picture of echocardiography at 34-week-old female from LL group.

At the end of the observation, under global anesthesia (100 mg/kg ketamine and 3 mg/kg xylazine) tissues (kidney, heart) were sampled for morphological and molecular investigation. Body and organ (kidney, heart and liver) weight were recorded. Blood samples were collected from the abdominal aorta in each group for further studies.

3.6. Radiotelemetric measurement of blood pressure

Pregnant rats at embryonic day 2 in group LD (n = 4), LL (n = 4), DD (n = 6) and 3:21-LD (n = 7) as well as offspring (female rats) at 34 ± 2 weeks of age 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 implantation of telemetrically device to monitor BP, HF and motor activity.

Under isoflurane (1-3%, 5% for induction) anesthesia and controlled temperature (37 ± 0,5 °C) in ventral abdominal incision the catheter of the telemetry sensor (model PA-C40; Data Science International) was implanted into the abdominal aorta at the level of the bifurcation, below the renal arteries. (Figure 10-11) Sensor was fixed using a small amount of tissue adhesive and small fiber patches, the transmitter itself was fixed intraperitoneally with non-absorbable suture with a simple interrupted pattern. After a 7-14 days recovery, measurements were taken under normal feeding regime (except FR-LD) under ambient temperature. Signals were sent to a telemetry receiver that was placed under the cage (Typ IV) every 5-10 min (ca. 10.000 readings/rat) and transmitted in a 10- minute average from 3 consecutive measurements with use of Dataquest system for further analysis.

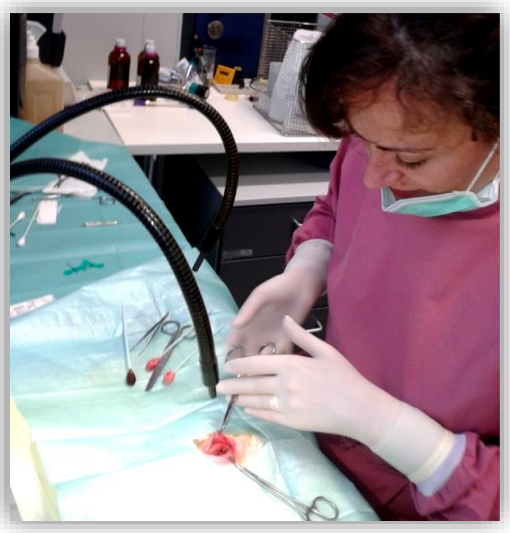


Figure 10. Implantation of the telemetric device (HD)

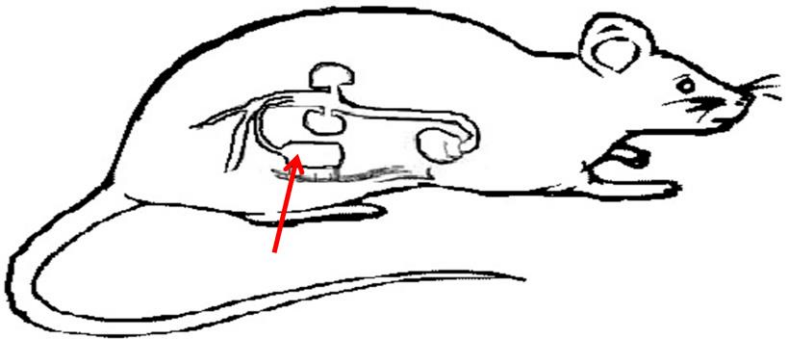


Figure 11. Drawing of a rat implanted with a transmitter (red arrow) capable of monitoring blood pressure and heart rate via the abdominal aorta, as well as a locomotor activity.

3.7. RNA extraction and quantitative real-time PCR

Total RNA was extracted from homogenized kidney tissue (10–50 mg) of male offspring (n = 7 / group) using RNeasy Mini Isolation Kit (Cat.No.74106, Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA concentrations were determined by spectrophotometry. The reverse transcription reaction was performed using 1 µg of total RNA and GeneAmp RNA-PCR Kit (Applied Biosystems, Darmstadt, Germany). All samples were run in duplicate and with a negative control. The quantitative PCR reactions, followed by melt curve analysis were run on a StepOnePlus Real-Time PCR System (Applied Biosystems). The number of the specific transcripts in each sample was calculated, using relative standard curves from each different target 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 (Applied Biosystems).

Table 1. Primersequences used for quantitative RT-PCR.

	FORWARD	REVERSE	AMPL
Clock	GCCATCCACCTATGAATATGTGAG	GTGCGCTGTATAGTTCCTTCGAA	102
Bmal1	AGCACCGTCCTTCCAATGG	TGCTCAGGGAACCGGAGA	103
Rev-erbα	GGTTATGTGGCGTCCTTGAAC	GAAGCTGCCATTGGAGCTGT	158
Cry1	ACCATCCGCTGCGTGTACA	GGCATCAAGGTCCTCAAGACA	105
Cry2	GCTGTCCTGCAGTGCTTTCTT	CAGGTATCGCCGGATGTAGTC	103
Per1	CAATAAGGCAGAGAGCGTGGT	TCATGATGATGTCCGACTCCG	104
Per2	GCTCCAGCGGAAATGAAAAC	TCCGCCTCTGTCATCATGAGT	151
αENaC	GCTGTTTCTCCAAGTGTCGGAA	CATCTCGAAGATCCAATCCTGG	103
SGK1	GAAGATCACGCCCCCATTTA	TGTGACAAGGATGCTGTCAGG	127
ENH3	TTGAGTCTTTCGTGACGCTGG	GCTCGATGATACGCACATGCT	168
AVPR2	CATTGCTGCCTGTCAGGTTCT	CAGCAGCATGAGCAACACAA	119
18S	AGTTGGTGGAGCGATTTGTC	GCTGAGCCAGTTCAGTGTAGC	205

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. The primer pairs were produced by Eurofins MWG Operon (Ebersberg, Germany). The primers and their sequences are listed in Table 1.

3.8. Statistical analysis

Data are presented as mean ± SD or mean ± SEM as indicated in the figures or tables. The statistical analysis was performed using SigmaPlot (version 11.0) program. Different parameters of individuals were compared by one-way ANOVA, 2-way ANOVA or 3-way ANOVA analysis (factors: prenatal light condition, gender, sampling time, gestational week) followed by multiple comparisons versus control group (vs LD) or pairwise multiple comparison procedures (Holm-Sidak method) to determine the difference between the groups. 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 significance of oscillations was tested with the null hypothesis 2A = 0 using an F-test with two degrees of freedom in the numerator representing φ and 2A. Statistical analyses were performed with R (version 3.0.3) and cosinor functions by Charles W. Berry program. Due to the exploratory nature of the current study, a standard significance level of α = 5% was used without correction for multiplicity.

4. RESULTS

4.1. Maternal outcomes (Experiment 1)

4.1.1. Effects of maternal circadian disturbance during pregnancy on the dams

The dams' body weight at the beginning of the pregnancy (at E2) and the mean liter size in the different groups did not differ significantly (Table 2). There was a significant difference ($p < 0,001$; one-way ANOVA) between groups (n = 6-10 dams/group) with respect to body weight at the end of the pregnancy (E20). The body weights were significantly lower of dams kept under **6:6-LD** ($p = 0,022$), **3:21-LD** ($p < 0,001$) light conditions and dams exposed to restricted feeding regime under normal light-dark cycle (**FR-LD**, $p = 0,016$). (See also Figure 17.)

Table 2. Body weight and litter size of dams

	Body weight (g)		Litter size
	at E2 (g)	at E20 (g)	Pups/Mother
LD	209,87 ± 9,95	368,89 ± 22,50	12,09 ± 2,29
LL	212,11 ± 17,04	367,59 ± 29,97	13,00 ± 1,51
DD	216,84 ± 15,63	359,42 ± 30,67	13,38 ± 1,81
6:6-LD	204,73 ± 15,73	346,05 ± 29,25 *	12,66 ± 1,80
3:21-LD	207,84 ± 17,33	332,57 ± 32,64 *	11,67 ± 2,56
FR-LD	209,87 ± 9,49	331,67 ± 13,11 *	11,83 ± 1,17
one-way	NS		NS

n= 6-12/ groups, values are means ± SD. NS stands for not significant, * $p < 0,05$ vs. LD.

4.1.2. Telemetric measurements of dams

Monitoring of the dams' locomotor activity (n = 4-6 / group) with telemetric devices revealed that dams at 3.GW maintained under **LD** and fed ad libitum were mostly active during the dark period of the LD cycle (ZT 12-24*;* 24 h mean of counts/min = *4,10*). Representative actograms of each group over a 24 h interval are shown in Figure 12.

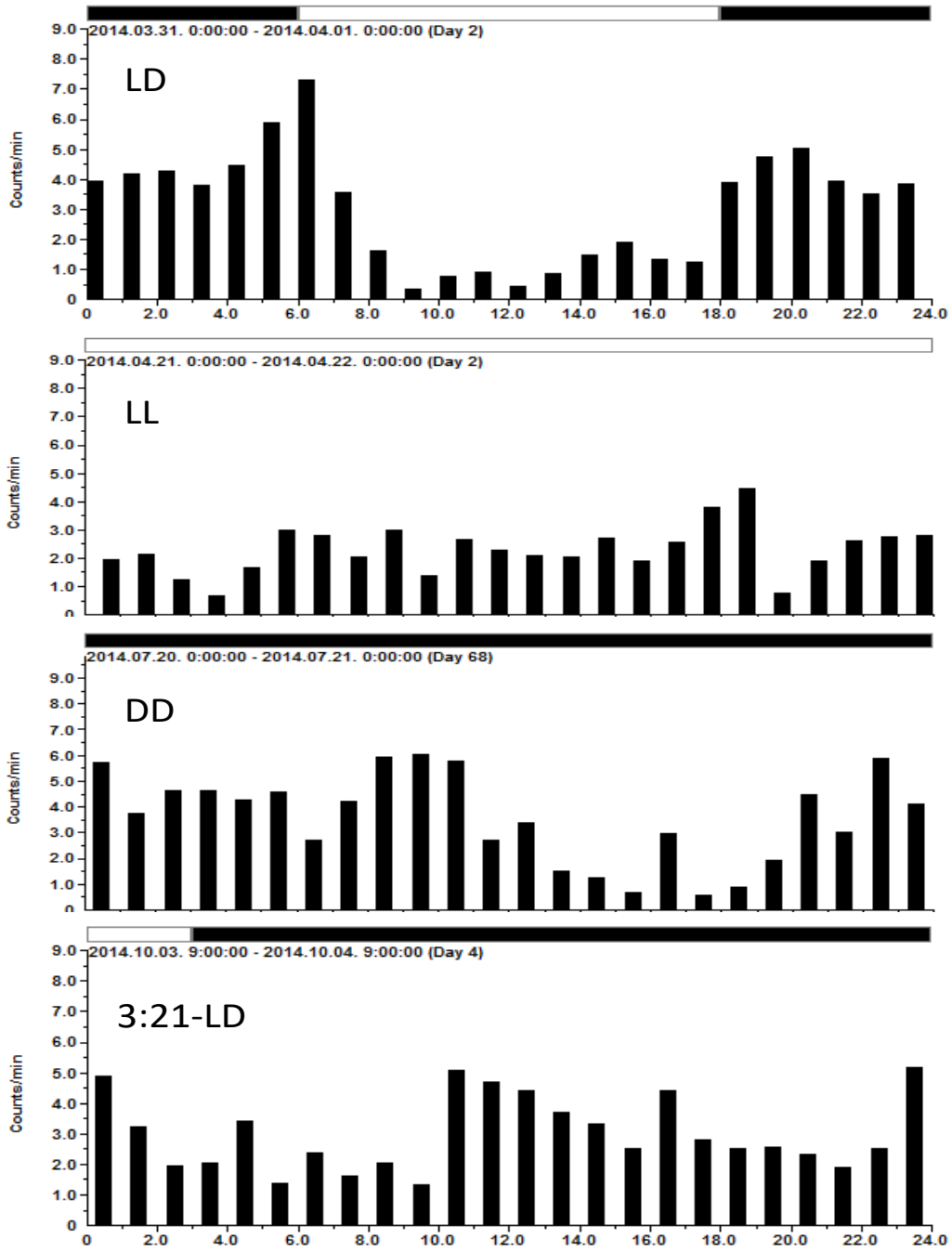


Figure 12. 24 h actograms of dams' (4-6/group) locomotor activity recorded with telemetric device at the 3. gestational week (3.GW) under different light-dark conditions (LD, LL, DD and 3:21-LD). In each group, the black marks indicate the mean locomotor activity (counts/min) presented in 1-hour average value. Dams maintained under LD (mean counts/day = 3,65 ± 3,61) became active during the dark period, and showed a reduced locomotors activity during the light period. Pregnant rat under DD (mean counts/ day = 3,14 ± 2,21) showed rhythmic behavior, while under LL (mean counts/ day = 2,06 ± 0,68) showed reduced activity with no daily variation. A 3 h light cycle under 3:21 - LD (mean counts/ day = 3,29 ± 1,33) condition induced a ca. 9 h rest period followed the light onset.

As expected, exposure to constant darkness (**DD**) did not affect the circadian locomotor activity, dams remained rhythmic over the entire gestational period (24 h mean of counts/min = *3,65*). While dams exposed to **LL** had reduced daily moving activity at 3.GW with no circadian variation (24 h mean of counts/min = *2,06*). At the 3.GW, the mean daily activity of dams under 3:21 LD schedule showed to be reduced as well (24 h mean of counts/min = *3,29*) with a shortened rest period driven by the light onset. The mean of the daily blood pressure and heart rate at the third week of the pregnancy did not significantly differ between the groups (LD, LL, DD, 3:21 LD).

4.1.3. Circadian variation in renal excretory function during pregnancy

To assess the circadian rhythm of urinary sodium, potassium, calcium and phosphate fractional excretion, urine was collected at 4-hour intervals from freely moving dams housed in metabolic cages over 24 h period (n = 8-12 / 4 h interval over 24 h period). As shown in Table 3, the daily variation of fractional sodium excretion was observed in

Table 3. Daily rhythms of renal excretory function at 3. GW under LD, LL and DD

	LD (n = 8)	LL (n = 12)	DD (n = 8)	2-way ANOVA	
	4-hours mean ± SEM			*Factor A*	*Factor B*
U-Volume (ml)	2,59 ± 0,33 ☒	2,39 ± 0,28 ☒	4,40 ± 0,34 ☒	NS	<0,001
U-Na/U-Crea (mmol/mmol)	21,75 ± 1,22 ☑	19,73 ± 1,04 ☑	21,19 ± 1,24 ☑	<0,001	NS
U-K/U-Crea (mmol/mmol)	39,56 ± 1,35 ☑	38,71 ± 1,16 ☒	32,04 ± 1,38 ☒	0,003	<0,001
U-Ca/U-Crea (mg/mg)	0,52 ± 0,07 ☒	0,42 ± 0,63 ☒	1,30 ± 0,06 ☒	NS	<0,001
U-P/U-Crea (mg/mg)	5,10 ± 0,17 ☑	4,26 ± 1,14 ☑	0,53 ± 0,17 ☒	<0,001	<0,001
Glu/ U-Crea (mg/mg)	0,36 ± 0,03 ☒	0,33 ± 0,02 ☒	0,48 ± 0,03 ☒	NS	<0,001
Albumin (mg/l)	3,27 ± 3,51 ☒	3,76 ± 3,01 ☒	10,71 ± 3,58 ☒	NS	NS
	LD (n = 8)	LL (n = 12)	DD (n = 8)	2-way ANOVA	

2-way ANOVA (Factor A: sampling time, Factor B: Prenatal light condition (LD, LL and DD)
☑ = significant difference, ☒ = no significant variation with respect to the sampling time within the group (LD, LL and DD)

all studied groups (in **LD, LL** and **DD** $p < 0,05$), while urine volume, Ca-, Glucose- and albumin excretion exhibited no daily variation at 3.GW. Potassium and phosphate excretion exhibited significant variation respect to the sampling time in dams kept under normal, **LD** condition (K^+ and P^+ $p < 0,05$). No significant variation in potassium and phosphate urinary excretion was observed in dams under **DD**. Dams under **LL** maintained the significant daily variation in the phosphate excretion ($p < 0,05$), but lost daily rhythm of the potassium. Daily variations of the sodium and potassium excretion under **LD** and **LL** conditions are presented in Figure 15-B/A.

Analysis of the urine samples over 24 h showed that dams at the 3. week of pregnancy under **DD** exhibit an increase in of urinary volume compared with dams under **LD** (DD vs. LD $p < 0,001$) (See Figure 13.) and have a tendency to hyperglycosuria (DD vs. LD $p < 0,001$) and hypercalciuria (DD vs. LD $p < 0,001$), as well as hypophosphaturia (DD vs. LD $p < 0,001$) and decrease in urinary potassium excretion (DD vs. LD $p < 0,001$). There is no significant difference between the groups with respect to sodium and albumin renal excretion. To determine the difference Holm-Sidak method was used followed 2-way ANOVA.

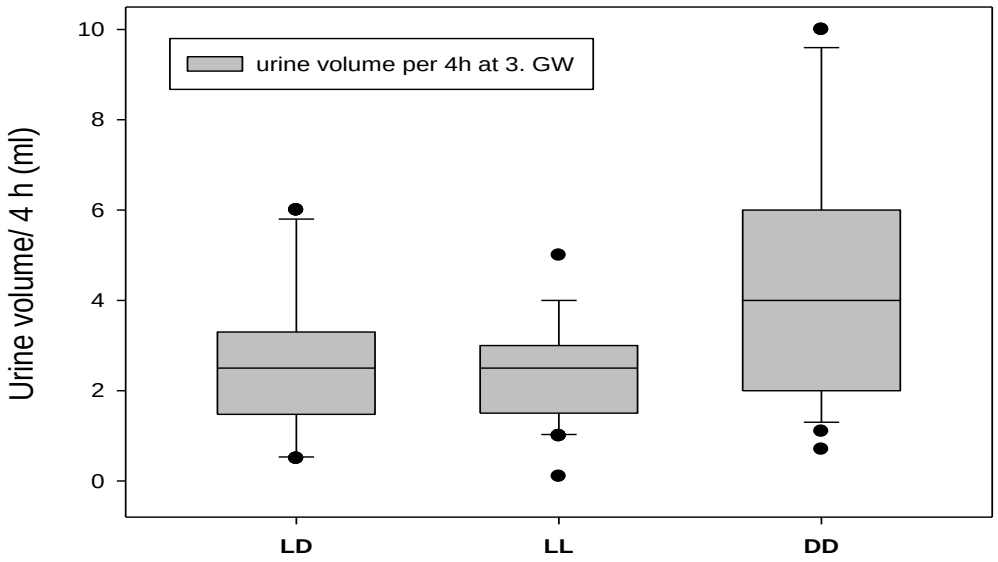


Figure 13. Fractional (sampling time 4 h interval over 24 h period) urine volume of dams at 3.GW under LD, LL and DD (n = 12 / 4h interval over 24 h period). Under DD there is significant difference in urine production compared to control. (DD vs. LD $p < 0,001$) See Table 3.

4.1.4. **Daily urinary aldosterone over the pregnancy**

In group **LD** (n = 8 / 4h over 24 h period) and **LL** (n = 12 / 4h over 24 h period) the urinary aldosterone excretion rate was measured at 4-hour intervals over a 24 h period at the 1. and the 3. gestational week (1.GW and 3.GW). Results are presented in Table 4. There was a significant increase in the urinary aldosterone fractional excretion at 3.GW compared to 1. GW in each investigated group. (3.GW vs. 1.GW in LD $p = 0{,}002$, in LL $p < 0{,}001$). There was no apparent difference in respect to the different light conditions. Significant difference ($p < 0{,}05$) regarding the sampling time was only observed in the LL group at 3.GW. (Figure 14-A)

Table 4. Urinary aldosterone excretion during pregnancy under LD and LL

U-Aldosterone/U- Creatinin (pg/mg) Mean ± SEM						
	LD (n = 8)		LL (n = 12)		3-way ANOVA	
	1.GW	3.GW	1.GW	3.GW		
					factorA / factorB	
24h	731,8 ± 172,0	1461,9 ± 172,0	886,2 ± 109,7	1633,0 ± 96,3	NS	p < 0,001
04:00	867,9 ± 326,4	1598,9 ± 291,9	1309,0 ± 268,6	1196,9 ± 268,6		
08:00	827,8 ± 326,3	720,1 ± 291,9	864,8 ± 257,2	1163,6 ± 268,6		
12:00	794,1 ± 326,3	1873,0 ± 376,8	873,6 ± 268,6	2226,7 ± 181,8		
16:00	498,8 ± 291,9	1375,3 ± 376,8	621,5 ± 296,9	1860,9 ± 247,1		
20:00	729,3 ± 326,3	1419,3 ± 291,9	556,2 ± 268,6	1547,6 ± 257,2		
00:00	731,1 ± 291,9	1914,8 ± 291,9	1062,2 ± 281,7	1114,0 ± 257,2		
	NS	NS	NS	p < 0,05	Sampling time (*factorC)*	
	3-way ANOVA (see factor A, B and C)					

3-way ANOVA (*factor A*: light conditions, factor B: gestational week (GW), factor C: sampling time).

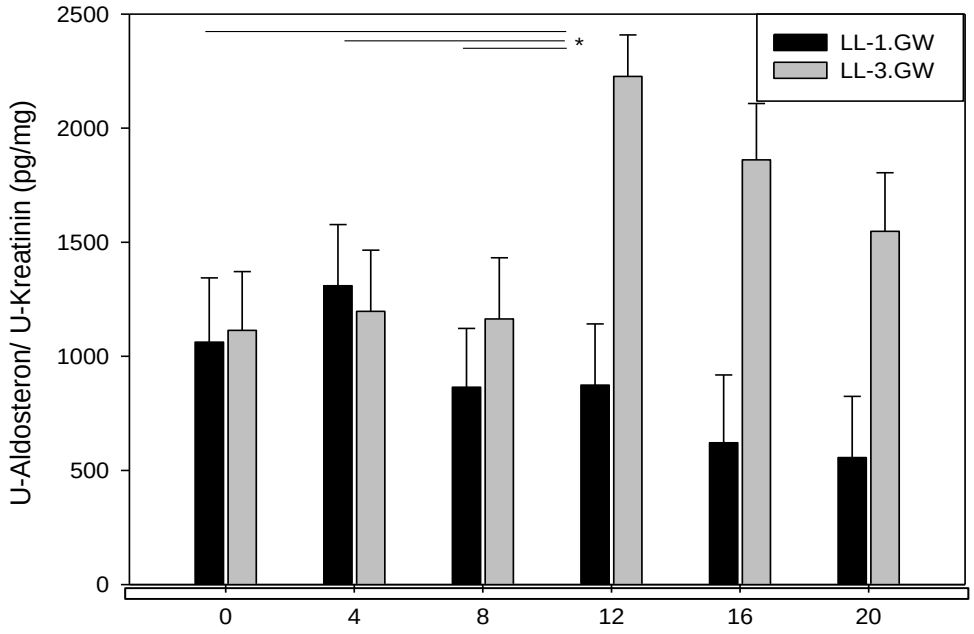


Figure 14-A Daily urinary aldosterone excretion of dams at 1.GW and 3.GW under LL (n = 12 / 4h interval over 24 h period). Just like observed under LD, under LL there is significant difference between 1.GW (marked with gray, 886,2 ± 109,7 pg/mg) and 3.GW (marked with black, 1633,0 ± 96,3 pg/mg) ($p < 0,001$). See Table 4. In LL, at 3.GW there is a significant daily variation in the urinary aldosterone concentration, 12:00 vs. 00:00 (*p = 0,008*), 12:00 vs. 04:00 (*p = 0,024*) and 12:00 vs. 08:00 (*p = 0,018*). Values are means ± SEM.

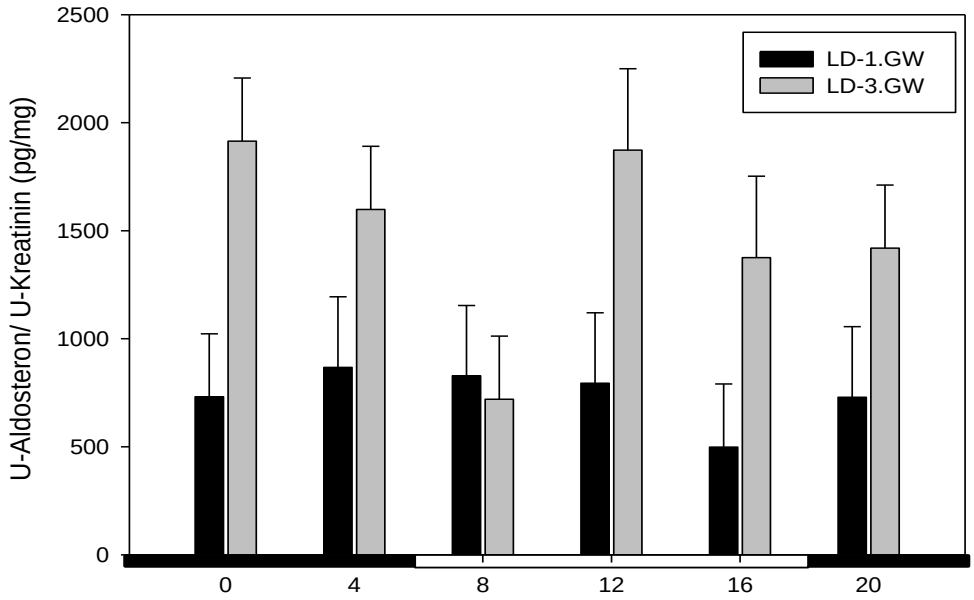


Figure 14-B Daily urinary aldosterone excretion of dams at 1. GW and 3.GW under LD (n=12/4h interval over 24 h period). For detailed description, see Figure 14-A.

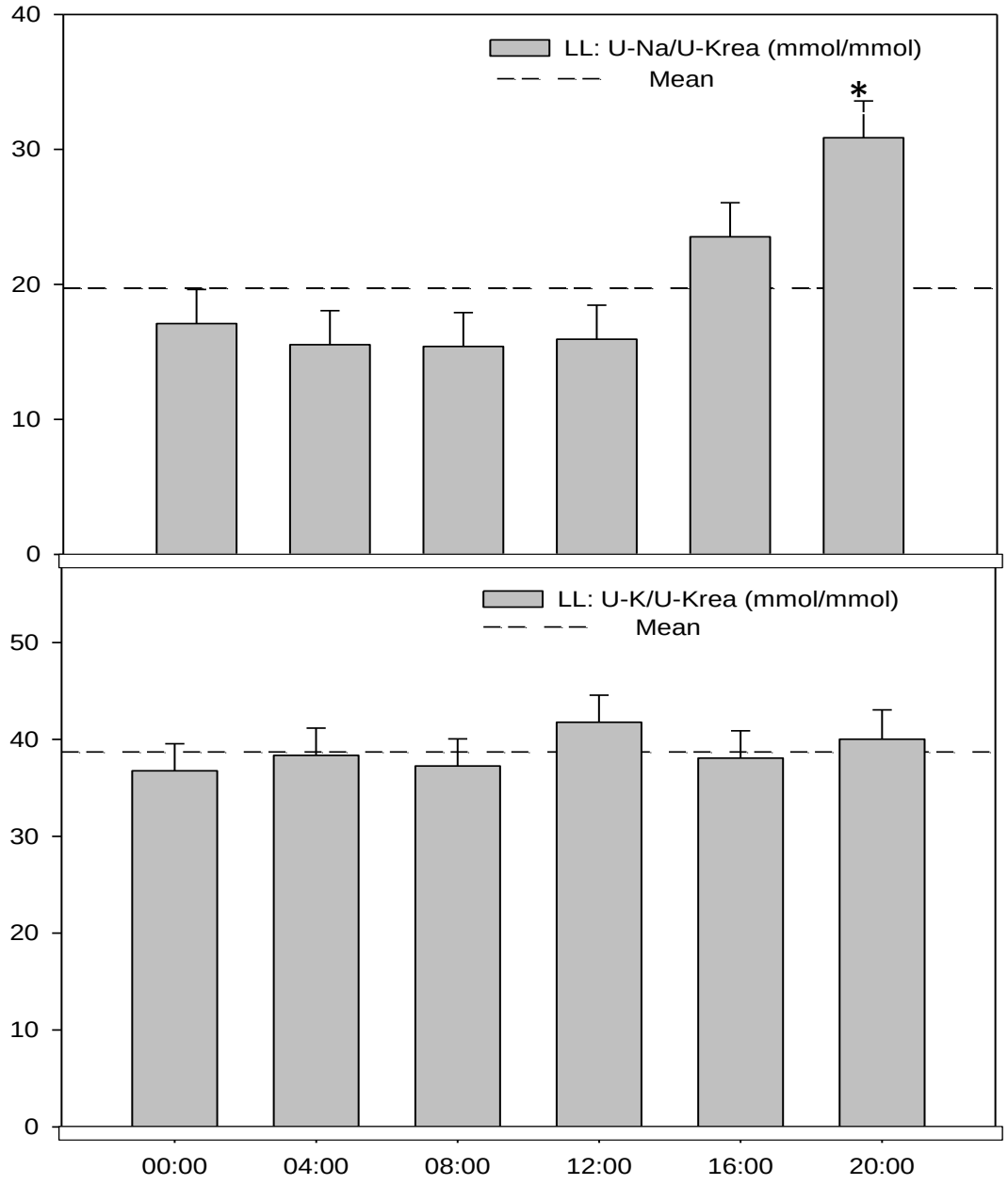


Figure 15-A Daily urinary sodium (U-Na) and potassium (U-K) excretion patterns at 3.GW of freely moving dams (n = 8) fed ab libitum, exposed to constant illumination (LL) during the pregnancy.

With a 2-WAY ANOVA analysis there is significant difference in respect to the sampling time of the sodium excretion, but not of the potassium. With the Holm-Sidak post hoc method, there could be a significant difference observed in the sodium excretion rate between the 20:00 vs. 00:00 ($p = 0{,}040$), 20:00 vs. 04:00 ($p = 0{,}001$), 20:00 vs. 08:00 ($p = 0{,}001$), 20:00 vs. 12:00 ($p = 0{,}002$) time points. Values are given as means ± SEM.

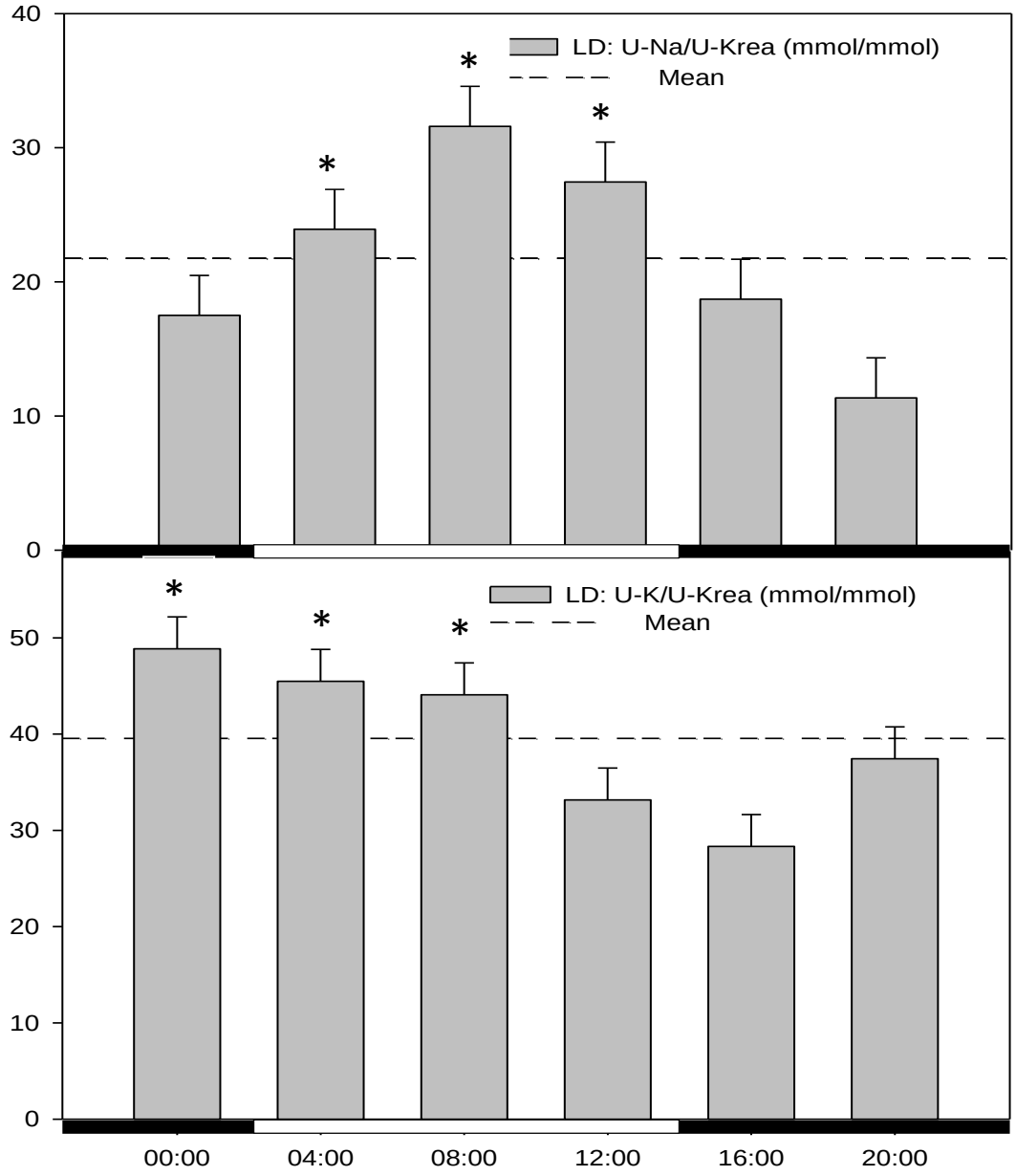


Figure 15-B Daily urinary sodium (U-Na) and potassium (U-K) excretion patterns at 3.GW of freely moving dams (n = 8) fed ab libitum, exposed to normal light-dark cycle (LD) during the pregnancy. With a 2-way ANOVA analysis there is significant difference respect to the sampling time of the sodium ($p < 0,001$) and potassium ($p = 0,003$) excretion. With the Holm-Sidak post hoc method, significant differences could be observed in the sodium excretion rate between the 20:00 vs. 04:00 ($p = 0,040$), 20:00 vs. 08:00 ($p < 0,001$), 20:00 vs. 12:00 ($p = 0,004$), 08:00 vs. 00:00 ($p = 0,016$) and 08:00 vs. 16:00 ($p = 0,035$) time points. Regarding the potassium excretion rate there is a significant difference between 00:00 vs. 12:00 ($p = 0,014$), 00:00 vs. 16:00 ($p < 0,001$), 16:00 vs.04:00 ($p = 0,006$) and 16:00 vs. 08:00 ($p = 0,015$) sampling time points. Values are given as means ± SEM.

4.1.5. Urinary melatonin excretion

The urinary melatonin (**6-SMT**) concentration was determined with a 4-hour interval over a 24 h period at the second gestation week (2.GW) in **LD** (n = 5 / 4h over 24 h period) and **DD** (n = 7 / 4h over 24 h period). The urinary melatonin excretion was calculated with respect to the urinary creatinine concentration (U-Mel/U-Crea). A significantly daily variation was observed in each group respectively to the sampling time (LD p = 0,02; DD p < 0,001). Dams kept under DD showed more robust circadian oscillation of the urinary melatonin excretion, urinary melatonin concentration at 4, 8 and 12 o'clock was significantly higher under DD than under control condition (DD vs. LD respectively p < 0,001, p = 0,005, p = 0,041). (Figure 16)

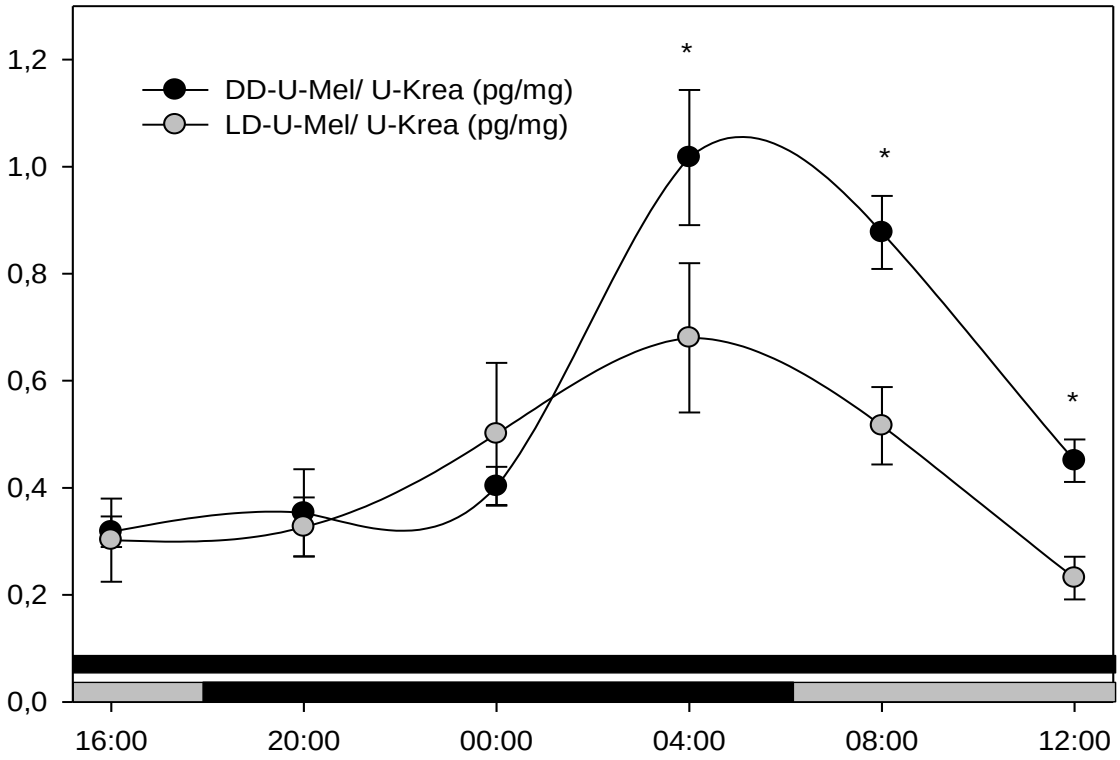


Figure 16. Urinary melatonin (melatonin-sulfat, 6-SMT) excretion at 2.GW under LD (n = 5) and DD (n= 7) conditions. **Under LD, light: ZT0-12= 06:00-18:00.** A significantly daily variation in each group was observed respectively to the sampling time (LD p = 0,02; DD p < 0,001). In the DD group the urinary melatonin concentration was significantly higher than in the LD group at 04:00 (p < 0,001), 08:00 (p = 0,005) and 12:00 (p = 0,041) sampling time points. Values are presented in mean ± SD.

4.2. Effects of prenatal maternal circadian disturbance (Experiment 1)

4.2.1. Intrauterine growth

The body weight and placenta weight of offspring at E20 analyzed with 2-way ANOVA analysis did not differ significantly between the investigated groups considering the light condition during the pregnancy. The body weight of males compared with that of females was significantly different (p = 0,008) at E20 with 2-way ANOVA, however by further analysis, only in the LD group was the difference significantly (**LD**: p = 0,013). (Table 5, Figure 17).

Table 5. Body weight (B-weight) and Placenta weight (P-weight) at E20 (for details see Materials and Methods, Experiment 1)

	E20						
	LD	LL	DD	6:6-LD	3:21-LD	FR-LD	
	MEAN ± SD (g)						*factor B*
B- weight							
Female	2,73 ± 0,06	2,76 ± 0,07	2,74 ± 0,07	2,74 ± 0,07	2,84 ± 0,10	2,95 ± 0,08	NS
Male	2,92 ± 0,05	2,93 ± 0,06	2,84 ± 0,06	2,82 ± 0,07	2,89 ± 0,08	3,05 ± 0,08	NS
factor A (p = 0,008)	p = 0,013	NS	NS	NS	NS	NS	
P-weight							
Female	0,49 ± 0,29	0,55 ± 0,33	0,49 ±0,34	0,53± 0,35	0,55± 0,47	0,49± 0,37	NS
Male	0,51 ± 0,23	0,55 ± 0,31	0,52± 0,34	1,90±0,36	0,53± 0,40	0,51± 0,40	NS
factor A	NS	NS	NS	NS	NS	NS	
2-WAY ANOVA (Factor A: gender and factor B: light condition)							

Body weight of the pups are presented in Figure 17.

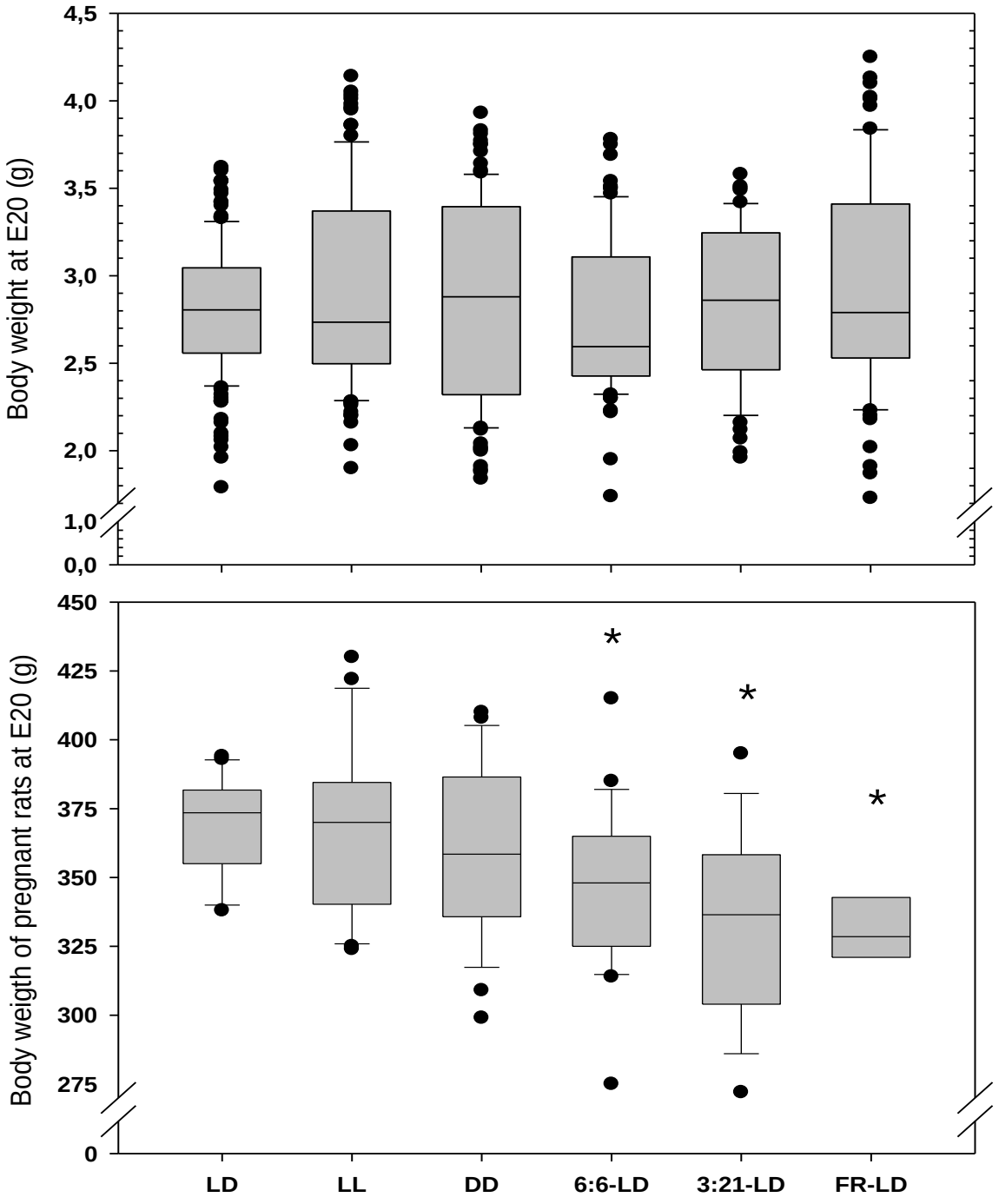


Figure 17. Body weight of pups and their dams at the end of the pregnancy (E20) under different light condition. The body weight at E20 of offspring (male and female) under different light conditions did not differ significantly. (Figure on the top.) The body weight of dams was significantly lower kept under 6:6-LD ($p = 0,022$), 3:21-LD ($p < 0,001$) light condition and dams exposed to restricted feeding regime under normal light-dark cycle (FR-LD, $p = 0,016$) compared to the control group, to the LD. (Figure below.)

4.2.2. Renal circadian gene variation at birth

LD: in offspring's kidney (male) at embryonic day 20 (E20) under **LD**, 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 (Table 6; Figs. 18-19 and Figs. 20A-22A). The peak of the oscillations was observed during the dark period (ZT15-19h). The mean amplitude of the oscillation at day E20 (mean 2A = 53 ± 22%) was smaller than later in life (Fig.23). The expression of Bmal1, Cry1, Cry2 and Per1 showed no significant variation. (Table 6)

FR-LD: renal clock gene expression of fetuses (male) from mothers exposed to food restriction (food available ZT0-12) during the entire pregnancy under LD showed altered daily profile of the investigated RNA expressions (*Cry2* was not studied in this group): the circadian expression of *Clock, Rev-erbα* and *AVPR2* were lost compare to LD, while the daily expression of *Per2* (*p < 0,001*), as well as of the clock-controlled genes *αENaC* (*p < 0,001*), *SGK1* (*p = 0,024*) and *NHE3* (*p < 0,001*) remained rhythmic. The peak for the *Per2* oscillations was observed at the beginning of the dark period (ZT14), for the renal clock-controlled gene at the end of the dark period (ZT22-24 h). The mean amplitude (mean 2A = 50 ± 15%) did not differ compared with the LD group. (Table 6; Figs. 18-19)

LL: among the renal core clock genes not only *Clock* (*p = 0,004*), *Per2* (*p < 0,001*) and *Rev-erbα* (*p = 0,027*), but *Cry2* (*p = 0,018*) and *Per1* (*p = 0,013*) showed circadian oscillation in fetuses (male) at E20 with perinatal constant light exposure **(LL)**. All investigated clock-controlled genes showed to be rhythmic *αENaC* (*p < 0,001*), *SGK1* (*p < 0,001*), *NHE3* (*p < 0,001*) and *AVPR2* (*p = 0,022*). (Table 6; Figs. 18-19). Under the constant light exposure, the peak of the circadian oscillations was observed at ZT12-17h. The amplitude of the rhythms was higher (mean 2A = 73 ± 25%) than in the group under LD. The expression of *Bmal1, Cry1* showed no significant variation. (Table 6; Figs. 18-19)

DD: there is no circadian rhythm of the investigated genes in the kidney of fetuses (male) at E20 from mothers that were kept under constants dark condition (**DD**). (Table 6; Figs. 18-19)

6:6-LD: the expression of clock gene, i.e. *Bmal1, Clock, Cry1, Per1, Per2* and *Rev-erbα,* as well as *αENaC, NHE3* and *AVPR2* from the clock-controlled genes showed no significant variation in the (male) fetal kidney at E20 from mother exposed to ultradian light-dark condition **(6:6-LD)** during the entire pregnancy (*Cry2* was not studied in this group). Only *SGK1* showed circadian oscillation ($p = 0,031$, mean 2A = 45%, acrophase at ZT20). (Table 6; Figs. 18-19)

3:21-LD: under 3:21 LD condition there is a significant circadian oscillation of the clock genes *Clock* ($p = 0,018$) and *Bmal1* ($p < 0,001$), as well as the clock-controlled genes *αENaC* ($p = 0,002$), *SGK1* ($p = 0,01$), *NHE3* ($p < 0,001$) and *AVPR2* ($p < 0,001$) in (male) fetuses at E20. The circadian oscillation of *Per2* and *Rev-erbα* was lost, the expression of *Cry1* and *Per1* showed no significant variation (*Cry2* was not studied in this group). The peak of the oscillations was observed during late dark period (ZT19-21h, ZT0 = 09:00 a.m.). The mean amplitude of the oscillation (mean 2A = 61 ± 21%) was higher than in LD. (Table 6; Figs. 18-19)

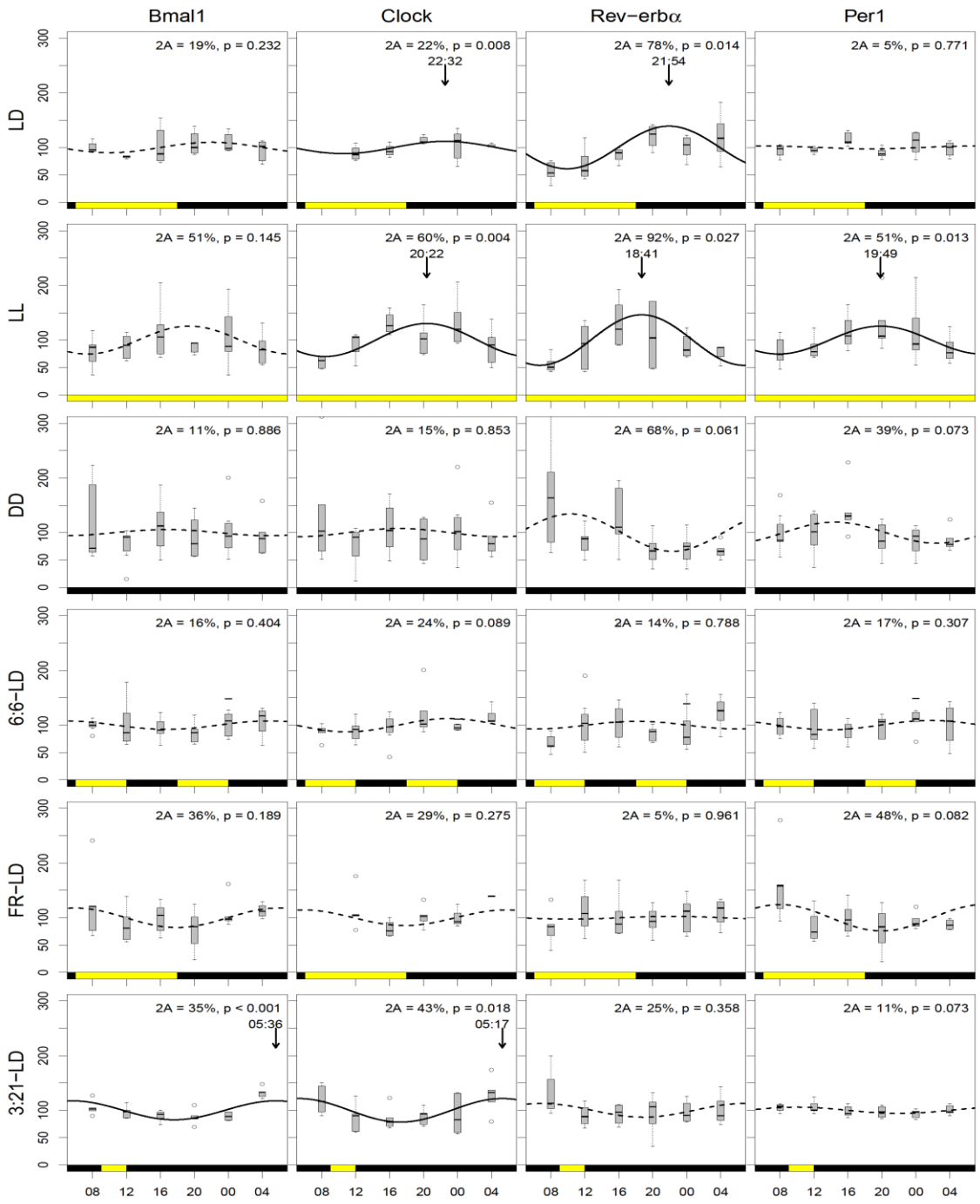


Figure 18. Daily pattern of renal clock gene, i.e. Bmal1, Clock, Rev-erbα and Per1 expression in fetuses at E20 from mother kept under different light-dark condition (LD, LL, DD, 6:6-LD, 3.21-LD) or under restricted feeding schedule (FR-LD) during the entire pregnancy. Real time PCR assay units expressed as % mean in 4-hour interval (mean ± SD) for comparison (n = 7 male fetuses / 4 h intervals over a 24 period in each group). For time effect, p values from ANOVA and analysis by the single cosinor procedure (best-fitting of a 24h cosine way to all data by least-squares linear regression, see cosine line, dotted when not significant), as well as the double amplitudes (mean 2A) and the acrophases (the ↓ arrows show the acrophases, ϕ) are listed.

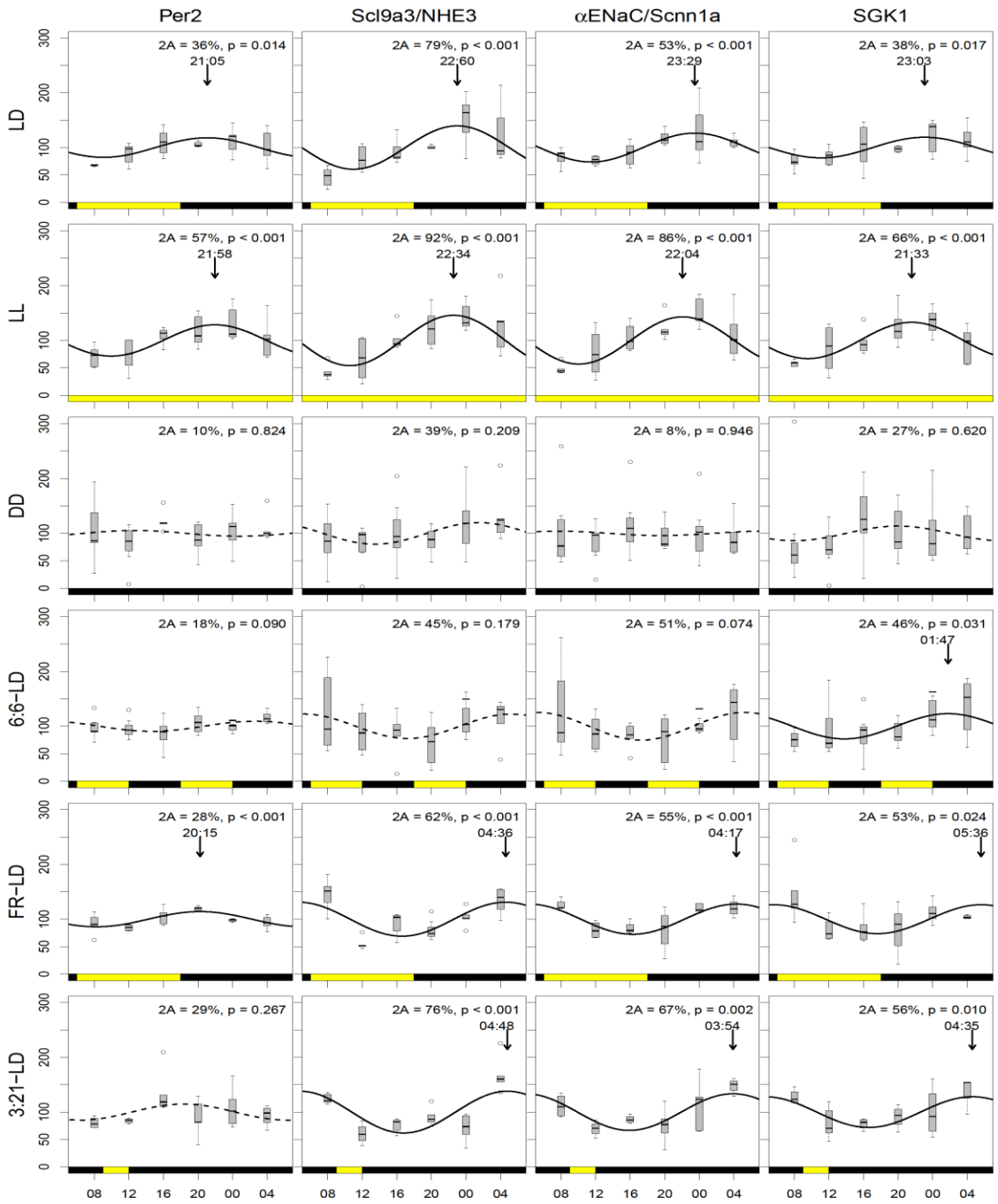


Figure 19. Daily pattern of renal clock and clock-controlled gene, i.e Per2, NHE3, αENaC, SGK1 expression in fetuses at E20 from mother kept under different light-dark condition (LD, LL, DD, 6:6-LD, 3.21-LD) or under restricted feeding schedule (FR-LD) during the entire pregnancy. See legend to Figure 18 for details.

Table 6. Cosinor analysis of renal circadian clock and clock-controlled gene expression at age E20 (n = 6-12 dams / group).

	LD-E20 (ZT0=6:00)			LL-E20 (ZT0=6:00)			DD-E20 (ZT0=6:00)			6:6-LD-E20 (ZT0=6:00)			FR-LD-E20 (ZT0=6:00)			3:21-LD-E20 (ZT0=9:00)		
	2A (%) / ϕ(ZT) / p			2A (%) / ϕ(ZT) / p			2A (%) / ϕ(ZT) / p			2A (%) / ϕ(ZT) / p			2A (%) / ϕ(ZT) / p			2A (%) / ϕ(ZT) / p		
Bmal1	19	16:03	NS	51	13:24	NS	11	22:43	NS	16	22:47	NS	36	23:57	NS	35	20:36	< 0,001
Clock	22	16:32	0,008	60	14:22	0,004	15	23:04	NS	24	17:00	NS	29	23:45	NS	43	20:17	0,018
Cry1	12	18:42	NS	26	14:34	NS	13	23:18	NS	9	18:11	NS	11	22:51	NS	10	15:08	NS
Cry2	27	13:31	NS	38	14:17	0,018	14	13:52	NS	-	-	-	-	-	-	-	-	-
Per1	5	00:40	NS	51	13:49	0,013	39	08:25	NS	17	19:43	NS	48	13:57	NS	11	13:01	NS
Per2	36	15:05	0,014	57	15:58	< 0,001	10	18:43	NS	18	20:39	NS	28	14:15	< 0,001	29	09:20	NS
Rev-erbα	78	15:54	0,014	92	12:41	0,027	68	04:19	NS	14	12:07	NS	5	16:16	NS	25	09:52	NS
αENaC	53	17:29	< 0,001	86	16:04	< 0,001	8	13:37	NS	51	23:15	NS	55	22:17	< 0,001	67	18:54	0,002
SGK1	38	17:03	0,017	66	15:33	< 0,001	27	13:58	NS	46	19:47	0,031	53	23:36	0,024	56	19:35	0,01
NHE3	79	17:00	< 0,001	92	16:34	< 0,001	39	19:28	NS	45	23:16	NS	62	22:36	< 0,001	76	19:48	< 0,001
AVPR2	66	19:08	0,003	119	14:33	0,022	20	15:34	NS	58	23:41	NS	54	22:14	0,21	91	20:19	< 0,001

Note — Two anesthetized pregnant rats with their fetuses (7-14 pups/mother) were sacrificed at 4-hour intervals to cover one entire circadian cycle at E20 in each investigated group (LD, LL, DD, 6:6-LD, FR-LD, 3:21-LD). Clock and clock controlled genes in the kidney of the male fetuses were further investigated. Circadian rhythms were analyzed by the single cosinor procedure (fit of a 24h cosine way to all data by least-squares linear regression). P signifies significance level of circadian rhythmicity. 2A: double amplitude expressed as % of 24-hour mean (mesor). ϕ: Acrophase (time of cosine wave peak), expressed as clock time or Zeitgeber time (hours after light onset, ZT0: 6:00).

4.3. Postnatal changes (Experiment 2)

4.3.1. Expression pattern circadian genes under LD photoperiod

In male offspring, at 1-week postpartum significant circadian oscillations were observed for all investigated 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 = 0,004), NHE3 (p < 0,001) and AVPR2 (p < 0,001)*] (Table 7 and Figs. 20B-22B). The amplitudes of rhythmic expression (mean 2A = 117 ± 25%) showed more than doubled compared to the mean amplitudes at E20. A phase shift from nighttime to early daytime (ZT2-ZT6) was observed for all genes except *Rev-erbα*, which peaked at nighttime (ZT18) (Table 7; Fig. 23).

At 4 weeks the circadian variation 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 = 0,010*) showed significant variation with time (Table 7; Figs. 20C-22C). The maximal expression of *NHE3* was observed further on in the light period (ZT9) and *Bmal1* peaked at the beginning of the light phase (ZT0). While the oscillation phase shifted back from day- to nighttime for *Per1, Per2, Cry1, αENaC* and *SGK1* (ZT14-19), whereas the phase of *Rev-erbα* was inverted from nighttime to daytime (ZT6). (Table 7; Figure 23). The amplitudes of the circadian expression of *Rev-erbα, Bmal1* and *Cry1* increased further (mean 2A = 167 ± 71%), whereas the amplitudes of the rhythmic oscillations of *Per1, Per2, αENaC, SGK1* and *NHE3* were reduced (mean 2A = 76 ± 18%, Fig. 23).

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*) (Table 7; Figs. 20D-22D). Compared to age 4 weeks the amplitudes of most investigated genes increased further on (Fig. 23). The phase was unchanged for *Rev-erbα* and *Cry1* (acrophase differences < 30 min), delayed by 1-2 hours for *Cry2, Per2* and *SGK1*, and advanced by 1-3h for *Bmal1* and *Per1*. (Table 7; Fig. 23)

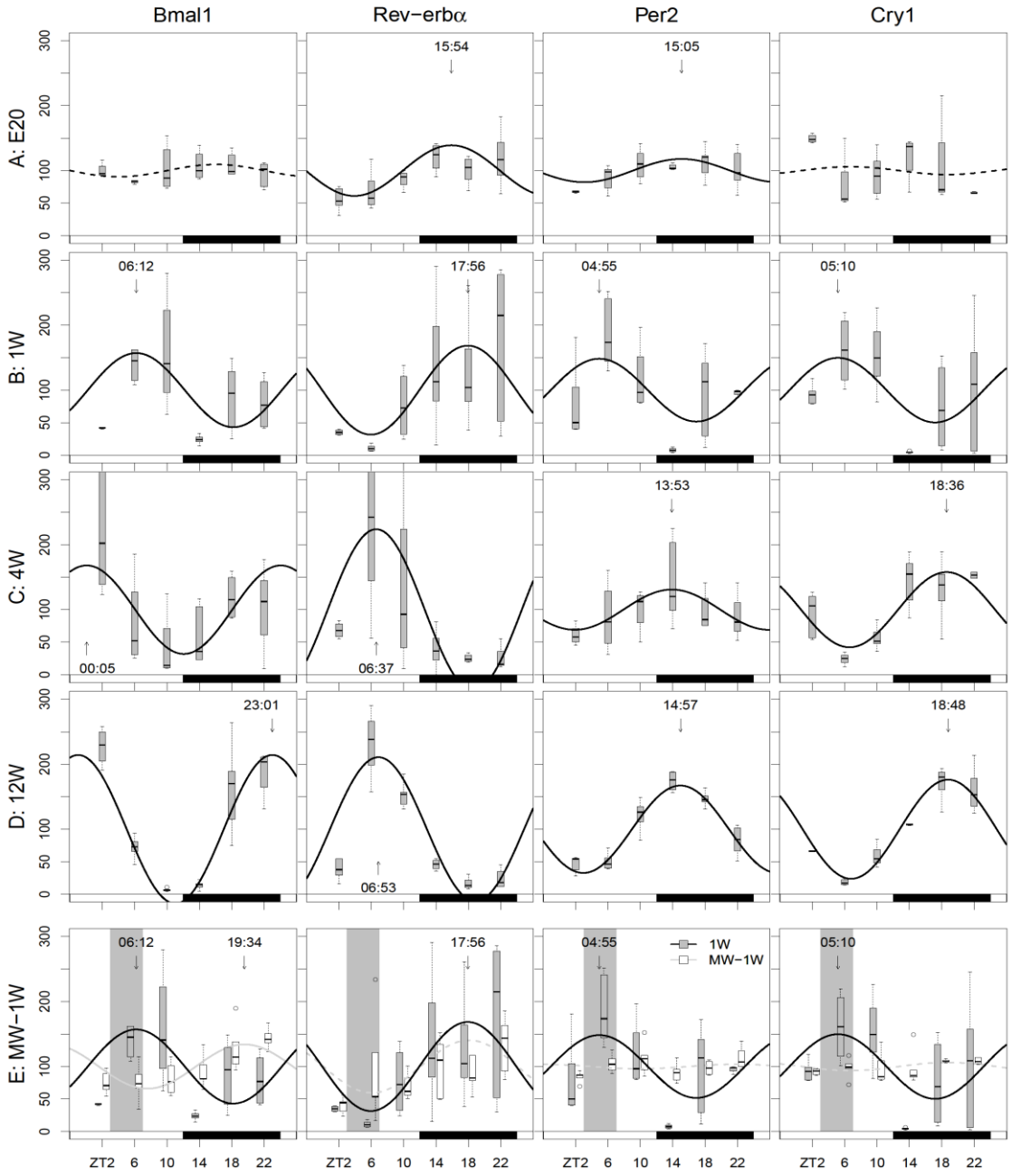


Figure 20. Postnatal development of clock gene expression in the kidney (A-D)
Male offspring (n = 7 / 4 h over a 24 period) were sacrificed every 4 hours (ZT4-24; ZT0 at 06:00 a.m.). Daily expression pattern of clock gene in the kidney was measured by rt-PCR (units expressed as % mean in 4-hour interval (mean ± SD)) at embryonic day 20 (E20), postnatal week 1 (1W), 4 (4W) and 12 (12W). The arrows show the acrophases (the ↓ arrows show the acrophases, ϕ, calculated by best-fitting of a 24h cosine way analysis, see cosine line, dotted when not significant).

Bottom panel (E): Circadian renal clock gene expression of 1-week-old pups (n = 7 / 4 h over a 24 period) after 7 days of daily absence of the mother (MA-1W) for 4 hours (ZT3–7).

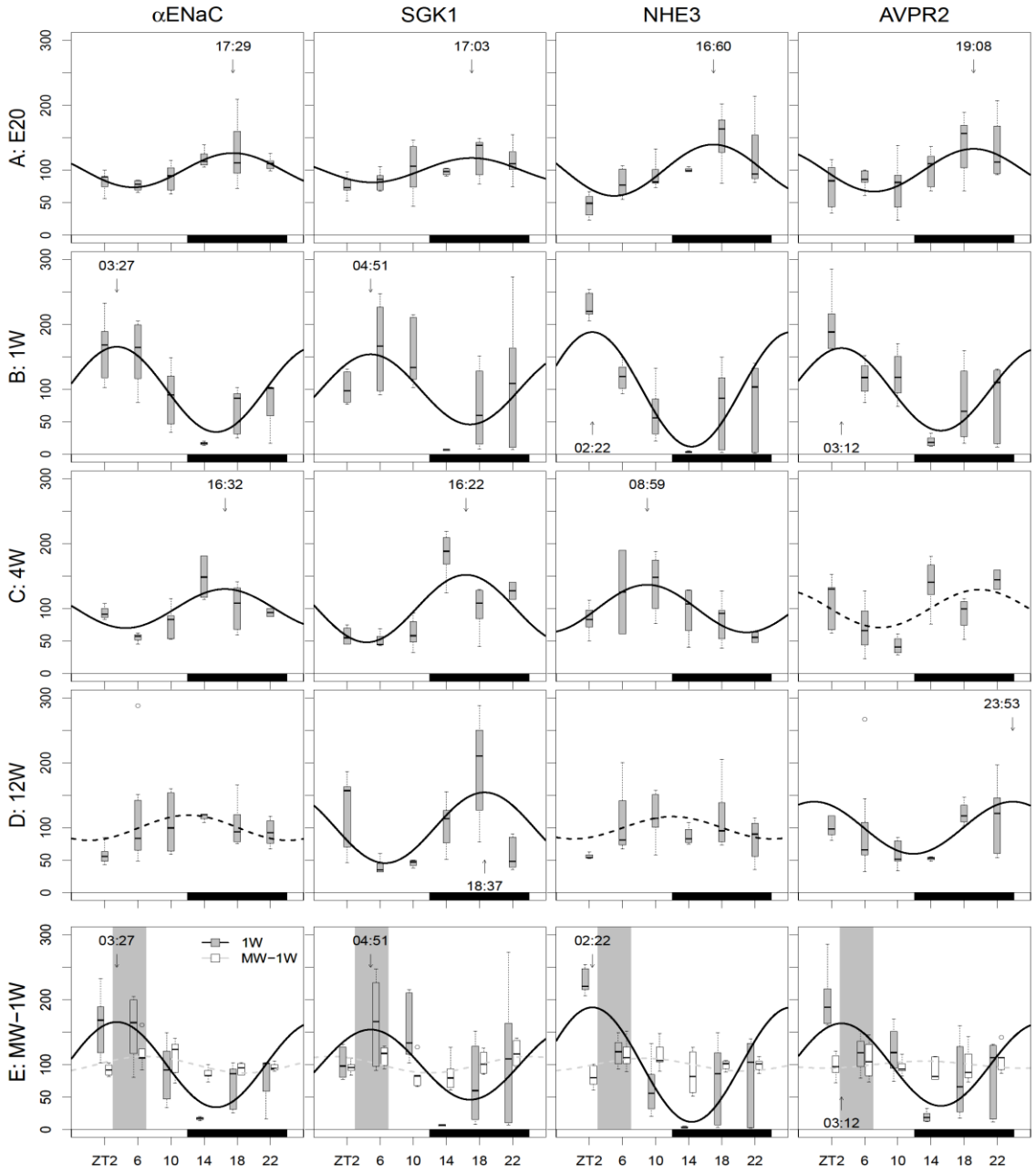


Figure 21. Postnatal development of clock controlled gene expression in the kidney (A-D) Male offspring (n = 7 / 4 h over a 24 period) were sacrificed every 4 hours (ZT4-24; ZT0 at 06:00 a.m.). Daily expression pattern of clock-controlled gene in the kidney was measured by rt-PCR (units expressed as % mean in 4-hour interval (mean ± SD)) at embryonic day 20 (E20), postnatal week 1 (1W), 4 (4W) and 12 (12W). The arrows show the acrophases (the ↓ arrows show the acrophases, ϕ, calculated by best-fitting of a 24h cosine way analysis, see cosine line, dotted when not significant).

Bottom panel (E): Circadian renal clock gene expression of 1-week-old pups (n= 7/4 h over a 24 period) after 7 days of daily absence of the mother (MA-1W) for 4 hours (ZT3–7).

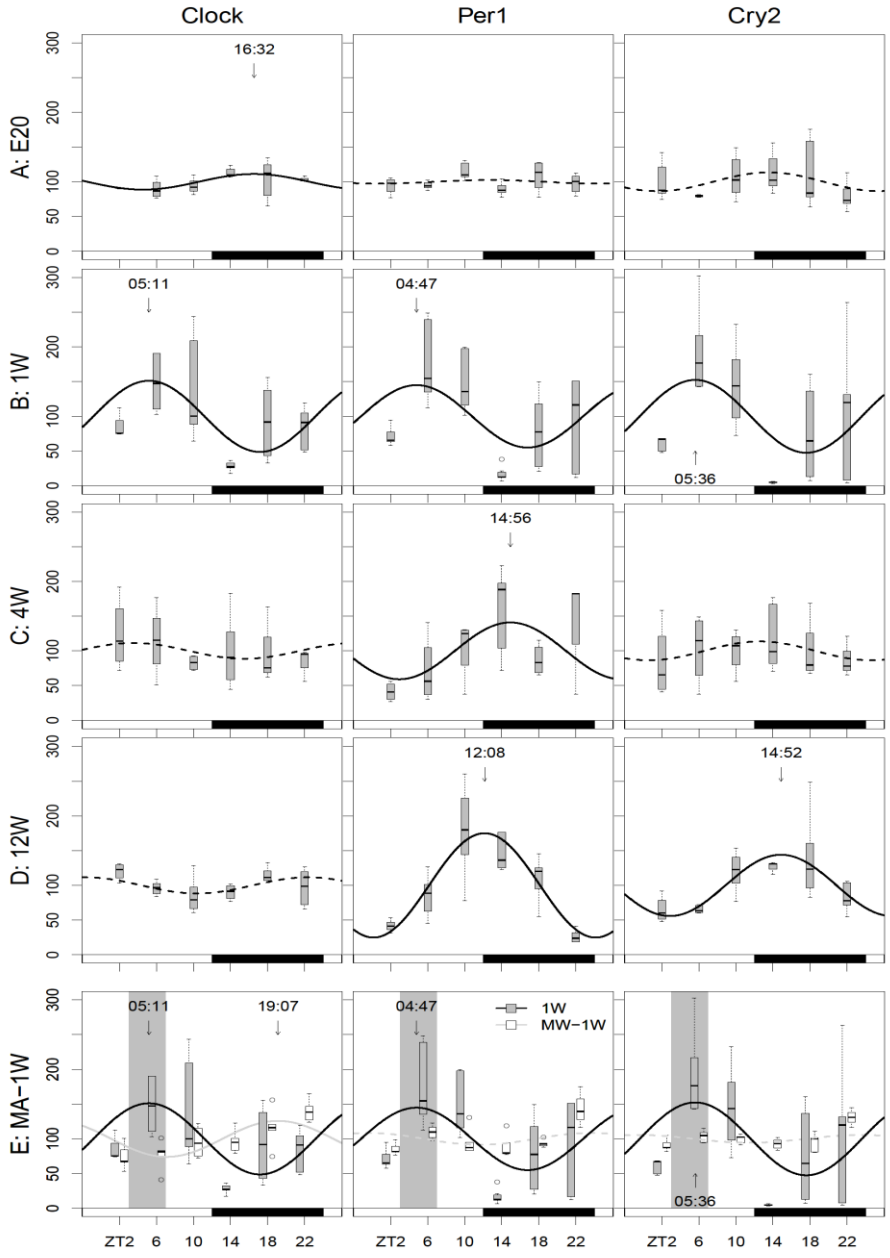


Figure 22. Postnatal development of clock gene expression in the kidney (A-D)
Male offspring (n = 7 / 4 h over a 24 period) were sacrificed every 4 hours (ZT4-24; ZT0 at 06:00 a.m.). Daily expression pattern of clock gene in the kidney was measured by rt-PCR (units expressed as % mean in 4-hour interval (mean ± SD)) at embryonic day 20 (E20), postnatal week 1 (1W), 4 (4W) and 12 (12W). The arrows show the acrophases (the ↓ arrows show the acrophases, ϕ, calculated by best-fitting of a 24h cosine way analysis, see cosine line, dotted when not significant).

Bottom panel (E): Circadian renal clock gene expression of 1-week-old pups (n = 7 / 4 h over a 24 period) after 7 days of daily absence of the mother (MA-1W) for 4 hours (ZT3–7).

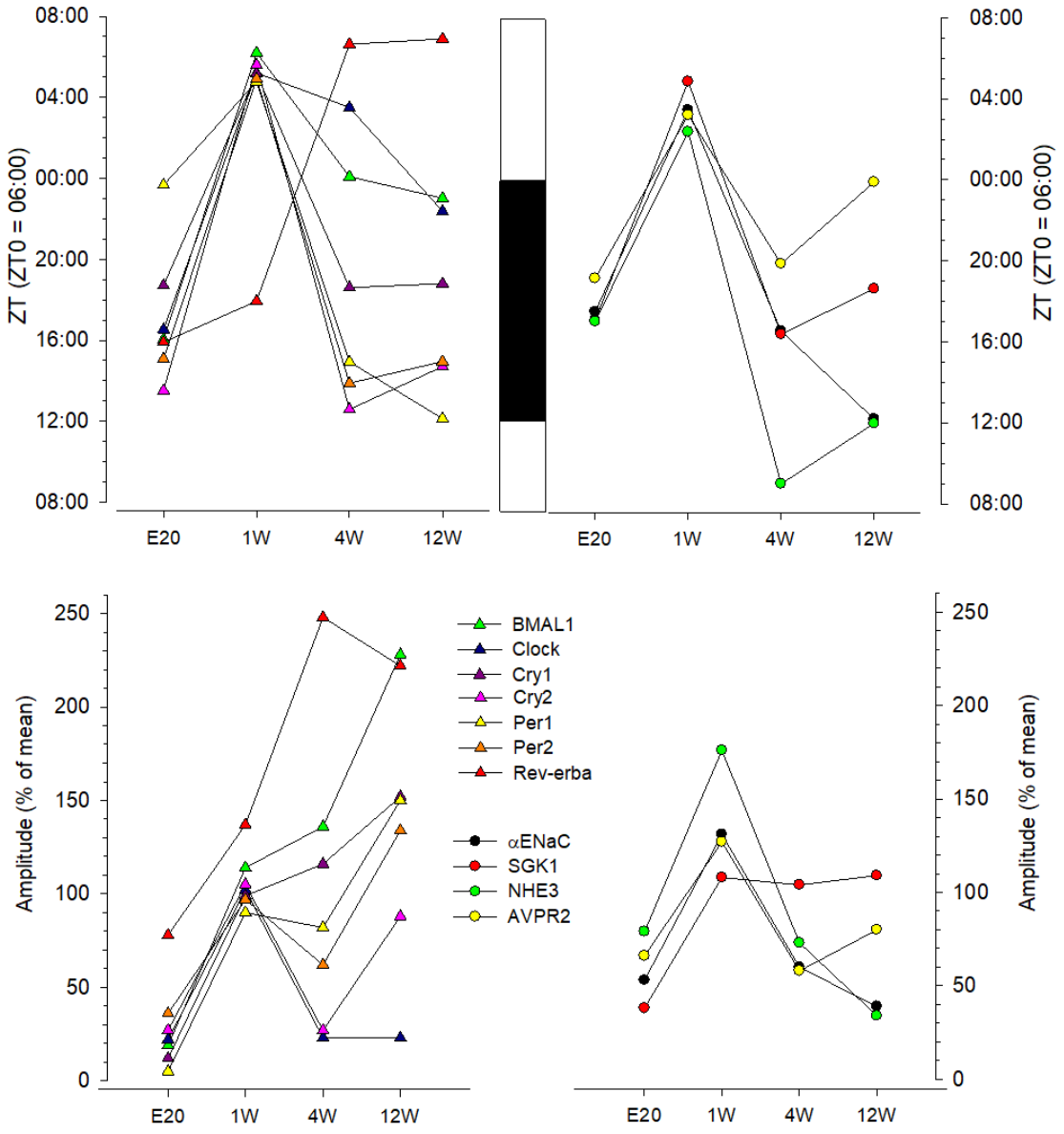
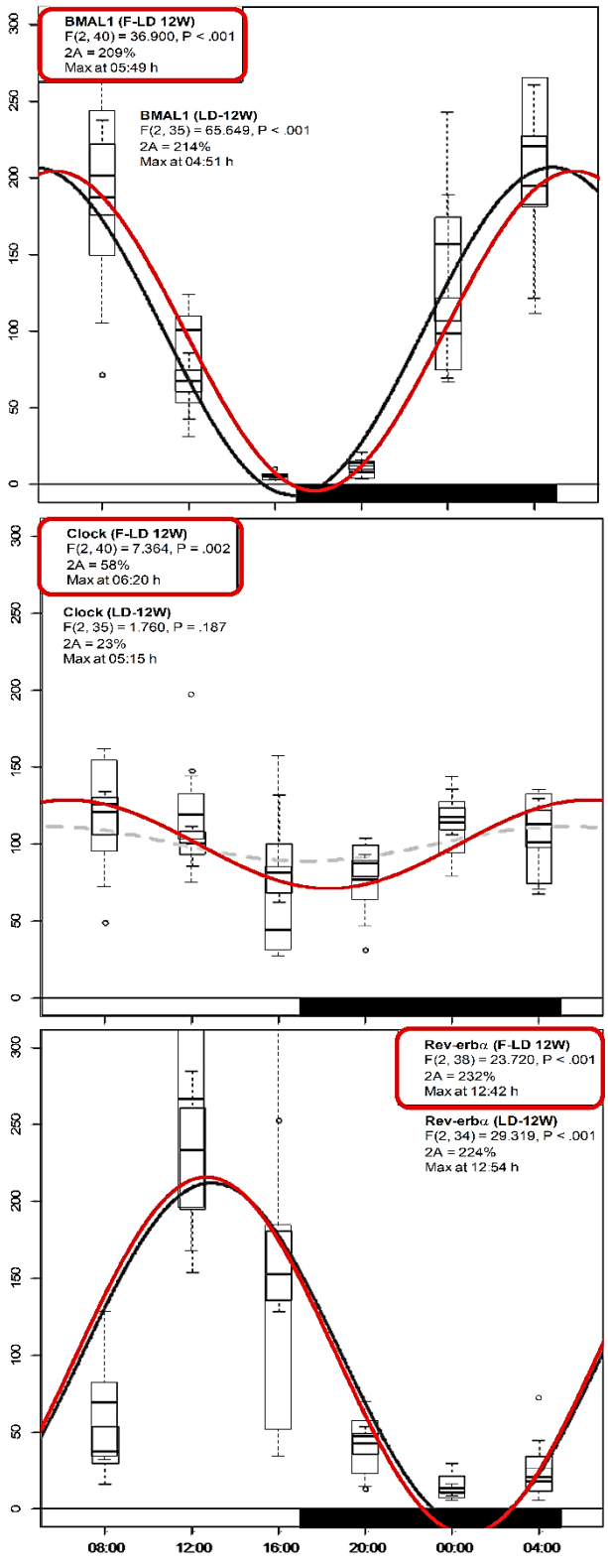


Figure 23. Changes in acrophase (figure on the top) and amplitude (figure below) of renal circadian gene expression during postnatal development. Clock genes are plotted on the left, clock-controlled genes on the right. 2A: double amplitude expressed as % of 24-hour mean (mesor). Acrophase (ϕ) is the time of cosine wave peak. ZT= Zeitgeber time, ZT0 is light onset, at 06:00 a.m. E20: embryonic day 20; 1W, 4W, 12W: postnatal age 1, 4 and 12 weeks.



To assess gender related differences in the renal clock rhythms we determined the daily gene expression patterns of *Bmal1, Clock and Rev-erbα* in females at 12W under control condition (LD) (n = 7 / 4 h over a 24 period) to compare to the expression pattern in males. The daily renal expression pattern of *Bmal1* ($p < 0,001$, 2A = 209%, ϕ = 05:49h (ZT)) and *Rev-erbα* ($p < 0,001$, 2A = 233%, ϕ = 12:42h (ZT)) in females showed the similar pattern as were observed in males, while the expression of *Clock* ($p = 0,02$, 2A = 58%, ϕ = 06:20h (ZT)) gene with very low amplitude has a circadian variation in females but not in males. See Figure 24.

← **Figure 24. Renal expression patterns of *Bmal1*, *Clock* and *Rev-erbα* genes in females vs. males at 12W**
Male and female offspring were sacrificed every 4 hours (n = 7 / 4 h over a 24 period). Daily expression pattern of clock gene in the kidney was measured by rt-PCR (units expressed as % mean in 4-hour interval (mean ± SD)) at 12 weeks of age. Acrophases (= max at, ϕ), calculated by best-fitting of a 24h cosine way analysis, see cosine line, dotted when not significant, females are marked with red, males with black. P values, double amplitude (2A) and acrophases (peak time) are listed in legends.

Table 7. Cosinor analysis of renal circadian gene expression under LD in rat at age E20, 1W, 4W and 12W (n = 7 / 4h).

	LD											
	E20			1W			4W			12W		
	2A (%)	ϕ(ZT)	p	2A (%)	ϕ(ZT)	p	2A (%)	ϕ(ZT)	p	2A (%)	ϕ(ZT)	p
Bmal1	19	16:03	NS	114	6:12	0,021	136	0:05	0,012	228	23:01	< 0,001
Clock	22	16:32	0,008	102	5:12	0,009	23	3:30	NS	23	22:22	NS
Cry1	12	18:42	NS	99	5:10	0,008	116	18:36	< 0,001	152	18:48	< 0,001
Cry2	27	13:31	NS	105	5:36	0,013	27	12:35	NS	88	14:52	< 0,001
Per1	5	00:40	NS	90	4:47	0,038	82	14:56	0,039	150	12:08	< 0,001
Per2	36	15:05	0,014	97	4:55	0,012	62	13:53	0,044	134	14:57	< 0,001
Rev-erbα	78	15:54	0,014	137	17:56	0,006	248	6:27	0,014	222	6:53	< 0,001
αENaC	53	17:29	< 0,001	131	3:27	< 0,001	60	16:32	0,010	39	12:08	NS
SGK1	38	17:03	0,017	108	4:51	0,004	104	16:22	< 0,001	109	18:37	0,004
NHE3	79	17:00	< 0,001	176	2:22	< 0,001	73	8:59	0,010	34	12:03	NS
AVPR2	66	19:08	0,003	127	3:12	< 0,001	58	19:51	NS	80	23:53	0,046

Note — Male offspring (n = 7) at 1W, 4W, 12W were sacrificed every 4 hours to cover one entire circadian cycle. Circadian rhythms were analyzed by the single cosinor procedure (fit of a 24h cosine way to all data by least-squares linear regression). P signifies significance level of circadian rhythmicity. 2A: double amplitude expressed as % of 24-hour mean (mesor). ϕ: Acrophase (time of cosine wave peak), expressed as clock time or Zeitgeber time (hours after light onset, ZT0: 6:00).

4.3.2. Postnatal behavioral changes

The relationship between feeding time and activity level and oscillatory gene expression is depicted in Figure 25. The 1-week-old offspring were fed mostly at daytime by breast feeding during the rest-period of the dams. The oscillation peaks of all studied genes (except *Rev-erbα*) were observed during this period (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 pattern 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). (Table 7; Figure 25)

At the 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 peaked during the dark period (ZT12-24), except *Rev-erbα* (ZT6), *Bmal1* (ZT0) and *NHE3* (ZT9). Feeding behavior remained constant from 4 to 12 weeks of age, whereas the activity maximum 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), *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 of the maximum feeding pattern (ZT19). (Table 7; Figure 25)

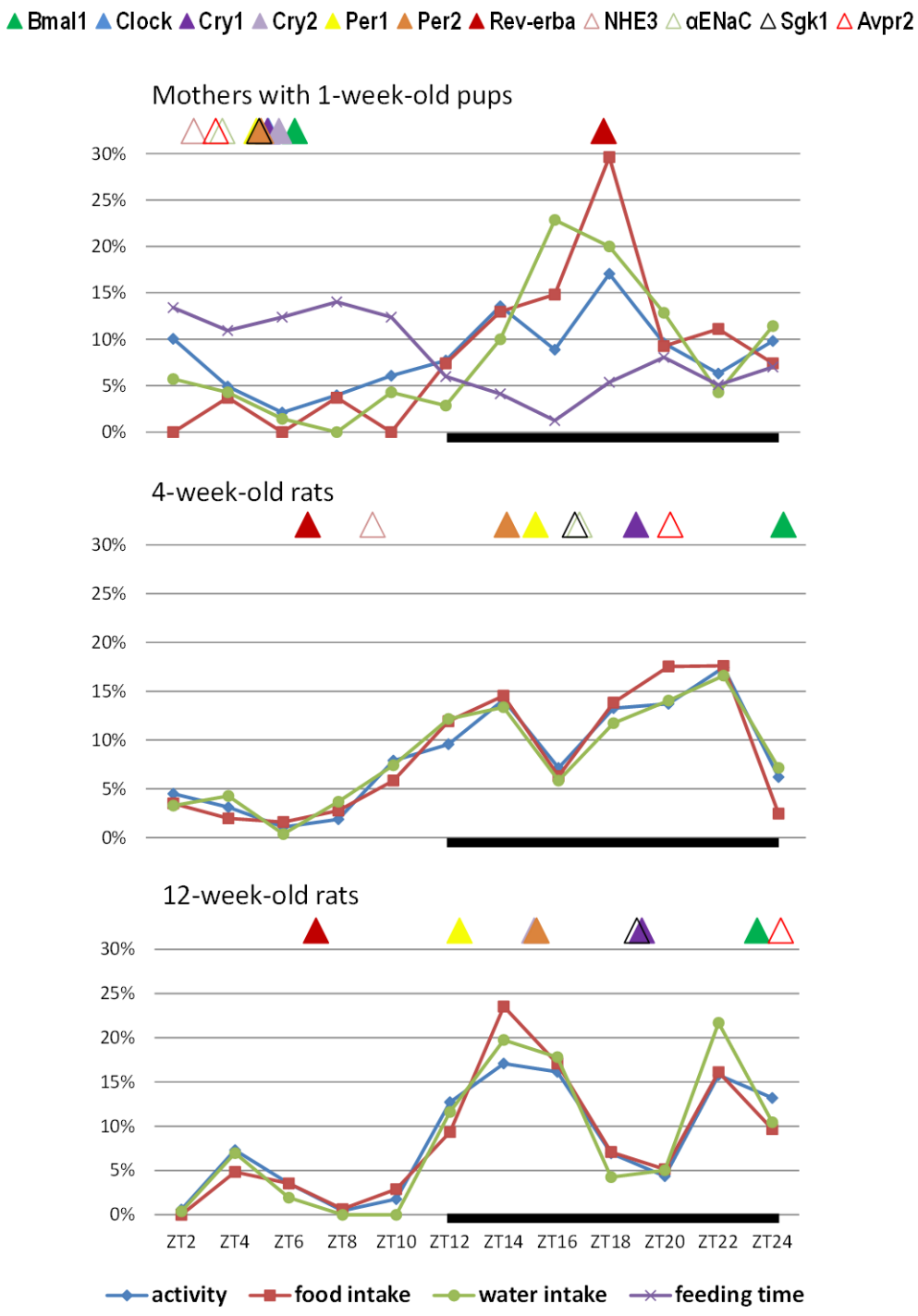


Figure 25. Patterns of daily locomotor activity, food and water intake and maternal feeding time (shown in 2 h interval as % of daily total, ZT0 is light onset (at 06:00 a.m.). The timing of maternal feeding showed a diurnal pattern: 1-week-old pups are fed during the rest period of the mother (light period). While the mothers and the rats at 4, 12-weeks-old were active during the dark period, food and water intake occurred mostly during the night. Triangles indicate acrophases of circadian gene expression.

4.3.3. The effects of timed maternal melatonin treatment

To assess whether maternal melatonin (i.e. through the breast milk) might have an effect as a Zeitgeber to the renal peripheral clock in the offspring, dams kept under normal light-dark condition (LD) were treated with exogenous melatonin or its vehicles (Me: 4,8 mg/kg ip. or 6% ethanol, at ZT3) over a week period starting directly after the delivery.

[To address the gene related differences at 1 week of age we used only females in this experiment to compare with the control group (1 week of age males with intrauterine LD condition). Results presented in Table 8.]

The renal circadian clockwork in the offspring (only in females, n = 5-7 / timepoints) was studied at age 1-week-old. As shown in Table 8, in females (**1W**♀) there is circadian variation of *Bmal1*, *Clock, Cry2, Per1*, *Per2, Rev-erbα,* as well as of *αENaC*, *SGK1* and *NHE3*-RNA expression (mean 2A = 61 ± 21%, acrophases ZT22-ZT3, for Per2 at ZT13). There is no circadian oscillation in *Cry1* and *AVPR2* expression compared with male (mean 2A = 117 ± 25%, mean acrophase ZT2-ZT6, for *Rev-erbα* at ZT18). After the melatonin treatment (**ME-1W**♀) of the mother a phase advance (ca. 4-8 h) of the *Bmal1, Clock, Cry2, Per1, αENaC* and *AVPR2* circadian expression was observed (acrophase ZT16-ZT19). The circadian expression of *Per2, Rev-erbα, SGK1* and *NHE3* were lost, while the *Cry1* expression showed circadian rhythm after timed melatonin treatment ($p < 0,001$, acrophase ZT18, mean 2A = 63%). We observed circadian alteration in the vehicle treated group (**Vech-1W**♀) as well: a phase delay for *Cry2, Per1, αENaC, NHE3* and *AVPR2* (ca. 4-11 h) and phase advance for *Per2* circadian expression observed (mean 2 A = 66,5 ± 40%) compared with the control female group. (Table 8) The Body weight of female pups which mothers were treated with melatonin was significantly higher compared to vehicle treated females (16,96 ± 0,30 g vs. 15,68 ± 0,31 g; $p = 0,022$) or non - treated parallel observed females (BW: 13,60 ± 0,88 g, $p = 0,004$).

4.3.4. Periodic maternal withdrawal at the first postnatal week period

The mothers fed and nursed their pups predominantly during their rest (light) period (Fig. 25). The daily separation of the pups from their mothers during the feeding period (ZT3-7) for 7-days postpartum disturbed the rhythmicity of the daily rhythmic feeding behavior of the mothers. See Figure 26. The intervention was associated with a 12-hour phase shift of the circadian expression rhythm of *Bmal1* ($p < 0{,}001$) and *Clock* ($p = 0{,}02$) and a loss of circadian rhythmicity for the other clock genes and all clock target genes studied (Table 8, Fig. 20E-22E).

We investigated whether the feeding restriction influenced the gained weight of the pups. The body weight of the male pups at 1 W with maternal withdrawal (17,30 ± 0,29 g, mean ± SEM) did not differ significantly compared with the parallel observed control group without maternal withdrawal (1 W males under LD, BW: 17,11 ± 0,32 g)

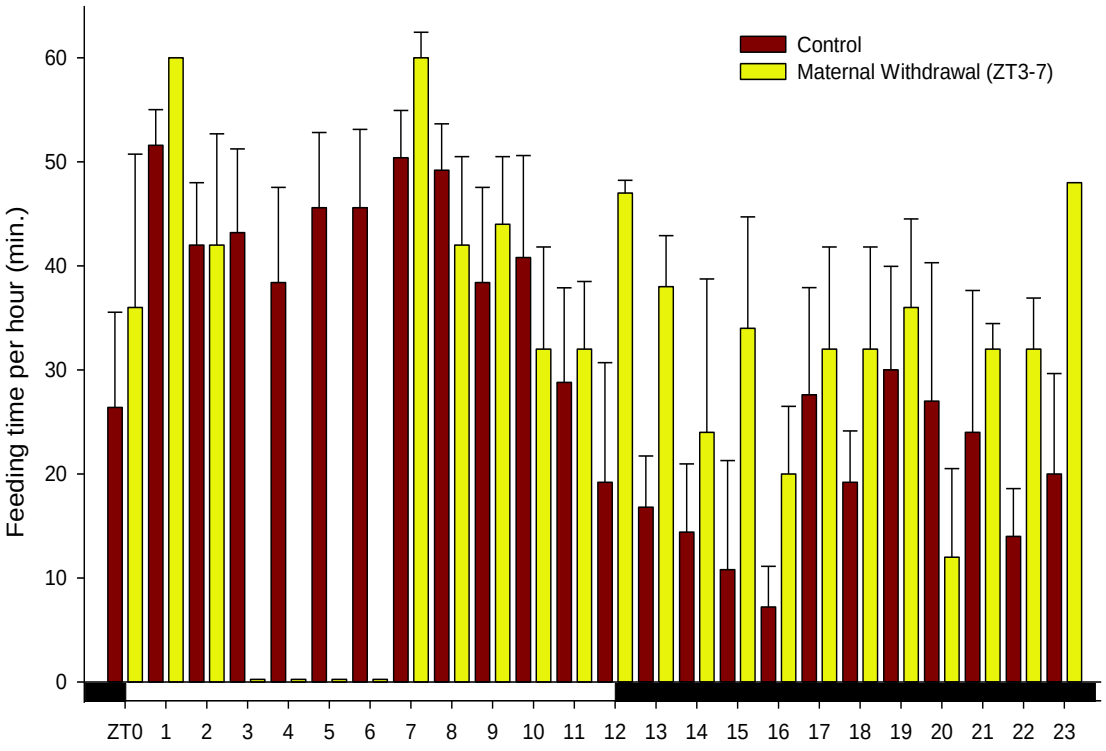


Figure 26. Feeding activity of the control mothers and mothers separated from their pups for 4 hours (ZT3-7, ZT0=06:00 h) per day. The observed feeding activity is shown in minutes/hour. Control mothers (n=3) fed their pups mostly during the resting period, whereas the withdrawal of mothers from their pups (n = 3) at ZT 3-7 for 7 days postpartum resulted in compensatory feeding throughout the late light and entire dark cycle.

Table 8. Cosinor analysis of circadian clock and clock-controlled tubular gene expression in rat kidney at 1W (n = 5-7 / timepoint).

	LD														
	1W (♀)			ME-1W (♀)			Vech-1W (♀)			1W (♂)			MW-1W (♂)		
	2A (%) / ϕ(ZT) / p			2A (%) / ϕ(ZT) / p			2A (%) / ϕ(ZT) / p			2A (%) / ϕ(ZT) / p			2A (%) / ϕ(ZT) / p		
Bmal1	43	22:42	0,039	74	19:07	< 0,001	36	00:16	NS	114	6:12	0,021	68	19:34	< 0,001
Clock	34	23:14	0,039	57	18:35	< 0,001	25	04:00	NS	102	5:12	0,009	52	19:07	0,002
Cry1	20	02:06	NS	63	18:27	< 0,001	17	17:26	NS	99	5:10	0,008	13	18:42	NS
Cry2	52	00:27	0,004	44	18:34	< 0,001	51	06:36	< 0,001	105	5:36	0,013	11	23:28	NS
Per1	56	01:03	0,014	45	16:13	< 0,001	48	06:00	0,017	90	4:47	0,038	17	23:38	NS
Per2	49	01:25	0,004	29	13:42	NS	42	07:03	0,037	97	4:55	0,012	7	21:55	NS
Rev-erbα	101	21:58	0,028	51	18:32	NS	68	20:35	NS	137	17:56	0,006	81	18:05	NS
αENaC	70	02:44	0,004	56	19:10	< 0,001	50	07:07	0,013	131	3:27	< 0,001	25	06:57	NS
SGK1	82	01:49	0,013	28	23:52	NS	32	07:46	NS	108	4:51	0,004	25	24:00	NS
NHE3	64	23:56	0,029	28	15:17	NS	60	05:08	0,008	176	2:22	< 0,001	20	20:33	NS
AVPR2	34	02:29	NS	43	17:52	0,039	148	21:25	< 0,001	127	3:12	< 0,001	11	13:07	NS

Note — Pups at 1W were sacrificed every 4 hours to cover one entire circadian cycle (n= 5-7/ 4 h interval over a 24 h period). Circadian rhythms of clock and clock controlled genes in the kidney were analyzed by the single cosinor procedure (fit of a 24h cosine way to all data by least-squares linear regression). P signifies significance level of circadian rhythmicity. 2A: double amplitude expressed as % of 24-hour mean (mesor). ϕ: Acrophase (time of cosine wave peak), expressed as clock time or Zeitgeber time (hours after light onset, ZT0= 6:00).

4.3.5. Postnatal changes of the gene expression pattern in offspring (LL and DD)

Dams kept under LL and DD during the pregnancy were returned to 12-12h light-dark cycles (ZT0 = 06:00 a.m.) with their offspring (n = 12 ± 2 / liter) directly after the delivery. At age 1 week, pups (n = 14 / time-point, 7 males in each group) were sacrificed at 4 h intervals over a 24 h period. Renal gene expression patterns (only) in male offspring were studied. At 1-week postpartum significant circadian oscillations were observed for all genes in DD group [*Clock (p < 0,001), Bmal1 (p < 0,001), Rev-erbα (p = 0,009), Per1 (p < 0,001), Per2 (p < 0,001), Cry1 (p < 0,001), Cry2 (p < 0,001), αENaC (p < 0,001), Sgk1 (p < 0,001), NHE3 (p < 0,001)* and *AVPR2* (*p < 0,001*) (Table 9)]. The amplitudes of rhythmic expression (mean 2A = 115 ± 28%) did not differ compared to the LD group (mean 2A = 117 ± 25%).

Table 9. Circadian gene expression in rat kidney at 1W (n = 7 / timepoint).

	1W								
	LD			LL			DD		
	2A (%)	ϕ(ZT)	p	2A (%)	ϕ(ZT)	p	2A (%)	ϕ(ZT)	p
Bmal1	114	6:12	0,021	82	05:27	< 0,001	152	21:38	< 0,001
Clock	102	5:12	0,009	38	05:27	0,001	99	22:54	< 0,001
Cry1	99	5:10	0,008	57	04:01	0,049	134	21:16	< 0,001
Cry2	105	5:36	0,013	61	05:58	< 0,001	125	22:41	< 0,001
Per1	90	4:47	0,038	35	07:01	0,029	74	22:20	< 0,001
Per2	97	4:55	0,012	54	05:04	< 0,001	110	22:01	< 0,001
Rev-erbα	137	17:56	0,006	44	04:31	NS	61	01:33	0,009
αENaC	131	3:27	< 0,001	21	00:59	NS	114	22:31	< 0,001
SGK1	108	4:51	0,004	24	02:07	NS	126	22:42	< 0,001
NHE3	176	2:22	< 0,001	42	03:51	NS	125	22:41	< 0,001
AVPR2	127	3:12	< 0,001	52	06:13	0,022	148	21:25	< 0,001

Circadian rhythms of clock and clock controlled genes in the kidney were analyzed by the single cosinor procedure. P signifies significance level of circadian rhythmicity. 2A: double amplitude expressed as % of 24-hour mean (mesor). ϕ: Acrophase (time of cosine wave peak), expressed as clock time or Zeitgeber time (hours after light onset, ZT0= 6:00)

The maximal expression levels of these genes were reached coherent at the end of the dark and the beginning of the light period (ZT21-ZT2). The circadian expression pattern for *Rev-erbα, αENaC, SGK1* and *NHE3* was lost in LL group, while the other investigated genes (*Clock (p < 0,001), Bmal1 (p = 0,001), Per1 (p = 0,029), Per2 (p < 0,001), Cry1 (p = 0,049), Cry2 (p < 0,001)* and *AVPR2* (*p = 0,022*) showed the same patterns as were observed in offspring with LD prenatal conditions (Table 9).

At 4 weeks, males with constant darkness exposure prenatal (DD) showed significant circadian variation of all investigated gene in the kidney [*Clock (p = 0,019), Bmal1 (p < 0,001), Rev-erbα (p = 0,001), Per1 (p = 0,006), Per2 (p < 0,001), Cry1 (p < 0,001), Cry2 (p < 0,001), αENaC (p < 0,001), Sgk1 (p < 0,001), NHE3 (p = 0,002)* and *AVPR2 (p = 0,026)* (Table 10)]. Although males with prenatal constant light exposure display circadian variation in the clock gene expression [*Clock (p < 0,001), Bmal1 (p < 0,001), Rev-erbα (p = 0,001), Per1 (p < 0,001), Per2 (p < 0,001), Cry1 (p < 0,001), Cry2 (p = 0,002)* (Table 10)], no circadian oscillation in the clock controlled tubular genes.
Bmal1 and *NHE3* RNA expressions peak at the light period in LD (ZT0 and ZT9), while in LL (ZT13 and ZT19) and in DD (ZT22 and ZT15) they peak at the end of the dark period. In all group the maximal expression of *SGK1* was observed in the dark period (LD, LL, DD ZT17-24). *Cry1*, *Per1*, *Per2*, *Rev-erbα, αENaC* expression show the same daily pattern in DD and an inverted oscillation in LL compared to LD. (Table 10). The amplitudes of the circadian expression of *Bmal1*, *Per1* and *Per2* in LL and DD showed increased compared to LD, whereas the amplitudes of the rhythmic oscillations of *Rev-erbα* in LL and DD was lower than in LD. *Cry1, αENaC, SGK1* and *NHE3* were higher in DD than in LD (DD: mean 2A = 109 ± 51%, LL: 113 ± 71%). (Table 10)

Table 10. Circadian gene expression in rat kidney at 4W (n = 7 / timepoint).

	4W								
	LD			LL			DD		
	2A (%)	ϕ(ZT)	p	2A (%)	ϕ(ZT)	p	2A (%)	ϕ(ZT)	p
Bmal1	136	0:05	0,012	217	12:57	< 0,001	183	21:48	< 0,001
Clock	23	3:30	NS	44	14:16	< 0,001	51	19:53	0,019
Cry1	116	18:36	< 0,001	99	08:16	< 0,001	151	18:35	< 0,001
Cry2	27	12:35	NS	44	22:53	0,002	87	15:09	< 0,001
Per1	82	14:56	0,039	99	21:44	< 0,001	84	15:07	0,006
Per2	62	13:53	0,044	86	01:51	< 0,001	116	15:00	< 0,001
Rev-erbα	248	6:27	0,014	208	19:24	< 0,001	203	08:19	0,001
αENaC	60	16:32	0,010	32	00:14	NS	101	16:59	< 0,001
SGK1	104	16:22	< 0,001	60	23:55	NS	117	19:01	< 0,001
NHE3	73	8:59	0,010	28	18:38	NS	53	14:45	0,002
AVPR2	58	19:51	NS	20	10:30	NS	60	16:13	0,026

Circadian rhythms of clock and clock controlled genes in the kidney were analyzed by the single cosinor procedure. P signifies significance level of circadian rhythmicity. 2A: double amplitude expressed as % of 24-hour mean (mesor). ϕ: Acrophase (time of cosine wave peak), expressed as clock time or Zeitgeber time (hours after light onset, ZT0= 6:00)

In adult rats 12 weeks after birth, in LL and DD profound rhythmic expression was observed for all investigated genes except *AVPR2* in DD. [**LL**: *Clock (p = 0,001), Bmal1 (p < 0,001), Rev-erbα (p < 0,001), Per1 (p = 0,006), Per2 (p < 0,001), Cry1 (p < 0,001), Cry2 (p < 0,001), αENaC (p < 0,001), Sgk1 (p = 0,006), NHE3 (p < 0,001) and AVPR2 (p < 0,001),* **DD***: Clock (p < 0,001), Bmal1 (p < 0,001), Rev-erbα (p < 0,001), Per1 (p = 0,003), Per2 (p < 0,001), Cry1 (p < 0,001), Cry2 (p < 0,001), αENaC (p < 0,001), Sgk1 (p < 0,001), NHE3 (p = 0,012)* (Table 11)] The phase of *Bmal1*, *Cry1*, *Cry2*, *Per1* and *Per2* genes expression patterns showed very similar pattern under each condition (Table 11, acrophase differences max ca. 2 h, *Bmal1* presented in Figure 27.), *Rev-erbα* and *SGK1* peaked as well in the same light period with 1-4 h delay/advance in different groups. The daily expression of Clock showed a circadian variation in LL and DD groups with the same acrophase (ZT24) and *αENaC* expression showed circadian pattern in LL (ZT13) as well in DD (ZT19), but no in LD.

Table 11. Circadian gene expression in rat kidney at 12W (n = 7 / timepoint).

	12W								
	LD			LL			DD		
	2A (%)	ϕ(ZT)	P	2A (%)	ϕ(ZT)	p	2A (%)	ϕ(ZT)	p
Bmal1	228	23:01	< 0,001	204	22:34	< 0,001	253	00:12	< 0,001
Clock	23	22:22	NS	26	23:33	0,001	79	23:36	< 0,001
Cry1	152	18:48	< 0,001	135	17:26	< 0,001	153	20:01	< 0,001
Cry2	88	14:52	< 0,001	135	12:51	< 0,001	91	15:25	< 0,001
Per1	150	12:08	< 0,001	148	12:16	< 0,001	115	16:10	0,003
Per2	134	14:57	< 0,001	128	14:28	< 0,001	122	17:00	< 0,001
Rev-erbα	222	06:53	< 0,001	228	08:05	< 0,001	190	09:37	< 0,001
αENaC	39	12:08	NS	139	12:59	< 0,001	74	18:43	< 0,001
SGK1	109	18:37	0,004	97	13:58	0,006	140	21:13	< 0,001
NHE3	34	12:03	NS	117	12:49	< 0,001	58	15:53	0,012
AVPR2	80	23:53	0,046	130	13:27	< 0,001	37	06:59	NS

Circadian rhythms of clock and clock controlled genes in the kidney were analyzed by the single cosinor procedure. P signifies significance level of circadian rhythmicity. 2A: double amplitude expressed as % of 24-hour mean (mesor). ϕ: Acrophase (time of cosine wave peak), expressed as clock time or Zeitgeber time (hours after light onset, ZT0= 6:00)

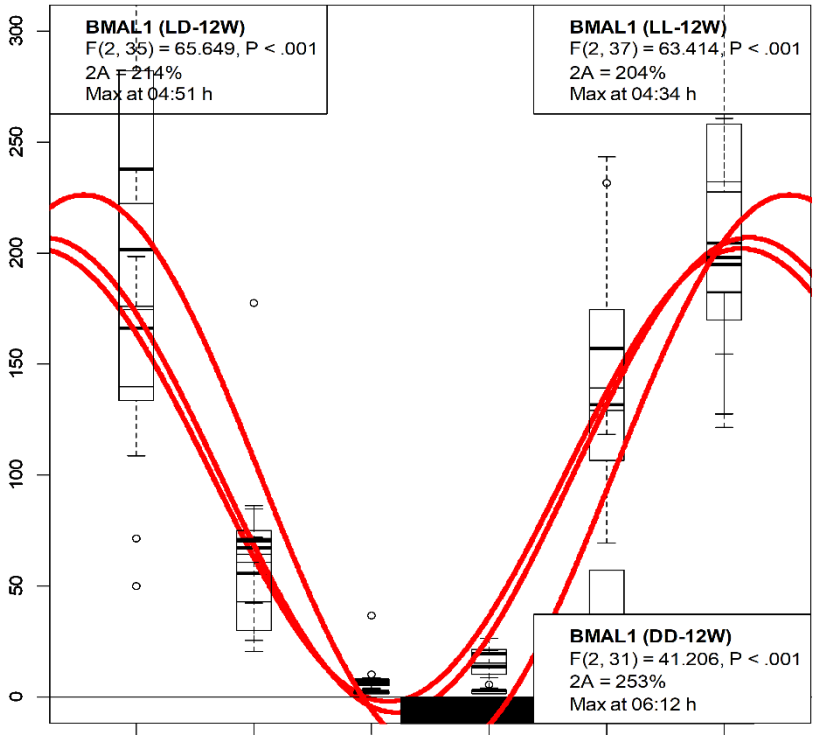


Figure 27. Daily gene expression patterns of Bmal1 in rat kidney at 12W followed different prenatal light exposure (LD, LL and DD). Male offspring (n = 7 / 4 h over a 24 period) were sacrificed every 4 hours (ZT4-24; ZT0 at 06:00 a.m.). Daily expression pattern of Bmal1 gene in the kidney was measured by rt-PCR (units expressed as % mean in 4-hour interval (mean ± SD)) at 12 weeks of age (12W) after a different prenatal light exposure. P values, double amplitude (2A) and acrophases (time of cosine wave peak) are listed in legends, see cosine line calculated by best-fitting of a 24h cosine way analysis.

4.4. Follow-up experiment (Experiment 3)

4.4.1. Long-lasting effects in offspring

By 2-way ANOVA there is a significant difference in mean values of the body weight among the different groups (n = 42 / group) respect to the prenatal light exposure of the mother ($p < 0,001$) as well as with respect to the gender ($p < 0,001$). With the Holm-Sidak method the body weight at **1W** of females and males in LL group was significantly higher than in groups LD ($p < 0,001$). Males in LL ($p < 0,001$), DD ($p < 0,001$) and 3:21-LD ($p = 0,018$) have also significantly higher body weight compared to males in LD. Table 12.

Table 12. The body weight (B-weight) at 1, 4, 12 and 34W of age of offspring from mothers, that were kept under LD, LL, DD, 6:6-LD and 3:21-LD light conditions.

Body- weight						
	LD	LL	DD	6:6-LD	3:21-LD	*factor B*
	1W [MEAN BW ± SD (g)]					
Female	13,6 ± 1,5	15,9 ± 2,1*	14,5 ± 1,5	14,4 ± 2,1	13,6 ± 1,8	< 0,001
Male	13,87 ± 1,8	16,3 ± 2,2*	15,6 ± 1,5*	14,8 ± 1,8	15,0 ± 1,7*	< 0,001
factor A	NS	NS	0,012	NS	< 0,001	
	4W [MEAN BW ± SD (g)]					
Female	75,9 ± 7,6	75,2 ± 8,9	76,9 ± 8,4	75,0 ± 4,5	76,3 ± 7,5	NS
Male	82,9 ± 10,5	81,7 ± 8,9	85,3 ± 7,7	82,0 ± 4,8	82,5 ± 8,2	NS
factor A	< 0,001	< 0,001	< 0,001	< 0,001	< 0,001	
	12W [MEAN BW ± SD (g)]					
Female	266,2 ± 20,0	286,1 ± 18,2*	282,4 ± 23,6*	262,7 ± 24,1	244,0 ± 14,7*	< 0,001
Male	438,3 ± 36,3	462,0 ± 27,2*	456,2 ± 32,9*	445,6 ± 43,0	424,0 ± 34,5	< 0,001
factor A	< 0,001	< 0,001	< 0,001	< 0,001	< 0,001	
	34W (± 2 W) [MEAN BW ± SD (g)]					
Female	342,2 ± 32,5	332,0 ± 13,4	400,9 ± 41,0*	360,6 ± 16,1*	379,5 ± 60*	< 0,001
Male	611,7 ± 23,4	628,5 ± 70,0	610,9 ± 82,4	710,6 ± 47,2*	583,1 ± 39,3	< 0,001

2-WAY ANOVA (Factor A: gender and factor B: light condition)

Note- B-weight of FR-LD is not presented: males mean body weight 651,3 ± 53,5 g, females mean body weight 376,5 ± 33,7 g. Values are means ± SD. See also Figures 28-30.

At **4W** of age, there is a significant difference analyzed with 2-way ANOVA with respect to the gender in each investigated group (n = 42 / group), however there is no difference between the groups respect to the light conditions during the pregnancy.

At **12W** of age there is a significant difference in mean body weight (g) by 2-way ANOVA between the investigated groups (n = 42 / group) regarding the prenatal light conditions ($p < 0,001$) and respect to the gender ($p < 0,001$). In females, the body weight in 3:21-LD group was significantly lower than in the control groups (vs. LD $p = 0,016$; with the Holm-Sidak post hoc method). The body weight of females in DD and LL is significantly higher than body weight in LD (LL vs. LD $p = 0,009$, DD vs. LD $p = 0,031$). Males in LL and DD were bigger at age 12 week than males in LD (LL vs. LD $p < 0,001$, DD vs. LD $p = 0,027$). (Figure 28)

The evaluation of the long-term cohort groups (at age of **34W** ± 2 W, n = 20 / group) explored that there is a significantly difference of the body weight in both genders respect to the prenatal light condition. Females from mothers kept under constant dark exposure (DD), ultradian light-dark cycle (6:6-LD), prolongated dark phase (3:21-LD) and under a restricted feeding regime during the pregnancy (FR-LD) have higher body weight compared to the control, LD prenatal light conditions (DD vs. LD $p < 0,05$, 6:6-LD vs. LD $p < 0,05$, 3:21-LD vs. LD $p < 0,05$, FR-LD vs. LD $p < 0,05$). (Figure 29 and 30)

The body weight of males in the different groups showed significantly difference with one-way ANOVA analysis ($p < 0,001$). Used a Holm Sidak post hoc method the body weight of males in 6:6-LD group was significantly higher than the body weight of males in LD ($p < 0,001$).

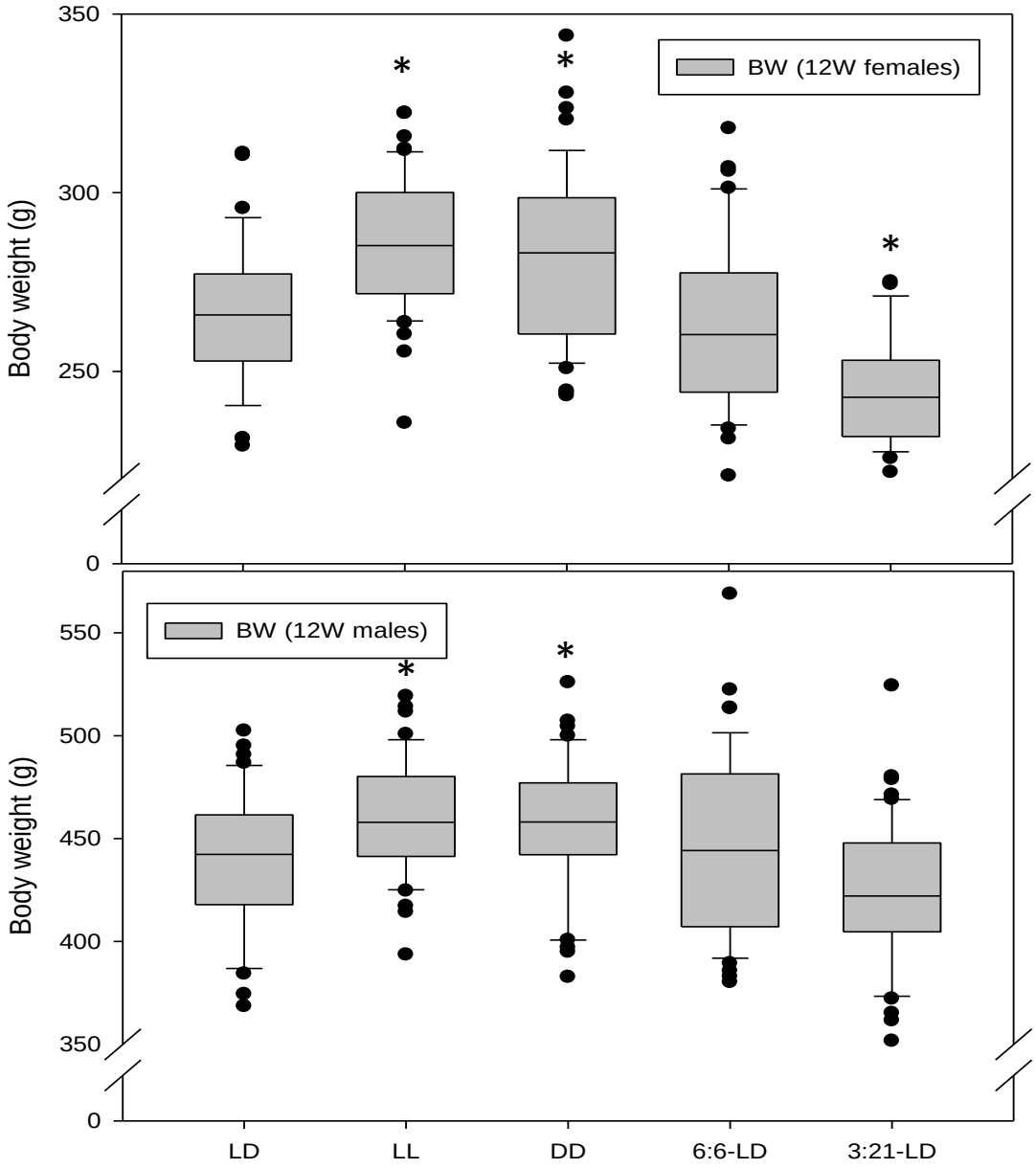


Figure 28. Body weight (BW) of offspring at 12 weeks of age (12W) exposed to different i.e. LD, LL, DD, 6:6-LD and 3:21-LD light-dark cycle prenatally.

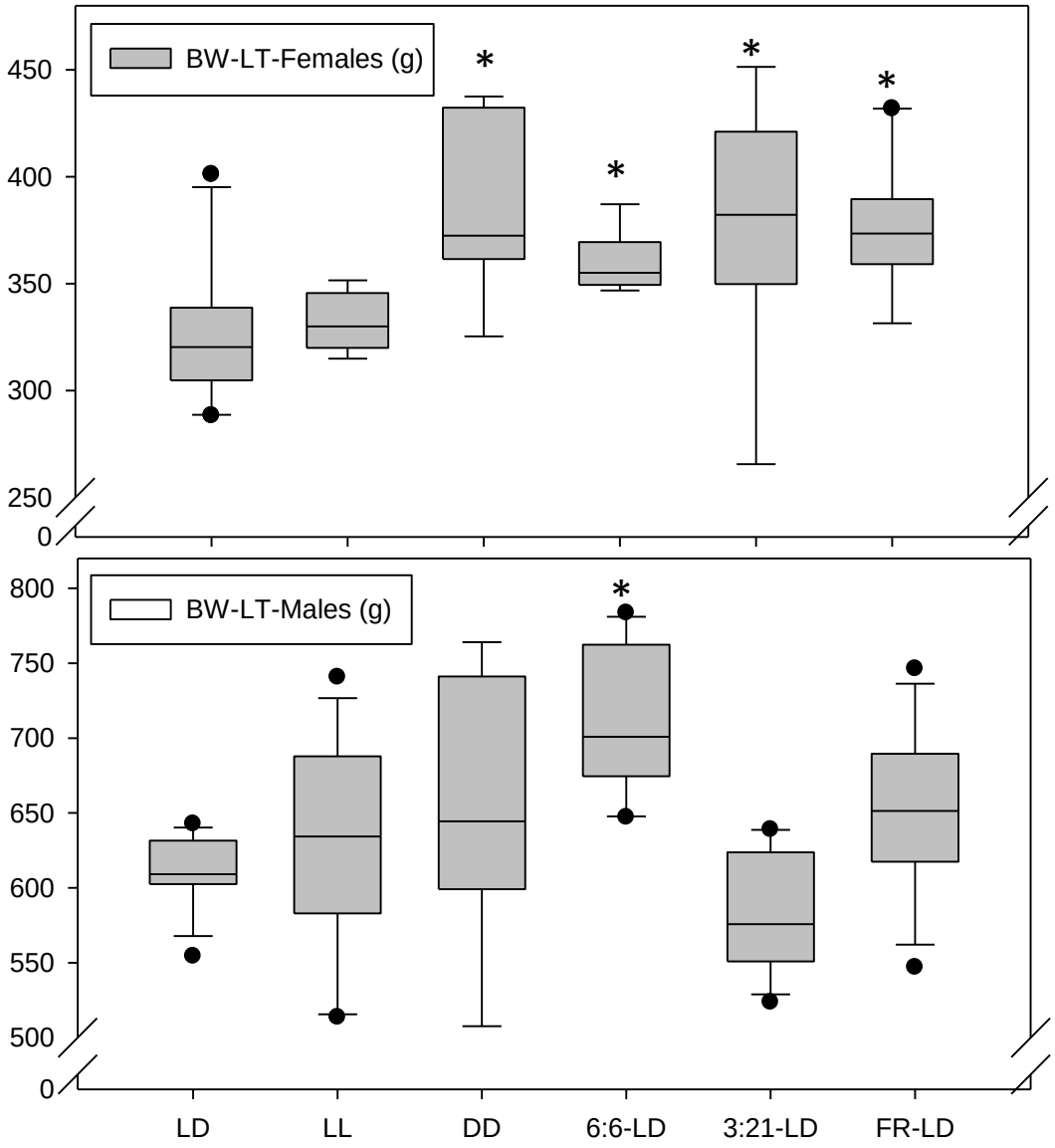


Figure 29. Body weight (BW) of the long-term groups (LT, offspring at 34 ± 2 weeks of age exposed to different prenatal conditions (for details see materials and methods).

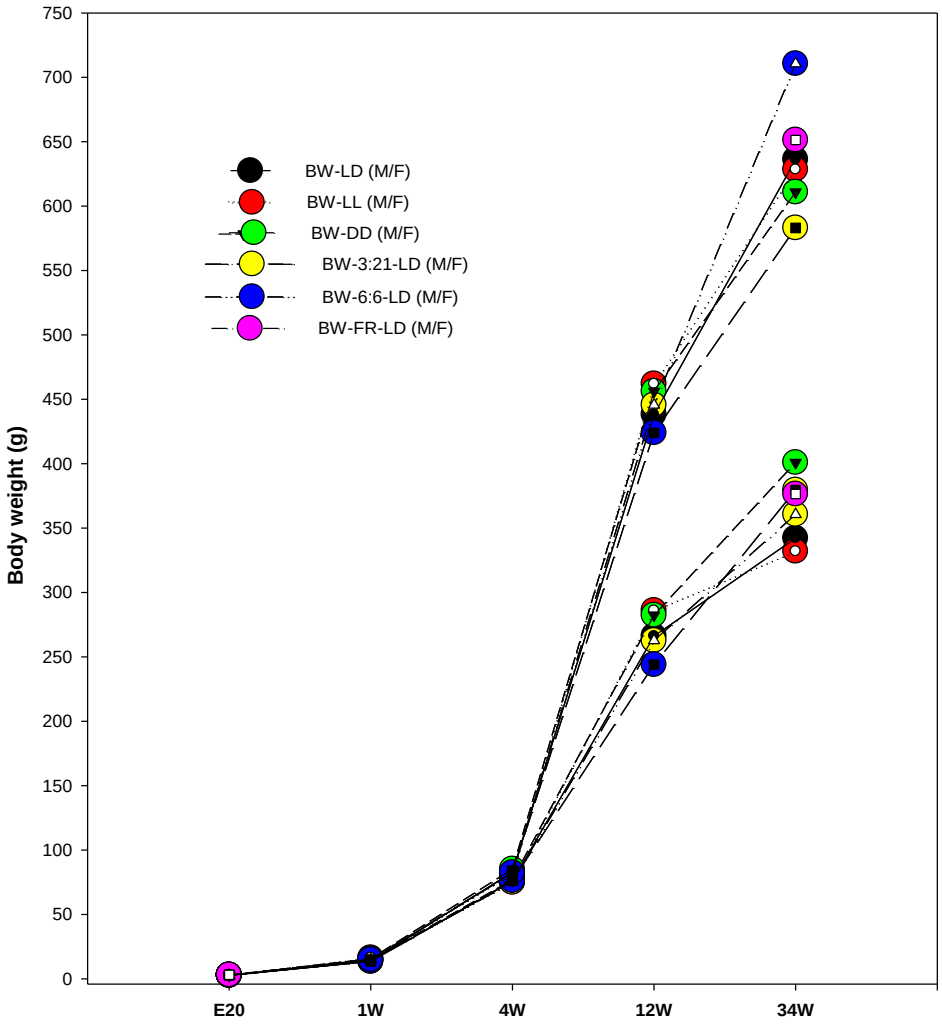


Figure 30. Body weight (g) of the offspring (male above and female below) at different age (E20, 1W, 4W, 12W and 34W of age) exposed to different prenatal conditions (for details see materials and methods).

4.4.2. Renal function

Daily sodium excretion

In this long-term cohort there is a significant difference with a 2-way ANOVA analysis regarding to the daily sodium excretion among the different groups ($p < 0,001$) and between the genders ($p < 0,001$). The Hol Sidak method with multiple comparison procedure was used to isolate group(s) that differs from the others. **Females** in 6:6-LD, 3:21-LD and FR-LD showed a decrease in the daily sodium excretion compared to LD (6:6-LD vs. LD $p < 0,001$, 3:21-LD vs. LD $p = 0,001$, FR-LD vs. LD $p < 0,001$). **Males** in 6:6-LD, 3:21-LD and FR-LD showed a decrease in the daily sodium excretion compared to LD (6:6-LD vs. LD $p < 0,001$, 3:21-LD vs. LD $p = 0,001$, FR-LD vs. LD $p = 0,019$). See Table 13.

Daily potassium excretion

There is a significant difference with a 2-way ANOVA analysis respect to the daily potassium excretion among the different groups ($p < 0,001$) and between the genders ($p < 0,001$). **Females** in 6:6-LD, 3:21-LD and FR-LD showed a decrease in the daily potassium excretion compared to LD (6:6-LD vs. LD $p = 0,002$, 3:21-LD vs. LD $p < 0,001$, FR-LD vs. LD $p = 0,013$). **Males** in 6:6-LD and 3:21-LD showed a decrease in the daily sodium excretion compared to LD (6:6-LD vs. LD $p = 0,021$; 3:21-LD vs. LD $p = 0,043$). (Table 13)

Phosphate excretion

There is a significant difference with a 2-way ANOVA analysis respect to the daily phosphate excretion among the different groups ($p < 0,001$) and between the genders ($p < 0,001$). By further analysis, significant differences between males and females were observed in LL and 3:21-LD only. **Females** in LL showed an increase in the daily phosphate excretion compared to LD (LL vs. LD $p = 0,023$). Phosphate excretion of **males** did not differ significantly. (Table 13)

Calcium excretion

There is a significant difference with a 2-way ANOVA analysis respect to the daily calcium excretion among the different groups ($p < 0,001$) and between the genders ($p <$

0,001). **Females** in LL showed an increase in the daily calcium excretion compared to the LD (LL vs. LD $p < 0,001$). Calcium excretion of **males** did not differ significantly.

Glucosuria

There is an increased glucosuria of males compared to females in LL (male vs. female $p = 0,013$) and FR-LD (male vs. female $p < 0,001$) group. In other groups (LD, DD, 6:6-LD and 3:21-LD) there is no significant difference respect to the gender. With respect to the prenatal light conditions, no significant difference of the urinary glucose excretion could be observed in the investigated groups.

Microalbuminuria

There is a significantly higher albumin excretion in females of LL group compared to the LD (LL vs. LD $p = 0,030$), but not in males. (Table 13)

Table 13. Renal excretory function in a long-term (LT) cohort groups at 34W of age LD, LL, DD, 6:6-LD, 3:21-LD and FR-LD.

U/24 h		LD-LT	LL-LT	DD-LT	6:6-LT	3:21-LT	FR-LT	*factor B*
U-Na/U-Crea	♀	22,1 ± 3,8	28,7 ± 6,7	22,7 ± 5,2	16,3 ± 2,7*	17,6 ± 3,8*	16,8 ± 2,8*	< 0,001
(mmol/mmol)	♂	16,5 ± 2,7	19,2 ± 2,6	16,2 ± 2,0	8,42 ± 1,20*	10,6 ± 1,9*	12,6 ± 1,4*	< 0,001
factor A		< 0,001	< 0,001	< 0,001	< 0,001	< 0,001	0,013	
U-K/ U-Crea	♀	32,2 ± 7,5	40,9 ± 3,8	33,9 ± 6,4	25,8 ± 5,3*	22,0 ± 4,5*	27,4 ± 3,5*	< 0,001
(mmol/mmol)	♂	22,1 ± 4,9	24,8 ± 5,6	24,3 ± 2,8	16,9 ± 0,6*	18,0 ± 2,4*	21,5 ± 2,1	< 0,001
factor A		< 0,001	< 0,001	< 0,001	< 0,001	NS	0,018	
U-P/ U-Crea	♀	0,83 ± 0,55	1,77 ± 0,98*	0,98 ± 0,41	0,73 ± 0,41	0,79 ± 0,67	0,54 ± 0,24	< 0,001
(mg/mg)	♂	0,49 ± 0,25	0,60 ± 0,48	0,83 ± 0,43	0,29 ± 0,15	0,13 ± 0,09	0,42 ± 0,19	NS
factor A		NS	< 0,001	NS	NS	0,013	NS	
U-Ca/ U-Crea	♀	0,45 ± 0,25	0,78 ± 0,13*	0,41 ± 0,11	0,19 ± 0,14	0,38 ± 0,18	0,25 ± 0,07	< 0,001
(mg/mg)	♂	0,11 ± 0,06	0,20 ± 0,13	0,09 ± 0,04	0,05 ± 0,01	0,06 ± 0,02	0,06 ± 0,03	NS
factor A		< 0,001	< 0,001	< 0,001	0,033	< 0,001	0,002	
U-Glu/ U-Crea	♀	0,38 ± 0,36	0,27 ± 0,23	0,62 ± 0,31	0,25 ± 0,22	0,63 ± 0,96	0,05 ± 0,09	NS
(mg/mg)	♂	0,41 ± 0,19	0,63 ± 0,26	0,53 ± 0,11	0,35 ± 0,30	0,36 ± 0,17	0,97 ± 0,86	NS
factor A		NS	0,013	NS	NS	NS	< 0,001	
Albumin (mg/l)	♀	3,66 ± 1,46	25,42 ± 35,87*	6,91 ± 10,81	3,28 ± 1,24	3,36 ± 2,10	2,50 ± 0,00	< 0,001
	♂	8,47 ± 7,30	9,33 ± 6,19	6,42 ± 5,63	2,60 ± 0,18	2,50 ± 0,00	4,23 ± 2,59	NS
factor A		NS	0,001	NS	NS	NS	NS	

2-WAY ANOVA ANALYSIS (Factor A: gender and factor B: light condition, Mean ± SD)

Table 14. Cardiovascular function in a long-term (LT) cohort groups at 34W of age LD, LL, DD, 6:6-LD, 3:21-LD and FR-LD.

	LD-LT	LL-LT	DD-LT	6:6-LT	3:21-LT	FR-LT	*factor B*
Systolic RR							
mmHg	120,36 ± 4,78	114,68 ± 4,41	116,63 ± 4,62	125,24 ± 4,29*	111,25 ± 4,79	137,02 ± 7,86*	< 0,001
DAY	117,31 ± 3,55	111,79 ± 3,78	114,14 ± 2,90	123,69 ± 4,75↔	107,71 ± 3,61	140,84 ± 4,40†	
NIGHT	123,43 ± 3,83	117,58 ± 2,86	119,12 ±4,75	126,79 ± 3,28↔	114,80 ± 2,74	133,20 ± 8,84†	
factor A	< 0,001	0,002	0,006	NS	< 0,001	< 0,001	
Pulse Pressure							
mmHg	35,21 ± 1,77	31,83 ± 1,70	38,43 ± 1,60*	34,03 ± 1,53	32,19 ± 1,55	40,66 ± 3,46*	< 0,001
(% of systolic RR)	(29,3)	(27,8)	(33,0)	(27,2)	(28,9)	(29,7)	
DAY	34,13 ± 1,20	30,74 ± 1,41	37,81 ± 1,41~	33,64 ± 1,69↔	31,15 ± 1,20	41,93 ±3,18†	
NIGHT	36,30 ± 1,59	32,91 ± 1,20	39,04 ± 1,61~	34,42 ± 1,29↔	33,23 ± 1,10	39,39 ± 3,38†	
factor A	0,005	0,005	NS	NS	0,007	0,001	

2-WAY ANOVA ANALYSIS (Factor A: daytime vs. nighttime and factor B: prenatal light condition), see Figure 31-33. MEAN ± SD.

* - significant different compared to LD, ↔ - no daily variation, † - inverted blood pressure fluctuation.

4.4.3. Telemetric measurements

Systolic blood pressure

There is a significant difference with a 2-way ANOVA analysis respect to the systolic blood pressure among the different groups ($p < 0,001$) and there is a diurnal variation as well (DAY vs. NIGHT $p < 0,001$). The Hol Sidak method with multiple comparison procedure was used to isolate which group(s) differs from the others. Systolic blood pressure of females at age 34 ±2 weeks (long-term, LT) from mothers that were kept under restricted feeding schedule (LT-FR-LD) and females in LT-6:6-LD showed significantly higher than in the control group (LT-FR-LD vs. LT-LD $p < 0,001$, LT-6:6-LD vs. LT-LD $p < 0,001$).

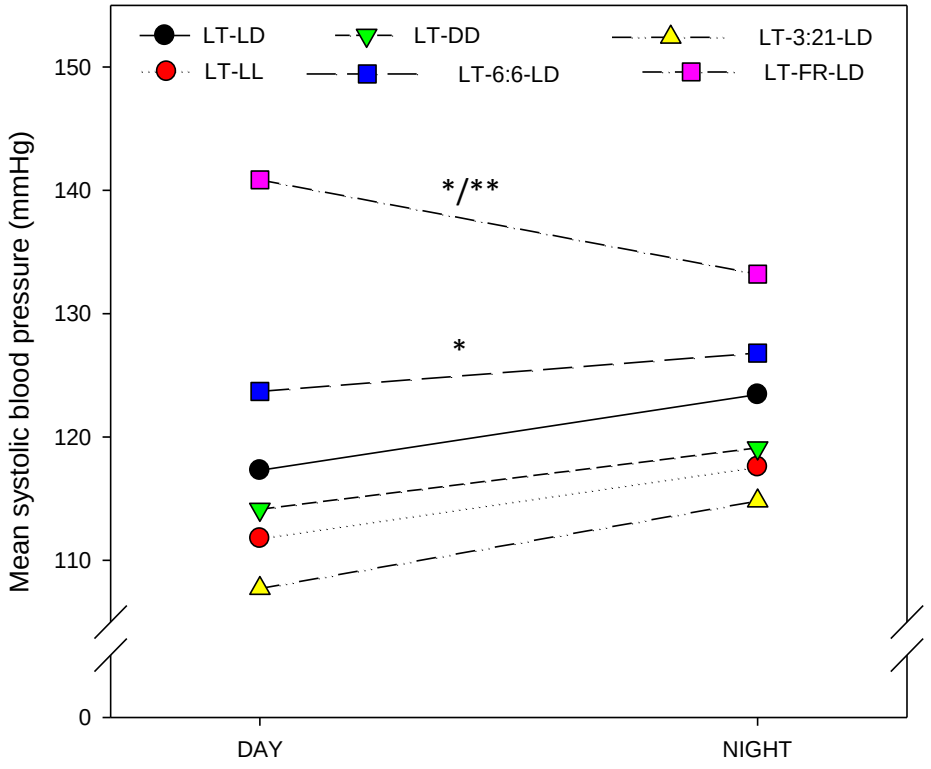


Figure 31. Systolic blood pressure measured by intra-aortal telemetry in female offspring at 34 ± 2 weeks of age (long-term, LT) after different prenatal light exposure (LD, LL, DD, 6:6-LD and 3:21-LD) and food restriction of the mother (FD-LD). (n = 5-11 / group) 7-10 days after the sensor implantation measurement were taken under normal light-dark cycle and feeding regime. Telemetric recordings containing 10-minute data collections from each rat (LT-LD, LT-LL, LT-DD, LT-6:6-LD, LT-3:21-LD and LT-FR-LD) were analyzed used Dataquest system.

There is no daily systolic blood pressure variation (daytime vs. nighttime, mean difference 2%) in LT-6:6-LD, while there is an inverted daily rhythm (daytime value 7 % higher than night time) in LT-FR-LD. (Table 14; Figure31)

Pulse pressure

There is a significant difference with a 2-way ANOVA analysis respect to the systolic pulse pressure among the different groups ($p < 0,001$) and between the genders ($p < 0,001$). The pulse pressure of females at age 34 ±2 weeks (long-term, LT) from mothers kept under restricted feeding schedule (FR-LD) and females in DD showed significantly higher than in the control group (LT-FR-LD vs. LT-LD $p < 0,001$, LT-DD vs. LT-LD $p < 0,001$).

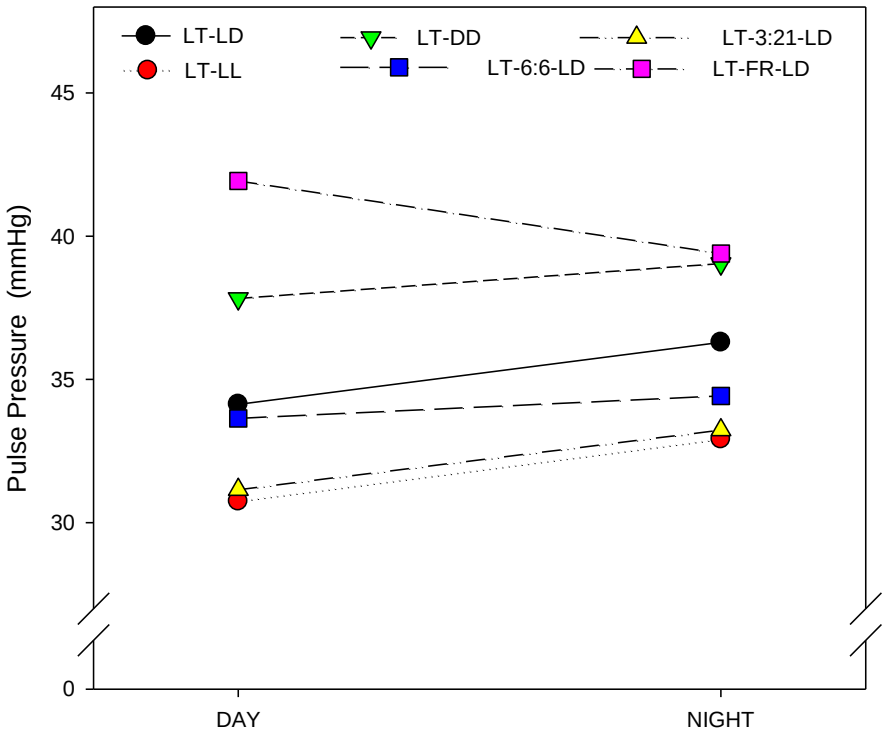


Figure 32. Systolic pulse pressure ($P_{systolic}$ - $P_{diastolic}$) measured by intra-aortal telemetry in female offspring at 34 ± 2 weeks of age (LT) after different prenatal light exposure (LD, LL, DD, 6:6-LD and 3:21-LD) and food restriction of the mother (FD-LD). (n = 5-11 / group) 7-10 days after the sensor implantation measurement were taken under normal light-dark cycle and feeding regime. Telemetric recordings containing 10-minute data collections from each rat (LT-LD, LT-LL, LT-DD, LT-6:6-LD, LT-3:21-LD and LT-FR-LD) were analyzed used Dataquest system.

There is an inverted daily rhythm (daytime higher than night time) in LT-FR-LD and no daily variation of pulse pressure (daytime vs. nighttime) could be observed in LT-DD and LT-6:6-LD. (Figure 32) The pulse pressure values were between 27,2 - 33% of the systolic value. (Table 14) Heart rate and motor activity did not significantly differ between the groups.

4.4.4. Echocardiography

Echocardiography showed no significant difference respect to the FS% in the different groups of females (DD were not investigated). However, the mean value of the FS% was lower in long-term LL (mean FS% in LL 50,55 ± 10,6), 6:6-LD (mean FS% in 6:6-LD 47,34 ± 12,40), 3:21-LD (mean FS% in 3:21-LD 45,48 ± 7,47) and FR-LD (mean FS% in FR-LD 49,94 ± 5,50) groups compared to LD (mean FS% in LD 57,79 ± 8,80,). (Figure 33)

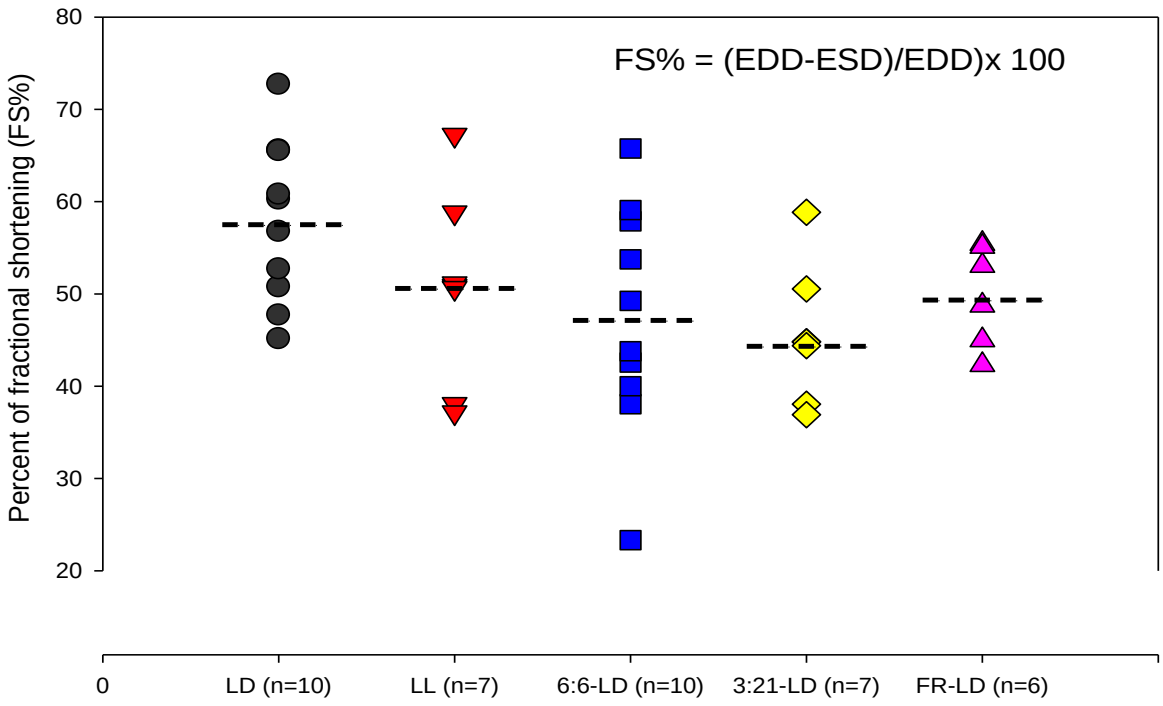


Figure 33. Echocardiography at week 34 ± 2 (long-term, LT): FS% values in LD, LL, 6:6-LD, 3:21-LD and FR-LD groups. With M-mode imaging the left ventricular end-diastolic (EDD) and end-systolic (ESD) internal diameter of female rats (n=6-10/group) in each group were measured and the percentage of fractional shortening (FS %) was calculated.

5. DISCUSSION

In the present work we investigated whether the maternal circadian disruption during the intrauterine period has direct or long-lasting effects on the peripheral circadian organization in the kidney. We hypothesized that this 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 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 (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. While in **experiment 2** we observed the postnatal changes of the renal clockwork, 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 focused on the renal, cardiovascular and metabolic function.

Above that, we aimed to explore the **gender related differences** in the circadian oscillation of the clock genes at different age (1W and 12W). Numerous studies have intensively discussed gender related differences in many circadian processes.[288] However, the sex differences have not been studied at the level of the molecular clock in the kidney previously. Indeed, mixed groups studying the clock gene expression pattern have been commonly used in rodent experiment

It is of note that in the present study we used whole kidney tissue to assess the clock and clock-controlled gene expression pattern. Thus, we were unable to investigate compartment-specific circadian regulation. Regional oscillatory gene activity in specific functional segments of the kidney has been successfully studied by in situ hybridization and tissue microdissection techniques.[239,247]

EXPERIMENT 1

The main finding of this study is the evidence for the presence of a functioning molecular clockwork in the late fetal kidney (at E20), characterized by circadian expression of its core molecular components and many clock-controlled genes around the time of birth.[289] Such circadian oscillations of *Per2* were reported in cultured explants of fetal mouse liver, kidney and heart tissue as early as at E18, while no circadian gene rhythmicity was observed *in vivo* at this stage of fetal development.[168] In another in vivo study, at E20 in the rat liver only Rev-erbα exhibited circadian rhythmicity at the end of intrauterine development.[165] In contrast to these findings we found three clock genes (*Clock, Per2* and *Rev-erbα*) to oscillate prior to birth in kidney tissue. Hence, the functional organization of the molecular clockwork appears to occur earlier in the kidney than in other peripheral organs. While rhythms which are directly controlled by the master clock do not yet function at birth, we assume that the rhythms observed at embryonic day 20 reflect intrinsic, autonomous oscillation of renal tissues.[159,290,291] The functional integrity of the intrarenal circadian pacemaker system at birth is also reflected by the oscillatory expression of key clock-controlled genes involved in the regulation of fluid and electrolyte homeostasis.

There were no studies to our knowledge addressing the intrarenal, molecular circadian clockworks alteration of the offspring followed prenatal modified light exposure or restricted feeding regime. We documented that the maternal circadian disturbance causes a temporary alteration of the renal circadian clockwork function in the offspring at the birth-time which might trigger adverse effects later in life. At this point, it is difficult to demonstrate the actual relevance of each alteration in the circadian genes. Nevertheless, our observation is agreement with the accumulated evidence that glucocorticoids, melatonin signaling and feeding cues contribute to the circadian regulation.[176,201,202]

Our study showed that maternal exposure to modified light conditions or food restriction (FR-LD) during the gestational period alters the internal temporal order of a wide range of functions in the mother e.g. locomotor activity (i.e. in LL and 3:21-LD).

LL: it is well established that constant illumination reduces nocturnal melatonin levels and induces alteration in the circadian behavioral rhythm of the adult animal.[101,135,136,292,293] Consistent with this fact we found that prenatal constant light exposure during the gestation period was associated with a decrease in overall daily activity and loss of the circadian moving pattern without altering the gained body weight during the gestation period of the

dams. However, our study did not investigate the melatonin pattern in this group, based on the previously studies we assumed that the melatonin rhythm providing entraining signals for the fetal tissues was weak or even lost.[292] Additionally, it has been reported that melatonin suppression induce precocious maturation of the fetal adrenal gland and leads to increased plasma cortisol concentration in newborn.[294] Thus, we focused our interest on the glucocorticoids (GCs) which are also key signals conveying maternal-fetal circadian information. It is well established that glucocorticoid signaling provides time cues for specific organs like the kidney.[87] In human and animal studies has been preciously documented that the maternal GC level is elevated in the late pregnancy.[295,296] Along the same lines, we found an elevated urinary aldosterone level towards the end of pregnancy of the mother under constant illumination and normal light-dark cycle (in LD and LL). In contrast to the control we documented a rhythmic maternal urinary aldosterone excretion pattern, without altering renal sodium excretion (maximal values reached at 12:00 h, ZT6) in the late gestation under constant illumination. However, in adults the GC are exhibited rhythmically with a peak around the beginning of the wakefulness and behavioral activity, the lack of its daily rhythms under normal condition may represent a true absence of circadian oscillation during the pregnancy.[86] Rhythm of the serum cortisol level has been previously reported only in mothers under restricted feeding during the pregnancy.[297] Thus, at this point, it is difficult to evaluate whether the rhythmic aldosterone synthesis in this group is triggered by the maternal stress axis.

Previous studies show that core clock component e.g. Per1 expression is also stimulated by restrain stress.[298] Gumz et al. presented that *Per1* which is involved in the basal and aldosterone-mediated regulation of the alpha *αENaC* activity is an early target of the aldosterone.[253] Given that mother exposed constant light displayed rhythmic aldosterone urinary excretion indicating rhythmic plasma level, it might not be surprising that their fetuses exhibited robust renal circadian oscillation of Per1 contrast to the control group at E20. Furthermore, all renal clock and clock-controlled genes which were expressed in circadian manner under LD were also showed rhythmic under this condition. Thus, it is more likely, that the sustained expression of clock gene in the kidney at late gestational period is driven by glucocorticoids rather than melatonin signaling pathway. Whether the fetal adrenal gland function contributes to the observed changes at the molecular level remain to be elucidate.

DD: dams kept under constant darkness displayed unaffected locomotor activity pattern without altering the gained body weight and robust urinary melatonin fluctuation. The

maintenance of circadian behavior and diurnal changes of the nocturnal melatonin fluctuation in individual rats is consistent with previous observations.[299] In human observational studies an increase in its night-time concentration has been previously presented.[300,301] However, the available data so far do not provide evidence for that: we found even a higher urinary melatonin peak under DD compared to control group.

Interestingly, we have observed a tendency to polyuria with elevated glucose and altered electrolyte excretion of dams at the late gestation period. While slight degree of nephrogenic diabetes insipidus is common phenotype in clock gene mutation, we assumed a temporal disruption of the endogenous renal clockwork regulation under constant darkness.[222,302]

Overall, our findings suggest defective maternal melatonin signaling in the fetal kidney considering the fact that the renal clock and clock controlled gene at E20 were not expressed in circadian manner. Melatonin excretion showed to be synchronized in the group so we assumed a sort of social entrainments between the individuals.[303] However, we cannot discard the possibility that arrhythmic of the investigated gene expressions may be due to a desynchronization of the fetuses. Worth to mention that development of the melatonin receptor in the fetal kidney is unknown in rat. As an example, in Siberian hamsters, melatonin-binding sites in the central nervous system were first apparent from the E10 in region specific manner.[304] The exact role of the maternal melatonin contributing to the maternal entrainment of the fetus for the kidney remained to be explored.[174,294,305]

6:6-LD**:** It has been previously documented that under ultradian cycle (to which mice cannot entrain) the endogenous circadian rhythm is disturbed.[306] That is leading to metabolic disorders, such as glucose intolerance and accelerated gained body weight in adult animals.[306] Controversially, study reported that rats exhibit persistent circadian locomotor activity with the same phase of the N-acetyltransferase enzyme activity in the pineal gland i.e. the melatonin synthesis directly controlled by the master clock even under 6:6-LD.[307] Along the same lines, Aschoff observational experiments showed persistent circadian rhythms under 4-4 hour rest -activity schedule.[308] In contrast to previous work with non-pregnant animal we observed reduced gained weight of the dams under ultradian light-dark cycle.[22]

Interestingly, only *Sgk1*which is regulated by different hormones such as glucocorticoids on the genomic level showed circadian expression in the fetal kidney at E20 under prenatal shortened light-dark cycle. Worth mentioning that Sgk1 is a well characterized aldosterone target and regulator of ENaC[309] and its mRNA level increases in Per1-knockdown cells under

the effect of aldosterone.[252] Whether the *Sgk1* level is triggered by rhythmic aldosterone levels under this condition remains to be investigated.

3:21-LD: To the best of our knowledge, this is the first study investigating the effect of prorogated dark phase during the gestation period on the fetal renal clockwork. By the evaluation of the locomotor activity of dams we observed shortened rest period (ca. 9h) of the dams induced by the short light period with no increased moving activity over a 24 h. Similar to the 6:6-LD group dams gained less weight during the pregnancy compare to the control group.

In the fetus at E20, only the positive limb of the circadian feedback loop, *Clock* and *Bmal1* and the tubular clock controlled genes expressions showed circadian variation. The Clock-Bmal1 heterodimer triggers the rhythmic expression *of Per1/2, Cry1/2 and Rev-erbα.* Per and Cry form complexes which translocate back into the nucleus inhibiting their own transcription.[222] However, we did not find oscillation of the negative limb, the expression of the Clock and Bmal1remained rhythmic, which indicate other regulatory process under this condition.

LD-FR: Feeding is known as a strong Zeitgeber. Several studies showed that the disruption of the circadian rhythm, e.g. sleep-wake cycle associated with phase shift of the feeding pattern has adverse effects on the metabolic functions.[310] On the other hand, the quality of the food, i.e. caloric overload has an effect on the clock gene expression pattern in peripheral organs (liver, kidney), without effecting the circadian behavior of the mice.[311,312] A fascinating report has shown that restricted feeding (RF) without altering the caloric intake could attenuate high-fat diet caused obesity.[313] A similar mechanisms could be involved in the development of lower gained body weight of dams exposed to prenatal time restriction feeding regime (food available at ZT0-12 ad lib) in our experiment.

It has recently become evident that the regular food intake also plays entraining effect of the circadian clock. Moreover, it has been previously reported that RF in pregnant rats kept under constant illumination is able to restore the maternal circadian behavior and entrain the fetal circadian clock, i.e. circadian rhythmicity of the Avp and c-fos in the fetal SCN.[170] It is well known, that nutrient sensing hormones i.e. the insulin secretion following the plasma glucose level according to the feeding behavior have pivotal role in the peripheral circadian control.[90] Specially, restricted feeding is always coupled with a fasting period, thus with altered corticosteroid secretion.[93,129] Thus, we must consider nonspecific cues such as stress. Our study revealed that prenatal food restriction to the rest period of the dam (food available at

ZT0-12 ad lib) was associated altered gene expression patterns of the offspring at E20. From the core circadian clock genes only *Per2* displayed daily variation with the same phase observed in LD which suggests that the *Per2* oscillation in the kidney may not be dominated by the food cue. In contrast to our observation in rat liver a short feeding stimulus associated to Per2 induction has been documented by Wu.[94] The renal clock controlled genes: *αENaC, SGK1* and *NHE3* exhibited daily rhythmicity with a similar peak at the end of the dark period which leads us to speculate with rather other exogenous control that feeding regime of the mother.

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.[314,315] Human studies are showing conflicting results as well.[24,25] A Danish study, for example has documented only a limited effects of shift work during pregnancy on the fetal outcome.[25]

EXPERIMENT 2

In the further experiment in the offspring 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). This is consistent with previous studies in other tissue showing only a weak rhythm around the birth time and developing the adult like pattern postnatal.[167,170,179] For example, the rhythmicity of the clock gene expression in the SCN gradually develops parallel to morphological maturation and the sympathogenesis of the SCN, which are completed by postnatal day 10 (P10) in rats.[82]

Our findings in the rat kidney differ somewhat from *in vivo* studies of the pre- and postnatal ontogeny of molecular clockworks in other organs. In the rat liver only Rev-erbα exhibited circadian rhythmicity at the end of intrauterine development and during the first 10 days of postnatal life, with temporary additional low-amplitude oscillations of Cry1 at E20, Bmal1 at P2, and Per1 at P10.[165] Only after the first postnatal month had the clock genes developed stable circadian rhythmicity. In the heart, significant circadian expression of Bmal1 developed between P2 and P5, and of Per2 by P14.[167] In contrast to these findings we found three clock

genes to oscillate prior to birth in kidney tissue, and all components of the molecular clockwork showed high-amplitude circadian expression by 1 week postnatal.

During the first week of life we observed not only an increase in amplitude but also a distinct phase shift of the circadian oscillations. The peak clock of the gene expression shifted from the dark to the light period, with acrophases tightly synchronized around ZT4 ± 2 (i.e. 4 hours after light onset). Most investigated genes increased at the same time, which leads us to speculate with rather an exogenous control of the intrarenal circadian pacemaker system, than a functioning intrinsic internal regulation of the transcription-translation feedback loops similar to adult pattern at this time. The only exception was the complementary expression pattern of Rev-erbα, a transcription factor which is cyclically inhibiting Bmal1 expression as part of the clockwork machinery.[52] One role of the Rev-erbα in mediating the early phase of feeding-induced entrainment of the liver clock has been reported.[90]

At 4 weeks, clock gene expression shifted again towards the adult pattern with acrophases timed to the dark period (and Rev-erbα reciprocally to daytime). The renal core clock gene expression pattern of all investigated gene at 12 W show the same pattern as previously reported by Liu et al..[316]

The observed postnatal phase shift of clock gene expression is in keeping with findings of two *ex vivo* tissue culture studies using tissues of rat pups transgenic for Per1- and Per2-luciferase constructs.[166,179] We sought to identify the mechanism of this early reversal of the molecular clockwork coupling to the light-dark cycle. Videomonitoring of mothers nursing one-week-old pups demonstrated that feeding occurred mostly during the light period, i.e. the resting period of the mother animals. After weaning (at age 4 weeks), pups had largely acquired the adult pattern of food intake with two peaks at the beginning and end of the dark period, consistent with previous studies.[317] Based on this observation it is likely that the 'reversed coupling' of clockwork gene expression during the nursing period results from nutrient cues at a time of development when synchronization to daylight via the SCN is not yet operational. Sudden large changes in feeding regime seemed to be similar potent as an alteration of the light-dark period.

This hypothesis is supported by previous experimental evidence. In adult rodents' food-induced phase resetting proceeds have been observed in various tissues including the kidney independent of SCN activity.[94,102] Restricted feeding induces phase shifts in the peripheral tissues, whereas the rhythms in the SCN remain unaffected.[91,92,98] The liver generally seems to

be most readily entrained by nutrition-related metabolic cues both in adult and neonatal animals.[101,103,179] Allowing mother rats to nurse their one-week-old offspring exclusively during the dark period induced a phase shift of hepatic Per1 expression.[179] In contrast to the profound impact on hepatic Per1 expression, maternal deprivation did not affect oscillations in the SCN. In adult liver tissue, clock gene oscillations were reset in response to changes in food intake within 3 to 5 days. A slower response was found in the kidney: partial resetting with variable phase shifts by 4 to 12 hours occurred after 7 days of selective daytime feeding.[94,318]

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 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.[319,320] It has been recently reported that short disruption in sleep pattern leads to rapid alteration of the Per2 circadian rhythm e.g. in the kidney.[320] Furthermore, restricted feeding regime is always coupled with a fasting period, thus with potentially altered corticosteroid secretion.[93,129] We considered that the complete re-entrainment of clock genes by the changed feeding pattern may have been prevented by the general stress induced by periodic maternal absence, which has been shown to induce stress-related genes such as corticotropin releasing hormone, arginine vasopressin and the glucocorticoid receptor.[201] The stress response may have interfered with the metabolic entrainment of clock gene expression in the kidney.

Subsequently a major part of the study was to modify other environmental signals, i.e. maternal melatonin pattern. The potential synchronizations effects of mother-infant interactions, e.g. the maternal nursing as a Zeitgeber signals were previously described. [180,321] Plasma melatonin circadian oscillation is one of the daily rhythms of the mother that associate with the daily activity pattern and potentially drives the fetal circadian clock during prenatal period. As the SCN in rats is still immature at birth, thus the circadian clock machinery

controlled by maternal signals is particularly vulnerable postpartum as well. Previous studies documented a desynchronized rhythm between the pups by pinealectomy of the mother.[322] We investigated the effect of the maternal melatonin treatment in reverse phase on the 1-week old pups' circadian clockwork machinery. We hypnotized that maternal breast feeding including melatonin has a potentially Zeitgeber effect on the pups during the postnatal period as well. We assumed that the overall effect of 7 day long maternal melatonin treatment during the light period (at ZT3) potentially caused misalignment of nocturnal melatonin rhythm in the mother, as it was previously presented by Zhdanova.[323]

Since we have observed effects of the vehicle (6% ethanol in saline = 4,8 mg/kg) as well, it was difficult to make any conclusions. But it is important to acknowledge this alteration of the renal circadian gene expression in the offspring caused maternal alcohol consumption. There are relatively few studies reporting the effect of alcohol intake on circadian gene expression in animal experiment.[324,325] The observed changes in molecular rhythms depend on the length and dose of alcohol exposure. High alcohol consumption is also associating with altered circadian phenotypes.[326] Furthermore, the nocturnal melatonin concentration might be affected by e.g. ethanol.[327] Because melatonin is common solved in water/salin-ethanol solution in animal experiment, we must emphasize the direct effects of the ethanol on the molecular clockwork machinery which we have observed.

Moreover, our study revealed that prenatal light exposure has long-lasting effect in the circadian clock regulation. We presented a gained renal oscillation of the *Clock* gene in the adult offspring from mother exposed to constant illumination and constant darkness. Except for Clock, however, we did not find robust changes in the core clock component between the different groups.

The expression of the clock-controlled genes regulating the kidneys' homeostatic functions closely followed the postnatal changes influenced by many factors, suggesting that the coupling of the molecular clockwork to kidney functions is already operational in early postnatal life. Of note, the misalignment causing transient alteration of the clockwork might contribute to the long-lasting effect. In fact, the circadian expression of genes regulating electrolyte and water transport was most marked during the nursing period and was attenuated after weaning. It is tempting to speculate that the coupling of tubular functions to circadian nutritional cues during early postnatal development serves to maintain fluid and electrolyte

homeostasis of intermittently nursed animals incapable of spontaneous nutrient and fluid intake.

EXPERIMENT 3

In the recent years, evidence has been provided for an important role of the perinatal milieu in the intrauterine programming of adult diseases. Maternal distress and adverse metabolic state e.g. inappropriate nutrient supply, high maternal salt intake, alcohol consumption have been associated with development of late-onset cardiovascular, renal and metabolic diseases.[185-187,189,328190-194] As such, impact of the prenatal maternal circadian dysfunction on the adult offspring has not been well characterized. We examined the long-term outcome of prenatal circadian disruption on multiple indices of renal, cardiovascular and metabolic function in male and female offspring. Our particularly interest was the blood pressure regulation in aged offspring.

In examination of body weight in adult offspring (12W) from mothers exposed to prenatal light exposure (LL) or kept under constant darkness (DD), both male and female had significant higher body weight compared to controls (LD). Our observation is consistent with previous work examining the maternal distress during the pregnancy that has been associated with long-term obesity risk.[329] [330] [331] However, our results are in contrast with the previous works in which the fetuses had also been exposed to intrauterine growth restriction: we have presented no significant differences in placenta-, fetal growth, or litter size (See experiment 1.) after prenatal maternal exposure to altered light-dark cycle or food restriction.

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. Several studies have reported that women have a higher prevalence of obesity than men.[332] Gender differences exist even at the epigenetic level which is in part underlying the fetal programming of adult diseases.[333] Furthermore, studies have demonstrated that sex hormones (e.g. estrogen) can influence epigenetic mechanisms, including post-translational histone modifications.[333] We should consider the acute effect of the hormonal differences as well: i.e. in human, after menopause, decreased estrogen levels lead to reduced metabolic activity

which might advance the development of the obesity. Lack of direct measurement of sex hormones excretion of the offspring at this period is a limitation of this study.

It is estimated that up to ca. 50 % of metabolic syndrome can be linked to an adverse fetal environment. Maternal obesity, diabetes, but even mild maternal overnutrition has been shown to alter the brain appetite regulators in the offspring which might predispose to an imbalance in appetite and sympathetic control.[334 335 336] Imbalanced food intake and obesity is often associated with leptin resistance.[337] It had been previously shown that leptin treatment in the neonatal rodent could e.g. alter the expression of appetite-regulating orexigenic neuropeptide-Y (NPY) in the hypothalamus[338], which is also known to be involved in the input mechanism of the SCN control.[339 73] (See Figure 2)

During the menopausal transition, there is an increase in the prevalence of the metabolic syndrome, elevated body weight, and hypertension.[340] Thus, we deliberately studied the blood pressure in female offspring which approach the endocrine equivalent of human perimenopause by ca. 8 months of age [341].

The most adverse effect was found in offspring from mothers with food restriction during the pregnancy. Females in this group displayed reduced urinary sodium excretion with a higher systolic blood pressure, as well as pulse pressure and inverted phase, i.e. rising blood pressure during the resting period. Our data are consistent with previous animal experiment in which the maternal chronic shift of photoperiod was associated with elevated blood pressure later in 90 days old (rat) offspring.[342] In other animal models, maternal dietary restriction, glucocorticoid administration or prenatal stress are also associated with elevated blood pressure and exaggerated stress responses in offspring.[204,343-345] Alwasel et al. has documented that dietary protein restriction during the pregnancy caused persistent hypertension in adult rats.[346] A similar effect has been reported using a rat model of maternal 50% food restriction from the second week of gestation until weaning.[347] A potential underlying mechanism for development high blood pressure in adult life after maternal dexamethasone exposure has been explained by a life-long reduction in renal 11β-HSD2 activity.[348] Reduced 11βHSD2 enzyme activity causes sodium retention and salt-dependent hypertension as a result of accelerated activation of the mineralocorticoid receptor by cortisol.[349]

We assumed that stress-related mechanisms could be involved by the prenatal alteration of the light-dark cycle that we have studied. Aged females gestated under ultradian light-dark condition (6:6-LD) exhibited higher systolic daily blood pressure and decrease in the urinary

sodium excretion demonstrating a potential adverse long-lasting effect of the prenatal circadian disturbance. Additionally, we observed increased pulse pressure without alteration in the systolic blood pressure in female offspring from mothers with prenatal exposure to constant darkness (DD). Of note, in a large cohort group of normotensive patients, the elevation in pulse pressure has been shown as a marker to predict global cardiovascular risk.[350] Moreover, we found impaired renal sodium excretion without blood pressure alteration in females with prenatal prolonged dark exposure (3:21-LD). The impact of renal sodium handling and the salt sensitivity of blood pressure in health and hypertension has been intensively studied.[351] And the 24 h urinary sodium excretion has been shown to associate with blood pressure. 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. 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. Such a long-lasting effect of the prenatal light exposure of the mother was preciously not reported to our knowledge. However, phosphate handling is regulated by three organs: parathyroid, kidney and bone through feedback loops, we assume the renal tubular dysfunction which is demonstrating the pivotal role of the altered circadian rhythm on the development of renal malfunction.

GENDER RELATED DIFFERENCES

We found no differences in the expression pattern of *Bmal1* and *Rev-erbα* at adult age (12W) in the kidney between females and males. (Figure 24) The core circadian component, *Clock* expressed a distinct circadian oscillation in female offspring, while we found no significant oscillation in male offspring.

Although gender related differences of the clock gene oscillations were observed at the postnatal period (1W). This data leads us to speculate with a gender specific response to the entraining signaling in the early postnatal period.

6. CONCLUSIONS

In conclusion, our observations support evidence that the maternal circadian disruption impacts the clockwork machinery in the fetus potentially causing misalignment of physiological function later in life. The weakness of this study which must be pointed out is the experimental gap linking the gene expression pattern to the physiological function. An important aim task of future studies will be to elucidate the temporal structure of signal transmission from the transcriptional to the functional protein level.

Hence, 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. There are many largely unexplored questions of this field. It will be of interest to explore the underlying mechanism and the involvement of the clockwork machinery in response to other endogenous and exogenous homeostatic challenges in health and disease.

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.

7. SUMMARY

Evidence exist that many behavioral and physiological processes, e.g. renal function are driven by the circadian clock involving the intrarenal molecular clockwork. At the same time, there is a growing evidence that the chronic disturbances in the temporal regulation of the internal clock e.g. (social-) jet lag, shift work are implicated in a range of pathophysiological dysfunctions including e.g. hypertension and obesity in adults. On the other hand, 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 rhythm of offspring and have potentially long-lasting effects later in life.

Here, we studied prenatal and postnatal circadian expressions of endogenously expressed genes encoding proteins that are known to contribute to renal function as well as the role of the prenatal maternal circadian misalignment in the clockwork regulation and renal function involving blood pressure regulation in the offspring later in life. Dams were exposed to altered light-dark cycle(s) (LD, LL, DD, 6:6-LD and 3:21-LD) or restricted feeding schedules (FR-LD) during the entire gestation (E2-20) period. The pregnancy outcomes and the effect of maternal circadian misalignment on the offspring were evaluated at different developmental stage(s) (E20, 1W, 4W, 12W, 34W).

The main finding of this study is the evidence for the presence of a functioning molecular clockwork in the late fetal kidney, earlier than in other tissues (at E20). We described a temporary alteration of the renal circadian clockwork machinery in the fetus induced by disturbed prenatal maternal environment. Furthermore, 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 found that the maternal circadian dysfunction e.g. altering the feeding behavior (FR-LD) induced an alteration of the circadian clockwork machinery at the time of birth which was associated with e.g. elevated blood pressure and gender specific obesity in the offspring later in life. Moreover, we documented several long-lasting effects in the offspring of the dams which were exposed to different light-dark schedule during the pregnancy.

8. ÖSSZEFOGLALÁS

Napjainkban egyre több adat bizonyítja, hogy számos élettani folyamat, pl. a veseműködés napszakos változása, a molekuláris cirkadián óra által generált endogén ritmusok fiziológiás megnyilvánulása. Ezzel párhuzamosan egyre több meggyőző bizonyíték gyűlt össze arra vonatkozóan, hogy a cirkadián óra deszinkronizációja felnőttekben olyan patofizilógiai elváltozásokat indukálnak, melyek súlyos kórképek (e.g. anyagcsere, kardiovaszkuláris és tumoros betegségek) kialakulásában játszanak szerepet. Mindezek mellett ismert, hogy a méhen belüli fejlődés során a nem megfelelő környezeti viszonyok kedvezőtlenül befolyásolják az organogenezist, így úgymond programozhatják egyes krónikus betegségek későbbi kialakulását. A fentieket figyelembe véve felmerül, hogy a cirkadián ritmus zavara, a várandós anya megváltozott táplálkozási és alvási mintázatával, befolyásolhatja a cirkadián óra szerveződését az utódokban, amely potenciálisan hosszan tartó hatásokkal bírhat.

Munkánk során vizsgáltuk a prenatális anyai cirkadián ritmus diszfunkciójának hatását az óragének (*Clock, Bmal1, Per1/2, Cry1/2* és *Rev-erbα*) szerveződésére az utódok veséjében, valamint a vesefunkcióra, vérnyomás-szabályozására későbbi élet során. Anyaállatokat a vemhességük során (**E2-20**) különböző megvilágítási ciklusnak vettettük alá (**LD**, **LL**, **DD**, **6:6-LD** és 3**:21-LD**), vagy a megváltoztattuk a táplálékfelvétel normál napi ritmus a táplálék fotoperiódushoz kapcsolt megvonásával (**FR-LD**). Ezt követően az utódokat különböző életkorban vizsgáltuk (**E20**, valamint 1, 4 és 12 hetes korban, **1W**, **4W**, **12W**, **34W**).

Ez a tanulmány mutatta be először, hogy a vesében lévő óragének már a késői gesztációs időszakban (**E20**) cirkadián oszcillációt mutatnak, mely tükrözi a fötális vese sejtjeinek belső, autonóm oszcillációját. Ezen túl, leírtuk az renális óragének napi expressziós mintázatának fejlődését (**1W, 4W, 12W**). Munkánk alátámasztja azon korábbi kutatások eredményeit, melyek bizonyítékokkal szolgálnak arra vonatkozóan, hogy az óragének expressziós mintázata a vesében összefügg a táplálék felvétel napi ritmusával.

Továbbá dokumentáltuk a vizsgált gének napi expressziós mintázatának sokrétű változásait a prenatálisan módosított fényviszonyok (**LL**, **DD**, **6:6-LD**, **3:21-LD**) vagy az időben korlátozott anyai táplálékfelvétel (**FR-LD**) hatására. Bár a patomechanizmus nem tisztázott, az anyai cirkadián óra zavara az intrauterin fejlődés során a vizsgált felnőtt utódokban elhízásra való hajlammal, magasvérnyomással és megváltozott vesefunkcióval járt.

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10. OWN PUBLICATIONS

I. Publication related to the topic of the Ph.D. dissertation:

1.Meszaros K, Pruess L, Szabo AJ, Gondan M, Ritz E, Schaefer F
Development of the circadian clockwork in the kidney.
KIDNEY INTERNATIONAL 86:(5) pp. 915-922. (2014)

II. Other publications:

2. 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
PEDIATRIC TRANSPLANTATION 17:(5) pp. 454-460. (2013)

3. Rusai K, Prokai A, Juanxing C, Meszaros K, Szalay B, Pasti K, Muller V, Heemann U, Lutz J, Tulassay T, Szabo AJ
Dexamethasone protects from renal ischemia/reperfusion injury: a possible association with SGK-1.
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4. Rusai K, Prokai A, Szebeni B, Meszaros K, Fekete A, Szalay B, Vannay A, Degrell P, Muller V, Tulassay T, Szabo AJ
Gender Differences in Serum and Glucocorticoid Regulated Kinase-1 (SGK-1) Expression during Renal Ischemia/Reperfusion Injury.
CELLULAR PHYSIOLOGY AND BIOCHEMISTRY 27:(6) pp. 727-738. (2011)

11. ACKNOWLEDGMENTS

I would like to thank everyone who has helped me make this work possible. Specially, I would like to acknowledge my advisors, Prof. Franz Schäfer and Prof. Eberhard Ritz, who allowed me to conduct the research in this dissertation under their supervision and would like to thank Prof. Attila Szabó for his help in directing the course of my PhD studies.

A special thanks to Dr. Éva Nyerges and Prof. Dr. Ulrich Luft, who encouraged me to carry on, and to my amazing husband for all his support are gratefully acknowledged.