Functional Analysis of the Adrenal Circadian Clock

Von der Naturwissenschaftlichen Fakultät der

Gottfried Wilhelm Leibniz Universität Hannover

zur Erlangung des Grades einer

Doktorin der Naturwissenschaften

Dr. rer. nat.

genehmigte Dissertation

von

Dipl.-Biol.

Silke Kießling

geboren am 22. Juli 1980, in Bremen

2010

Referent: Prof. Dr. Stephan Steinlechner

Korreferent: Prof. Dr. Gregor Eichele

Tag der Promotion: 5.5.2010

Meinen Eltern

"Zeit ist das, was wir haben, wenn wir unsere Uhren wegwerfen"

Jürgen Aschoff (Begründer der Chronobiologie)

Contents

Figure List

Supplemental figure list

Table list

Supplemental table list

Summary

Keywords: Adrenal Circadian Clock, Glucocorticoids, Jet lag

The daily rotation of the Earth around its axis influences all live on our planet, from unicellular organisms to humans. Endogenous clocks have evolved to anticipate and synchronize physiology and behavior to daily recurring environmental changes ("entrainment"). In mammals, a central circadian pacemaker is localized in the *suprachiasmatic nuclei* (SCN) of the hypothalamus, directly entrained by light. The SCN orchestrate subordinate clocks found throughout the body – via both humoral and neuronal pathways – to coordinate the overall entrainment of the organism. In the absence of external time information circadian rhythms are sustained with an endogenous period length of approximately 24 hrs (hence the term circadian: lat. "circa" = approximately and "dies" = day). Environmental timing cues (so called *Zeitgeber*) like the light/dark cycle can phase shift the endogenous oscillator to synchronize the animal to external time. Circadian clocks are built from a set of so-called "clock genes" organized in a system of transcriptional/ translational feedback loops and creating stabilized transcriptional rhythms of ca. 24 hrs period.

In the first project we characterize the function of the circadian clock in the adrenal, an important endocrine gland that synchronizes physiological and metabolic rhythms via the regulation of circadian glucocorticoid (GC) synthesis. Using a transgenic mouse model with a combined disruption of specific "clock" genes (*Per2* and *Cry1*) that lacks circadian clock function, we analyzed the impact of either the SCN or the adrenal clock in the regulation of GC rhythms. We show that the adrenal pacemaker, which itself is light entrainable, modulates/gates rather than controls the glucocorticoid production in response to a hormonal signal (ACTH), ultimately controlled by the SCN.

The second project addresses the molecular events underlying circadian disruption during jet lag. Rapid travel across several time zones causes jet lag that is characterized by a transient disturbance of numerous physiological parameters such as the sleep-wake cycle, hormone levels and metabolism. To better understand the molecular manifestations of jet lag, we monitored central and peripheral circadian clocks under jet lag conditions in an attempt to identify factors that regulate the adaptation of the circadian timing system to a new time zone. We show that jet lag in mice causes a transient disruption of multiple core clock genes, on the level of the SCN itself and in a similar fashion in peripheral oscillators. In addition we observed transient de-synchronization between different tissues. This global

"uncoupling" provides a mechanistic rationale for the wide spectrum of jet lag-associated physiological effects. We further used a combination of mouse genetics and adrenal tissue transplantation to show that the adrenal circadian clock influences the kinetics of re-entrainment of running-wheel activity during jet lag by controlling the rhythmic release of GCs. Adrenal clock-controlled GC release affects the regulation of activity rhythms, which is primarily controlled by the SCN, indicating a feedback of the adrenal clock to the SCN pacemaker. Chronopharmacological manipulation of GC phase prior to jet lag is sufficient to substantially accelerate behavioral re-entrainment and, thus, shorten the duration of jet lag. This strategy might constitute a simple and more targeted approach to alleviate the various symptoms of jet lag that thousands of travelers and shift workers suffer from each day.

Taken together this work provides new insights into the connection between the SCN and a peripheral clock, the adrenal. It describes the molecular events underlying circadian desynchrony during jet lag and highlights the potential of GC rhythms as new targets to alleviate jet lag associated syndromes.

Zusammenfassung

Schlagwörter: Zirkadiane Nebennierenuhr, Glukokortikoide, Jet lag

Die tägliche Rotation der Erde um ihre eigene Achse beeinflusst das gesamte Leben auf diesem Planeten, von Einzellern bis zum Menschen. Um die damit einhergehenden periodischen Umweltveränderungen zu antizipieren und Physiologie und Verhalten daran anzupassen, verfügen die meisten Lebewesen über sog. "zirkadiane Uhren". Der zentrale zirkadiane Schrittmacher bei Säugern sitzt im *Nucleus suprachiasmaticus* (SCN) des Hypothalamus. Der SCN wird direkt durch den Tag/Nacht-Zyklus synchronisiert und kontrolliert untergeordnete Uhren in verschiedensten Geweben des Körpers durch humorale und neuronale Verbindungen, um so den Gesamtrhythmus des Organismus zu koordinieren. In Abwesenheit externer Zeitsignale bleibt der interne zirkadiane Rhythmus erhalten, allerdings mit einer Periodenlänge von nur ungefähr 24 Stunden (daher der Ausdruck „zirkadian", vom Lateinischen *circa dies* = *ungefähr ein Tag*). Die Anpassung der inneren Uhr erfolgt durch sogenannte *Zeitgeber* wie dem Licht/Dunkel-Wechsel. Diese können die Phase des endogenen Oszillators verschieben, um so den Organismus mit der externen Zeit zu synchronisieren. Das molekulare Uhrwerk besteht aus einem System von Genen. Diese sogenannten „Uhrengene" sind durch transkriptionelle/translationelle Rückkopplungsschleifen miteinander verbunden, wodurch eine stabile Oszillation von ca. 24 Stunden generiert wird.

Im ersten Teil des Projektes wird die Funktion der zirkadianen Uhr in der Nebenniere untersucht. Die Nebenniere, ein wichtiges endokrines Organ, beeinflusst viele physiologische Stoffwechselrhythmen durch die Produktion von Steroidhormonen z.B. den Glukokortikoiden. Mit Hilfe eines Mausmodels mit gewebsspezifischen Mutationen in zwei wichtigen Uhrengenen (*Per2 und Cry1*) habe ich den Einfluss einer funktionellen SCN- bzw. einer funktionellen Nebennierenuhr auf die Regulation der zirkadianen Glukokortikoidsynthese untersucht. Ich konnte zeigen, dass die Nebennierenuhr, welche ähnlich der des SCNs ebenfalls lichtreguliert ist, eher modulierend als kontrollierend auf die rhythmische Produktion von Glukokortikoiden wirkt, welche durch ein ultimativ vom SCN kontrolliertes Hormon (ACTH) iniziiert wird.

Im zweiten Teil des Projektes soll der dem Jetlag zugrunde liegende molekulare Mechanismus aufgeklärt werden. Schnelles Reisen über mehrere Zeitzonen verursacht Jetlag, welcher durch die übergangsweise Störung diverser physiologischer Paramter wie des Schlaf/Wach-Ryhthmus, des Hormonspiegels und des Stoffwechsel charakterisiert ist. Die dem Jetlag-Syndrom zugrundeliegenden molekularen Vorgänge wurden analysiert, indem ich

die Reaktion des zirkadianen Systems nach einer Phasenverschiebung beobachtete. Ziel war es, auf diese Weise Faktoren zu identifizieren, welche die Adaptationsgeschwindigkeit des zirkadianen Systems regulieren können. Ich konnte zeigen, dass durch Jetlag eine vorübergehende Verschiebung verschiedener Uhrenkomponenten verursacht wird, die auf der Ebene des SCNs und auch in ähnlicher Weise im peripheren Uhrwerk auftritt. Solch eine Desynchronisierung liegt auch zwischen einzelnen Geweben vor. Die resultierende globale Entkopplung aller Uhren und ihrer Komponenten liefert eine mechanistische Begründung für das weite Spektrum der Jetlag-assoziierten physiologischen Effekte. Ferner nutzte ich eine Kombination aus Mausgenetik und Nebennierentransplantations-Technik, um den Einfluss der Nebennierenuhr auf die Kinetik der Aktivitätsanpassung während des Jetlags zu ermitteln. Ich konnte so zeigen, dass die durch die Nebenniere kontrollierte Glukokortikoidfreisetzung die Anpassung des Aktivitätsrhythmus reguliert, welcher primär durch den SCN gesteuert wird. Das deutet auf ein Feedback von der Nebennierenuhr auf den SCN Schrittmacher hin. Chronopharmakologische Manipulation der Phase des Glukokortikoidrhythmus ist ausreichend, um die Anpassung der Aktivität wesentlich zu beschleunigen und die Dauer des Jetlags zu verkürzen. Diese Strategie bietet einen einfachen aber gezielten Ansatz zur Milderung der durch Jetlag verursachten Symptome, welche tausende Reisende und Schichtarbeiter erleiden müssen.

Zusammengefasst betrachtet, bietet diese Arbeit neue Einblicke in die Verbindung zwischen dem SCN und einer peripheren Uhr, der Nebenniere. Sie beschreibt den molekularen Mechanismus, welcher der desynchronisierenden Wirkung des Jetlags zugrunde liegt und zeigt das Potential der Glukokortikoidsynthese als neues Angriffsziel zur Behandlung von Jetlag-assoziierten Syndromen.

Abbreviations

° C, degrees Celsius
τ, internal period length
(v/v), volume per volume
(w/v), weight per volume
11-Deoci-Cortisol, 11-deoxycorticosterone
17-OH-preg, 17-OH progesterone
^{35}S-UTP, sulphur-35-sulphate labeled uridine triphosphate
5-HT, serotonin
a, adrenal
AC, adenylate cyclase
ACTH, adrenocorticotropic hormone
Adcy5, adenylate cyclase 5
ARC, arcuate nucleus
ATP, adenosine triphosphate
AVP, arginine vasopressin
Bmal1, brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1
bHLH, basic helix-loop-helic
BIT, brain immunoglobulin-like molecule with tyrosine-based activation motifs
C, corticotrope cell of the pituitary
ca., circa
cAMP, cyclic adenosine monophosphate
CCG, clock controlled gene
cDNA, copy DNA
CEH, cholesteryl esters hydrolase
cGMP, cyclic guanosine monophosphate
CKK, cholecystokinin
CLC, cardiotrophin-like cytokine
Clock, circadian locomotor output cycles kaput
Cm, centimeters
CNS, central nervous system
cpm, counts per minute
CRE, cAMP responsive elements
CREB, CRE binding protein
CRH, corticotrophin-releasing hormone
Cry1/2, Chryptochrome1/2
CSCC, cholesterol side chain cleavage
CT, circadian time
CTP, cytidine triphosphate
CYP, *P450-cytochromes*
(11A1/21A1/11B1/11B2/17)+HSD3β
Dbp, D-albumine binding protein
DD, constant darkness
Dec1/2, basic helix-loop-helix family member e40 1/2
DHEA, dehydroepiandrosterone
Dil., diluent
DMH, dorsomedial hypothalamic nucleus
DMV, dorsal motor nucleus of the vagus
DNA, deoxy-ribonucleic acid
dNTS, deoxy-nucleotide triphosphate
DTT, dithiothreitol
E4BP4, nuclear factor, interleukin 3, regulated
EDTA, ethylene-diamine-tetra-acetic acid
Ef1α, elongation factor 1 alpha
engl., english
ER, endoplasmatic reticulum
et al., and co-workers
EtOH, ethanol
Fig., figure
fw, forward
g, gram
gr., Greek
GC, glucocorticoid
Glu, glutamate
GTP, guanosine triphosphate
hrs, hrs
h, host
HCl, hydrochloric acid
HPA, hypothalamus-pituitary-adrenal axis
HSA, hypothalamus-spinal cord-adrenal axis
I, interneuron
IGL, intergeniculate leaflet
IML, intermediolateral column of the spinal chord
i.p., intra peritoneal
ISH, *in situ hybridization*
kb, kilobases
kg, kilogram
l, liter
lat., Latin
LD, light dark cycle
LD12:12, 12h dark, 12h light
Ldlr, low-density lipoprotein receptor
LDL, low-density lipoprotein
LL, constant light
LS, intermediate lateral septal nucleus
M/Mol, moles per liter
MAPK, mitogen-activated protein kinase
Mc2R, *melanocortin2 receptor*
MET, Metyrapone
beta-MeSH, ß-mercaptoethanol
mg, milligram
MgCl, magnesium chloride
min, minutes
µg, microgram
µl, microliter

µM/µMol, micromoles per liter
ml, milliliters
mm, millimeters
mM/mMol, millimole per liter
MPO, medial preoptic area
mpPVN, medioparvocellular PVN
mRNA, messenger ribonucleic acid
mut, mutant
n, sample size
ng, nanogram
NaCl, sodium chloride
NADH (NADPH), nicotinamide adenine dinucleotide (phosphate)
Neo, neomycin resistance gene
NH_4Ac, ammonium acetate
NPY, neuropeptide Y
NTS, nucleus of the solitary tract
OP, operation
PACAP, pituitary adenylate cyclase-activating peptide
PAG, periaquaeductal gray
PAS, period / aryl hydrocarbon receptor nucleartranslocator / singleminded
PBN, parabrachial nucleus
PBS, phosphate buffered saline
PCR, polymerase chain reaction
Per1/2, Period1/2
PFA, paraformaldehyde
PHA, posterior hypothalamic area
PK2, prokineticin 2
PKA, protein kinase A
pM/pMol, picomoles per liter
Por , P450 oxidoreductase
PS_{50}, 50% phase shift
PT, paratenial nucleus
PVN, paraventricular nucleus of the hypothalamus
PVT, paraventricular nucleus of the thalamus
qPCR, quantitative real-time PCR
rev, reverse
RGC, retinal ganglion cells
RHT, retinohypothalamic tract
RIA, Radio-immunoassay
RNA, ribonucleic acid
Rorα, retioid acid receptor-related orphan receptor alpha
rpm, rounds per minute
RT, room temperature
RT-PCR, reverse transcription polymerase chain reaction
sec, seconds
SCN, suprachiasmatic nuclei
SDS, sodium dodecylsulfate
SEM, standard error of the mean
SGC, small granule chromaffin cells
SN, supernatant
Sp1, trans-acting transcription factor 1
SPZ, sub-paraventricular zone
SSC, standard sodium chloride / sodium citrate bufferStAR, steroidogenic acute regulatory protein
Tgfα, transforming growth factor alpha
Tris, Tris -(hydroxymethy)-aminomethane
TTL, transcriptional/ translational feedback loop
vol., volume
vs., *versus*
vSCN, ventral SCN
vlSCN, ventrolateral SCN
VMH, ventromedial nuclei of the hypothalamus
W, watt
WT, wild-type
zf., zona fasciculata
zg., zona glomerulosa
zr., zona reticularis
ZT *Zeitgeber* time
ZT_{max}, peak ZT

Chapter 1

Introduction

1.1. The biological clock

The regular changes of day and night caused by the Earth's rotation around its axis influence all life on this planet, from single cells to higher life forms (Edery 2000; Devlin and Kay 2001). Especially for plants the sun light is a very strong power source fuelling the photosynthesis machinery. In 1729 the astronomer Jean-Jaques Dortous de Mairan initially discovered the diurnal leave movement of *Mimosa*, which initiated the beginning of a complete new research direction. These opening-closing rhythms did not merely reflect the response to changed environmental conditions, but rhythmicity persisted with nearly 24 hrs ("*circadian*") period when plants were kept in constant darkness. But how does a plant time leave movements in isolation from external time information ("*Zeitgeber*" = engl. timing cue; in this case the sun, Aschoff 1951)? The answer is: by an internal endogenous clock.

Biological clocks are the subject of modern chronobiology (gr. "chronos" = time, "bios" = life and "logos" = subject), the research on biological rhythms. Internal clocks have been described in almost all *phyla*, but it took more than 100 years to reveal the existence of circadian rhythms in *Homo sapiens* (Dunlap, Loros et al. 1999). In 1845 Davy discovered for the first time that the body temperature (T_b) in humans fluctuates during the day and can be influenced by physical exertion and exposure to extreme temperatures. In 1866 Ogle revealed that the circadian rhythm of T_b showed anticipation with a rise prior to awaking and a drop before falling asleep. Since then, rhythms have been described in all areas of biology, such as in development, lifespan and lifestyle (Schibler and Naef 2005), behavioral rhythms but also rhythms in single cells, enzyme activity or even at the level of transcription (Pittendrigh 1993).

For many species sunlight is the most important Zeitgeber (Daan and Pittendrigh 1976). Except for plants and photosynthetic prokaryotes the succession of light and dark is not an important environmental factor by itself, but rather it is the presence or absence of prey or predators, the availability of food or temperature differences obviously tied to the solar cycle (Pando and Sassone-Corsi 2001; Roenneberg and Merrow 2002). To anticipate these re-occurring changes – and thereby enhancing the chance of survival and reproduction –

internal time-measurement mechanisms have evolved, so called circadian clocks. Another advantage of an endogenous oscillator is the persistence of an endogenously generated rhythm under constant conditions, e.g. in subterranean animals that are isolated (at least for some time) from the outer time information.

A second facet of circadian timekeeping is endogenous synchronization. Minimizing energy consumption increases evolutionary fitness. If most organs necessary for metabolism are synchronized with each other, active at the same time, digestion becomes more efficient. The internal clock is the link between pathways involved in metabolism and catabolism and via temporal organization of the interplay promotes maximal efficiency (Akhtar, Reddy et al. 2002; Panda, Antoch et al. 2002; Storch, Lipan et al. 2002).

1.2. Properties of biological oscillators

The simplest concept of a biological clock is that an endogenous oscillator receives time information from the environment through an input pathway and drives physiological rhythms via an output pathway. A functional biological oscillator has to fulfill different key properties.

i) Sustainment: In order to be assigned to be circadian, the biological clock produces persistent rhythms close to 24 hrs (circadian: lat. "circa" = approximately and "dies" = day), even under constant conditions with a free-running period (tau/τ). Other biological rhythms are described with periods ranging from seconds to decades. Those with shorter periods (<20 hrs) are called ultradian. If periods become longer than 30 hrs , they are termed infradian. Not all of these rhythms are self-sustained like the circadian clock.

ii) Resetting: An oscillator has to respond rapidly to environmental timing cues, so called *Zeitgebers*, to maintain its phase relationship with environmental cycles ("resetting"). Under natural conditions, the differences between the external and the internal phase are adjusted by the sunlight every day in a process called entrainment (Aschoff and Pohl 1978). In the absence of an external Zeitgeber, animals follow their internal clocks. As a rule of thumb, in nocturnal animals (like mice) endogenous clocks run at periods shorter than 24 hrs while in diurnal animals (such as humans) clocks are slightly slower (between 24 and 26 hrs) (Daan and Pittendrigh 1976). Possible Zeitgeber include light, temperature, humidity, food availability (Mistlberger 1994; Challet, Solberg et al. 1998), social cues (Levine, Funes et al. 2002), sound (Menaker and Eskin 1966) and magnetic fields (Griefahn, Kunemund et al. 2002).

iii) Temperature compensation: Normally, chemical reactions run faster at higher temperatures. If temperature-dependent, the circadian clock would run faster at high than at low temperatures and would not be a reliable predictor of day time. Therefore mechanisms have evolved to keep the length of the circadian period relatively constant over a wide range of temperatures (Reyes, Pendergast et al. 2008). This is of special importance in species that do not have their own temperature regulation, such as plants or invertebrates.

1.3. A central pacemaker in the brain – the suprachiasmatic nuclei

In mammals a "master-clock" resides in the ventrolateral hypothalamus (Klein, Moore et al. 1991). Lesion experiments by two independent groups located it in a small bilateral set of neurons, the suprachiasmatic nuclei (SCN) (Moore and Eichler 1972; Stephan and Zucker 1972). The SCN drive rhythms in behavior and physiology, including hormonal secretion, the sleep-wake cycle, cardiovascular activity, T_b, acuity of the sensory system, renal plasma flow, intestinal peristaltic activity and detoxification (Ralph, Foster et al. 1990; Sollars, Kimble et al. 1995; Panda, Antoch et al. 2002; Schibler and Sassone-Corsi 2002; Storch, Lipan et al. 2002; Schibler, Ripperger et al. 2003; Sujino, Masumoto et al. 2003).

The SCN are composed of ca. 10,000 tightly packed neurons. SCN electrical activity follows a circadian rhythm that persists in explant cultures in vitro (Shibata and Moore 1993) and even single SCN cells are capable of governing circadian rhythmicity (Welsh, Logothetis et al. 1995). These cell autonomous rhythms are believed to be synchronized by the release of neuropeptides (Colwell, Michel et al. 2003; Cutler, Haraura et al. 2003; Yamaguchi, Isejima et al. 2003).

1.3.1. Clock input

The most important photoreceptors responsible for the entrainment of circadian rhythms are not localised in the rods and cones, harboring the visual photoreceptor-system (Freedman, Lucas et al. 1999; Berson, Dunn et al. 2002). Rather, a subset of directly light sensitive retinal ganglion cells, expressing the photopigment melanopsin (ipRGCs) (Provencio, Rollag et al. 2002) (**Figure 1**), acts as time sensor for the SCN clock.

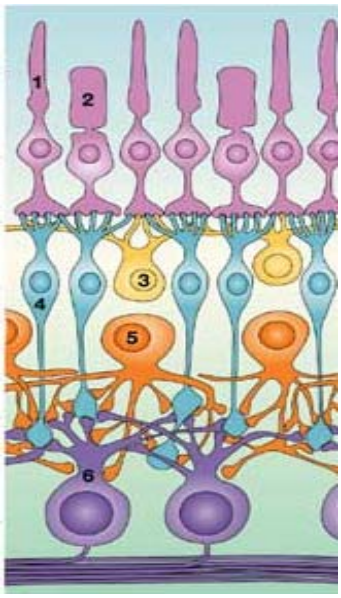


Figure 1: Anatomical structure of the photorecptors of the retina. Rods (1), cones (2), horizontal cells (3), bipolar cells (4), amacrine cells (5) and retinal ganglion cells (RGCs)(6)(Wassle 2004).

But ipRGCs alone are not exclusively responsible for the detection of light; a subset of visual photoreceptors also impacts on the entrainmet of the circadian system (Hattar, Lucas et al. 2003). However, the photic input for the clock (ipRGCs) is anatomically separated from image-forming visual pathways (non-ipRGCs) (Guler, Ecker et al. 2008).

Monosynaptic projections from ipRGCs reach neurons (Berson 2007; Hankins, Peirson et al. 2008) in the ventrolateral part of the SCN (vlSCN), via the retino-hypothalamic tract (RHT) (Moore and Lenn 1972; Johnson, Morin et al. 1988). Neurotransmitters of the RHT are glutamate (Glu) and PACAP (pituitary adenylate cyclase-activating peptide) (Hirota and Fukada 2004). Upon binding of Glu/PACAP at SCN neurons a signalling cascade is activated (**Figure 2**), which leads to the activation of calcium dependent kinases and proteases (activation of calmodulin, MAP kinases and PKA) and the phosphorylation of the transcription factor CREB (cAMP response element binding protein). P-CREB induces the expression of clock genes, e.g. *Per* (period) 1 and 2, via the CRE element (cAMP response element) in their promotors (Xia, Dudek et al. 1996; Obrietan, Impey et al. 1998; Gillette and Tischkau 1999; Gau, Lemberger et al. 2002; Hirota and Fukada 2004; Antle and Silver 2005; Maywood, O'Neill et al. 2007).

At the beginning of the dark phase, light exposure causes phase delays of the circadian clock (Daan and Pittendrigh 1976) with an increase of *Per2* and *Per1* expression, while during the second half of the night, a phase advance is induced which is correlated with an increase of *Per1* , but not *Per2*, expression. cGMP dependent protein-kinase II (PKGII) seems to be required for the photic induction of *Per2* that delays the resetting of the clock (Oster, Werner et al. 2003).

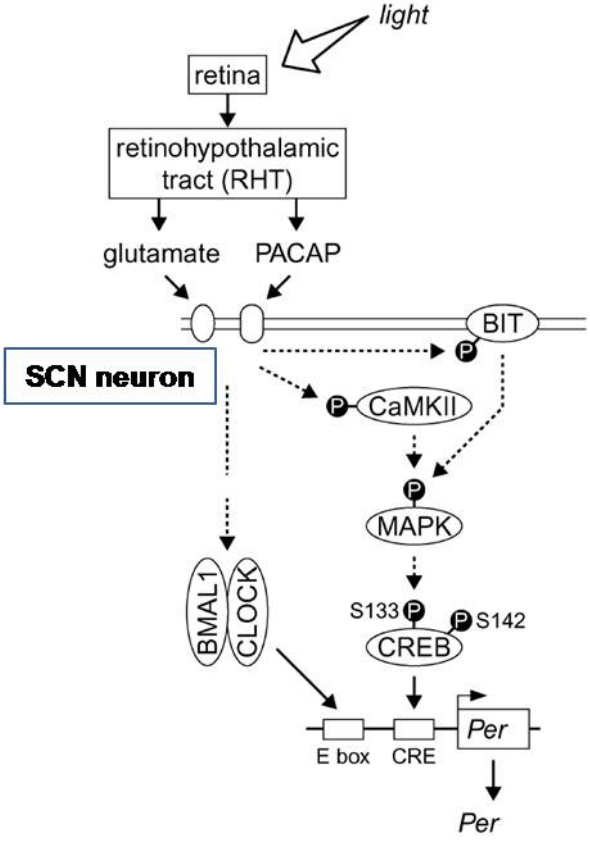


Figure 2: Photic input and the signal transduction cascades in the SCN neuron. Solid and dashed line depicts direct and indirect pathways, respectively. Abbreviations: BIT, brain immunoglobulin-like molecule with tyrosine-based activation motifs; CaMKII, calcium-calmoduline kinase II; CRE, cAMP response element; CREB, CRE-binding protein; PACAP, pituitary adenylate cyclase-activating peptide; Modified from (Hirota and Fukada 2004).

Apart from light there exist so-called "non-photic" Zeitgeber which are also able to reset the circadian clock. Such input is transmitted via neuropeptide Y (NPY) expressing cells in the intergeniculate leaflet of the thalamus (IGL) (Biello, Janik et al. 1994; Janik and Mrosovsky 1994; Maywood, Smith et al. 1997). ipRGCs also project to the IGL, indicating a role for this structure in the integration of photic and non-photic signals (Juhl, Hannibal et al. 2007). The SCN also receives serotonergic (5-HT) input from the raphe nuclei (Bosler and Beaudet 1985; Francois-Bellan and Bosler 1992) that modulates the photic signal response in the SCN (Klein, Moore et al. 1991). In addition, signaling cues from the cortex, the forebrain and the hypothalamus reach dorsomedial parts of the SCN (Moga and Moore 1997; Mendoza, Clesse et al. 2008).

1.3.1.1. Molecular clockwork/TTL

In the SCN each neuron is believed to harbor its own circadian clockwork (Welsh, Logothetis et al. 1995; Herzog and Tosini 2001). These cellular clocks are coupled, resulting in an integrated and robust rhythmicity of the whole nucleus (Low-Zeddies and Takahashi 2001). Cellular clocks are based on transcriptional/translational feedback loops (TTLs). The first clock gene, *Per*, was identified in a mutagenesis screen in *Drosophila melanogaster* in 1971 (Konopka and Benzer 1971). From there on other clock genes were identified in different model organisms such as cyanobacteria, fungi, flies, mice, and humans (Rosbash 2009). Basic principles of the molecular clockwork are reviewed in Panda et al. (Panda, Antoch et al. 2002). In the following sections I will focus on the circadian system of mammals.

The molecular clockwork is composed of interacting autoregulatory transcriptional/ translational feedback loops (TTLs) that drive recurrent changes of RNA and protein levels of key clock components (Reppert and Weaver 2002). In the mammalian system, each TTL can be subdivided into a positive and a negative limb (**Figure 3**). The positive limb of the core TTL consists of the helix-loop-helix (bHLH)-PAS (*Period - Arnt – Singleminded*) transcription factors CLOCK and BMAL1 (ARNTL). CLOCK/BMAL1 heterodimers bind to enhancer elements termed E-boxes and directly activate the transcription of three *Period* genes (*Per1*, *Per2* and *Per3*), two *Cryptochrome* genes (*Cry 1* and *Cry 2*), members of the blue-light photoreceptor/ photolyase family (Jin, Shearman et al. 1999; Kume, Zylka et al. 1999), and the orphan nuclear receptors *Rev-Erbα* and *Rorα* at the beginning of the day. PER and CRY proteins are synthesized in the ER and accumulate in the cytoplasm to a critical level in the course of the day, before they heterodimerize and translocate into the nucleus in the beginning of the night.

There, the PER/CRY complex interacts negatively with CLOCK/BMAL1, attenuating the activation of their own genes. Via this feedback PERs and CRYs constitute the negative limb of the core TTL (Figure 3; King and Takahashi 2000; Albrecht and Eichele 2003). During the night, while *Per/Cry* transcription is low, PER/CRY protein levels gradually decrease due to degradation while at the same time *Bmal1* transcription is activated (see below). Around dawn the critical relation between BMAL1 and PER/ CRY levels is reached and *Per/Cry* transcription is re-initiated. Rhythmicity of *Bmal1* expression is mediated by an accessory loop, involving Rev-Erbα and Rorα, both activated through E-Box elements and CLOCK-BMAL1. REV-ERBα inhibits and RORα activates the transcription of *Bmal1* by competitive binding to RORE elements in the *Bmal1* promoter (Preitner, Damiola et al. 2002; Akashi and Takumi 2005). Other accessory loops based on the transcription factors DEC1/2 and DBP/E4BP4 have been described. Together, these are believed to stabilize the rhythm of the core TTL and to provide further means of output from the molecular clock (Hastings 2000).

A number of posttranslational events, affecting protein stability, degradation or nuclear accumulation, are responsible for the delay of several hrs between *mRNA* and protein peaks for genes involved in the stabilization of the circadian oscillator. Posttranscriptional modifications like phosphorylation, sumoylation, ubiquitylation, intracellular transport and degradation seem to be critical for the generation of oscillations in clock gene products and the stabilisation of a 24h period (Kume, Zylka et al. 1999; Lee, Etchegaray et al. 2001; Miyazaki, Mesaki et al. 2001; Vielhaber, Duricka et al. 2001; Yagita, Tamanini et al. 2002; Yu, Nomura et al. 2002; Cardone, Hirayama et al. 2005).

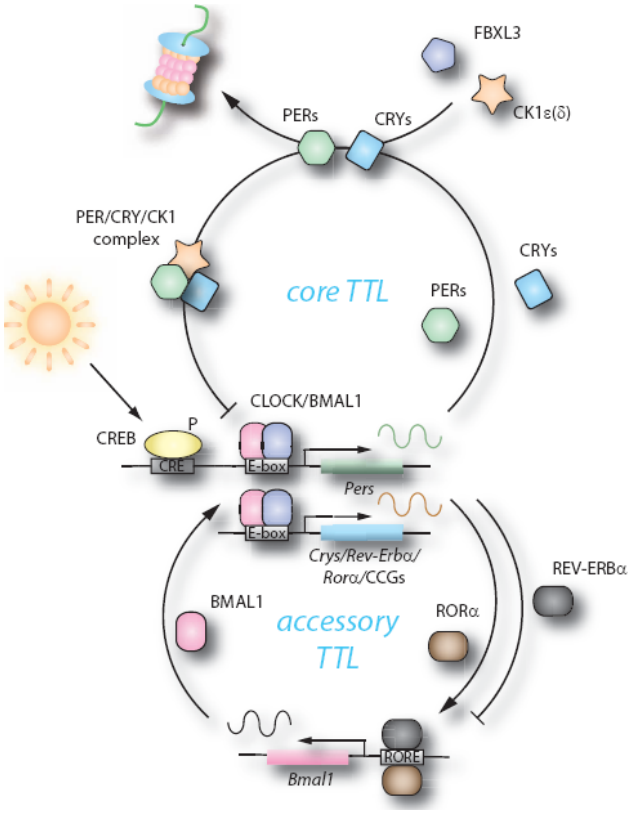


Figure 3: Model of the mammalian circadian clockwork within an individual SCN neuron. The heterodimer of CLOCK/ BMAL1 activates E-box-containing clock genes, such as *Per* and *Cry,* and clock controlled genes (CCGs). PER and CRY prot eins form a multimeric complex which inhibit CLOCK/ BMAL1. Both loops are linked by Rev-erbα, activated by CLOCK/ BMAL1, but inhibit Bmal1 transcription. *Casein kinase 1e* phosphorylates the PER proteins which leads to their degradation ultimately. For details see text (Modified from (Oster 2006)).

1.3.1.2. Clock output genes

E-boxes are not only found in the promoter regions of clock genes (**Figure 3**), but also regulate so called *clock controlled genes* (CCGs). Other CCGs contain ROREs (binding REV-ERB *Rev-Erbα* /ROR *Rorα*) or D-box elements (binding DBP/E4BP). A combination of these elements is used to fine tune the expression phase of CCGs. Circadian clocks are believed to provide time information primarily by controlling rhythmic transcriptional activation of 1st- and 2nd-order CCGs (Reppert and Weaver 2002). While rhythmic regulation of core clock genes is conserved among most organs, CCGs are tissue specific and reflect the physiological function of the organ in question (Panda and Hogenesch 2004). In any mammalian tissue, up to 10% of the whole transcriptome shows daily oscillations (Panda, Antoch et al. 2002; Storch, Lipan et al. 2002)

1.3.1.3. Clock mutants / Clock knockouts

Alteration of at least parts of the molecular clockwork via disruption of specific clock genes or its functions results in various phenotypes of different strength, affecting the period length and/or clock resetting. Mice with a targeted deletion in the *Bmal1* gene show an impaired entrainment to a LD cycle (Bunger, Wilsbacher et al. 2000). Upon release into constant darkness (DD) the animals immediately become arrhythmic, indicating a complete disruption of the circadian clockwork reflected in disturbed clock gene expression rhythms. A semidominant mutation of *Clock* lengthens circadian period and also abolishes persistence of rhythmicity (Vitaterna, King et al. 1994). Therefore, either CLOCK or BMAL1 alone are not sufficient to activate E-box driven transcription and keep the circadian oscillator running.

Per and *Cry* genes - as output of the negative limb of the core loop - are also essential for maintaining a functional circadian clock. Single gene deletions do not cause dramatic changes, but shortened or lengthened free-running rhythms of mutant animals have been reported (van der Horst, Muijtjens et al. 1999; Zheng, Larkin et al. 1999; Bae, Jin et al. 2001; Cermakian, Monaco et al. 2001; Zheng, Albrecht et al. 2001). Disruption of both *Pers* or both *Cry* genes causes immediate arrhythmicity in constant conditions (Vitaterna, King et al. 1994; van der Horst, Muijtjens et al. 1999; Bae, Jin et al. 2001; Zheng, Albrecht et al. 2001). The same holds true for *Per2/Cry1* double mutant animals showing disrupted locomotor activity and clock gene expression rhythms in the SCN and several other tissues (Oster, Yasui et al. 2002). The two *Per* genes have an additional role in SCN resetting by light. While *Per1* expression can be induced throughout the night, *Per2* is light sensitive only at the beginning of the dark phase (Albrecht, Sun et al. 1997). It has been demonstrated that *Per* mutant mice show impaired resetting of their activity rhythms in response to nocturnal light pulses. Whereas *Per2* mutants have deficiencies in clock delaying after a light

pulse at the beginning of the night (Albrecht, Zheng et al. 2001), *Per1* mutants are not able to phase advance after light exposure just before the light onset. Both facts taken together point to the *Pers* as a link between the input pathways to the clock and the central oscillator and suggest a non-redundant role of both genes in the mammalian circadian clock (Zheng, Albrecht et al. 2001).

1.3.2. SCN output

Efferent projections from the SCN have been well characterized. Neurons project mostly to intra-, but also to extra hypothalamic areas (**Figure 4**). SCN axons reach the preoptic area (MPO), the paraventricular nucleus of the hypothalamus (PVN), the sub-paraventricular zone (SPZ), the dorsomedial nucleus of the hypothalamus (DMH), the posterior hypothalamic area (PHA), the periaquaeductal gray (PAG), the arcuate (ARC) and the ventromedial nuclei (VMH). Outside the hypothalamus the SCN innervates the paraventricular nucleus of the thalamus (PVT), paratenial nucleus (PT), the intermediate lateral septal nucleus (LS) and the intergeniculate leaflet of the lateral geniculate nucleus (IGL) (Watts 1991).

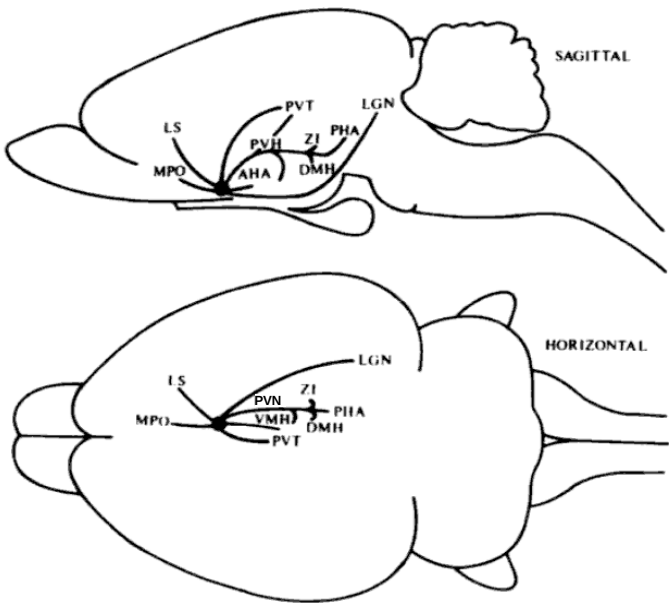


Figure 4: The efferent projections of the supra-chiasmatic nucleus. Modified from Watts (Watts 1991).

The rhythmic information from the SCN is transported to different brain regions which are all able to translate these information into physiological meaningful signals, such as hormone release (reviewed by Kalsbeek, Palm et al. 2006). Different projections along multiple pathways, reaching various brain regions enable integration of other environmental cues and allow for simultaneous changes in brain output signals (Buijs, Kalsbeek et al. 1993; Saper, Lu et al. 2005).

Information from the SCN is mainly translated directly, but also indirectly through intermediate neurons to the paraventricular nucleus of the hypothalamus (PVN), where it is converted into hormonal and autonomic signals (Buijs and Kalsbeek 2001). These reach peripheral organs such as the adrenal gland, thyroid, liver, fat tissue and gonads. From there, both visceral sensory and hormonal information feeds back to the hypothalamus,

allowing the organism to adjust and balance peripheral light/dark information with metabolic information from the peripheral organs (**Figure 5**)(reviewed by Buijs and Kalsbeek 2001).

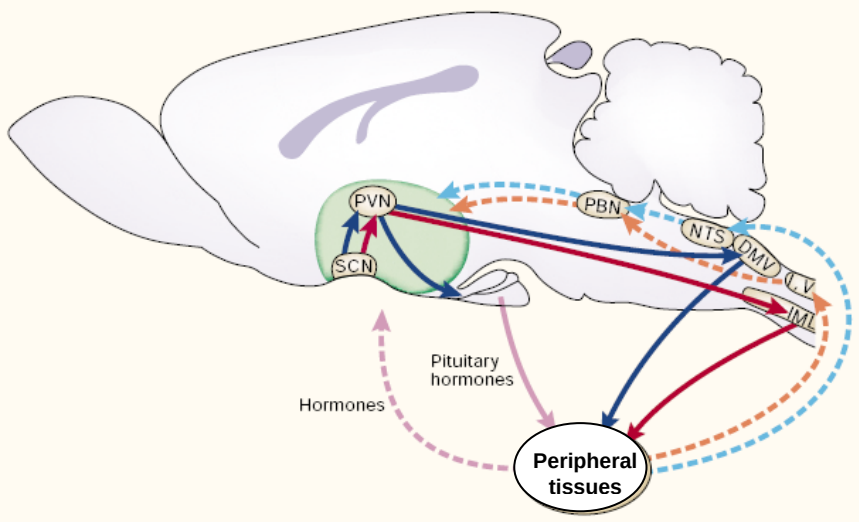


Figure 5: Interaction between peripheral tissues and SCN, an illustration of the main pathways by which periphery and central clocks might communicate with the central nervous system. The SCN translates its signals mainly by the paraventricular nucleus of the hypothalamus (PVN) into a hormonal and autonomic signal. These hormonal, parasympathetic and sympathetic signals reach peripheral organs, which themselves feedback via both visceral sensory and hormonal signals. These connections provide the hypothalamus with information that allows the organism to adjust and balance peripheral light–dark information with metabolic information from the peripheral organs. Abbreviations: NTS, nucleus of the solitary tract; PBN, parabrachial nucleus; DMV, dorsal motor nucleus of the vagus; IML, intermediolateral columns (modified from Buijs and Kalsbeek 2001).

1.4. Peripheral oscillators

In mammals, the circadian clock of the suprachiasmatic nucleus (SCN) acts as the central pacemaker driving circadian rhythms of behavior (Ko and Takahashi 2006). Because circadian clocks influence most if not all aspects of physiology and behavior, it is not surprising that circadian oscillators exist in various mammalian tissues. Clock genes are rhythmically expressed throughout the animal (reviewed in Balsalobre, Damiola et al. 1998; Balsalobre 2002), e.g. in the eye, heart, kidney, lung (Oishi, Sakamoto et al. 1998), liver (Zylka, Shearman et al. 1998; Lee, Etchegaray et al. 2001), muscle (Zylka, Shearman et al. 1998; Nonaka, Emoto et al. 2001), vasculature (McNamara, Seo et al. 2001) and testis (Zylka, Shearman et al. 1998).

Importantly, these gene expression rhythms persist in explant cultures (Whitmore, Foulkes et al. 1998; Yamazaki, Numano et al. 2000; Yoo, Yamazaki et al. 2004; Kaneko, Hernandez-Borsetti et al. 2006), indicating the existence of autonomous circadian rhythms in

peripheral tissues. In the adrenal gland (Andrews and Folk 1964; Andrews 1971) and the heart (Tharp and Folk 1965) such autonomy of rhythmicity had been described even before the discovery of clock genes themselves. More recently, autonomous peripheral circadian clocks have also been described in additional brain regions, such as the cerebral cortex (Yan, 2000; Abe 2002), the retina (Tosini and Menaker 1996) and in many peripheral tissues (for review see Schibler, Ripperger et al. 2003), e.g. liver (Yamazaki, Numano et al. 2000), kidney (Yoo, Yamazaki et al. 2004), heart, muscle (Damiola, Le Minh et al. 2000) and pancreas (Damiola, Le Minh et al. 2000; Oishi, Murai et al. 2000; Liu, Cai et al. 2007).

1.4.1. Synchronization between SCN and peripheral clocks

The concept of endogenous persistent clocks in peripheral tissues changed our view of the central pacemaker, which no longer is considered to directly drive peripheral rhythms, but rather to entrain and coordinate the many autonomous peripheral oscillators throughout the body. Without continuous input from the SCN, a gradual desynchronization between the uncoupled cells of peripheral oscillators is observed, resulting in an overall loss of rhythmicity within a short time (reviewed in (Schibler, Ripperger et al. 2003)). But, how do the central and the peripheral clocks interact to control the physiology of the whole animal? And how does the master clock keep control over all the different tissue oscillators? Encapsulated SCN grafts - unable to form synaptic contacts with host tissues - restore circadian activity/rest cycles in previously arrhythmic, SCN-lesioned recipient animals (Silver, LeSauter et al. 1996), indicating that paracrine factors mediate circadian coordination within the brain. Since circadian gene expression in the periphery was demonstrated to be delayed by about 4h, even if the basic clock mechanisms are the same in peripheral clocks and in the SCN (Balsalobre, Damiola et al. 1998; Allen, Rappe et al. 2001), synchronization via diffusible factors is favored (Silver, LeSauter et al. 1996; Oishi, Sakamoto et al. 1998). The general mechanism by which peripheral clocks are reset still remains unclear, but a number of pathways affecting the periphery have been described:

i) Possible candidates affecting the rest-activity rhythm are transforming growth factor alpha (TGFα, Kramer, Yang et al. 2001) and cardiotrophin-like cytokine (CLC) (Kraves and Weitz 2006), both rhythmically secreted from the SCN (**Figure 6**). Another peptide, prokineticin 2 (PK2) is also rhythmically released from the SCN and acutely induced by light (Cheng, Bullock et al. 2002). The SCN may coordinate circadian behavior, maybe also body temperature and physiology by PK2 (Prosser, Bradley et al. 2007).

ii) Most major organs must adapt their physiology to food and water absorption, therefore one dominant entrainment pathway involves feeding time, which in turn

might also be determined by SCN-driven rest-activity cycles. Diffusible candidates reviewed by Schibler and colleagues (2001) include food or food-induced metabolites such as NADH/NADPH (Rutter, Reick et al. 2001), heme and carbon monoxide (Dioum, Rutter et al. 2002) and feeding or fasting controlled hormones such as ghrelin (LeSauter, Hoque et al. 2009), secretin, gastrin, and cholecystokinin (CKK)(Rehfeld 1998).

iii) It has been postulated that both endogenous and environmental temperature cycles can participate in the synchronization of peripheral clocks in mammals (Brown, Zumbrunn et al. 2002). Abnormal environmental temperature cycles were shown to decouple peripheral oscillators from the central pacemaker in the SCN. Moreover, rhythms of temperature fluctuation, comparable to the daily cycling, can sustain or entrain circadian rhythmicity in cells. This mechanism could fine-tune peripheral clocks to their environment.

iv) Neural signals seem important for some output pathways (see below; Buijs and Kalsbeek 2001).

v) Freely circulating diffusible signals, such as hormones (e.g. glucocorticoids), cytokines, salts, or small-molecular-weight food metabolites (Pando, Morse et al. 2002) are also likely candidates affecting peripheral clocks.

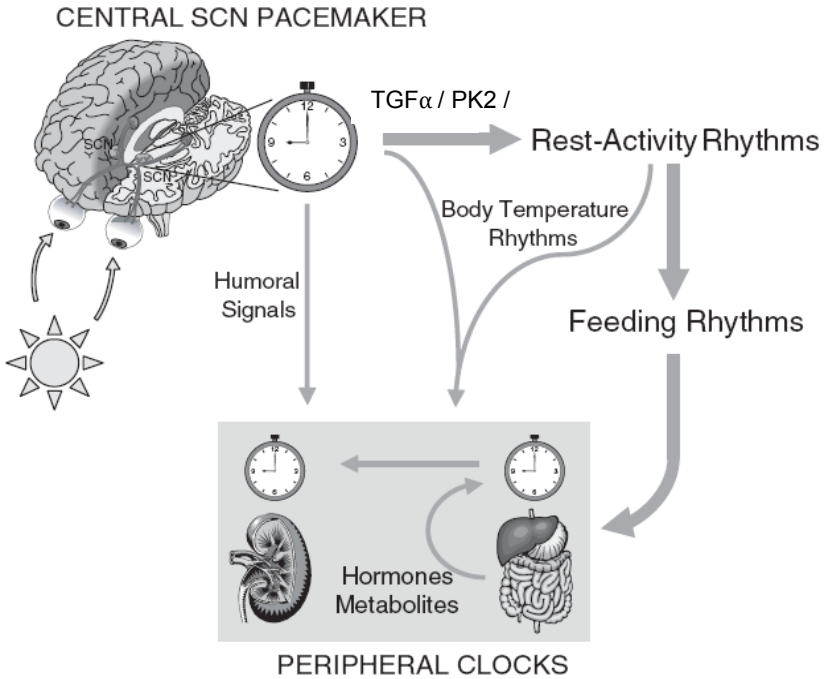


Figure 6: Hierarchical organization of circadian clocks. Even if each cell harbors a cell-autonomous circadian oscillator, these different oscillators must be synchronized to the external light information. In mammals and higher vertebrates, peripheral oscillators are synchronized by a central clock localized in the SCN, which is light entrainable (modified from Schibler, Ripperger et al. 2003).

Because of the importance of such diffusible signals in my present study, the possible role of adrenal glucocorticoids is described in more detail in the next sections. I will first focus on the communication between the SCN and the adrenal, regulating the circadian production of glucocorticoids.

1.4.1.2. SCN-adrenal connections

The circadian release of glucocorticoids and the hormone adrenocorticotropic hormone (ACTH) are under the control of the SCN, because SCN lesions abolish both rhythms (Moore and Eichler 1972; Abe, Kroning et al. 1979; Szafarczyk, Ixart et al. 1979; Cascio, Shinsako et al. 1987). Since SCN grafts are able to rescue circadian locomotor rhythms, but fail to restore the circadian endocrine rhythms, including those of glucocorticoids, axonal connections seem to be required for their circadian control (Meyer-Bernstein and Morin 1999).

Signaling between the SCN and the adrenal gland employs both, hormonal signaling which proceeds along the hypothalamus/pituitary/adrenal (HPA) axis, and also neuronal mechanisms involving the pre-autonomic nervous system (**Figure 7**). The major relay for all these pathways is the PVN, an integrative center concerned with neuroendocrine, autonomic and behavioral processes. Especially the medioparvocellular PVN (mpPVN) is of special interest, regulating the secretions of corticotropin-releasing hormone (CRH) from the PVN (Buijs and Kalsbeek 2001), which evokes rhythmic secretion of ACTH from hypophysial corticotroph cells (Simpson and Waterman 1988).

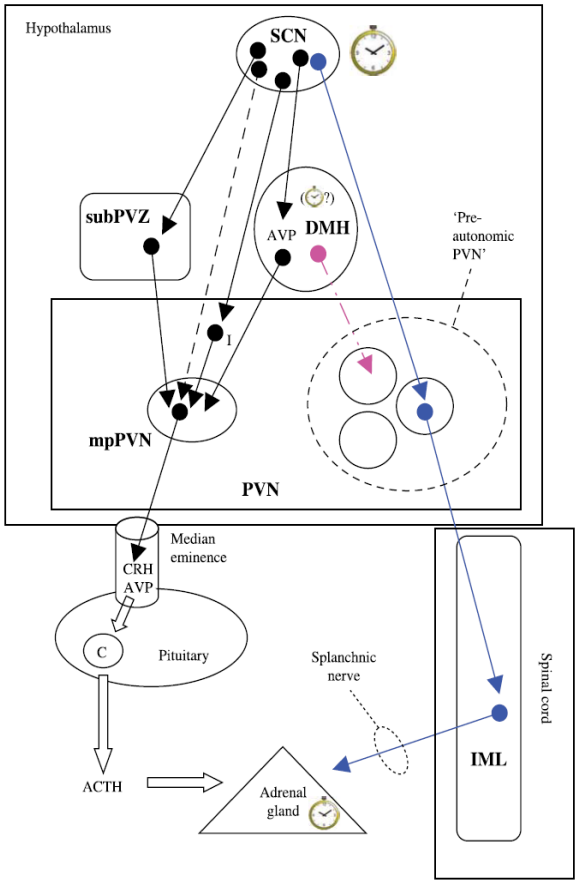


Figure 7: Neural pathways in circadian control of glucocorticoid release. See text for details. Black arrows: neural projections targeting the HPA axis. Blue arrows: neural projections via the autonomic nervous system. Purple arrow: projection from the DMH to the pre-autonomic PVN. Open arrows: humoral. Clocks symbolise autonomous pacemakers. Abbreviations: SCN, suprachiasmatic nucleus; PVZ, subpara-ventricular zone; DMH, dorsomedial hypothalamus; PVN, paraventricular nucleus; mpPVN, medioparvo-cellular division of the PVN; I, interneuron of the PVN; C, corticotrophic cell of the pituitary; IML, intermediolateral column of the spinal cord; AVP, arginine vasopressine; CRH, corticotrophin-releasing hormone; ACTH, adreno-corticotropic hormone. From (Dickmeis 2009).

A multisynaptic SCN-adrenal pathway (Buijs, Wortel et al. 1999; reviewed in Engeland and Arnhold 2005) has been described, passing through neurons of the pre-autonomic PVN connected with sympathetic preganglionic neurons in the intermediolateral column (IML) of the spinal cord (Buijs, Wortel et al. 1999), which then innervate the adrenal via the splanchnic nerve (**Figure 7**). This neuronal pathway transmits light information to the adrenal, followed by an HPA-axis independent corticosterone release (Niijima 1992; Niijima, Nagai et al. 1993; Buijs, Wortel et al. 1999; Ishida, Mutoh et al. 2005). The signal might be transmitted through the innervation of the adrenal medulla and then to the cortex via adrenalin release (Ishida, Mutoh et al. 2005). The adrenal cortex receives autonomic signals from the medulla, and in addition direct pre- and postganglionic, sympathetic and parasympathetic connections from sensory excitatory vagus nerve innervations have been found (Coupland, Parker et al. 1989; Parker, Mohamed et al. 1990). A neuronal control mechanism prepares the endocrine organs for the arrival of hormones (Buijs, van Eden et al. 2003), by modulating the sensitivity of the adrenal to the ACTH signal (reviewed in (Dallman, Engeland et al. 1978; Dijkstra, Binnekade et al. 1996; Ulrich-Lai and Engeland 2000; Sage, Maurel et al. 2002; Engeland and Arnhold 2005).

In summary, the rhythmic corticosterone secretion from the adrenal is controlled slowly via the hormonally regulated HPA-axis and a second "HSA-axis" (hypothalamus-spinal cord-adrenal axis), which is the neural complement, transmitting the light information via the SCN directly to the adrenal.

1.5. Glucocortoicoids (GCs)

1.5.1. Glucocorticoids and the circadian clock

Many hormones (including glucocorticoids, GCs) show circadian rhythms of blood concentrations, making them good candidates to coordinate central and peripheral rhythms (Hastings, O'Neill et al. 2007; Haus 2007). The circadian peak in GC release is locked to the beginning of the activity phase, i.e. the early morning in diurnal and the early night in nocturnal animals (Kalsbeek, Kreier et al. 2007). Notably, GCs have been implicated in the regulation of peripheral circadian rhythms and are themselves subject to circadian regulation at many levels of organization. GCs can induce circadian gene expression in fibroblasts and induce phase shifts of the clock in liver, kidney and heart (Balsalobre, Brown et al. 2000) (reviewed by (Schibler, Ripperger et al. 2003)).

GCs, produced and secreted in the adrenal, have many different functions in body homeostasis and stress responses (Sage, Maurel et al. 2001; Kalsbeek, Ruiter et al. 2003; Atkinson, Wood et al. 2006; Papadimitriou and Priftis 2009; So, Bernal et al. 2009). The

adrenal anatomy, steroid-hormone synthesis, especially GC-synthesis, and its regulation will be reviewed in the next sections.

1.5.2. Adrenal gland anatomy

Both adrenals are located at the upper poles of the kidneys and function as endocrine glands. The adrenal has two anatomically and functionally different components: the cortex and the medulla.

1.5.2.1. Cortex

The adrenal cortex can be separated into three parts: *zona glomerulosa*, *zona fasciculata* and *zona reticularis* (**Figure 8**). All these zones have functions in different aspects of steroid biosynthesis (Rehman et al., 2003). Mineralocorticoids (mainly aldosterone) are produced in the *zona glomerulosa*. Glucocorticoids (like cortisol, corticosterone) are synthesized in the *zona fasciculata*. Located next to the medulla are the cells of the *zona reticularis*, producing small amounts of androgens, such as testosterone, oestradiol and dehydroepiandrosterone (DHEA). Rats and mice produce corticosterone, instead of cortisol which is the major GC secreted in humans (Keidel 1985).

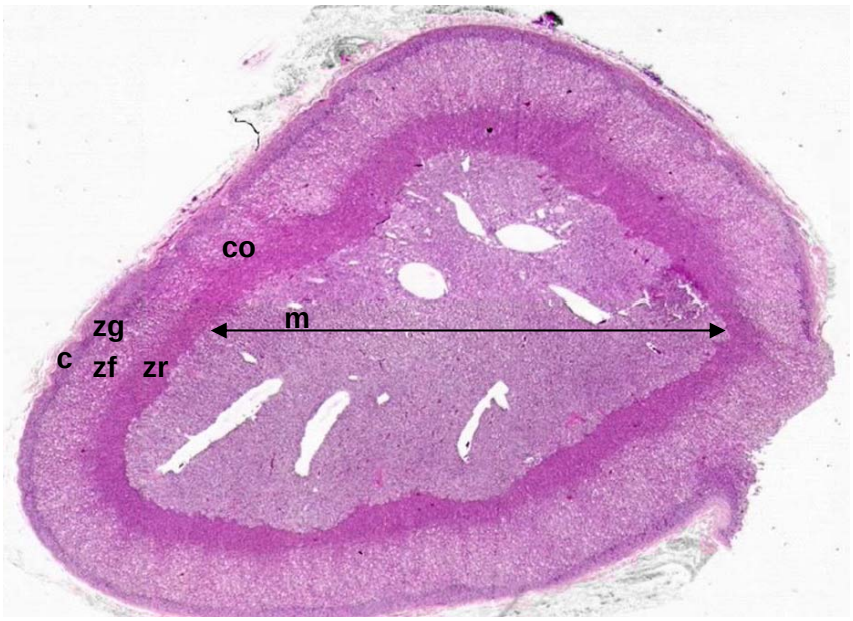


Figure 8: Histological profile of the adrenal gland (stained with eosin). The layered histoarchitecture of the adrenal: adrenal capsule (c); zona glomerulosa (zg); zona fasciculata (zf); zona reticularis (zr), cortex (co) and medulla (m). Aldosterone is produced in *glomerulosa*- cells, corticosterone and cortisol in *fasciculata*-cells, androgens in *reticularis* cells of the cortex. In the chromaffin- cells of the medulla catecholamines are produced.

1.5.2.3. Medulla

The medulla consists of paraneural chromaffin cells, which evoke endocrine and paracrine action potentials via secretion of catecholamines. The medulla harbors different cell types retaining adrenaline and noradrenaline and a third type of chromaffin cells, the SGCs (*small-granule chromaffin cells*), containing axon-like appendages. Since the medulla can be seen as a sympathetic ganglion in which the postganglionic cells have lost their axons, it is not surprising that autonomic innervation of the adrenal was found (Ishida, Mutoh et al. 2005) (see 1.2.1.3.).

1.5.3. Glucocorticoid biosynthesis

GC rhythms are regulated by the SCN via the hypothalamus/pituitary/adrenal axis and via the autonomous nervous system (see 1.4.1.2.)(Perreau-Lenz, Pevet et al. 2004). Corticoid (gluco- and mineral corticoid) secretion is mediated by ACTH (Papadimitriou and Priftis 2009; So, Bernal et al. 2009), which acts in the *zona fasciculata*. ACTH can stimulate the production of glucocorticoids, but on the other hand also be a potent stimulator of aldosterone secretion (Muller 1998).

1.5.3.1. Supply of cholesterol

Cholesterol is the precursor for corticoid production. Under normal conditions, endogenous cholesterol is primarily produced by the liver and transported to the adrenal via blood-borne lipoprotein complexes (mainly LDL)(Rudney and Sexton 1986). It has, however, been shown that the adrenal can substitute for low blood cholesterol by *de novo* biosynthesis to sustain corticoid production (Heikkila, Kahri et al. 1989) in a circadian fashion (Oster, Damerow et al. 2006). In small amounts, cholesterol is esterified and stored in lipid droplets (**Figure 9**). Cholesteryl ester hydrolase (CEH) converts the stored cholesteryl-ester to cholesterol. The rate-

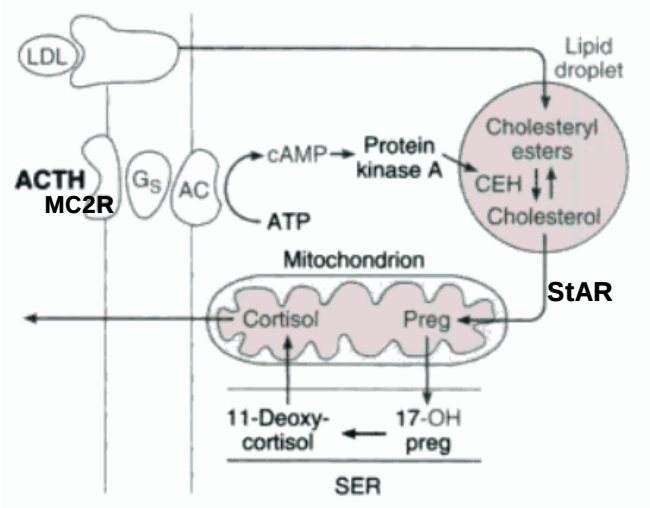


Figure 9: Mechanism for ACTH induced glucocorticoid synthesis. ACTH binds to its Gs-protein coupled receptor (MC2R) and activates adenylyl cyclase (AC). Abbreviations: activates protein kinase A (PKA); cholesteryl esters hydrolase (CEH); pregnenolon, Preg; endoplasmic reticulum (SER); 17-OH progesterone (17-OH preg); 11-deoxycorticosterone (11-Deoxy-cortisol). Arrows denote signaling pathways and regulators. (Modified from Barrett, Barman et al. 2009).

limiting step of steroidogenesis is the transfer of cholesterol, incorporated into lipid droplets, to the mitochondrial inner membrane mediated by the steroidogenic acute regulatory protein (StAR) (Stocco 1999; Miller 2007; Clark, Wells et al. 1994; Lin, Sugawara et al. 1995). In this compartment steroidogenesis takes place (Foster 2004).

1.5.3.2. Glucocorticoid biosynthesis pathway

ACTH binds to the *melanocortin2 receptor* (MC2R), mainly expressed in the *zona glomerulosa* and *fasciculata* (Abdel-Malek 2001). Binding of ACTH activates cAMP-dependent protein kinase A (PKA) via G_s-protein coupled signaling (Fakunding, Chow et al. 1979; Dwivedi and Pandey 2000)(**Figure 9**).

During steroidogenesis, the modification of cholesterol at different positions is catalyzed by *P450-cytochromes* (CYPs) under the consumption of NADPH. After transport to the inner mitochondrial membrane, cholesterine is converted by CYP11A1 (also known as CSCC = cholesterol side chain cleavage) to pregnenolone (**Figure 10**). After moving to the endoplasmic reticulum pregnenolone is dehydrogenated and converted to 17-OH progesterone by cytochrome P450 steroid hydroxylases (HSD3b6) (Payne and Hales 2004). This part of the pathway is shared for aldosterone and corticosterone biosynthesis. Next, progesterone is converted to 11-deoxycorticosterone via 21-hydroxylation (by CYP21A1), followed by 11β-hydroxylation (CYP11B1) to corticosterone. In the *zona glomerulosa* no 11β-hydroxylase is expressed; instead 11-deoxycorticosterone is converted by the aldosterone synthase (CYP11B2) into aldosterone.

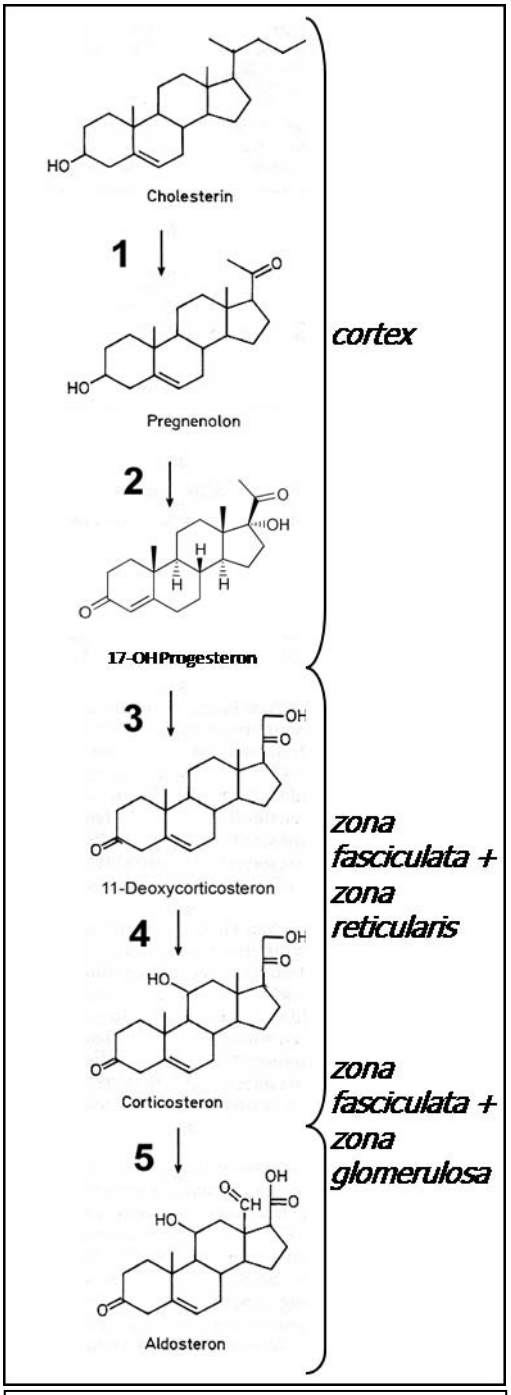


Figure 10: Steroidhormone synthesis. Stored cholesterine is release and converted to corticosterone, cortisol (not shown), aldosteron and androgens (not shown), such as testosteron and oestrogen (not shown) via different *P450-cytochromes* (CYPs). 1: CYP11A1, 2: HSD3β6, 3: CYP21A1, 4: CYP11B1, 5: CYP11B2.

1.5.3.3. Regulation of glucocorticoid biosynthesis

Secretion of corticoids from the adrenal follows a circadian pattern (Reinberg, Touitou et al. 1982). Although ACTH is a principal factor stimulating the biosynthesis and secretion of glucocorticoids, it is not the only factor regulating the plasma level of GCs. Discrepancies between the level of ACTH and the secretion of GCs are often observed, suggesting that other factors may also be involved (Kaneko, Kaneko et al. 1981). A direct modulation of GC output at the level of the adrenal gland has been implicated by studies using splanchnic nerve transection (Edwards, Jones et al. 1986; Jasper and Engeland 1994; Jasper and Engeland 1997). The neural input of the splanchnic nerve activates sympatho-adrenomedullary activity resulting in the production of components in the medulla, which might get involved in the steroid hormone synthesis (Edwards, Jones et al. 1986; Ehrhart-Bornstein, Hinson et al. 1998). Additionally, the circadian rhythm of GCs is regulated by a gating mechanism residing in the adrenal cortical clock itself (see Oster, Damerow et al. 2006, above).

1.6. Clock disorders

1.6.1. Metabolic effects

A major function of the circadian system is the regulation of the metabolic machinery to prepare the body for temporal variations in the abundance of nutrients (Kovac, Husse et al. 2009). Since sleep-wake alterations in shift workers cause a higher risk of obesity, metabolic syndrome and diabetis II (Young and Bray 2007), metabolic phenotypes might be the consequence of a misalignment between the body´s physiology and the internal clockwork. In different circadian clock mutant mice defective metabolic regulations have been documented. *Bmal1* deficient and *Clock* mutant mice both show impaired glucose homeostasis (Rudic, McNamara et al. 2004). *Clock* mutation affects the daily activity distribution combined with attenuated diurnal food intake. These animals are hyperphagic and obese, with increased fat mass especially fat accumulation in the liver (Turek, Joshu et al. 2005). Also components of the negative limb of the core TTL, such as *Per2* are linked to a higher metabolic syndrome risk (Englund, Kovanen et al. 2009). It seems clear, that major metabolic pathways are under circadian control.

1.6.2. Jet lag

Jet lag is the phenomenon experienced when the synchronization of the body and the external environmental cues is disrupted (= external desynchronization) - for example when traveling between time zones (Moore-Ede, Kass et al. 1977; Arendt and Marks 1982;

Comperatore and Krueger 1990; Nagano, Adachi et al. 2003; Davidson, Castanon-Cervantes et al. 2009). In addition to the external desynchronization, jet lag evokes an internal desynchronization inside the body. The SCN clock and the peripheral clocks do not "tick" at the same phase anymore. At the level of the master clock itself - the SCN - regions with different resetting kinetics can also be distinguished (Davidson, Castanon-Cervantes et al. 2009). It can take several days to readjust to the new external time, when re-synchronization of the complete system occurs.

Although the term "jet lag" refers to disturbances in a variety of symptoms, jet-lagged travelers mostly complain of night time insomnia, reduced alertness (Haimov and Arendt 1999), reduced performance (Tapp and Natelson 1989), loss of appetite, depressed mood, poor psychomotor coordination, gastrointestinal disturbances and reduced cognitive skills (Cho, Ennaceur et al. 2000). The severity of these symptoms depends on the length and the direction of travel (Haimov and Arendt 1999; Waterhouse, Reilly et al. 2007). Whereas most humans experience mild symptoms of discomfort during a single jet lag episode, the effects of chronic de-synchronization may likely be profound. Repetitive jet lag has been shown to accelerate malignant growth (Filipski, Delaunay et al. 2004) and to produce temporal lobe atrophy, spatial cognitive deficits (Cho, Ennaceur et al. 2000; Cho 2001) and even hasten death in aged mice (Davidson, Sellix et al. 2006) and cardiomyopathy in hamsters (Penev, Kolker et al. 1998).

Similar to jet lag caused by traveling, shift work produces chronic sleep disruption lasting for the duration of shift work exposure (Lemmer 2007). The term "social jet lag" was coined to explain the discrepancy between biological internal timing, defined by the endogenous period of the clock, and the social timing - the preferred time to sleep or to be active/awake dependent on the lifestyle (Wittmann, Dinich et al. 2006).

1.7. Aims

The main objectives of this project were to (i) analyze the role of the adrenal clock in the production of glucocorticoids (GCs) and (ii) its impact on resetting, especially during jet lag.

(i) The existence of circadian clocks outside the SCN has been demonstrated by several independent studies (reviewed in (Cuninkova and Brown 2008)), but the physiological function of these *peripheral oscillators* is still poorly understood (Durgan, Trexler et al. 2006; McDearmon, Patel et al. 2006; Storch, Paz et al. 2007). Also, little is known about how the SCN synchronizes other clocks (Schibler, Ripperger et al. 2003). A sensitivity of the adrenal to light exposure has been shown (Ishida, Mutoh et al. 2005). However, if this photic signal

is sufficient to entrain the adrenal circadian clock remains unclear. Although it has been demonstrated that such an adrenal clock exists (Bittman, Doherty et al. 2003; Ishida, Mutoh et al. 2005), its role in the regulation of diurnal GC rhythms has not been defined. For this thesis I developed a transplantation model to assess adrenal clock function in the living animal and analyze its impact on the regulation of behavior and physiology, i.e. locomotor activity and the regulation of GC synthesis.

(ii) Rapid phased shifts of the light-dark cycle as experienced when travelling across multiple time zones cause a transient desynchronization of internal and external time termed *jet lag*. Even though many papers have described the complex symptomatic of the jet lag syndrome (Waterhouse, Reilly et al. 2007; Arendt 2009), little is known about the underlying molecular mechanisms. Even though it is generally believed that circadian clocks are disrupted during jet lag, it remains to be shown if this disruption is mainly observed at the molecular level, e.g. between different clock genes (Reddy, Field et al. 2002), or if de-phasing of different tissue oscillators is the major cause of jet lag symptoms (Yamazaki, Numano et al. 2000). If the latter is true, then it would be interesting to see if there are differences between clocks and if certain tissues are contributing to the overall process of re-alignment to the new time zone. This is of particular interest for the adrenal, as adrenal GCs have the capacity to reset other peripheral clocks in vivo (Balsalobre, Brown et al. 2000) and influence behavioral resetting during jet lag (Sage, Ganem et al. 2004). In this thesis I monitored the molecular changes that underlie re-entrainment of the circadian timing system during jet lag in the living animal. Differences in resetting kinetics were analyzed for different clock genes and between various tissues. Using the transplantation model mentioned above and pharmacological manipulations I assessed the role of the adrenal clock and GC secretion rhythms in behavioral and physiological re-entrainment, hoping to identify new targets for the acceleration of clock resetting and the alleviation of jet lag.

Chapter 2

Results

2.1. Publication: “The circadian rhythm of glucocorticoids is regulated by a gating mechanism residing in the adrenal cortical clock”

Henrik Oster, Sebastian Damerow, Silke Kiessling, Vladimira Jakubcakova, Diya Abraham, Jiong Tian, Matthias W. Hoffmann, and Gregor Eichele

Cell Metabolism 2006, 4: 163-173

Summary

In mammals, the master clock of the *suprachiasmatic nuclei* (SCN) and subordinate clocks found throughout the body coordinate circadian rhythms of behavior and physiology. We characterize the clock of the adrenal, an important endocrine gland that synchronizes physiological and metabolic rhythms. Clock gene expression was detected in the outer adrenal cortex prefiguring a role of the clock in regulating gluco- and mineral corticoid biogenesis. In *Per2/Cry1* double mutant mice, which lack a circadian clock, hypothalamic-pituitary-adrenal axis regulation was defective. Organ culture and tissue transplantation suggest that the adrenal pacemaker gates glucocorticoid production in response to adrenocorticotropin (ACTH). *In vivo* the adrenal circadian clock can be entrained by light. Transcriptome profiling identified rhythmically expressed genes located at diverse nodes of steroid biogenesis that may mediate gating of the ACTH response by the adrenal clock.

Introduction

The axial rotation of the earth causes extensive yet periodic variations in a wide range of environmental conditions. Most organisms have evolved circadian clocks to optimally adjust their behavior and physiology to such recurring changes (Harmer, Panda et al. 2001). In mammals, the master circadian clock resides in the *suprachiasmatic nuclei* (SCN) of the ventral hypothalamus (Rusak and Zucker 1979; Ralph, Foster et al. 1990). To synchronize physiology with external time, the SCN clock emits timing signals to a series of peripheral circadian clocks found in most organs (Schibler, Ripperger et al. 2003)). An important goal of contemporary chronobiological research is to understand how central and peripheral circadian pacemakers communicate and how peripheral clocks regulate behavior and physiology.

Signaling between the SCN and the periphery employs both hormonal and neuronal mechanisms. In the case of the adrenal gland, hormonal signaling proceeds along the hypothalamus/pituitary/adrenal (HPA) axis. Specifically, the SCN activates rhythmic release of corticotropin releasing hormone (CRH) from the paraventricular nucleus (PVN) that evokes circadian adrenocorticotropin hormone (ACTH) release from hypophysial adrenocorticotrophs (Simpson and Waterman 1988). ACTH in turn, regulates circadian corticoid release from the *zona glomerulosa* and the *zona fasciculata* of the adrenal cortex. In addition, neuronal signals generated by the SCN propagate through the autonomic nervous system to the adrenal cortex to contribute to the circadian regulation of glucocorticoid production (Buijs, van Eden et al. 2003). Transection of the splanchnic nerve renders the cortex more responsive to ACTH while simultaneously dampening diurnal rhythms of corticoid secretion (Jasper and Engeland 1994; Jasper and Engeland 1997). The important work of Ishida et al. (2005) shows that photic signals from the SCN are transduced through the autonomous nervous system to the adrenal, since denervation of the adrenal abolishes photic induction of the clock gene *Per1* in the adrenal cortex. These neuroendocrinological data, in conjunction with the circadian expression pattern of *Per1* and *Per2* in the adrenal gland of rodents (Bittman et al., 2003; Torres-Farfan et al., 2006; Ishida et al., 2005) and rhythmic expression of clock-controlled genes in the adrenal of primates (Lemos et al.; 2006) provide *bona fide* evidence for the presence of a peripheral circadian clock in the adrenal gland. Among peripheral clocks the one in the adrenal is particularly interesting since adrenal corticoids have been implicated in the synchronization of subordinated oscillators (Balsalobre, Brown et al. 2000) and were demonstrated to control metabolic rhythms in many other organs including liver (Oishi, Amagai et al. 2005), kidney (Nicholson, Levine et al. 1976) and brain (Casanueva and Dieguez 1999).

The availability of mutant mice lacking a circadian clock (van der Horst, Muijtjens et al. 1999; Bunger, Wilsbacher et al. 2000; Bae, Jin et al. 2001; Zheng, Albrecht et al. 2001; Oster, Yasui et al. 2002), combined with the ease with which adrenal tissue can be cultured (Lindhe, Lund et al. 2001) and transplanted (Ulrich-Lai and Engeland 2000; Musholt, Klebs et al. 2002), offer new opportunities to capture the physiological function of the adrenal circadian pacemaker. In this study we demonstrate that a light-regulated circadian clock resides in the adrenal cortex and this clock gates the physiological response of this organ to ACTH stimulation *via* the control of genes encoding proteins of the complex steroidogenic network. Our work thus identifies a mechanism that bridges the gap between peripheral clock gene oscillations and the control of corticoid-dependent physiological rhythms in the adrenal gland and possibly in many other organs.

Results

Localization of a peripheral circadian clock to the outer adrenal cortex

We carried out a circadian microarray profiling with wild-type murine adrenals to identify rhythmically expressed genes. Mice were entrained to a standard light/dark cycle (LD12:12) and transferred to constant darkness (DD; top bar in Fig. 1A). On the second day in DD, adrenals were removed at 4 hour intervals for a period of 48 hrs and gene expression was assessed with Affymetrix MG430v2.0 microarrays. Using the COSOPT algorithm (Straume 2004) we identified 1,606 annotated genes (*http://www.geneontology.org/; as of April 2006*) with significant circadian expression rhythms (Oster et al., unpublished data). All microarray data are accessible at the NCBI gene expression omnibus (GEO) data base (accession no. GSE4253). In keeping with the theme of the present study we focus on genes directly implicated in the circadian clock and those involved in adrenal glucocorticoid metabolism.

The majority of canonical clock genes exhibit a circadian expression pattern in the adrenal. Microarray data revealed circadian rhythms for the expression of *Bmal1* (Fig. 1B), *Cry1* (Fig. 1D), *Per1* (Fig. 1F), *Per2* (Fig. 1G), *Per3* (Fig. 1H) and *Rev-Erbα* (Fig. 1I). *Clock* and *Cry2* transcripts were also detected but did not show an obvious circadian rhythm (Fig. 1C, E). For the first day of the experiment, clock gene expression was validated by qPCR using a different set of adrenals, with results closely matching those obtained from the microarrays (Fig. 1B-I, open circles). The adrenal gland consists of a catecholamine-producing medulla enclosed within a three-layered cortex (Fig. 1B'). *In situ* hybridization was carried out on adrenals collected 46 and 58 hrs after "lights off" (Fig. 1A) to characterize the expression sites of several clock genes most of which were strongly expressed in the *zona glomerulosa* and the *zona fasciculata* (Fig. 1B'-I'). *Bmal1* (Fig. 1B'), *Cry1* (Fig. 1D'), *Per1* (Fig. 1F'), *Per3* (Fig. 1H') and *Rev-Erbα* (Fig. 1I') were temporally regulated in these layers while *Clock* (Fig. 1C') and *Cry2* (Fig.1 E') were not. For *Per2*, we did not detect a difference in expression for the two time points selected (Fig. 1G') but when adjacent sections were probed for PER2 protein, a clear differential expression was seen in the outer cortical layers (Fig. 1G''). In the *zona reticularis,* only *Bmal1*, *Per3* and *Rev-Erbα* showed temporally regulated expression (Fig. 1B', H' and I'), while in the medulla only *Bmal1*, *Per1* and *Per3* displayed a clear expression difference between both time points examined (Fig. 1B', F' and H'). Taken together, microarray and qPCR in combination with *in situ* hybridization demonstrate that canonical circadian clock genes are expressed in the *zona glomerulosa* and the *zona fasciculata* and – with the exception of *Clock* and *Cry2* – show a robust circadian rhythm of expression. These findings suggest that the corticoid-producing outer layers of the cortex are chief sites of the adrenal circadian pacemaker.

The adrenal circadian clock defines ACTH responsiveness

Although the adrenal cortex rhythmically expresses most canonical clock genes, this does not uncover how this pacemaker regulates the circadian properties of this organ. To begin to address this, we analyzed adrenal clock regulation in *Per2/Cry1* double mutant animals that have a disrupted circadian clock (Oster, Yasui et al. 2002). When we monitored clock gene transcription in the *Per2/Cry1*-deficient adrenal gland, we found that *Bmal1*, *Per1*, *Per3* and *Rev-Erbα,* all rhythmically expressed in the wild-type adrenal (Figs. 1B, F, H and I), are devoid of circadian transcriptional regulation (Figs. 2A and B).

We next examined how HPA axis hormones were regulated in mutant mice. In wild-type animals kept either under LD or DD conditions, plasma ACTH levels peaked ~2 hrs prior to the onset of the (subjective) night (Fig. 2C, D), consistent with previous work (Cheifetz 1971). ACTH plasma levels of *Per2/Cry1* double mutant animals were steady-state at ~130ng/ml which is the mean of wild-type zenith and nadir (Fig. 2C, D). Plasma corticosterone concentration profiles of wild-type mice trailed those of ACTH by ~2 hrs peaking at the onset of the (subjective) night (Fig. 2E, F). Mutant mice lacked not only an overt corticosterone rhythm in serum and feces (Fig. 2E-H) but corticoid levels hovered around nadir concentrations suggesting that the mutant adrenal cannot properly respond to hypophysial ACTH. Hence, in the absence of a functional clock input and output signals in the adrenal become to a certain degree uncoupled.

In the intact animal, however, one cannot determine whether the adrenal clock itself or rhythmic serum ACTH causes the rhythmic production of corticosterone. To address this issue, adrenal tissue slices from wild-type or mutant mice were cultured and stimulated with 20 nM ACTH at two opposite time points of the circadian cycle. At 38 hrs after "lights off" (Fig. 1A), when plasma corticosterone levels were minimal in the wild-type animal (Fig. 2F), ACTH evoked a moderate increase of corticosterone in wild-type and *Per2/Cry1* adrenal slices (Fig. 3A). ACTH treatment of slices from wild-type adrenals collected at 50 hrs (zenith of plasma corticosterone, Fig. 2F) evoked a more marked corticosterone release into the medium (Fig. 3B). By contrast, mutant adrenal slices treated with ACTH at this time point exhibited basal level corticosterone release reminiscent of that seen at the 38 hr time point. Fig. 3C compares the initial rates of corticosterone secretion averaged for two experiments similar to those presented in Figs. 3A and B. A repeated stimulation after 24 hrs of culturing slices in fresh medium gave results similar to those shown in Fig. 3C (data not shown), indicating that the differential response observed in the wild-type was not an unspecific effect caused by preparation and culture procedures but most likely reflected an intrinsic rhythm of response. Our organ culture experiments suggest that the adrenal contains a circadian clock that defines – i.e. gates – a time window during which the adrenal most

effectively responds to ACTH. Tissue from *Per2/Cry1* mutant mice lacks this clock and, therefore, is unable to differentially respond to ACTH.

Clock regulation of selective components of the corticosterone biosynthetic pathway

The transcriptional nature of the circadian timing system predicts that the adrenal clock may exert its control on adrenal physiology *via* rhythmic transcriptional regulation of clock controlled genes. We used our microarray data set to identify those genes implicated in adrenal corticoid metabolism that exhibit a circadian expression pattern. Current gene ontology annotations only partly reflect the complex regulatory networks underlying physiological processes. We therefore assembled from the literature a more comprehensive representation of the genetic network that controls adrenal corticosterone metabolism (Fig. 4). 33 genes placed at various sites within the network (indicated by gray boxes) are rhythmically expressed (suppl. Table 1). The low-density lipoprotein receptor (*Ldlr*) and scavenger receptors (*Scrab2*) regulate the supply of the steroid precursor cholesterol from blood. Other gene products such as P450 oxidoreductase (*Por*) and trans-acting transcription factor 1 (*Sp1*) control the activity of steroidogenic enzymes and thereby directly influence steroid biosynthesis. The biggest cohort showing circadian regulations consisted of genes encoding proteins regulating the transport of storage cholesterol to the inner mitochondrial membrane where corticoid production occurs. These include carrier proteins (*Slc25a*) and regulators thereof (*Bzrp*), transcriptional regulators of steroidogenic acute regulatory protein (*Jun*, *Sp1*) and mediators of the ACTH receptor signaling pathway (*Mc2r*, *Adcy5*, several G-proteins, protein kinase A and protein phosphatase 1 subunits). For the 20 genes identified by COSOPT as being most rhythmic, a validation of the microarray data by qPCR was carried out and both methods yielded very similar results (Fig. 5). Several of these genes show expression rhythms paralleling glucocorticoid secretion into the blood (compare Fig. 2F and Fig. 5A-D, H, I, L-P and T). However, *mRNA* production and the availability of a functional protein located in the appropriate cellular compartment may lag behind transcript peaks by several hrs (Reppert and Weaver 2001). Regardless of the caveat, the surprising result of our transcriptome analysis is that within the intricate network of steroid biosynthesis numerous genes distributed over the entire network seem to be candidate targets of the circadian transcriptional machinery.

The adrenal clock regulates corticosterone rhythms *in vivo*

Our comparison of mutant and wild-type *in vitro* slice cultures revealed a "gating" mechanism in the sense that ACTH stimulation triggers corticosterone release in a temporally

controlled fashion. This led us to ask how this gating controls the regulation of circadian corticosterone secretion *in vivo*. To address this question we transplanted *Per2/Cry1* (arrhythmic) adrenals into wild-type adrenalectomized hosts and *vice versa* (Fig. 6A). We first examined whether the SCN pacemaker was affected in grafted mice by analyzing SCN clock gene expression as well as locomotor activity rhythms. In wild-type hosts carrying wild-type grafts, both *Per1* expression in the SCN and activity rhythms were those seen with un-operated animals (Fig. 6B). *Per1* expression peaked during the subjective day and the free-running period length (τ_{DD}) was 23.4 $\pm$ 0.1 hrs (mean $\pm$ SEM, n = 5). In both of the cross-genotype recombinations, *Per1* expression and activity rhythms were not significantly affected by the genotype of the graft (Fig. 6C, D). Wild-type hosts carrying a mutant adrenal showed wild-type LD and DD rhythms (τ_{DD} = 23.6 $\pm$ 0.1 hrs; mean $\pm$ SEM, n = 5) and rhythmic expression of *Per1* in the SCN (Fig. 6C). By contrast, mutant hosts carrying wild-type adrenals became immediately arrhythmic when released into DD. Moreover, *Per1* transcription in the SCN was blunted, as was previously reported for un-operated *Per2/Cry1* double deficient animals (Oster, Yasui et al. 2002). From these data we conclude that there is no feedback of the adrenal clock on the SCN pacemaker.

Next, grafted mice were examined for HPA axis regulation. We detected rhythmic ACTH levels in wild-type hosts carrying either wild-type or mutant adrenal grafts in both LD (Fig. 7A) and DD (Fig. 7B). In mutant hosts, a wild-type adrenal was sufficient to restore circadian ACTH secretion in both lighting conditions (Fig. 7A, B). Hence, ACTH secretion from the pituitary paralleled the *Per1* expression pattern in the SCN and the behavioral phenotype. By contrast, corticoid rhythms were clearly affected by the genotype of the adrenal transplant. In LD all host/graft combinations showed consistent corticosterone excretion rhythms. However, when either a mutant host or a mutant graft was used, the amplitudes of corticosterone secretion rhythms were dampened by 40-50% compared to the wild-type host/wild-type graft combination (Fig. 7C). These results indicate that the LD cycle can evoke rhythmic corticoid production from a wild-type adrenal in the absence of a functional SCN clock. In addition, they reinforce our finding that the genotype of the adrenal determines sensitivity of the steroidogenic response to ACTH.

In DD, mutant adrenals transplanted into a wild-type host released corticoids rhythmically but the amplitude was reduced when compared to that of control operated recipients (Fig. 7D). This suggests that the adrenal clockwork influences the amplitude of the corticoid rhythm in response to ACTH which is rhythmic in the wild-type host (Fig. 2D and 7B). Surprisingly, corticoid excretion rhythms were completely abolished in *Per2/Cry1* animals that received wild-type adrenals indicating that without a properly functioning SCN, a wild-type

graft cannot sustain rhythmicity of corticoid production (Fig. 7D) in the absence of an entraining light stimulus.

To monitor the molecular status of the clock in the grafts, expression levels of the clock genes *Per1* and *Rev-Erbα* were measured by qPCR. In LD, adrenal clock gene rhythms were paralleling rhythmic hormone secretion (Fig. 7E). Of note, this parallelism was also seen with wild-type adrenal grafts transplanted into arrhythmic hosts (Fig. 7E, mt_h/wt_a) which indicates that the LD cycle can entrain a peripheral clock independently of a functional SCN pacemaker. In DD, when combining wild-type hosts and wild-type grafts, both *Per1* and *Rev-Erbα* showed strong differences in expression levels between the two time points examined (Fig. 7F, wt_h/wt_a). In the case of grafting mutant adrenals into a wild-type host, both clock genes were expressed at similar levels at both times (Fig. 7F, wt_h/mt_a). This suggests that the rhythmic SCN and ACTH signals cannot restore the molecular clock in the grafted mutant adrenal. The observed circadian corticoid rhythm is likely to be driven by the host's SCN pacemaker *via* rhythmic ACTH release from the pituitary. When a wild-type adrenal was transplanted into a mutant host, rhythmic expression of both clock genes was also largely abolished (Fig. 7F, mt_h/wt_a). Thus, a wild-type adrenal cannot autonomously maintain a circadian rhythm in a clock-deficient host.

It was suggested that adrenal corticoids may serve as a signal that synchronizes peripheral clocks with the SCN pacemaker (Balsalobre et al., 2000). To begin to test this possibility we monitored *Per1* and *Rev-Erbα* expression in kidneys of transplanted animals. In both LD and DD conditions kidney clock gene transcription was not affected by the genotype of the adrenal graft but it fully reflected the genotype of the host. This would suggest that rhythmic corticosterone *per se* is not sufficient to re-establish rhythmicity in a mutant kidney clock (suppl. Fig. 1).

Taken together our transplantation studies show that the adrenal clock can be light-entrained even in the absence of a functional SCN pacemaker. The adrenal clock does not seem to feed back on the SCN; rather it modulates circadian corticosterone rhythms by gating the sensitivity of the adrenal to ACTH.

Discussion

To thrive in a rhythmically changing environment, organisms have evolved endogenous clocks that synchronize their physiology to the outside world. In mammals, peripheral clocks residing in endocrine glands are prime candidates for transducing rhythmic cues from the SCN to target tissues *via* a rhythmic release of hormones into the bloodstream. The adrenal, for example, discharges corticoids in a circadian fashion and by this mechanism may control the circadian component of stress response, energy homeostasis, mineral balance and reproduction (Zimmermann and Critchlow 1967; Kawasaki, Uezono et al. 1983; Strubbe and van Dijk 2002; Kennaway 2005). How the peripheral clock of the adrenal carries out this control was subject of this study.

The adrenal clockwork

The rhythmic expression of a limited number of clock genes in the adrenal gland was previously reported for rodents (Bittman, Doherty et al. 2003; Ishida, Mutoh et al. 2005) and monkeys (Lemos, Downs et al. 2006) suggesting the existence of an adrenal circadian oscillator. Using qPCR, microarray and *in situ* hybridization approaches we have characterized the circadian expression of canonical clock genes in this gland (Fig.1). We show that clock genes are expressed in a rhythmic pattern in the *zona glomerulosa* and *zona fasciculata*, the sites of adrenal corticoid production (Parker and Schimmer 2001). While some clock genes like *Per1*, *Per3* and *Bmal1* are also expressed in the adrenal medulla (Bittman, Doherty et al. 2003; Torres-Farfan, Seron-Ferre et al. 2006), others including *Per2*, the *Cry*s and *Clock* are – if at all – only weakly expressed in this tissue (Bittman, Doherty et al. 2003; Torres-Farfan, Seron-Ferre et al. 2006). These and our ISH data thus suggest that a canonical circadian clockwork in the adrenal primarily resides in the outer layers of the cortex, a notion consistent with the rhythmic synthesis of gluco- and mineral corticoids in these layers (Kemppainen and Behrend 1997).

The role of the peripheral clockwork in regulating ACTH responsiveness of the adrenal

We demonstrate that circadian rhythms of ACTH, corticosterone and of clock gene expression in the adrenal are abolished in *Per2/Cry1* double mutant animals (Fig. 2) consistent with the disruption of locomotor activity and clock gene expression rhythms in the SCN and several other tissues in these mice (Oster, Yasui et al. 2002). Since in the intact *Per2/Cry1*-deficient animal all tissues are mutant, one cannot determine whether the absence of hormonal rhythms is caused by a deficient SCN or by a deficient adrenal clock.

We therefore analyzed corticoid production in adrenal slice cultures to characterize the adrenal clock in the absence of a SCN. We found that the adrenal shows a "gated" sensitivity to ACTH which is maintained in the absence of external cues but critically depends on the presence of a functional adrenal clockwork since slices from *Per2/Cry1*-deficient adrenals lack gating (Fig. 3).

Using transcriptional profiling we identify circadian expression patterns of multiple genes involved in the regulation of adrenal steroid metabolism (Fig. 4, 5). These genes encompass most facets of the steroidogenic regulatory network including the ACTH signaling cascade. This and the fact that none of the previously identified rate-limiting components of steroid biosynthesis (e.g. steroidogenic acute regulatory protein (*Star)* or cytochrome P450 side-chain cleavage enzyme (*Cyp11a1*)) show circadian transcription rhythms suggest that the adrenal clock exerts its control on corticoid production not through a restricted set of targets but instead uses multiple molecular routes that collectively steer circadian rhythms of adrenal glucocorticoid production. It is presently not clear which adrenal gene products confer gating of the ACTH response. One attractive candidate is the ACTH receptor and the downstream signaling cascade (like adenylate cyclase or protein kinase A) which directly transmits the ACTH signal to the steroidogenic machinery. In addition, the supply of the steroid precursor cholesterol from the blood may be controlled *via* circadian regulation of the corresponding lipoprotein receptor (*Ldlr*) and transporter proteins (*Scarb2*).

The role of the adrenal clockwork in corticosterone regulation *in vivo*

We have begun to examine the impact of ACTH gating on the endocrine system *in vivo* using an adrenal transplantation model. We show that the genotype of the transplanted adrenal does neither affect *Per1* expression in the SCN nor locomotor activity rhythms of the animal (Fig. 6). A likely explanation of this result is that the SCN does not express glucocorticoid receptors (Rosenfeld, Van Eekelen et al. 1988).

In arrhythmic *Per2/Cry1* hosts kept in LD a wild-type adrenal restores corticosterone rhythmicity (Fig. 7C). In addition, transplantation of wild-type adrenal grafts into a wild-type animal evokes stronger corticoid rhythms than when mutant grafts are used (Fig. 7C, D). Both observations illustrate the consequences of a gated adrenal response to ACTH. In the mutant host, a constant ACTH signal is translated into rhythmic corticosterone by the rhythmic wild-type adrenal. In the wild-type host, high amplitude corticosterone rhythms are achieved because ACTH secretion and adrenal sensitivity rhythms are synchronized, both peaking around the day/night transition (Figs. 2, 3). By contrast, the disrupted clockwork in *Per2/Cry1* adrenal transplants placed into a wild-type mouse hampers synchronization of the

two pacemakers. This results in a less efficient reading of the ACTH stimulus and a dampened corticoid rhythm.

A previous report using transplanted *Clock*-mutant fibroblasts has shown that genetic disruption of a peripheral clock cannot be rescued by a functional SCN pacemaker (Pando, Morse et al. 2002). In accordance with this finding we show that rhythmicity in clock gene expression in mutant adrenal grafts is not restored by the SCN of the wild-type host (Fig. 7E, F). This result and the dampened amplitude of rhythmic corticoid excretion by these mice indicate that even though the peripheral clock of the adrenal clearly influences corticoid rhythms it is not the rhythm generator.

When wild-type adrenal grafts are placed into a mutant host, corticoid rhythms cannot be rescued under DD (Fig. 7B) but under LD conditions (Fig. 7C). This is also reflected in clock gene expression in the transplants (Fig. 7E, F). From these data we draw two conclusions: First, the adrenal clock can be made rhythmic by the light/dark cycle in the absence of a functional SCN clock. Second, this rhythm does not persist when mice are transferred to constant darkness. Ishida et al. (2005) have demonstrated that *Per* gene expression in and corticosterone release from the adrenal can be directly activated by a light pulse. This activation requires functional nerve connections between the SCN and the adrenal. It has also been shown that the sensitivity of the adrenal to ACTH stimulation is regulated *via* the splanchnic nerve (Jasper and Engeland 1994; Jasper and Engeland 1997). Together with our finding that the adrenal clock gates the ACTH response these data suggest that light may directly entrain the adrenal clock *via* the autonomic nervous system thereby influencing circadian hormone secretion.

Previous studies using organ cultures have shown that clock gene rhythms can persist for weeks in the absence of an external timing signal (Yamazaki, Numano et al. 2000; Welsh, Yoo et al. 2004; Yoo, Yamazaki et al. 2004), a finding that is also reflected in the persistence of rhythmic gating of the ACTH response observed in wild-type adrenal slice cultures (Fig. 3). In marked contrast, our transplantation data indicate that *in vivo* adrenal clock gene expression and corticosterone production rhythms critically depend on the input from the SCN. Circadian clock gene and corticoid excretion rhythms are lost in mutant hosts carrying wild-type adrenals within 48 hrs after release into DD (Fig. 7). Somewhat contrary to the prevailing view our data suggest that peripheral clocks are not primarily self-sustained generators of rhythms but more likely modulate and stabilize circadian rhythms of physiology. Hence, the principal rhythm generator is the SCN (Schibler and Sassone-Corsi 2002; Davidson, Yamazaki et al. 2003).

The SCN/adrenal/corticosterone regulatory network examined in the present work may serve as a paradigm for the organization of other physiological rhythms but at the same time our transplantation studies highlight the specificity of some components of this pathway. This may explain why the quest for a singular signaling molecule transmitting time information from the SCN to the body has so far not been successful.

Experimental procedures

Animals

All animal experiments were conducted in compliance with the German Law on Animal Welfare (TierSchG §1-11). To identify rhythmically expressed genes by microarray analysis 4 months old male C57BL/6J animals were used. Wild-type and *Per2*^{Brdm1}/*Cry1*$^{-/-}$ animals used for expression analysis, slice cultures and for transplantation experiments were derived from double heterozygous breeding pairs on a mixed 129Sv/C57BL/6J background (Oster, Yasui et al. 2002). General mouse handling and behavioral monitoring was performed as described (Jud, Schmutz et al. 2005).

Microarray hybridization and data analysis

Animals were entrained to a 12 hrs light: 12 hrs dark cycle (LD) for two weeks, released into constant darkness (DD) and sacrificed by cervical dislocation under a 15W safety red light at the indicated time points (see Fig. 1A). Eyes were removed prior to tissue dissection and tissue was stored in RNA*later* (Ambion, Huntingdon, UK) at -20 ° C until use. Total RNA samples from whole adrenal preparations were prepared using RNeasy Micro Kit (QIAGEN, Hilden, Germany) and integrity was assessed by analyzing aliquots on an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA). All subsequent experimental procedures, array hybridization (MG430v2.0 chips, Affymetrix, Santa Clara, CA) and analytical steps were performed by the RZPD (German Resource Center for Genome Research, Berlin, Germany, *http://www.rzpd.de/protocols*). One adrenal was used per chip. Chips were hybridized in duplicates. Primary data processing and sample comparison was performed using dChip 1.3 software (Li and Wong 2001). Fluorescence values were normalized according to a MM / PM difference model for each experiment. Only those probe sets qualified as "expressed" in all replicates for at least one time point (including both 24hr cycles in the wild-type experiment) were used for further analysis. Rhythmic genes were identified using the COSOPT algorithm (Straume 2004) with a pMMC-ß cut-off of 0.1 and a period length restriction of 22 to 26 hrs.

Quantitative real-time PCR (qPCR)

Total RNA from whole adrenals was prepared as described for microarrays. cDNA was synthesized using the Thermoscript RT Kit (Invitrogen, Paisley, UK). qPCR was performed on an iCycler (Bio-Rad, Hercules, CA) with iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's protocol. Primer sequences are found in supplemental Table 2. Single well amplification efficiency estimation and relative quantification of expression levels were performed as described (Ramakers, Ruijter et al. 2003).

In situ hybridization and immunohistochemistry

In situ hybridization using digoxigenin or ^{35}S-labeled probes on frozen sections was performed as described (Oster, Baeriswyl et al. 2003; Yaylaoglu, Titmus et al. 2005). All probe templates were generated by PCR from E 14.5 mouse embryo and adult brain cDNA. Primer sequences are found in supplemental Table 2. Immunohistochemistry on paraffin embedded sections was performed as described (Oster, Baeriswyl et al. 2003). The *anti*PER2 antibody (Alpha Diagnostic, San Antonio, TX) was 1,000 fold diluted. For signal amplification and visualization Ni-amplified DAB staining with the Vectastain Elite Kit (Vector, Burlingame, CA) was used according to the manufacturer's protocol.

Hormone measurements

Animals were sacrificed by cervical dislocation, decapitated and blood was collected from the jugular vein. Plasma extracts were prepared using Microvette 300 coated tubes (Sarstedt, Nümbrecht, Germany) and stored at -80 ° C. To rule out stress induced effects on hormone levels, animals were put into wheel-running cages equipped with a metal grid floor two days prior to sampling. On the following two days feces were collected at 4 hrs intervals and stored at -80 ° C until processing. Corticoid extraction was performed as described (Cavigelli, Monfort et al. 2005). ACTH and corticosterone/corticoid sample concentrations were determined by radioimmunoassay (RIA) using commercial available kits from DiaSorin (Cat.# 27130) and MP Biomedicals (Cat.# 07-120103).

Adrenal slice cultures

After entrainment to LD mice were released into DD and sacrificed by cervical dislocation. Adrenals were dissected and embedded in 4% low melting agarose on ice. After solidification agarose blocks were trimmed and adrenals sectioned at 200µm thickness with a vibratome (Model 765, Campden, Lafayette, IN). Adrenal slices were placed on top of Millicell-CM culture plate inserts (Millipore, Bedford, MA) and cultivated in FDMEM / 0.1% DMSO / 50µM MeSH / 2% FCS / 0.1% Gentamycin / 0.12 mg/ml Penicillin / 0.12 mg/ml Streptomycin at 37 ° C / 5 % CO_2. ACTH stimulation (20 nM) was performed by adding ACTH to the medium. Medium was aspirated and corticosterone levels were evaluated by RIA. For each time point a separate slice was used to circumvent corticoid feedback after medium withdrawal.

The formula used for calculation of the initial release rate (v_0) was $v_0 = -3*\ln(1-(conc-conc_0)/(conc_{max}-conc_0))*time^{-1}$ with conc = corticosterone concentration, $conc_0$ = conc at time = 0 and $conc_{max}$ = conc at time = ∞. All calculations were done with GraphPad Prism 4 software (GraphPad Software, San Diego, CA).

Adrenal gland transplantations

Transplantations of adrenal gland fragments were performed as described (Musholt, Klebs et al. 2002). Animals were anaesthetized using Ketamin / Rompun (100/10 mg/kg *i.p.*) and adrenals were dissected following median laparotomy. After removal of fat, each gland was divided into four pieces. Fragments from wild-type mice or *Per2/Cry1* mutants were transplanted underneath the kidney capsule of adrenalectomized animals of the opposite genotype. Always both adrenals from one donor were transplanted into a single host animal. The capsule of the host kidney was lifted with forceps and incised and the graft was pushed underneath the capsule up to the dorsal pole of the kidney (Fig. 6A). The incision was closed by bipolar electro coagulation. Animals recovered for two weeks prior to transfer to wheel-running cages. Hormone measurements and expression analyses were performed after two additional weeks of entrainment.

References

Bae, K., Jin, X., Maywood, E. S., Hastings, M. H., Reppert, S. M., and Weaver, D. R. (2001). Differential functions of *mPer1*, *mPer2*, and *mPer3* in the SCN circadian clock. Neuron *30*, 525-536.

Balsalobre, A., Brown, S. A., Marcacci, L., Tronche, F., Kellendonk, C., Reichardt, H. M., Schütz, G., and Schibler, U. (2000). Resetting of circadian time in peripheral tissues by glucocorticoid signaling. Science *289*, 2344-2347.

Bittman, E. L., Doherty, L., Huang, L., and Paroskie, A. (2003). *Period* gene expression in mouse endocrine tissues. Am J Physiol Regul Integr Comp Physiol *285*, R561-569.

Buijs, R. M., van Eden, C. G., Goncharuk, V. D., and Kalsbeek, A. (2003). The biological clock tunes the organs of the body: timing by hormones and the autonomic nervous system. J Endocrinol *177*, 17-26.

Bunger, M. K., Wilsbacher, L. D., Moran, S. M., Clendenin, C., Radcliffe, L. A., Hogenesch, J. B., Simon, M. C., Takahashi, J. S., and Bradfield, C. A. (2000). *Mop3* is an essential component of the master circadian pacemaker in mammals. Cell *103*, 1009-1017.

Casanueva, F. F., and Dieguez, C. (1999). Neuroendocrine regulation and actions of leptin. Front Neuroendocrinol *20*, 317-363.

Cavigelli, S. A., Monfort, S. L., Whitney, T. K., Mechref, Y. S., Novotny, M., and McClintock, M. K. (2005). Frequent serial fecal corticoid measures from rats reflect circadian and ovarian corticosterone rhythms. J Endocrinol *184*, 153-163.

Cheifetz, P. N. (1971). The daily rhythm of the secretion of corticotrophin and corticosterone in rats and mice. J Endocrinol *49*, 11-12.

Davidson, A. J., Yamazaki, S., and Menaker, M. (2003). SCN: ringmaster of the circadian circus or conductor of the circadian orchestra? Novartis Found Symp *253*, 110-121; discussion 121-115, 281-114.

Delarue, C., Contesse, V., Lenglet, S., Sicard, F., Perraudin, V., Lefebvre, H., Kodjo, M., Leboulenger, F., Yon, L., Gallo-Payet, N., and Vaudry, H. (2001). Role of neurotransmitters and neuropeptides in the regulation of the adrenal cortex. Rev Endocr Metab Disord *2*, 253-267.

Gallo-Payet, N., and Payet, M. D. (2003). Mechanism of action of ACTH: beyond cAMP. Microsc Res Tech *61*, 275-287.

Harmer, S. L., Panda, S., and Kay, S. A. (2001). Molecular bases of circadian rhythms. Annu Rev Cell Dev Biol *17*, 215-253.

Ishida, A., Mutoh, T., Ueyama, T., Bando, H., Masubuchi, S., Nakahara, D., Tsujimoto, G., and Okamura, H. (2005). Light activates the adrenal gland: timing of gene expression and glucocorticoid release. Cell Metab *2*, 297-307.

Jasper, M. S., and Engeland, W. C. (1994). Splanchnic neural activity modulates ultradian and circadian rhythms in adrenocortical secretion in awake rats. Neuroendocrinology *59*, 97-109.

Jasper, M. S., and Engeland, W. C. (1997). Splanchnicotomy increases adrenal sensitivity to ACTH in nonstressed rats. Am J Physiol *273*, E363-368.

Jud, C., Schmutz, I., Hampp, G., Oster, H., and Albrecht, U. (2005). A guideline for analyzing circadian wheel-running behavior in rodents under different lighting conditions. Biol Proced Online *7*, 101-116.

Kawasaki, T., Uezono, K., Ueno, M., Omae, T., Matsuoka, M., Haus, E., and Halberg, F. (1983). Comparison of circadian rhythms of the renin-angiotensin-aldosterone system and electrolytes in clinically healthy young women in Fukuoka (Japan) and Minnesota (USA). Acta Endocrinol (Copenh) *102*, 246-251.

Kemppainen, R. J., and Behrend, E. N. (1997). Adrenal physiology. Vet Clin North Am Small Anim Pract *27*, 173-186.

Kennaway, D. J. (2005). The role of circadian rhythmicity in reproduction. Hum Reprod Update *11*, 91-101.

Lemos, D. R., Downs, J. L., and Urbanski, H. F. (2006). Twenty-four hour rhythmic gene expression in the rhesus macaque adrenal gland. Mol Endocrinol. *20*, 1164-76.

Li, C., and Wong, W. H. (2001). Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. Proc Natl Acad Sci U S A *98*, 31-36.

Lindhe, O., Lund, B. O., Bergman, A., and Brandt, I. (2001). Irreversible binding and adrenocorticolytic activity of the DDT metabolite 3-methylsulfonyl-DDE examined in tissue-slice culture. Environ Health Perspect *109*, 105-110.

Manna, P. R., and Stocco, D. M. (2005). Regulation of the steroidogenic acute regulatory protein expression: functional and physiological consequences. Curr Drug Targets Immune Endocr Metabol Disord *5*, 93-108.

Musholt, T. J., Klebs, S. H., Musholt, P. B., Ellerkamp, V., Klempnauer, J., and Hoffmann, M. W. (2002). Transplantation of adrenal tissue fragments in a murine model: functional capacities of syngeneic and allogeneic grafts. World J Surg *26*, 950-957.

Nicholson, W. E., Levine, J. H., and Orth, D. N. (1976). Hormonal regulation of renal ornithine decarboxylase activity in the rat. Endocrinology *98*, 123-128.

Oishi, K., Amagai, N., Shirai, H., Kadota, K., Ohkura, N., and Ishida, N. (2005). Genome-wide Expression Analysis Reveals 100 Adrenal Gland-dependent Circadian Genes in the Mouse Liver. DNA Res *12*, 191-202.

Oster, H., Baeriswyl, S., Van Der Horst, G. T., and Albrecht, U. (2003). Loss of circadian rhythmicity in aging *mPer1*$^{-/-}$*mCry2*$^{-/-}$ mutant mice. Genes Dev *17*, 1366-1379.

Oster, H., Yasui, A., van der Horst, G. T., and Albrecht, U. (2002). Disruption of *mCry2* restores circadian rhythmicity in *mPer2* mutant mice. Genes Dev *16*, 2633-2638.

Pando, M. P., Morse, D., Cermakian, N., and Sassone-Corsi, P. (2002). Phenotypic rescue of a peripheral clock genetic defect via SCN hierarchical dominance. Cell *110*, 107-117.

Parker, K. L., and Schimmer, B. P. (1997). Steroidogenic factor 1: a key determinant of endocrine development and function. Endocr Rev *18*, 361-377.

Parker, K. L., and Schimmer, B. P. (2001). Genetics of the development and function of the adrenal cortex. Rev Endocr Metab Disord *2*, 245-252.

Payne, A. H., and Hales, D. B. (2004). Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. Endocr Rev *25*, 947-970.

Ralph, M. R., Foster, R. G., Davis, F. C., and Menaker, M. (1990). Transplanted suprachiasmatic nucleus determines circadian period. Science *247*, 975-978.

Ramakers, C., Ruijter, J. M., Deprez, R. H., and Moorman, A. F. (2003). Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci Lett *339*, 62-66.

Reppert, S. M., and Weaver, D. R. (2001). Molecular analysis of mammalian circadian rhythms. Annu Rev Physiol *63*, 647-676.

Richards, J. S. (2001). New signaling pathways for hormones and cyclic adenosine 3',5'-monophosphate action in endocrine cells. Mol Endocrinol *15*, 209-218.

Rosenfeld, P., Van Eekelen, J. A., Levine, S., and De Kloet, E. R. (1988). Ontogeny of the type 2 glucocorticoid receptor in discrete rat brain regions: an immunocytochemical study. Brain Res *470*, 119-127.

Rusak, B., and Zucker, I. (1979). Neural regulation of circadian rhythms. Physiol Rev *59*, 449-526.

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

Schibler, U., and Sassone-Corsi, P. (2002). A web of circadian pacemakers. Cell *111*, 919-922.

Sewer, M. B., and Waterman, M. R. (2001). Insights into the transcriptional regulation of steroidogenic enzymes and StAR. Rev Endocr Metab Disord *2*, 269-274.

Simpson, E. R., and Waterman, M. R. (1988). Regulation of the synthesis of steroidogenic enzymes in adrenal cortical cells by ACTH. Annu Rev Physiol *50*, 427-440.

Stocco, D. M. (2001). StAR protein and the regulation of steroid hormone biosynthesis. Annu Rev Physiol *63*, 193-213.

Stocco, D. M., Wang, X., Jo, Y., and Manna, P. R. (2005). Multiple signaling pathways regulating steroidogenesis and steroidogenic acute regulatory protein expression: more complicated than we thought. Mol Endocrinol *19*, 2647-2659.

Straume, M. (2004). DNA microarray time series analysis: automated statistical assessment of circadian rhythms in gene expression patterning. Methods Enzymol *383*, 149-166.

Strubbe, J. H., and van Dijk, G. (2002). The temporal organization of ingestive behaviour and its interaction with regulation of energy balance. Neurosci Biobehav Rev *26*, 485-498.

Torres-Farfan, C., Seron-Ferre, M., Dinet, V., and Korf, H. W. (2006). Immunocytochemical demonstration of day/night changes of clock gene protein levels in the murine adrenal gland: differences between melatonin-proficient (C3H) and melatonin-deficient (C57BL) mice. J Pineal Res *40*, 64-70.

Ulrich-Lai, Y. M., and Engeland, W. C. (2000). Rat adrenal transplants are reinnervated: an invalid model of denervated adrenal cortical tissue. J Neuroendocrinol *12*, 881-893.

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

Welsh, D. K., Yoo, S. H., Liu, A. C., Takahashi, J. S., and Kay, S. A. (2004). Bioluminescence imaging of individual fibroblasts reveals persistent, independently phased circadian rhythms of clock gene expression. Curr Biol *14*, 2289-2295.

Yamazaki, S., Numano, R., Abe, M., Hida, A., Takahashi, R., Ueda, M., Block, G. D., Sakaki, Y., Menaker, M., and Tei, H. (2000). Resetting central and peripheral circadian oscillators in transgenic rats. Science *288*, 682-685.

Yaylaoglu, M. B., Titmus, A., Visel, A., Alvarez-Bolado, G., Thaller, C., and Eichele, G. (2005). Comprehensive expression atlas of fibroblast growth factors and their receptors generated by a novel robotic *in situ* hybridization platform. Dev Dyn *234*, 371-386.

Yoo, S. H., Yamazaki, S., Lowrey, P. L., Shimomura, K., Ko, C. H., Buhr, E. D., Siepka, S. M., Hong, H. K., Oh, W. J., Yoo, O. J.*, et al.* (2004). PERIOD2:LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. Proc Natl Acad Sci U S A *101*, 5339-5346.

Zheng, B., Albrecht, U., Kaasik, K., Sage, M., Lu, W., Vaishnav, S., Li, Q., Sun, Z. S., Eichele, G., Bradley, A., and Lee, C. C. (2001). Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. Cell *105*, 683-694.

Zimmermann, E., and Critchlow, V. (1967). Effects of diurnal variation in plasma corticosterone levels on adrenocortical response to stress. Proc Soc Exp Biol Med *125*, 658-663.

Figure legends

Figure 1: *Robust circadian expression of clock genes in the murine adrenal cortex.*

A) Diagram of experimental conditions and sampling time points used in this study. Top: zebra bar represents the light/dark schedule to which mice were initially exposed; white segments indicate lights on whereas black segments signify darkness. Prior to "lights off" animals were kept at least for 14 days under LD12:12 conditions. Center: wild-type locomotor activity pattern relative to *Zeitgeber* (ZT) and circadian times (CT). Rest/activity patterns echo light conditions in that activity phases (black segments) alternate with resting phases (gray segments). Bottom: Tissue sampling times relative to the "lights off" event and to absolute time. **(B-I)** Circadian expression profiles (mean $\pm$ SEM) of canonical clock genes in the adrenal gland determined by qPCR (3 different adrenals per time point) and microarray analysis (two chips per time point each with a different adrenal). For an explanation of time axis see Fig. 1A, top. (**B'-F', H', I')** *In situ* hybridization micrographs of wild-type adrenals probed for different clock genes at 46 hrs (left panel) and 58 hrs after "lights off" (right panel). **G'')** Differential expression of PER2 protein revealed by immunohistochemistry in the adrenal cortex and medulla. **B'** (left panel) The layered histoarchitecture of the adrenal: adrenal capsule (c); *zona glomerulosa* (zg); *zona fasciculata* (zf); *zona reticularis* (zr) and *medulla* (m). Bar in B' is 0.2 mm.

Figure 2: *Per2/Cry1 mutant mice are defective in adrenal clock gene and HPA axis rhythmicity.*

A, B) *Bmal1*, *Per1* (A), *Per3* and *Rev-Erbα* (B) expression rhythms in the adrenal of *Per2/Cry1* double mutant animals in DD as determined by qPCR. Values were normalized to the average wild-type expression level as shown in Fig. 1. Absence of rhythmic transcription was also seen for all clock genes of Fig. 1 (data not shown). All values are mean $\pm$ SEM (n = 4 to 6). Gray and black bars delineate rest and active phases in wild-type control animals. **C)** Diurnal rhythm of plasma ACTH in wild-type and *Per2/Cry1* double mutant animals. **D)** Circadian rhythm of plasma ACTH. **E)** Diurnal rhythm of plasma corticosterone. **F)** Circadian profile of plasma corticosterone. Diurnal **G)** and circadian **H)** profiles of fecal corticoid excretion show distinct rhythmicity for wild-type but not for mutant mice. All data presented are mean $\pm$ SEM (n = 3 to 5). White and black bars indicate light and dark phases; gray and black bars indicate rest and activity phases in wild-type mice.

Figure 3: *A peripheral clockwork residing in the adrenal cortex gates the ability of ACTH to evoke corticosterone release.*

A, B) Corticosterone release into culture medium by wild-type and *Per2/Cry1* mutant adrenal tissue culture slices in response to ACTH stimulation at 38 hrs (A) and 50 hrs (B) after "lights off" (n = 6). **C)** Initial rate of corticosterone release for wild-type and *Per2/Cry1* mutant adrenal slices derived from two experiments one of which is shown in A and B. All data shown are mean $\pm$ SEM.

Figure 4: *Circadian clock regulated genes involved in the control of adrenal corticosterone biosynthesis.*

Network of enzymes and regulators of corticosterone production (modified from (Parker and Schimmer 1997; Delarue, Contesse et al. 2001; Richards 2001; Sewer and Waterman 2001; Stocco 2001; Gallo-Payet and Payet 2003; Payne and Hales 2004; Manna and Stocco 2005; Stocco, Wang et al. 2005)). Small gray boxes indicate genes identified as rhythmically expressed in our wild-type microarray time series. For gene descriptions see supplemental Table 1. The big gray box highlights the corticosterone biosynthesis pathway.

Figure 5: *Rhythmic expression of genes encoding regulators of steroidogenesis.*

Microarray (solid lines) and qPCR (dotted lines) time courses for 20 genes identified as rhythmically expressed – by COSOPT - from a wild-type microarray time series. Expression values are normalized to the average *mRNA* level. All values are mean $\pm$ SEM (n = 3 for qPCR and 2 for microarrays). Gray and black bars depict subjective day and night.

Figure 6: *The functionality of the adrenal clock does neither affect Per1 rhythms in the SCN nor locomotor activity*.

A) Schematic representation of the surgical procedure employed to transplant *Per2/Cry1* double mutant adrenals into wild-type adrenalectomized animals and *vice versa*. The inset cartoon illustrates how the adrenal grafts are implanted underneath the kidney capsule of the host. **B-D)** Contact autoradiograph scans of brain sections probed for *Per1* expression 46 and 58 hrs after "lights off" (upper panels) and representative double-plotted actograms for the different genotypes in LD and DD conditions (lower panels). **B)** Auto-grafted wild-type animals, **C)** wild-type hosts that obtained *Per2/Cry1* mutant adrenal transplants and **D)** *Per2/Cry1* double mutants with implanted wild-type adrenals. The arrow in B points at the SCN.

Figure 7: *HPA axis regulation and adrenal clock gene expression in adrenorecipient mice.*

A, B) Plasma ACTH regulation in grafted animals in LD (A) and DD (B). Numbers below the x-axis depict the time points at which animals were sacrificed (n = 3). **C, D)** Corticoid metabolite excretion rhythms of adrenal-grafted animals in LD (C) and DD (D). Data are double-plotted for better visualization (n = 4). **E, F)** *Per1* and *Rev-Erbα* expression in adrenal grafts in LD (E) and DD (F). Relative expression values are normalized to 46 hrs in the control operated animals (n = 3). All data are mean $\pm$ SEM. Abbreviations: wt_h – wild-type host; mt_h – mutant host; wt_a – wild-type adrenal; mt_a – mutant adrenal.

Acknowledgements

The authors thank Dr. G.T.J. van der Horst for the gift of *Cry1* mutant mice, Dr. M. Straume for providing the COSOPT analysis software and Dr. U. Albrecht for donating the template for the *Per3 in situ* probe and for critical comments on the manuscript. This work was supported by the EU grant QLG3-CT-2002-01829 and the Max-Planck-Society.

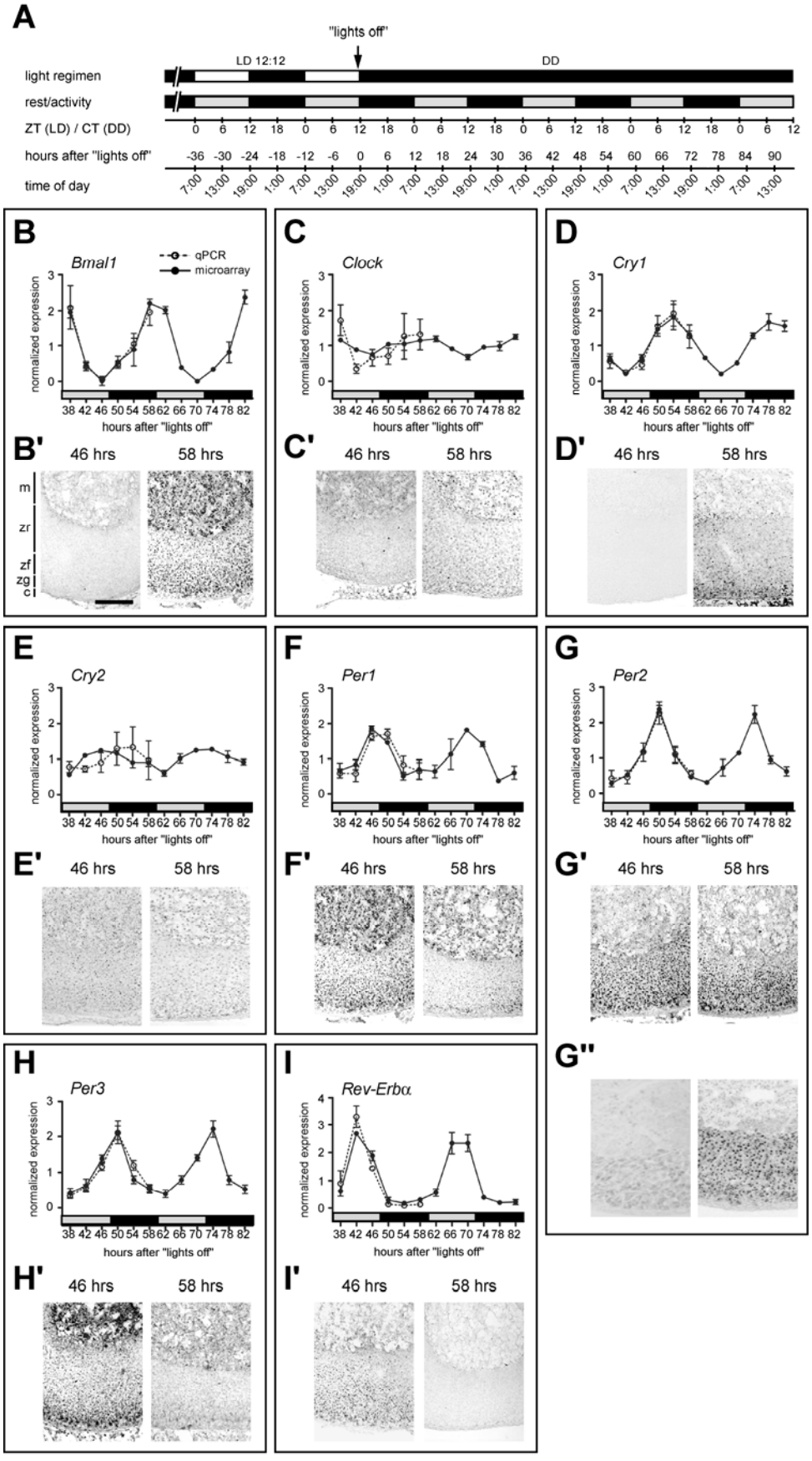


Figure 1: Robust circadian expression of clock genes in the murine adrenal cortex.

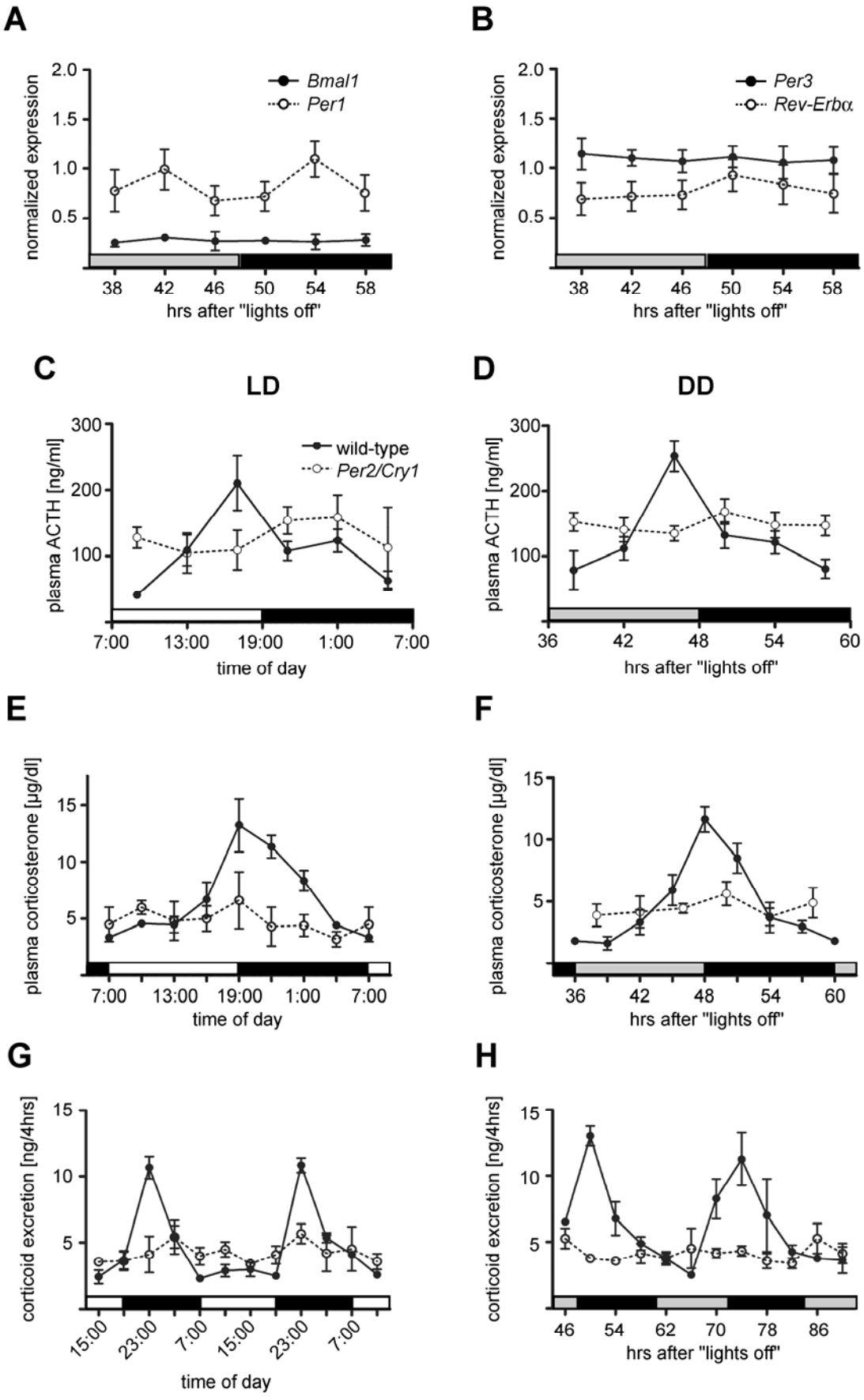


Figure 2: *Per2/Cry1* mutant mice are defective in adrenal clock gene and HPA axis rhythmicity.

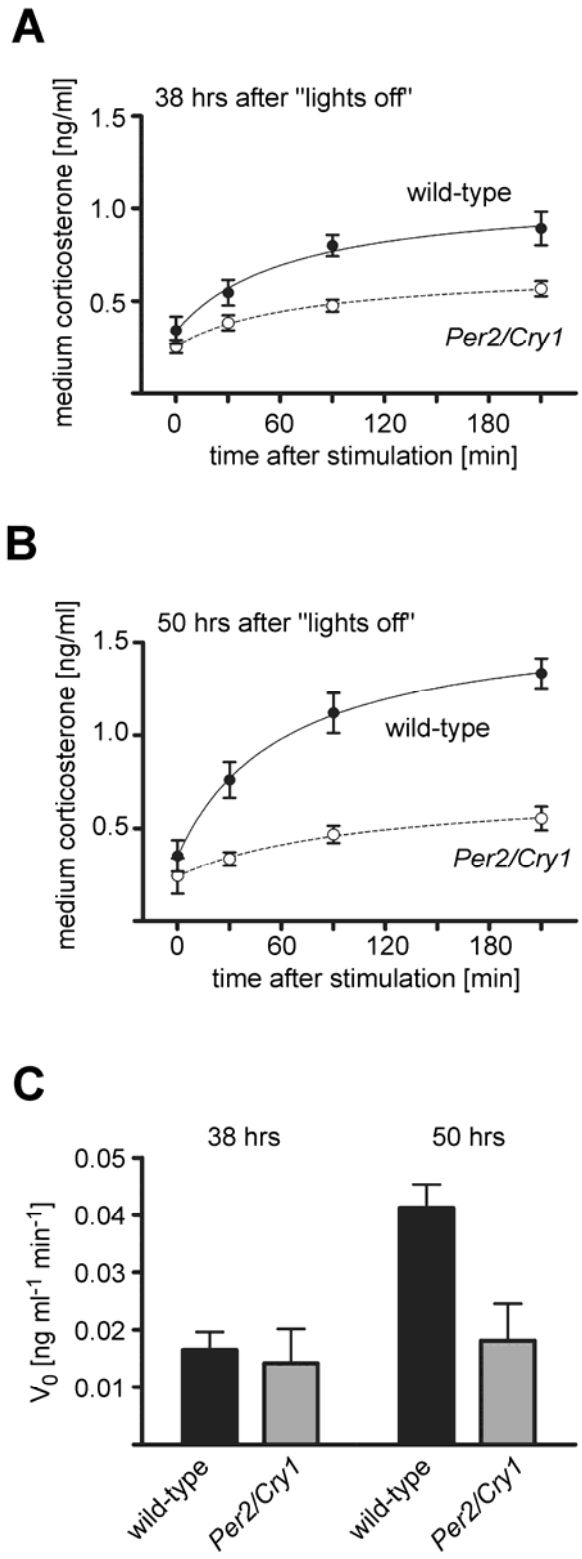


Figure 3: A peripheral clockwork residing in the adrenal cortex gates the ability of ACTH to evoke corticosterone release.

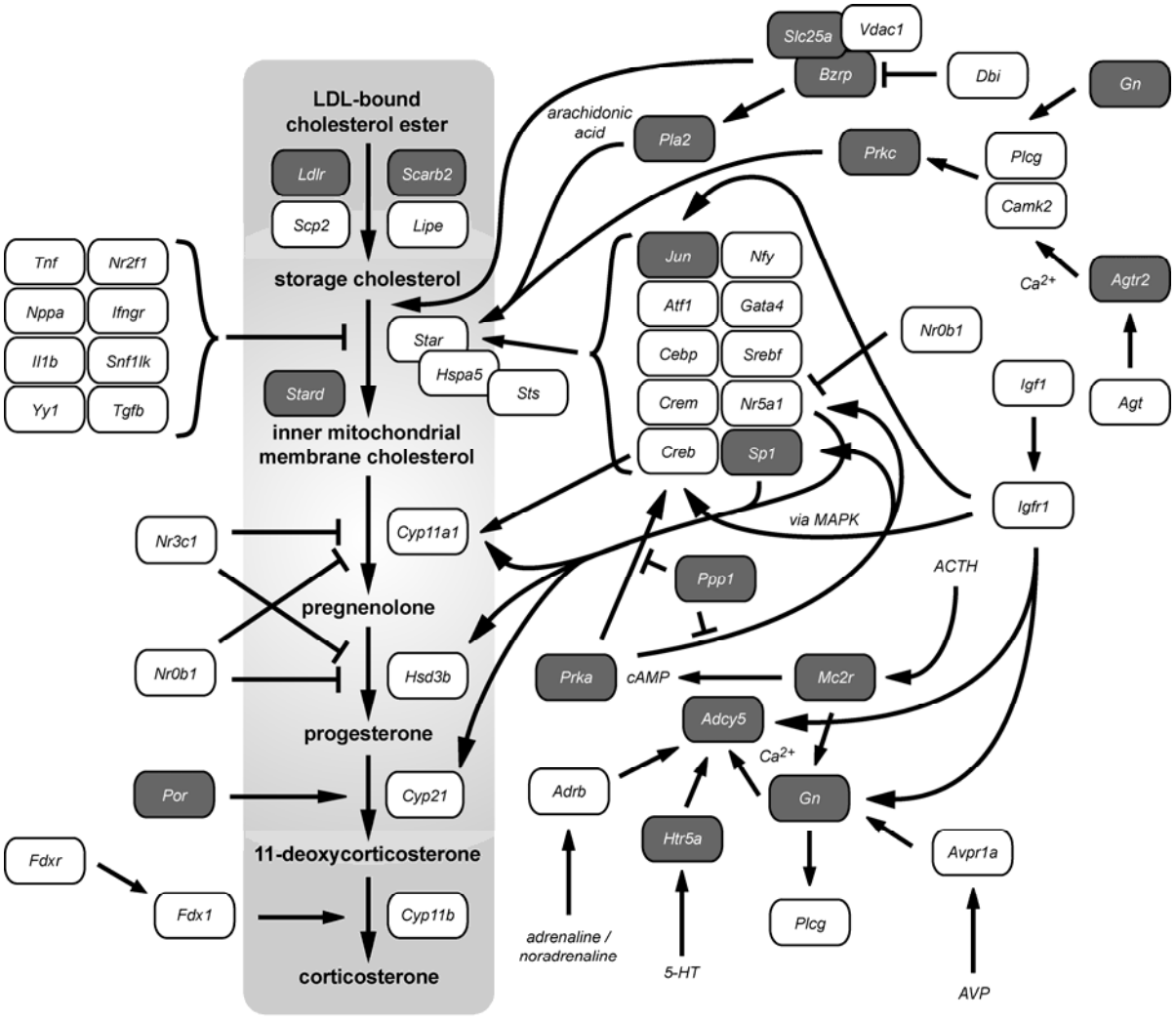


Figure 4: Circadian clock regulated genes involved in the control of adrenal corticosterone biosynthesis.

Figure 5: Rhythmic expression of genes encoding regulators of steroidogenesis.

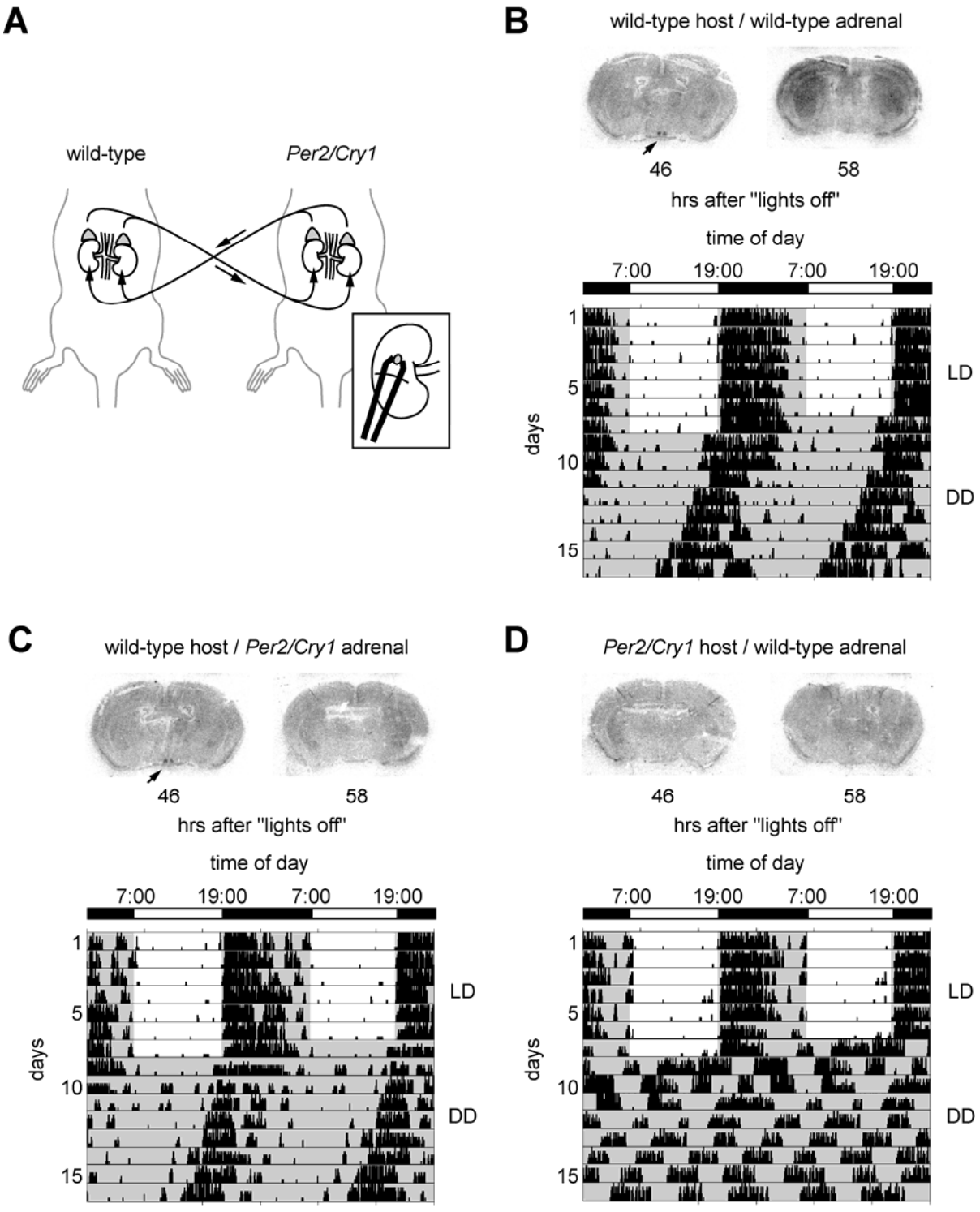


Figure 6: The functionality of the adrenal clock does neither affect *Per1* rhythms in the SCN nor locomotor activity.

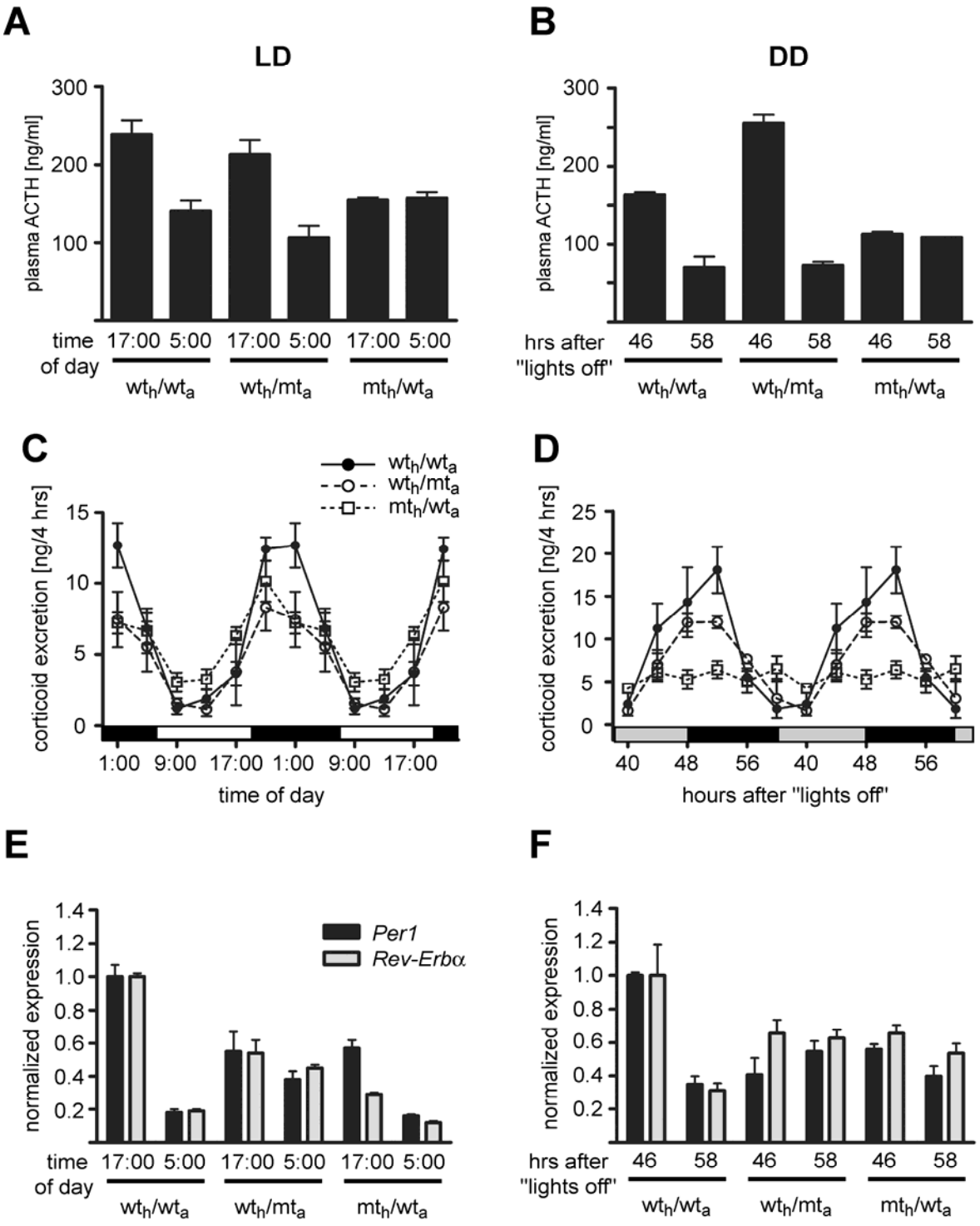
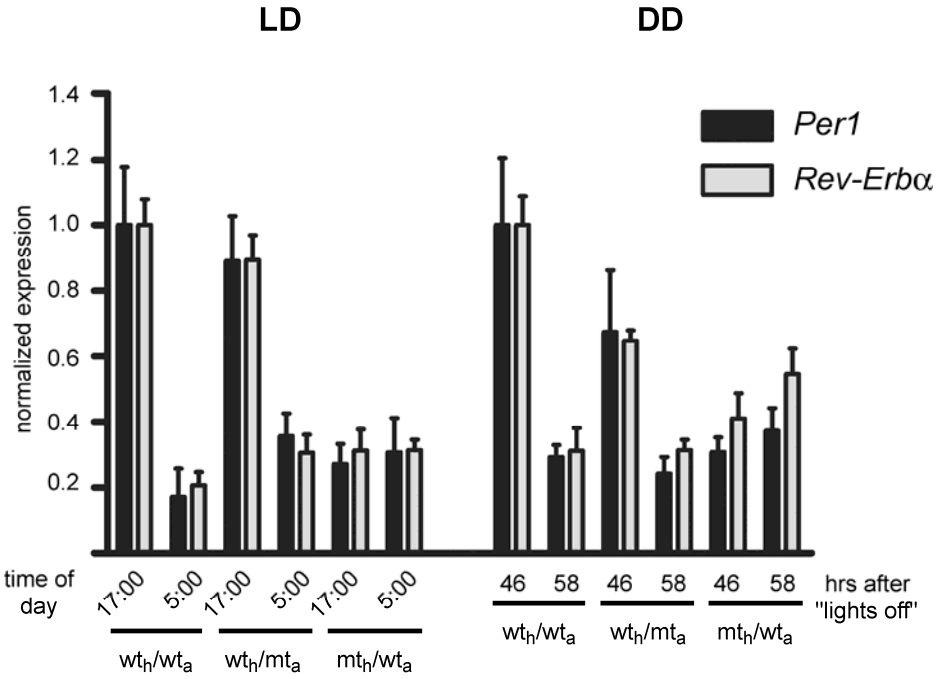


Figure 7: HPA axis regulation and adrenal clock gene expression in adrenorecipient mice.



Suppl. Figure 1: Clock gene expression in the kidneys of adrenorecipient mice.

Per1 and *Rev-Erbα* expression in host kidneys of grafted animals in LD and DD. Relative expression values are normalized to 46 hrs of control operated animals. All data are mean ± SEM (n = 3). For abbreviations see Fig. 7.

Supplemental table 1: Regulators of corticosterone biosynthesis showing a circadian expression rhythm in the adrenal gland

probe set	gene title	gene symbol	Entrez ID
1455296_at	adenylate cyclase 5	*Adcy5*	224129
1452878_at	angiotensin II receptor, type 2	*Agtr2*	11609
1438948_x_at	benzodiazepine receptor, peripheral	*Bzrp*	12257
1430295_at	guanine nucleotide binding protein, alpha 13	*Gna13*	14674
1420385_at	guanine nucleotide binding protein, alpha 14	*Gna14*	14675
1454959_s_at	guanine nucleotide binding protein, alpha inhibiting 1	*Gnai1*	14677
1421152_a_a	guanine nucleotide binding protein, alpha o	*Gnao1*	14681
1450115_at	guanine nucleotide binding protein, alpha q	*Gnaq*	14682
1419470_at	guanine nucleotide binding protein, beta 4	*Gnb4*	14696
1428156_at	guanine nucleotide binding protein, gamma 2	*Gng2*	14702
1417409_at	jun oncogene	*Jun*	16476
1421821_at	low density lipoprotein receptor	*Ldlr*	16835
1422926_at	melanocortin 2 receptor	*Mc2r*	17200
1416933_at	P450 (cytochrome) oxidoreductase	*Por*	18984
1434852_at	phospholipase A2, group IIF	*Pla2g2f*	26971
1422147_a_a	phospholipase A2, group VI	*Pla2g6*	53357
1447720_x_at	protein kinase A catalytic	*Prkaca*	18747
1425550_a_a	protein kinase A regulatory, type I alpha	*Prkar1a*	19084
1440132_s_at	protein kinase A regulatory, type I beta	*Prkar1b*	19085
1430640_a_a	protein kinase A regulatory, type II beta	*Prkar2b*	19088
1437861_s_at	protein kinase C, epsilon	*Prkce*	18754
1417410_s_at	protein kinase C, iota	*Prkci*	18759
1454848_at	protein phosphatase 1, regulatory (inhibitor) subunit 12C	*Ppp1r12c*	232807
1430286_s_at	protein phosphatase 1, regulatory (inhibitor) subunit 14C	*Ppp1r14c*	76142
1454704_at	scavenger receptor class B, member 2	*Scarb2*	12492
1452604_at	serologically defined colon cancer antigen 13	*Stard13*	243362
1443276_at	serotonin receptor 5A	*Htr5a*	15563
1438360_x_at	solute carrier family 25, member 5	*Slc25a5*	11740
1416954_at	solute carrier family 25, member 10	*Slc25a10*	27376
1449481_at	solute carrier family 25, member 13	*Slc25a13*	50799
1455011_at	StAR-related lipid transfer (START) domain containing 4	*Stard4*	170459
1422821_s_at	StAR-related lipid transfer (START) domain containing 5	*Stard5*	170460
1454852_at	trans-acting transcription factor 1	*Sp1*	20683

Supplemental table 2: Primer pairs used for the generation of cDNA templates for *in situ* hybridization probes and for qPCR.

A. Primer sequences used for *in situ* hybridization template generation

Gene	forward primer (5' to 3')	reverse primer (5' to 3')
Per1	ACCAGTCAGTGTAGCTTCAGCTCC	ATCCATCCAGTTCTGAGAAGAGCG
Per2	CACCATAGTTTTCTGGCGGT	CTGTGTTCGTCAGTGTGTTT
Per3	CAGTGAACACGAAGACCGAAA	GAATTCAGGATCAAAGCAGCCT
Cry1	CCATCGTCAATCATGCAGAG	CAAGAAGCAGCCCTGGTAAG
Cry2	CCATCGTCAATCATGCAGAG	CAAGAAGCAGCCCTGGTAAG
Clock	ACAGCAGCTTCCTTCAGTTCAGC	TGGGGACTTGAGAATGACACTGC
Bmal1	CTGTAGAATGAAGTGCAACAGGC	AGCTACCAATGATGCTTCTGTGC
Rev-Erbα	CTGAGGAAACGTAGCAG	TTGGAGTCCAGGGTCG

B. Primer sequences used for qPCR

gene	forward primer (5' to 3')	reverse primer (5' to 3')
Adcy5	ATCCTGAGCTGTACCCAGAAGC	CATCTCCTTTGACACCTGGTTG
Agtr2	CGGGAGCTGAGTAAGCTGATTT	GATTATCAAAAGGACGGCTGCT
Bmal1	CCTAATTCTCAGGGCAGCAGAT	TCCAGTCTTGGCATCAATGAGT
Clock	GTGGTGACTGCCTATCCTACCT	AAGGAGGGAAAGTGCTCTGTTG
Cry1	GTCATTGCAGGAAAATGGGAAG	TAAAGAGGCGGAGAGACAAAGG
Cry2	AGATGGCCTCAGGTTTTCTCAG	TTCAGGCCCACTCTACCTTCTC
Dbp	AATGACCTTTGAACCTGATCCCGCT	GCTCCAGTACTTCTCATCCTTCTGT
Gna13	TCCAGCTGGGTGAGTCTGTAAA	TTCTGATCTCTGGCCACCTACA
Gna14	GGAGAGCAAAGCCCTGTTTAGA	GACATCTTGCTTTGGTCCTGTG
Gnai1	TATCCAGTCCATCATTGCCATC	GTACTCCCGGGATCTGTTGAAG
Gnaq	CCTGGTTCCAGAACTCCTCTGT	GCACGTGAAGTGGGAGTAGATG
Htr5a	AGCCTTCCTACACCGTGTTCTC	ACGTATCCCCTTCTGTCTGGAA
Jun	GAGCATTTGGAGAGTCCCTTCT	CCTGTGCGAACTGGTATGAGTA
Ldlr	TTCAAGTGTCACAGTGGGGAGT	AGAGCCAATCTTGAGGTCCTTG
Mc2r	CACAAATGATTCTGCTGCTTCC	TTATTTCTTGCGGTGTCATTGG
Per1	TGGCTCAAGTGGCAATGAGTC	GGCTCGAGCTGACTGTTCACT
Per2	GCCAAGTTTGTGGAGTTCCTG	CTTGCACCTTGACCAGGTAGG
Pla2g2f	TCCAGCCTGGGTATGAAGAAAT	CCCTGCTCAAAGAGCTTCTCAT
Por	AAGGAACCAGCAGGAGAGAATG	CTCGCGGTACAGATAGTCCTCA
Ppp1r12c	ACTGGCTCAGAAACAGGAGGAC	CTTCACCTCCCTCATCCTCATC
Prkar1b	CCCTCTGACTACTTTGGGGAGA	TAACGCTGGATGTTCCTCTTCA
Prkar2b	AGAGAACGGAGCTGTGGAAATC	TGTTCCAAACAGCGCAACTAAT
Prkce	CCCCCTTCAAGCCGAGAAT	GTATTGGCTCTTCCCGCGTAA
Rev-Erbα	AGCTCAACTCCCTGGCACTTAC	CTTCTCGGAATGCATGTTGTTC
Scarb2	TCCTCTGTTGACTGTCGTGGAT	AGAAACACGTACTCCCCGTCAT
Slc25a13	CCTACAGGTTTGGACTGGGTTC	TAGCGGAGCACCTTCTTAAAGC
Sp1	TGAATGCTGCTCAACTCTCCTC	CACCAGATCCATGAAGACCAAG
Stard4	CTGGATGTTCTGGAGCACTTTG	TCTGGCTTGGACTGTCTTTGAG

2.2. Publication: “A role for adrenal glucocorticoids in the circadian resynchronization during jet lag”

Silke Kießling, Henrik Oster and Gregor Eichele

under review

Abstract

The term *Jet lag* comprises a range of psycho- and physiopathological symptoms that arise from temporal misalignment of the endogenous circadian clock with external time. Repeated jet lag exposure, encountered by business travelers and airline personnel as well as shift workers, has been correlated with immune deficiency, mood disorders, elevated cancer risk and anatomical anomalies of the forebrain. Here we characterized the molecular response of the murine circadian system in an established experimental paradigm for jet lag. Unexpectedly, strong heterogeneity of entrainment kinetics was found not only between different organs, but also within the molecular clockwork of each tissue. Manipulation of the adrenal circadian clock, in particular phase-shifting of adrenal glucocorticoid rhythms, regulates the speed of behavioral re-entrainment. This key role of adrenal glucocorticoid phasing for resetting of the circadian system provides a novel mechanism-based approach for the therapy of jet lag and jet lag-associated diseases.

Introduction

Jet lag comprises a set of physiological and psychological perturbations experienced when internal circadian rhythms and external time are out of synchrony, e.g. after traveling in a jet plane across multiple time zones (Arendt and Marks 1982; Comperatore and Krueger 1990). Jet lag is characterized by decreased alertness, nighttime insomnia, poor overall performance (Tapp and Natelson 1989), impaired cognitive skills (Cho, Ennaceur et al. 2000), loss of appetite, depressed mood, reduced psychomotor coordination and gastrointestinal disturbances (Waterhouse, Reilly et al. 2007). Severity and extent of these symptoms depend on the direction and speed of travel and the number of time zones crossed (Haimov and Arendt 1999; Waterhouse, Reilly et al. 2007; Srinivasan, Spence et al. 2008; Arendt 2009). Individuals exposed to chronic jet lag may experience accelerated malignant growth (Filipski, Delaunay et al. 2004) and temporal lobe atrophy combined with spatial cognitive deficits (Cho 2001). Rodents subjected to chronic jet lag suffer from cardiomyopathies (Penev, Kolker et al. 1998) and hastened death upon aging (Davidson, Sellix et al. 2006).

The pathogenesis of the jet lag syndrome resides in a perturbation of the endogenous circadian timing system. Circadian clocks are genetically controlled cellular oscillators which are driven by interlocked positive and negative transcriptional/translational feedback loops. Clock transcriptional activators CLOCK and BMAL1 turn on *Period* (*Per1*, *Per2, Per3*) and *Cryptochromes* (*Cry1* and *Cry2*) genes. PER and CRY proteins are negative regulators repressing CLOCK/BMAL1-mediated transactivation (Dunlap 1999; van der Horst, Muijtjens et al. 1999; Reppert and Weaver 2001). A second loop involves positive and negative regulation of *Bmal1* expression through ROR *Ror*α and REV-ERB *Rev-Erb*α, respectively (Preitner, Damiola et al. 2002). The transcription factor DBP regulates rhythmic activation of downstream target genes (Ripperger, Shearman et al. 2000), thereby serving as relay mediating the output of the circadian oscillator.

In addition to a master pacemaker of the hypothalamic suprachiasmatic nuclei (SCN) that synchronizes internal rhythms to the light/dark (LD) cycle, rhythmic expression of clock genes also occurs in many other tissues (Welsh, Yoo et al. 2004). These so called peripheral clocks are found in e.g. the cerebral cortex (Yan, Miyake et al. 2000; Abe, Honma et al. 2001), the retina (Tosini and Menaker 1996), the liver (Yamazaki, Numano et al. 2000), the kidney (Yoo, Yamazaki et al. 2004) and the pancreas (Damiola, Le Minh et al. 2000; Oishi, Fukui et al. 2000; Schibler, Ripperger et al. 2003; Liu, Cai et al. 2007). The SCN appears to synchronize peripheral oscillators through hormonal and neuronal pathways (Schibler, Ripperger et al. 2003; Perreau-Lenz, Pevet et al. 2004). Peripheral clocks translate clock time into physiologically meaningful signals via transcriptional regulation of clock controlled genes

(Panda, Antoch et al. 2002; Storch, Lipan et al. 2002). Therefore, the temporal disorganization of the circadian system during jet lag might disrupt the overall physiological coordination and, hence, be the cause of most jet lag-associated symptoms (Arendt 2009). Of particular relevance for the present work, adrenal glucocorticoids (GC), the release of which is controlled by the SCN and an adrenal clock (Ishida, Mutoh et al. 2005; Oster, Damerow et al. 2006; Son, Chung et al. 2008), show robust circadian rhythms in the blood and are able to synchronize peripheral oscillators both in vitro and in vivo (Balsalobre, Brown et al. 2000).

Yamazaki et al. have found that *Per1* expression rhythms re-entrain faster in the SCN than in any of the other organs examined (Yamazaki, Numano et al. 2000). Similar findings were reported by Davidson et al. for *Per2* (Davidson, Castanon-Cervantes et al. 2009). Reddy et al. compared re-entrainment of *Per1*, *Per2* and *Cry1* in the SCN in a rodent jet lag paradigm. They found a dissociation of *Per* and *Cry* gene expression rhythms and postulate that the slower *Cry* acts as a rate-limiting factor for behavioral adaptation (Reddy, Field et al. 2002). Collectively these findings suggest that the SCN pacemaker entrains peripheral oscillators throughout the body and that this adaptive phase control is transiently lost during jet lag.

It remains unclear if internal de-synchronization of clock gene rhythms during jet lag is preserved in peripheral organs and if this de-synchronization extends to other clock genes. Moreover, it remains to be determined which factors mediate re-entrainment of the peripheral clocks and how these clocks communicate during this process. We found that within a given tissue there was hierarchy of entrainment of clock genes, that this hierarchy was organotypic and that a main mediator of re-entrainment was the adrenal glucocorticoid corticosterone.

Results

Differential response of circadian clock gene expression in the SCN during jet lag.

Mice were entrained to a 12 h light: 12 h dark cycle (LD 12:12) and characteristically show running-wheel activity restricted to the dark phase (*Zeitgeber* time [ZT] 12 to 24) as expected for nocturnal animals. Advancing the LD cycle by 6 hrs which simulates eastward traveling, evoked a gradual adaptation of running-wheel activity to the changed light regimen. This transition was completed after 8-9 days (Figure 1A). We determined locomotor activity onsets before and after the shift. Onset resetting followed a sigmoid curve with a 50% phase shift (PS_{50}) reached at 4.0 ± 0.1 days (Figure 1B).

Because locomotor activity is driven by clock gene expression rhythms in the SCN (Stephan and Zucker 1972; Ralph, Foster et al. 1990; Sollars, Kimble et al. 1995; Sujino, Masumoto et al. 2003), adaptation to a new time zone should be reflected in corresponding changes in clock gene expression. Hence, we determined by *in situ* hybridization the jet lag-evoked change in the transcription profiles of five canonical clock genes (see Introduction). Results of densitometric quantifications of autoradiographs of coronal sections through the SCN are depicted in Figure 2A. Before the shift (day 0), transcripts of *Per1*, *Per2* and *Dbp* showed rhythmic expression with peak levels around the middle of the day (ZT 4-10) (Figure 2A, top row). *Bmal1* transcript levels were highest around ZT 16, while *Rev-Erbα mRNA* levels peaked around the night/day transition (ZT 0). 12 days after the shift, the transcript rhythms had fully adjusted to the new LD cycle (Figure 2A, bottom row). Circadian transcript profiles of the different clock genes at intermediary time points showed marked differences in adaptation characteristics. For example, *Per1* expression showed a transient peak broadening at ZT 2 to 10 at day 3, followed by a gradual adaptation to the pre-shift phasing by day 4 (Figure 2A). To quantify the kinetics of adaptation of clock gene expression, we modeled experimental *mRNA* profiles by sine wave regression using the Prism software (black curves in Figure 2A). The time point of peak expression for each gene and day was determined and plotted. To facilitate a direct comparison of clock gene expression resetting, peak times at days 3, 4 and 12 were plotted relative to peak time of day 0 (Figure 2B). Both, resetting of activity onsets (Figure 1B) and of peak expression followed sigmoid kinetics with full re-entrainment reached between day 4 (*Per2*) and day 8 (*Rev-Erbα*). PS_{50} values reveal a marked difference in adaptation kinetics amongst the clock genes (Figure 2C). Consistent with previous work, *Period* genes efficiently adapt to the new light schedule (Yamazaki, Numano et al. 2000). Importantly, other emblematic clock genes have a considerably slower rate of adaptation, more closely reflecting that seen with running-wheel activity (Figure 2C).

Peripheral oscillators vary in their rate of adjustment during jet lag.

In order to monitor adaptation of peripheral clocks, we analyzed clock gene expression in mice subjected to jet lag conditions. We prepared cDNA samples and tissue sections from somatosensory cortex, adrenal, liver, kidney and pancreas at day 0, 2, 3, 4, 8, and 12 following a 6 h advance of the LD cycle and quantified clock gene expression by combining data from quantitative real-time PCR (qPCR) and *in situ* hybridization. Expression maxima were determined by sine wave regression (Supplemental Figure 1). Bar graphs of Figure 3 A-E show PS_{50} values for all tissues examined. Somatosensory cortex and SCN show a similar pattern of clock gene adaptation (compare Figures 2C and 3A) and in both cases there was a rapid response of the two *Per* genes followed by a slower one for *Dbp*, *Bmal1* and *Rev-Erbα*. The fast rate of adjustment of *Per* genes was not as pronounced in non-neuronal tissues. *Dbp*, *Rev-Erbα* and *Per1, 2* peak time shifts of adrenal, kidney and liver were comparable (Figure 3B to D). The pancreas stands out because *Rev-Erbα* adjusts the fastest and *Per1* and *Per2* the slowest in this tissue (Figure 3E).

In summary, the clock gene expression rhythms in the SCN and in peripheral oscillators adapt to an advanced light schedule with different rates. Moreover, such adaptation varies among different organs. This misalignment in expression rhythms is thus a molecular hallmark of jet lag and a likely cause of its discomfort.

The adrenal clock regulates endocrine and behavioral re-entrainment during jet lag.

Adrenal GC release mediates clock resetting in the periphery (Balsalobre, Brown et al. 2000) and adrenalectomized rats show rapid phase shifting behavior (Sage, Ganem et al. 2004). We now show that the adrenal circadian clock and the SCN adapt to the new light schedule at comparable rate (compare Figures 2C and 3B). Together these findings raise the possibility that the adrenal clock contributes in a critical way to the regulation of jet lag adjustment. To examine this possibility, we investigated the role of the adrenal circadian oscillator in phase advance. First, we transplanted adrenals from clock deficient *Per2|Cry1* double mutant animals into adrenalectomized wild-type hosts (Oster, Damerow et al. 2006), creating adrenal clock-deficient mice, here referred to as $host^{WT}/adrenal^{Per2/Cry1}$ ($h^{WT}/a^{P2/C1}$). Animals were subjected to a 6 h phase advance and the re-entrainment of locomotor activity was compared to that of sham operated wild-type controls ($host^{WT}/adrenal^{WT}$, h^{WT}/a^{WT}). In control animals wheel-running activity took ~9 days to entrain to the new light dark cycle, whereas $h^{WT}/a^{P2/C1}$ mice adapted significantly faster, reaching full re-entrainment around day 7 (Figure 4A, B). On average, PS_{50} values of activity onsets were accelerated by 28% in animals lacking an adrenal clock (from 4.95 ± 0.07 to 3.55 ± 0.08 days).

The rhythmic secretion of GCs from the adrenal is regulated by both SCN and adrenal circadian clocks (Ishida, Mutoh et al. 2005; Oster, Damerow et al. 2006; Son, Chung et al. 2008). We therefore analyzed whether re-entrainment kinetics of adrenal corticosterone rhythms parallels those of behavioral re-entrainment in $h^{WT}/a^{P2/C1}$ animals. Corticosterone excretion rhythms were measured by collecting feces at 4 hrs intervals starting on the day prior to the phase shift (day 0) and ending on day 5. Additional 4 hrs interval samples were collected at day 12 post shift. Re-entrainment kinetics was assessed by determining the shift of the sine-fitted peak time of corticosterone concentration (Supplemental Figure 2). We found that the re-entrainment of hormonal rhythms was significantly accelerated in mice lacking an adrenal clock (Figure 4C). On average, the PS_{50} values were reduced by 36% in $h^{WT}/a^{P2/C1}$ animals, from 2.47 ± 0.17 days to 1.57± 0.10 days.

Irrespective of the genetic makeup of the adrenal, hormonal resetting preceded that of activity (compare Figures 4B and C). This becomes particularly obvious if one plots locomotor activity against hormonal resetting for transient days 1 to 5 for each animal (Figure 4D). This figure reveals a strong correlation between both parameters ($R^2 = 0.79$ h^{WT}/a^{WT}; $R^2 = 0.87$ $h^{WT}/a^{P2/C1}$). Almost all data points are located above the normal (y = x, dashed line), reiterating that corticosterone concentration rhythms shift more rapidly than locomotor behavior at all times.

In summary, the lack of an adrenal circadian clock accelerates phase-shifting of an important endocrine parameter and of locomotor activity. Since the GC phase shift precedes that of activity, it is possible that hormonal cues are causal for behavioral adaptation. If so, a manipulation of corticoid circadian rhythms might affect behavioral adaptation, as was suggested by work in adrenalectomized rats (41).

Glucocorticoid rhythms regulate re-entrainment of locomotor activity.

To manipulate endogenous GC circadian profiles in mice, we used the steroid 11β-hydroxylase inhibitor metyrapone (MET). MET was administered by two different methods (Figure 5B). One group of animals obtained MET during their active phase (night), the other during their rest phase (day). Treatment 1 was achieved by dissolving MET in the drinking water which is consumed by mice predominantly during the night (Edgar, Kilduff et al. 1991) (MET_d). An advantage of giving MET in the drinking water is avoidance of treatment-induced entrainment. Treatment 2 was achieved by daily intra-peritoneal injection of MET shortly after the onset of light (ZT 2-4) (MET_i). While such injection caused transient drowsiness of the mice, it had no effect on the onsets of wheel-running activity (Figure 5B, middle panel).

On day 0 (i.e. prior to the phase shift) relative to control animals, MET_i mice consistently showed a shift of the corticosterone maximal concentration from ZT 16 to ZT 23 (positive Δ ZT_{max}). By contrast, MET_d showed peak corticosterone concentration at ZT 14 (negative Δ ZT_{max} in Figure 5A). Of note, MET affected GC peak times, but does not significantly change the amplitude of GC excretion rhythms. Sample actograms (Figure 5B) emphasize that at the behavioral level MET_i mice re-entrained slower than non-treated mice, whereas MET_d animals showed an accelerated activity resetting (Figure 5B).

To compare the relationship between endocrine and behavioral parameters, we plotted for each individual mouse the maximal pre-jet lag shift (Δ ZT_{max} in Figure 5A) against the change in PS_{50} value of activity onsets (Figure 5C). This graph re-emphasizes that MET_i treatment forward shifts corticosterone peak concentration for up to 6 hrs, and leads to a slower re-entrainment (data points in top right quadrant). By contrast, MET_d back-shifts the corticosterone peak concentration time for up to 3.5 hrs, and similarly accelerates behavioral re-entrainment (data points in bottom left quadrant). Our data thus indicate that GC phasing influences behavioral re-entrainment in response to jet lag.

Discussion

Jet lag, also referred to as "desynchronosis" (Comperatore and Krueger 1990), arises from a transient misalignment of the endogenous circadian timing system with external time. We have determined organ-specific expression profiles of key circadian clock genes to molecularly characterize circadian re-synchronization during jet lag. Each of the organs and clock genes examined shows a characteristic time course of adjusting gene expression from the pre- to the post-jet lag state (Figure 6). Therefore, jet lag evokes a global de-synchronization of clock gene expression rhythms that gradually returns to the robust clockwork typical for the normal rhythm of the circadian pacemaker. We show that during this process the circadian clock of the adrenal has a special role in that adrenal clock-controlled glucocorticoids regulate the re-entrainment of locomotor activity rhythms. By timed application of the steroid 11β-hydroxylase inhibitor metyrapone, the phase of the endogenous GC rhythm was shifted which, in turn, evoked a predictable change in behavioral re-entrainment. Our study thus not only substantiates the importance of GC rhythms in jet lag adaptation, but also establishes an informative experimental animal model to explore the treatment of jet lag and its associated symptoms.

Cellular and system wide de-synchronization of circadian clocks during jet lag.

To date, surprisingly little is known about the molecular processes underlying re-synchronization of internal and external rhythms during jet lag. Pioneering studies with a *Per*-driven luciferase reporter have provided inroads to understanding the mechanism of jet lag, and propose that overall clock resetting is initiated at the level of the SCN (Yamazaki, Numano et al. 2000), with rapid re-entrainment of *Per* gene rhythms followed by that of C*ryptochromes* (Reddy, Field et al. 2002). Another dimension of perturbation was shown within the SCN clock, where cells can be separated in ventral and dorsal regions with different resetting kinetics during the period of desynchrony (Davidson, Castanon-Cervantes et al. 2009). Our data now show that in the SCN and in peripheral oscillators, the process of jet lag is characterized by a marked heterogeneity of phase resetting of clock genes that operate in the positive and negative branches of the clock. The consequence is a transient misalignment of the transcriptional feedback loops driving the circadian molecular clock which results in deregulation of tissue-specific oscillators (Panda, Antoch et al. 2002; Storch, Lipan et al. 2002). This global organ specific "uncoupling" provides a mechanistic rationale for a wide spectrum of jet lag-associated physiological effects.

The order of clock gene resetting varied considerably among different organs (Figure 6), indicating tissue-specific pathways of re-entrainment. We found that the pancreas showed

slow resetting kinetics of most circadian clock genes. This agrees with the finding that this organ has an exceptionally robust pacemaker (Peschke and Peschke 1998; Muhlbauer, Wolgast et al. 2004). Unlike in the SCN, where light-regulated *Per* genes reset first, in the pancreas, *Rev-Erbα* adapts fastest. Several studies have characterized the nuclear orphan receptor *Rev-Erbα* as a link between metabolic and circadian regulation (Ramakrishnan and Muscat 2006; Yin, Wang et al. 2006; Raghuram, Stayrook et al. 2007; Meng, McMaster et al. 2008) and hence, *Rev-Erbα* may be a metabolic sensor impinging on the circadian clock in the pancreas.

The adrenal clock regulates re-entrainment during jet lag.

We and others have shown that the adrenal clock gates the response of the steroidogenic machinery to adrenocorticotropic hormone and thereby influences the rhythm of GC secretion into the blood (Oster, Damerow et al. 2006; Son, Chung et al. 2008). Together with the capability of GCs in the resetting of peripheral oscillators (Balsalobre, Brown et al. 2000) these findings suggest a critical role for the adrenal clock in the overall resetting process (Le Minh, Damiola et al. 2001; Schibler, Ripperger et al. 2003). Here we show that adrenal clock genes display a rapid entrainment response, comparable to that of the SCN. This likely reflects a direct photic input to the adrenal via the autonomic nervous system (Ishida, Mutoh et al. 2005; Oster, Damerow et al. 2006). Moreover, our behavioral data demonstrate a key regulatory function of the adrenal circadian clock in behavioral re-entrainment during jet lag. Given this function of adrenal GCs as a candidate Zeitgeber (Balsalobre, Brown et al. 2000), the accelerated behavioral and hormonal resetting observed in adrenal clock-deficient mice might be the consequence of a disrupted adrenal feedback on the SCN (Stephan and Zucker 1972; Ralph, Foster et al. 1990; Sollars, Kimble et al. 1995; Sujino, Masumoto et al. 2003), mediated by the release of GCs. This is reminiscent of the dysregulation of GC feedback on the CNS, suspected to be a critical force underlying neurophysiologic disorders, such as major depression (Mizoguchi, Ikeda et al. 2008). Intriguingly, depression-associated symptoms are hallmarks of the jet lag syndrome (Arendt and Marks 1982).

Although GCs mediate various timing signals, the role they have in the case of jet lag differs from what is seen e.g. in a food restriction paradigm, in which case the SCN clock is not involved (Damiola, Le Minh et al. 2000). We are left with the question of how GCs affect behavioral rhythms in the jet lag paradigm. Because SCN neurons apparently do not express GC receptor (Rosenfeld, Van Eekelen et al. 1988; Balsalobre, Brown et al. 2000) GCs may target other brain regions which in turn relay to the SCN. High levels of GC receptors are

found in the cerebral cortex, the hypothalamic arcuate nucleus and the hippocampus (Gofflot, Chartoire et al. 2007).

Glucocorticoid rhythm phasing regulates re-entrainment during jet lag.

Common strategies to alleviate jet lag syndrome aim at adjusting the body clock to the new time zone prior to travel (Waterhouse, Reilly et al. 2007). Most treatments are based on pre-flight plans including long term light conditioning, sometimes in combination with timed melatonin administration (Arendt 2009). Earlier studies have addressed GC function in re-entrainment during jet lag in nocturnal as well as diurnal animals (Sage, Ganem et al. 2004; Mohawk, Cashen et al. 2005), focusing on the regulation of the amplitude of diurnal GC secretion. We show, to our knowledge, for the first time that the phasing of adrenal GC release is a key regulator of physiological and behavioral re-entrainment during jet lag. Normally the adrenal clock serves as a stabilizer of SCN-controlled circadian rhythms. In the case of jet lag, this stabilizing influence becomes a liability, preventing a rapid adaptation to the new time zone. This view is supported by our observation that incapacitating the adrenal clock results in a faster re-entrainment.

Chronopharmacological manipulation of GC phase prior to jet lag by timed administration of MET is sufficient to substantially either accelerate or slow down behavioral re-entrainment and, thus, prolong or shorten the duration of jet lag. In another context, GC administration was shown to promote hippocampal synaptic plasticity when high levels of the hormone and stimulation coincide in time (Wiegert, Joels et al. 2006), while suppressing it when given a few hrs before stimulation (Alfarez, Wiegert et al. 2002; Pu, Krugers et al. 2007). Both cases provide good examples for the clinical importance of timing in the design of therapeutic strategies. It has recently been shown that sildenafil enhances circadian responses to light and accelerates re-entrainment after phase advances of the light-dark cycle (Agostino, Plano et al. 2007). It will be important to examine whether this phosphodiesterase inhibitor also acts in a phase-sensitive fashion.

Considering the adverse side effects of complete GC suppression or GC rhythm dampening (Sage, Ganem et al. 2004), re-phasing of physiological GC rhythms by timed inhibition of GC synthesis is a practical treatment that could be tested in humans. In addition, the mouse model presented in our study offers a strategy for screening for new chronopharmacological agents that alleviate the various symptoms of jet lag affecting a large number of travelers and shift workers each day (Wittmann, Dinich et al. 2006).

Methods

Animals. For all experiments male wild-type (C57BL/6J-129/SvHsD mixed background) and homozygous *Per2/Cry1* double mutant mice (*Per2*^{tm1Brd} (Zheng, Larkin et al. 1999) and *Cry1*^{tm1Jhjh} (van der Horst, Muijtjens et al. 1999)) of two to three months of age were used as described (Oster, Yasui et al. 2002). All animal experiments were done with prior permission from the Office of Consumer Protection and Food Safety of the State of Lower Saxony and in accordance with the German Law of Animal Welfare. Mice were housed in small groups of ≤ 5 animals under 12 h light: 12 h dark (LD) conditions with food and water *ad libitum*.

Behavioral analysis. Handling and activity measurements during experiments were performed as described (Jud, Schmutz et al. 2005). Wheel-running activity was analyzed using the ClockLab software (Actimetrics). For the jet lag experiment (a 6 hrs rapid advance of the LD cycle), animals were single-housed in running-wheel equipped cages ≥ 2 weeks in LD ("lights on" – *Zeitgeber* time ZT 0; light intensities of 350 Lux). On day one of the jet lag, the "lights off" time (ZT 12) was shifted from 6:00 pm to 12 am. Animals synchronized to the new light regimen for another two weeks during which time wheel-running activity was recorded. Individual activity onsets before and after the shift were determined by visual inspection and averaged over the whole cohort to asses re-entrainment rates.

Quantitative real-time PCR (qPCR). Animals were sacrificed at the indicated time points by cervical dislocation before (day 0) and at four different days after the phase advance of the LD cycle (day 2, 3, 4 and 8). Eyes were removed prior to tissue dissection under a 15 W red safety light at time points falling into the dark phase (Albrecht, Sun et al. 1997). Tissue samples were dissected and stored frozen in RNAlater (Ambion). Total RNA samples from adrenal, kidney, liver and pancreas were prepared using RNeasy Micro and Mini Kits (Quiagen). cDNA was synthesized using Thermoscript RT Kit (Invitrogen). qPCR was performed on an iCycler thermocycler (Bio-Rad) with iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer´s protocol. Primer sequences and cycle conditions are detailed in (Oster, Damerow et al. 2006; Oster, Damerow et al. 2006). *Ef1α* was used as standard and single well amplification efficiency estimates and relative quantification of expression levels were performed as described (Ramakers, Ruijter et al. 2003).

In situ hybridization (ISH). Animals were sacrificed and brains, kidneys and adrenals dissected. Tissues were fixed, dehydrated, paraffin embedded, 8 µm sections were prepared (Jakubcakova, Oster et al. 2007) that were stored at – 80 ° C. Sections were hybridized with ^{35}S-UTP labeled antisense RNA probes for clock gene transcripts (Oster, Damerow et al. 2006). Relative quantification of expression levels was performed by densitometric analysis

of autoradiograph films using the Scion Image program (Scion Corporation) (Oster, Yasui et al. 2002).

Adrenal transplantations. Transplantations of adrenal fragments were performed as described (Musholt, Klebs et al. 2002; Oster, Damerow et al. 2006). Briefly, male wild-type and *Per2/Cry1* double mutant mice at the age of 6-8 weeks were anaesthetized by i.p. injection of 100/10 mg/kg Ketamin/Rompun. Adrenals were dissected from mutant animals following medial laparotomy and transplanted underneath the capsule of the kidney of an adrenalectomized wild-type host animal. Both adrenals were transplanted into a single host. To control for the transplantation procedure, wild-type animals received their own wild-type adrenal transplants following adrenal-ectomy. After the surgery, animals recovered for eight weeks under standard LD conditions to ensure complete re-innervations of the transplanted tissues (Ulrich-Lai and Engeland 2000).

Hormone measurements. Fecal samples were collected at 4 hrs intervals before and following the jet lag treatment using wheel cages equipped with wire grid floors. To rule out stress induced effects, animals were transferred to the collection cages 3 days prior to the first sampling interval. Fecal samples were stored at -80 ° C. Corticoid metabolite extraction and quantification by radio immune assay (RIA MP Biomedical) were performed as described (Abraham, Dallmann et al. 2006).

Pharmacological treatments. Administration of metyrapone (MET, Alliance UK) to wild-type mice was done for 16 days prior to the jet lag procedure (see above). One group of animals (MET_i) was injected i.p. at ZT 2-4 with MET dissolved in water (200 mg/kg body weight per day). Injections were stopped one day before the LD shift (day -1). A second group of animals (MET_d) received MET with their drinking water for 16 days before and during the whole jet lag period. Four groups of 3 to 4 MET_d animals each received different MET dosages (0.6g/l, 1.0g/l, 1.4g/l and 1.8g/l per day). At day 16 of the treatment (that is one day before the jet lag) and at day four thereafter fecal samples were collected to analyze the phase shift of corticoid excretion rhythms. Untreated wild-type littermates were used as controls for all experiments.

Data analysis and statistics. All data analyses were performed using the Prism software (GraphPad). To test for diurnal variation and rhythm peak time points a sine wave equation

$$y = BaseLine + Amplitude \times \sin\left(Frequency \times x + PhaseShift\right)$$

was fitted to the data. For gene expression and corticosterone data frequency was fixed to 24 hrs. Maxima were calculated using the axis section of the second derivative. To determine the resetting half point (PS_{50}) a sigmoid dose-response curve with variable slope was fitted to the sine wave maxima (corticosterone) or onset time points (locomotor activity) for each group

$$y = Bottom + (Top - Bottom) \div (1 + 10 \wedge ((\log EC\ 50 - x) \times HillSlope))$$

To test if the best-fit PS_{50} values differ between data sets, data were compared by extra sum-of-squares F test using p values of less than 0.05 as a threshold. Maxima of gene expression or corticosterone at specific days were compared by Mann-Whitney rank sum test even if normality tests and equal variance tests were positive, reflecting the small sample sizes (3 to 9). A statistically significant difference was assumed with p values of less than 0.05. Correlation between phase-shift activity onsets and corticosterone maxima as well as correlation between PS_{50} values of activity onset and hormone maxima shifts were performed using linear regression. The quality of fit was estimated by R^2-determination. Departure from linearity was tested with runs test. Normality and homoscedasticity tests were passed for all data sets.

References

1. Arendt, J., and Marks, V. 1982. Physiological changes underlying jet lag. *Br Med J (Clin Res Ed)* 284:144-146.
2. Comperatore, C.A., and Krueger, G.P. 1990. Circadian rhythm desynchronosis, jet lag, shift lag, and coping strategies. *Occup Med* 5:323-341.
3. Tapp, W.N., and Natelson, B.H. 1989. Circadian rhythms and patterns of performance before and after simulated jet lag. *Am J Physiol* 257:R796-803.
4. Cho, K., Ennaceur, A., Cole, J.C., and Suh, C.K. 2000. Chronic jet lag produces cognitive deficits. *J Neurosci* 20:RC66.
5. Waterhouse, J., Reilly, T., Atkinson, G., and Edwards, B. 2007. Jet lag: trends and coping strategies. *Lancet* 369:1117-1129.
6. Arendt, J. 2009. Managing jet lag: Some of the problems and possible new solutions. *Sleep Med Rev* 13:249-256.
7. Haimov, I., and Arendt, J. 1999. The prevention and treatment of jet lag. *Sleep Med Rev* 3:229-240.
8. Srinivasan, V., Spence, D.W., Pandi-Perumal, S.R., Trakht, I., and Cardinali, D.P. 2008. Jet lag: therapeutic use of melatonin and possible application of melatonin analogs. *Travel Med Infect Dis* 6:17-28.
9. Filipski, E., Delaunay, F., King, V.M., Wu, M.W., Claustrat, B., Grechez-Cassiau, A., Guettier, C., Hastings, M.H., and Francis, L. 2004. Effects of chronic jet lag on tumor progression in mice. *Cancer Res* 64:7879-7885.
10. Cho, K. 2001. Chronic 'jet lag' produces temporal lobe atrophy and spatial cognitive deficits. *Nat Neurosci* 4:567-568.
11. Penev, P.D., Kolker, D.E., Zee, P.C., and Turek, F.W. 1998. Chronic circadian desynchronization decreases the survival of animals with cardiomyopathic heart disease. *Am J Physiol* 275:H2334-2337.
12. Davidson, A.J., Sellix, M.T., Daniel, J., Yamazaki, S., Menaker, M., and Block, G.D. 2006. Chronic jet-lag increases mortality in aged mice. *Curr Biol* 16:R914-916.
13. Dunlap, J.C. 1999. Molecular bases for circadian clocks. *Cell* 96:271-290.
14. Reppert, S.M., and Weaver, D.R. 2001. Molecular analysis of mammalian circadian rhythms. *Annu Rev Physiol* 63:647-676.
15. van der Horst, G.T., Muijtjens, M., Kobayashi, K., Takano, R., Kanno, S., Takao, M., de Wit, J., Verkerk, A., Eker, A.P., van Leenen, D., et al. 1999. Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. *Nature* 398:627-630.
16. Preitner, N., Damiola, F., Lopez-Molina, L., Zakany, J., Duboule, D., Albrecht, U., and Schibler, U. 2002. The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell* 110:251-260.
17. Ripperger, J.A., Shearman, L.P., Reppert, S.M., and Schibler, U. 2000. CLOCK, an essential pacemaker component, controls expression of the circadian transcription factor DBP. *Genes Dev* 14:679-689.
18. Welsh, D.K., Yoo, S.H., Liu, A.C., Takahashi, J.S., and Kay, S.A. 2004. Bioluminescence imaging of individual fibroblasts reveals persistent, independently phased circadian rhythms of clock gene expression. *Curr Biol* 14:2289-2295.
19. Yan, L., Miyake, S., and Okamura, H. 2000. Distribution and circadian expression of dbp in SCN and extra-SCN areas in the mouse brain. *J Neurosci Res* 59:291-295.
20. Abe, H., Honma, S., Namihira, M., Masubuchi, S., Ikeda, M., Ebihara, S., and Honma, K. 2001. Clock gene expressions in the suprachiasmatic nucleus and other areas of the brain during rhythm splitting in CS mice. *Brain Res Mol Brain Res* 87:92-99.
21. Tosini, G., and Menaker, M. 1996. Circadian rhythms in cultured mammalian retina. *Science* 272:419-421.

22. Yamazaki, S., Numano, R., Abe, M., Hida, A., Takahashi, R., Ueda, M., Block, G.D., Sakaki, Y., Menaker, M., and Tei, H. 2000. Resetting central and peripheral circadian oscillators in transgenic rats. *Science* 288:682-685.
23. Yoo, S.H., Yamazaki, S., Lowrey, P.L., Shimomura, K., Ko, C.H., Buhr, E.D., Siepka, S.M., Hong, H.K., Oh, W.J., Yoo, O.J., et al. 2004. PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. *Proc Natl Acad Sci U S A* 101:5339-5346.
24. Damiola, F., Le Minh, N., Preitner, N., Kornmann, B., Fleury-Olela, F., and Schibler, U. 2000. Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes Dev* 14:2950-2961.
25. Liu, S., Cai, Y., Sothern, R.B., Guan, Y., and Chan, P. 2007. Chronobiological analysis of circadian patterns in transcription of seven key clock genes in six peripheral tissues in mice. *Chronobiol Int* 24:793-820.
26. Oishi, K., Fukui, H., and Ishida, N. 2000. Rhythmic expression of BMAL1 mRNA is altered in Clock mutant mice: differential regulation in the suprachiasmatic nucleus and peripheral tissues. *Biochem Biophys Res Commun* 268:164-171.
27. Schibler, U., Ripperger, J., and Brown, S.A. 2003. Peripheral circadian oscillators in mammals: time and food. *J Biol Rhythms* 18:250-260.
28. Perreau-Lenz, S., Pevet, P., Buijs, R.M., and Kalsbeek, A. 2004. The biological clock: the bodyguard of temporal homeostasis. *Chronobiol Int* 21:1-25.
29. Storch, K.F., Lipan, O., Leykin, I., Viswanathan, N., Davis, F.C., Wong, W.H., and Weitz, C.J. 2002. Extensive and divergent circadian gene expression in liver and heart. *Nature* 417:78-83.
30. Panda, S., Antoch, M.P., Miller, B.H., Su, A.I., Schook, A.B., Straume, M., Schultz, P.G., Kay, S.A., Takahashi, J.S., and Hogenesch, J.B. 2002. Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell* 109:307-320.
31. Son, G.H., Chung, S., Choe, H.K., Kim, H.D., Baik, S.M., Lee, H., Lee, H.W., Choi, S., Sun, W., Kim, H., et al. 2008. Adrenal peripheral clock controls the autonomous circadian rhythm of glucocorticoid by causing rhythmic steroid production. *Proc Natl Acad Sci U S A* 105:20970-20975.
32. Oster, H., Damerow, S., Kiessling, S., Jakubcakova, V., Abraham, D., Tian, J., Hoffmann, M.W., and Eichele, G. 2006. The circadian rhythm of glucocorticoids is regulated by a gating mechanism residing in the adrenal cortical clock. *Cell Metab* 4:163-173.
33. Ishida, A., Mutoh, T., Ueyama, T., Bando, H., Masubuchi, S., Nakahara, D., Tsujimoto, G., and Okamura, H. 2005. Light activates the adrenal gland: timing of gene expression and glucocorticoid release. *Cell Metab* 2:297-307.
34. Balsalobre, A., Brown, S.A., Marcacci, L., Tronche, F., Kellendonk, C., Reichardt, H.M., Schutz, G., and Schibler, U. 2000. Resetting of circadian time in peripheral tissues by glucocorticoid signaling. *Science* 289:2344-2347.
35. Davidson, A.J., Castanon-Cervantes, O., Leise, T.L., Molyneux, P.C., and Harrington, M.E. 2009. Visualizing jet lag in the mouse suprachiasmatic nucleus and peripheral circadian timing system. *Eur J Neurosci* 29:171-180.
36. Reddy, A.B., Field, M.D., Maywood, E.S., and Hastings, M.H. 2002. Differential resynchronisation of circadian clock gene expression within the suprachiasmatic nuclei of mice subjected to experimental jet lag. *J Neurosci* 22:7326-7330.
37. Stephan, F.K., and Zucker, I. 1972. Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions. *Proc Natl Acad Sci U S A* 69:1583-1586.
38. Sujino, M., Masumoto, K.H., Yamaguchi, S., van der Horst, G.T., Okamura, H., and Inouye, S.T. 2003. Suprachiasmatic nucleus grafts restore circadian behavioral rhythms of genetically arrhythmic mice. *Curr Biol* 13:664-668.

39. Ralph, M.R., Foster, R.G., Davis, F.C., and Menaker, M. 1990. Transplanted suprachiasmatic nucleus determines circadian period. *Science* 247:975-978.
40. Sollars, P.J., Kimble, D.P., and Pickard, G.E. 1995. Restoration of circadian behavior by anterior hypothalamic heterografts. *J Neurosci* 15:2109-2122.
41. Sage, D., Ganem, J., Guillaumond, F., Laforge-Anglade, G., Francois-Bellan, A.M., Bosler, O., and Becquet, D. 2004. Influence of the corticosterone rhythm on photic entrainment of locomotor activity in rats. *J Biol Rhythms* 19:144-156.
42. Edgar, D.M., Kilduff, T.S., Martin, C.E., and Dement, W.C. 1991. Influence of running wheel activity on free-running sleep/wake and drinking circadian rhythms in mice. *Physiol Behav* 50:373-378.
43. Muhlbauer, E., Wolgast, S., Finckh, U., Peschke, D., and Peschke, E. 2004. Indication of circadian oscillations in the rat pancreas. *FEBS Lett* 564:91-96.
44. Peschke, E., and Peschke, D. 1998. Evidence for a circadian rhythm of insulin release from perifused rat pancreatic islets. *Diabetologia* 41:1085-1092.
45. Raghuram, S., Stayrook, K.R., Huang, P., Rogers, P.M., Nosie, A.K., McClure, D.B., Burris, L.L., Khorasanizadeh, S., Burris, T.P., and Rastinejad, F. 2007. Identification of heme as the ligand for the orphan nuclear receptors REV-ERBalpha and REV-ERBbeta. *Nat Struct Mol Biol* 14:1207-1213.
46. Yin, L., Wang, J., Klein, P.S., and Lazar, M.A. 2006. Nuclear receptor Rev-erbalpha is a critical lithium-sensitive component of the circadian clock. *Science* 311:1002-1005.
47. Meng, Q.J., McMaster, A., Beesley, S., Lu, W.Q., Gibbs, J., Parks, D., Collins, J., Farrow, S., Donn, R., Ray, D., et al. 2008. Ligand modulation of REV-ERBalpha function resets the peripheral circadian clock in a phasic manner. *J Cell Sci* 121:3629-3635.
48. Ramakrishnan, S.N., and Muscat, G.E. 2006. The orphan Rev-erb nuclear receptors: a link between metabolism, circadian rhythm and inflammation? *Nucl Recept Signal* 4:e009.
49. Le Minh, N., Damiola, F., Tronche, F., Schutz, G., and Schibler, U. 2001. Glucocorticoid hormones inhibit food-induced phase-shifting of peripheral circadian oscillators. *EMBO J* 20:7128-7136.
50. Mizoguchi, K., Ikeda, R., Shoji, H., Tanaka, Y., and Tabira, T. 2008. Suppression of glucocorticoid secretion induces a behaviorally depressive state in rotarod performance in rat. *Pharmacol Biochem Behav* 90:730-734.
51. Rosenfeld, P., Van Eekelen, J.A., Levine, S., and De Kloet, E.R. 1988. Ontogeny of the type 2 glucocorticoid receptor in discrete rat brain regions: an immunocytochemical study. *Brain Res* 470:119-127.
52. Gofflot, F., Chartoire, N., Vasseur, L., Heikkinen, S., Dembele, D., Le Merrer, J., and Auwerx, J. 2007. Systematic gene expression mapping clusters nuclear receptors according to their function in the brain. *Cell* 131:405-418.
53. Mohawk, J.A., Cashen, K., and Lee, T.M. 2005. Inhibiting cortisol response accelerates recovery from a photic phase shift. *Am J Physiol Regul Integr Comp Physiol* 288:R221-228.
54. Wiegert, O., Joels, M., and Krugers, H. 2006. Timing is essential for rapid effects of corticosterone on synaptic potentiation in the mouse hippocampus. *Learn Mem* 13:110-113.
55. Alfarez, D.N., Wiegert, O., Joels, M., and Krugers, H.J. 2002. Corticosterone and stress reduce synaptic potentiation in mouse hippocampal slices with mild stimulation. *Neuroscience* 115:1119-1126.
56. Pu, Z., Krugers, H.J., and Joels, M. 2007. Corticosterone time-dependently modulates beta-adrenergic effects on long-term potentiation in the hippocampal dentate gyrus. *Learn Mem* 14:359-367.

57. Agostino, P.V., Plano, S.A., and Golombek, D.A. 2007. Sildenafil accelerates reentrainment of circadian rhythms after advancing light schedules. *Proc Natl Acad Sci U S A* 104:9834-9839.
58. Wittmann, M., Dinich, J., Merrow, M., and Roenneberg, T. 2006. Social jetlag: misalignment of biological and social time. *Chronobiol Int* 23:497-509.
59. Zheng, B., Larkin, D.W., Albrecht, U., Sun, Z.S., Sage, M., Eichele, G., Lee, C.C., and Bradley, A. 1999. The mPer2 gene encodes a functional component of the mammalian circadian clock. *Nature* 400:169-173.
60. Oster, H., Yasui, A., van der Horst, G.T., and Albrecht, U. 2002. Disruption of mCry2 restores circadian rhythmicity in mPer2 mutant mice. *Genes Dev* 16:2633-2638.
61. Jud, C., Schmutz, I., Hampp, G., Oster, H., and Albrecht, U. 2005. A guideline for analyzing circadian wheel-running behavior in rodents under different lighting conditions. *Biol Proced Online* 7:101-116.
62. Albrecht, U., Sun, Z.S., Eichele, G., and Lee, C.C. 1997. A differential response of two putative mammalian circadian regulators, mper1 and mper2, to light. *Cell* 91:1055-1064.
63. Oster, H., Damerow, S., Hut, R.A., and Eichele, G. 2006. Transcriptional profiling in the adrenal gland reveals circadian regulation of hormone biosynthesis genes and nucleosome assembly genes. *J Biol Rhythms* 21:350-361.
64. Ramakers, C., Ruijter, J.M., Deprez, R.H., and Moorman, A.F. 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci Lett* 339:62-66.
65. Jakubcakova, V., Oster, H., Tamanini, F., Cadenas, C., Leitges, M., van der Horst, G.T., and Eichele, G. 2007. Light entrainment of the mammalian circadian clock by a PRKCA-dependent posttranslational mechanism. *Neuron* 54:831-843.
66. Musholt, T.J., Klebs, S.H., Musholt, P.B., Ellerkamp, V., Klempnauer, J., and Hoffmann, M.W. 2002. Transplantation of adrenal tissue fragments in a murine model: functional capacities of syngeneic and allogeneic grafts. *World J Surg* 26:950-957.
67. Ulrich-Lai, Y.M., and Engeland, W.C. 2000. Rat adrenal transplants are reinnervated: an invalid model of denervated adrenal cortical tissue. *J Neuroendocrinol* 12:881-893.
68. Abraham, D., Dallmann, R., Steinlechner, S., Albrecht, U., Eichele, G., and Oster, H. 2006. Restoration of circadian rhythmicity in circadian clock-deficient mice in constant light. *J Biol Rhythms* 21:169-176.

Acknowledgements

This work was supported by the Max Planck Society. H.O. is an Emmy Noether fellow of the DFG.

Figures

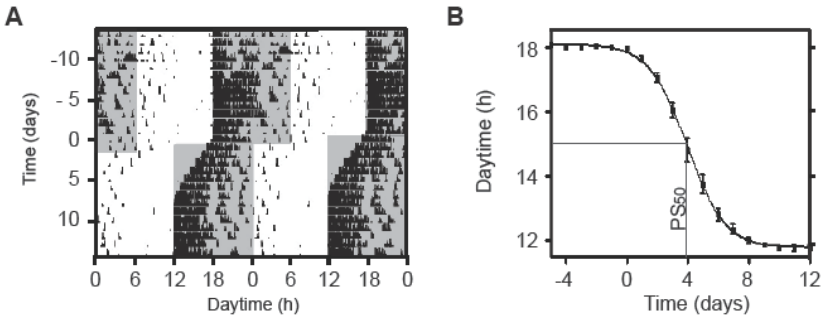


Figure 1. *Behavioral entrainment during jet lag.*

A) Representative double-plot actogram of a wild type mouse before and after a 6 h phase advance applied at day 1. Vertical bars represent wheel-running activity, white and gray shadings depict lighting conditions. **B)** Average onsets of wild type mice (n = 9) during jet lag. The PS_{50} value is defined as the time at which half of the phase shift was completed (4.0 ± 0.1 days). All values are average ± SEM.

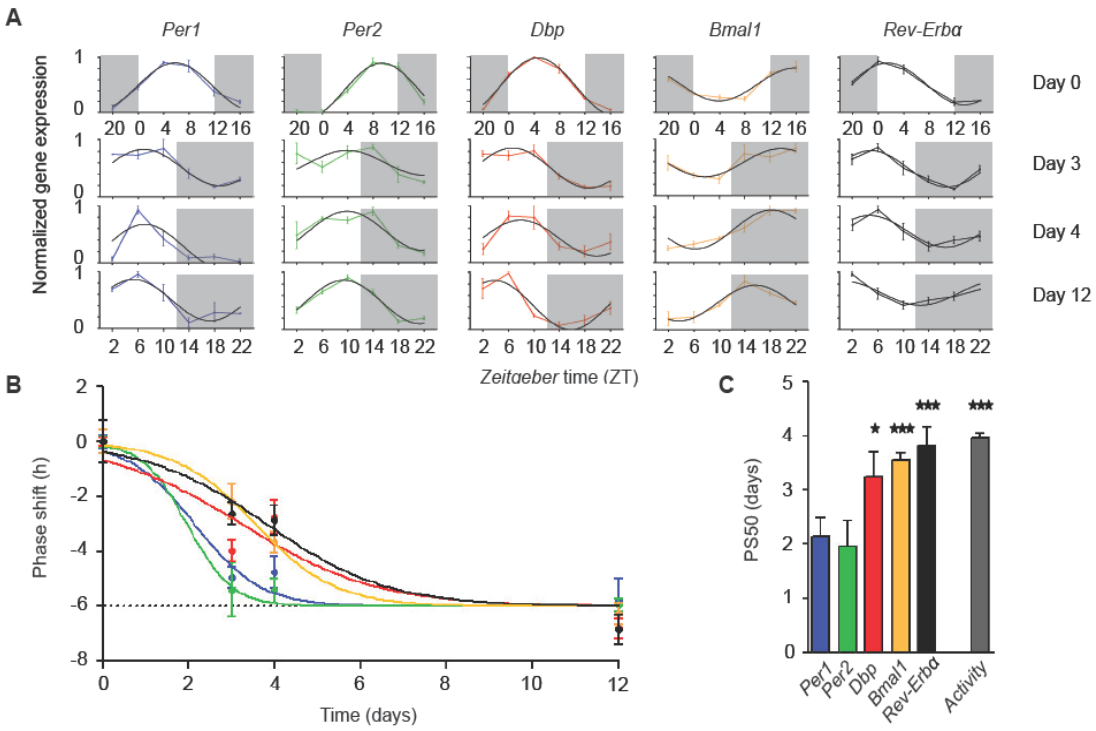


Figure 2. *Resetting of clock genes during jet lag in the SCN.*

A) Diurnal *mRNA* profiles (average ± SEM) of 5 different clock genes (in color) at days 0, 3, 4 and 12 after the LD shift superimposed with the fitted sine-waves (black). Light and dark phases are marked by white or grey shading. 3 animals were used per time point. **B)** Shifts of gene expression peak times obtained from the ISH data in Figure 2A show that adaptation to the new light schedule varies for the five genes. Color code is the same as in C. **C)** PS_{50} values (average ± SEM) of clock genes in the SCN (from Figure 2B) and PS_{50} of activity (from Figure 1B) differ. The significance of differences between PS_{50} values tested against *Per2* are indicated by asterisks (* $p \leq 0.05$; *** $p < 0.001$).

PS_{50}values were: 2.1 ± 0.5 days (*Per1*), 2.0 ± 0.8 days (*Per2*), 3.2 ± 0.5 days (*Dbp*), 3.6 ± 0.2 days (*Bmal1*), 3.8 ± 0.5 (*Rev-Erbα*). Both *Per* peak expression times shifted similarly fast ($p = 0.8217$ $F = 0.05212$ [1, 20] *Per1* vs. *Per2*), and significantly faster than the other three clock genes ($p = 0.0457$, $F = 4.488$ [1, 20] *Dbp* vs. *Per1*; $p = 0.0005$, $F = 16.60$ [1, 20] *Bmal1* vs. *Per1*; $p = 0.0014$, $F = 13.25$ [1, 20] *Rev-Erbα* vs. *Per1*; $p = 0.0130$, $F = 7.304$ [1, 20] *Dbp* vs. *Per2*; $p < 0.0001$ $F = 24.10$ [1, 20] *Bmal1* vs. *Per2*; p = 0,0003, $F = 18.72$ [1, 20] *Rev-Erbα* vs. *Per2*). Both *Per* genes also shifted faster than activity ($p = 0.0001$, 15.87 [1, 170] vs. *Per1*; $p < 0.0001$ $F = 26.16$ [1, 170] activity onset vs. *Per2*. *Dbp*, *Bmal1* and *Rev-Erbα* showed delayed adaptation resembling that seen with activity ($p = 0.3222$, $F = 0.9856$ [1, 170] vs. *Dbp*; $p = 0.4494$ $F = 0.5747$ [1, 170] vs. *Bmal1*; $p = 0.9129$ $F = 0.01200$ [1, 170] activity onset vs. *Rev-Erbα*).

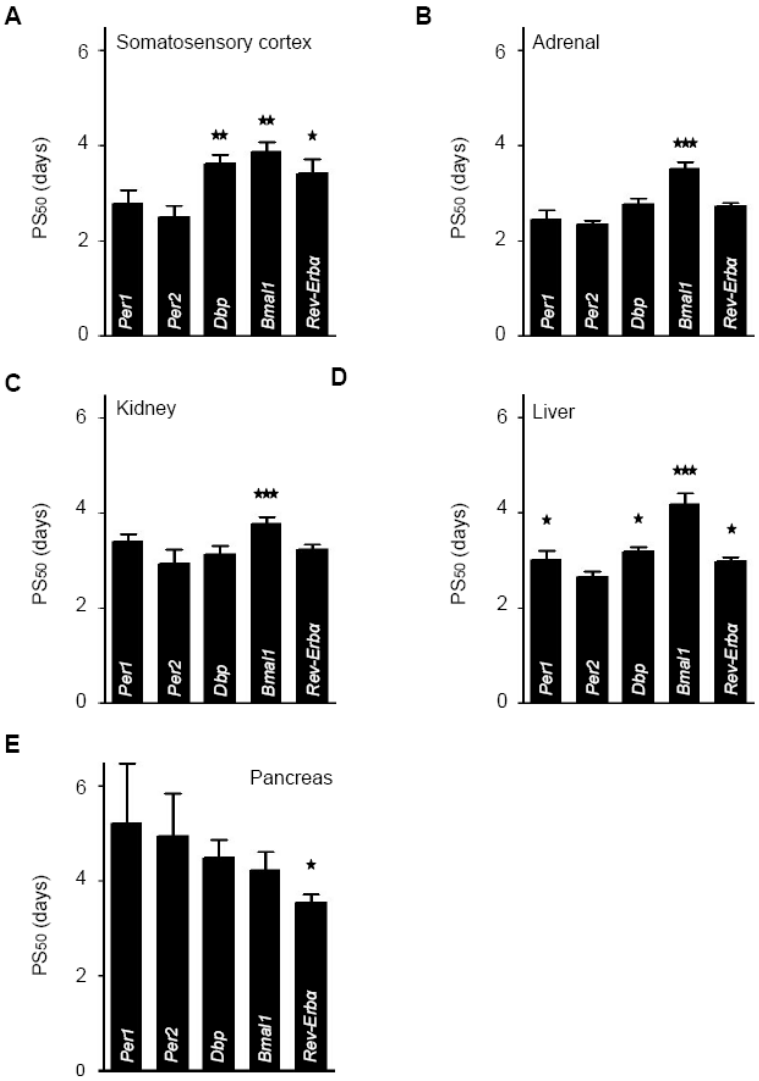


Figure 3. *Clock gene resetting kinetics in several peripheral tissues following a 6 hrs LD phase advance.*

Resetting is represented by PS_{50} values (averages ± SEM). Significant differences between PS_{50} values of genes compared with *Per2* are indicated by asterisks above the respective column (* $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$). Evaluation of PS_{50} values by the raw data (Supplement Figure 1; n = 3 animals per time point) was done by sine-waves fitting as described in Figure 2. **(A)** In the somatosensory cortex comparable (p = 0.5724, F = 0.3293 [1, 20]) and rapid adaptation of the two *Per* genes is followed by slower adaptation of *Dbp*, *Bmal1* and *Rev-Erbα* (p = 0.0098, F = 8.138 [1, 20] *Dbp* vs. *Per2*; p = 0.0039, F = 10.61 [1, 20] *Bmal1* vs. *Per2*; p = 0.0498, F = 4.361 [1, 20] *Rev-Erbα* vs. *Per2*). **(B)** In adrenal, *Per1* and *Per2* both showed comparable (p = 0.2915, F = 1.140 [1, 44]) and fast adaptation, while *Dbp* and *Rev-Erbα* rhythms shifted at a similar, but slightly slower rate (p = 0.0976, F = 2.865 [1, 44] *Dbp* vs. *Per2*; p = 0.0609, F = 3.701 [1, 44] *Rev-Erbα* vs. *Per2*). The *Bmal1* peak time showed the slowest adaptation with a PS_{50} value of 3.5 ± 0.2 days ($p < 0.0001$, F = 28.47 [1, 44] vs. *Per2)*. **(C)** A similar hierarchy was observed for kidney with fast adaptation *for Per*, *Dbp* and *Rev-Erbα* and a slow one for *Bmal1* (p = 0.2150, F = 1.586 [1, 41] *Per1* vs. *Per2*; p = 0.4240, F = 0.6520 [1, 41] *Dbp* vs. *Per2*; p = 0.2277, F = 1.500 [1, 41] *Rev-Erbα* vs. *Per2*; p = 0.0019, F = 11.08 [1, 41] *Bmal1* vs. *Per2*). **(D)** In liver, *Per2* expression shifted significantly faster than that of the other clock genes (p = 0.0321, F = 5.128 [1, 26] vs. *Per1*; p = 0.0148, F = 6.821 [1, 26] vs. *Dbp*; p = 0.0479, F = 4.311 [1, 26] vs. *Rev-Erbα*; $p < 0.0001$, F = 38.28 [1, 26] vs. *Bmal1*). *Per1*, *Dbp* and *Rev-Erbα* followed at comparable speed, while *Bmal1* adapted slowest with a PS_{50} value of 4.2 ± 0.2 days ($p < 0.0001$ F = 29.10 [1, 26] vs. *Per1*; F = 27.49 [1, 26] vs. *Dbp*; F = 40.08 [1, 26] vs. *Rev-Erbα*). **(E)** In pancreas, *Per1* and *Per2* shifting was slow with PS_{50} values of 5.3 ± 2.0 days and 4.9 ± 1.5 days, respectively (p = 0.6461, F = 0.5576 [3, 40]) followed by *Dbp* and *Bmal1* (p = 0.7558, F = 0.09938 [1, 20] *Dbp* vs. *Per2*; p = 0.6123, F = 0.2650 [1, 20] *Bmal1* vs. *Per2*). *Rev-Erbα* adaptation was fastest in this tissue with a PS_{50} value of 3.6 ± 0.3 days (p = 0.0059, F = 9.298 [1, 20] vs. *Per1*; p = 0.0078, F = 8.573 [1, 20] vs. *Per2*; p = 0.0410, F = 4.712 [1, 20] vs. *Dbp*; p = 0.1636, F = 2.077 [1, 20] vs. *Bmal1*).

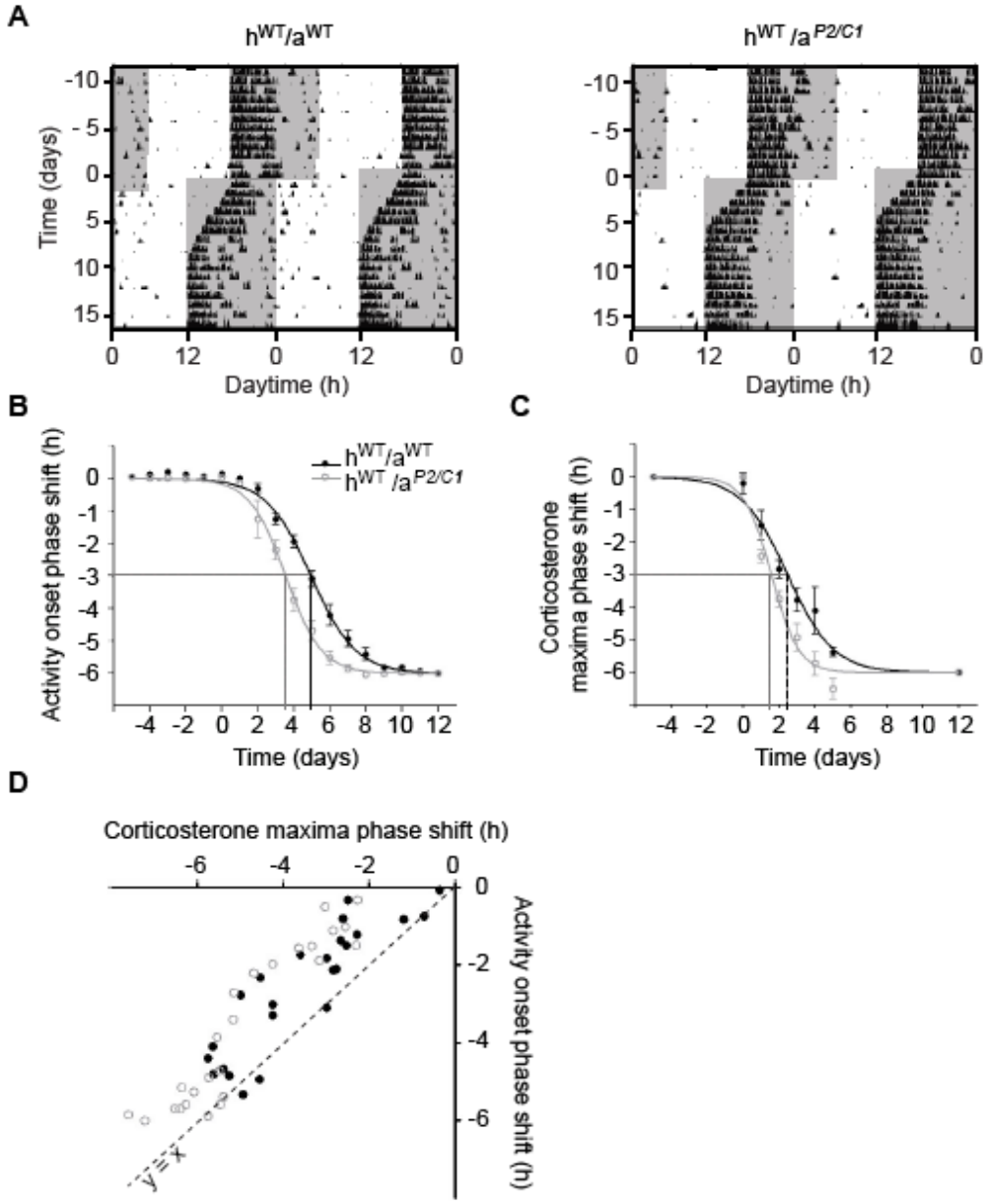


Figure 4. *Influence of adrenal clock function on activity re-entrainment after a 6 hrs phase advance of the LD cycle.*

(A) Representative double-plot actograms of a wild type sham operated animal (host^{WT}/adrenal^{WT} [h^{WT}/a^{WT}]; left) and an adrenalectomized wild type animal carrying *Per2*/*Cry1* mutant adrenal transplants (host^{WT}/adrenal$^{Per2/Cry1}$ [h^{WT}/a$^{P2/C1}$]; right). Light and dark phases are marked in white and grey. **(B)** Resetting kinetics of activity onsets (average ± SEM). The curves differ significantly between days 3 and 8 ($0.003 \leq p \geq 0.016$). On average, PS_{50} values of activity resetting were reduced by 28.4 % for h^{WT}/a$^{P2/C1}$ (4.95 ± 0.07 days for h^{WT}/a^{WT} and 3.55 ± 0.08 days for h^{WT}/a$^{P2/C1}$; $p < 0.0001$; $F = 161.4$ [1, 266]). n = 9 for h^{WT}/a^{WT} and n = 8 for h^{WT}/a$^{P2/C1}$. **(C)** Resetting kinetics of corticosterone excretion maxima peak times (average ± SEM). The curves differ significantly between days 2 and 5 ($0.030 \leq p \geq 0.004$). On average, PS_{50} values of corticosterone resetting were reduced by 36.5 % in h^{WT}/a$^{P2/C1}$ (2.47 ± 0.17 days for h^{WT}/a^{WT} and 1.57 ± 0.10 days; for h^{WT}/a$^{P2/C1}$; $p < 0.0001$; $F = 22.05$ [1, 84]. n = 5 for h^{WT}/a^{WT} and n = 6 for h^{WT}/a$^{P2/C1}$. (**D)** Corticosterone maxima and activity onset phase shifts are plotted against each other for individual h^{WT}/a^{WT} and h^{WT}/a$^{P2/C1}$ mice. Because nearly all individual experimental values are located above the diagonal (dashed line), corticosterone concentration rhythms shift more rapidly than locomotor behavior at all times. A strong correlation between both factors for both groups was found ($R^2 = 0.79$ h^{WT}/a^{WT}; $R^2 = 0.87$ h^{WT}/a$^{P2/C1}$). h^{WT}/a^{WT} are represented by black circles and h^{WT}/a$^{P2/C1}$ by open circles.

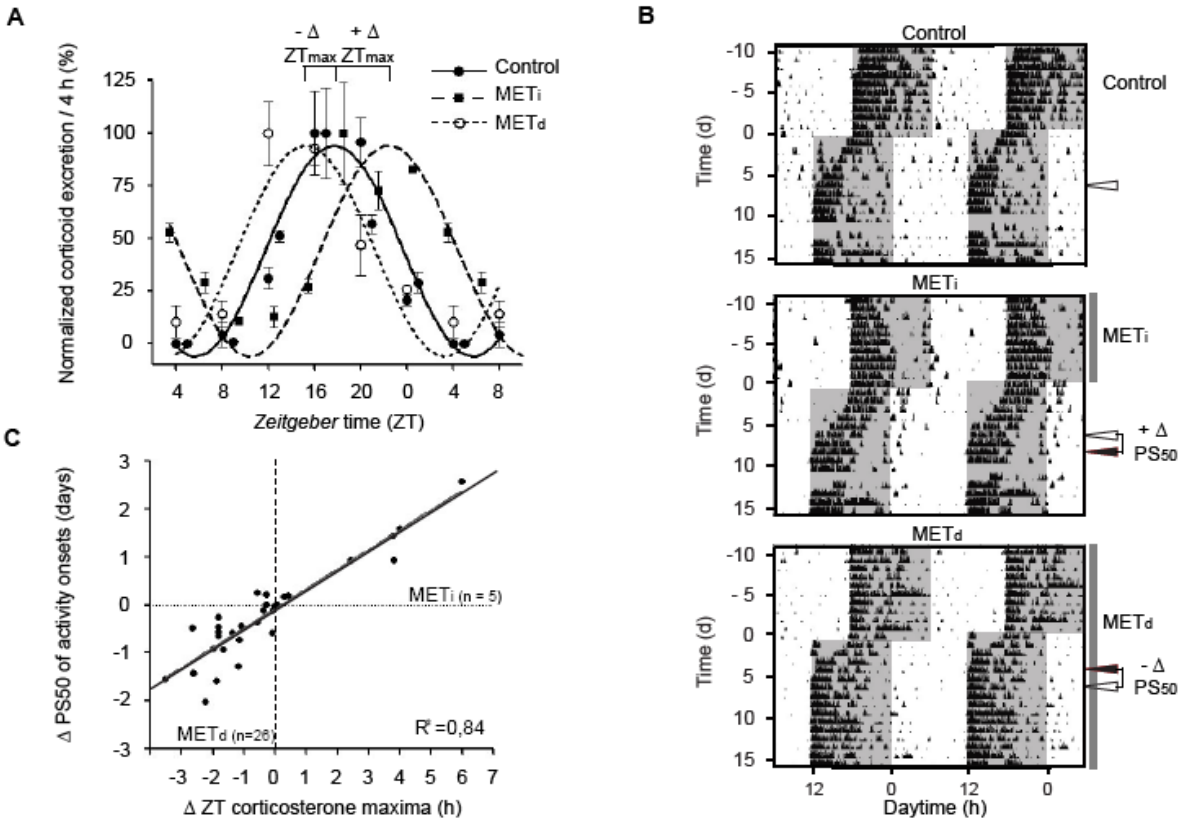


Figure 5. *Shifting corticosterone rhythms prior to jet lag affects behavioral resetting kinetics.*

(A) Advanced (MET_d) and delayed (MET_i) corticosterone excretion rhythms of three representative wild type mice after Metyrapone (11β-hydroxylase inhibitor) treatment. ΔZT_{max} defines the pre-jet lag shift of the corticosterone peak time in treated mice relative to the peak time in non-treated control mice. **(B)** Representative double-plot actograms of an untreated mouse (top), an MET_i mouse (middle) and an MET_d mouse (bottom) 2 weeks before and 2 weeks after a 6 hrs phase advance of the LD cycle. The duration of MET treatment is represented by grey bars on the right. Light and dark phases are marked in white and grey. White arrows indicate the day when re-entrainment of the non-treated control mouse was finished. The black arrow depicts the day at which this representative MET_i or MET_d mouse reached its re-entrainment. ΔPS_{50} defines the difference of the activity onset PS_{50} values in comparison to the PS_{50} values of the control animals. **(C)** Correlation line ($y = 0.42x - 0.08$, $R^2 = 0.84$; black line; MET_i: n= 5; MET_d: n= 26; control: n=6) between the pre-jet lag shift of corticosterone maxima and the corresponding PS_{50} values of activity onset resetting after the LD shift of individual treated animals.

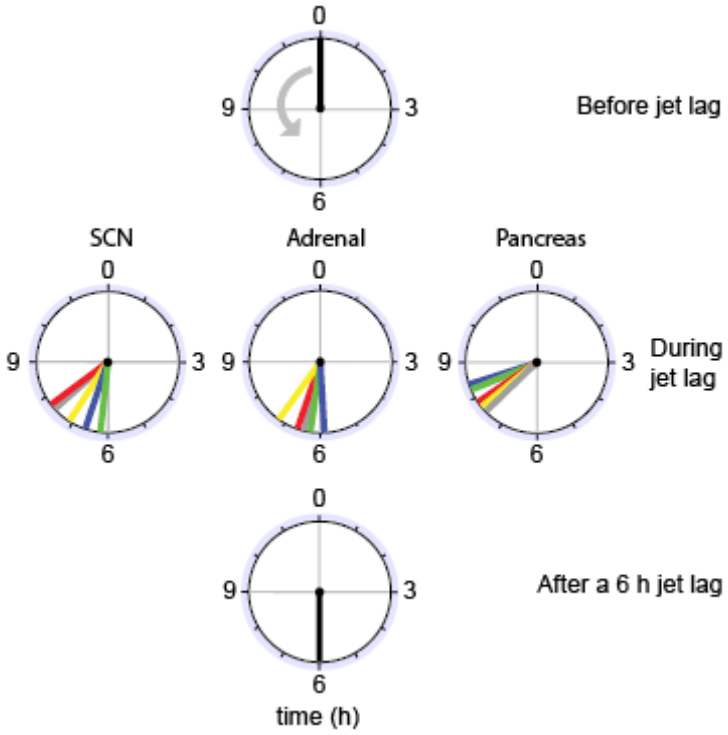
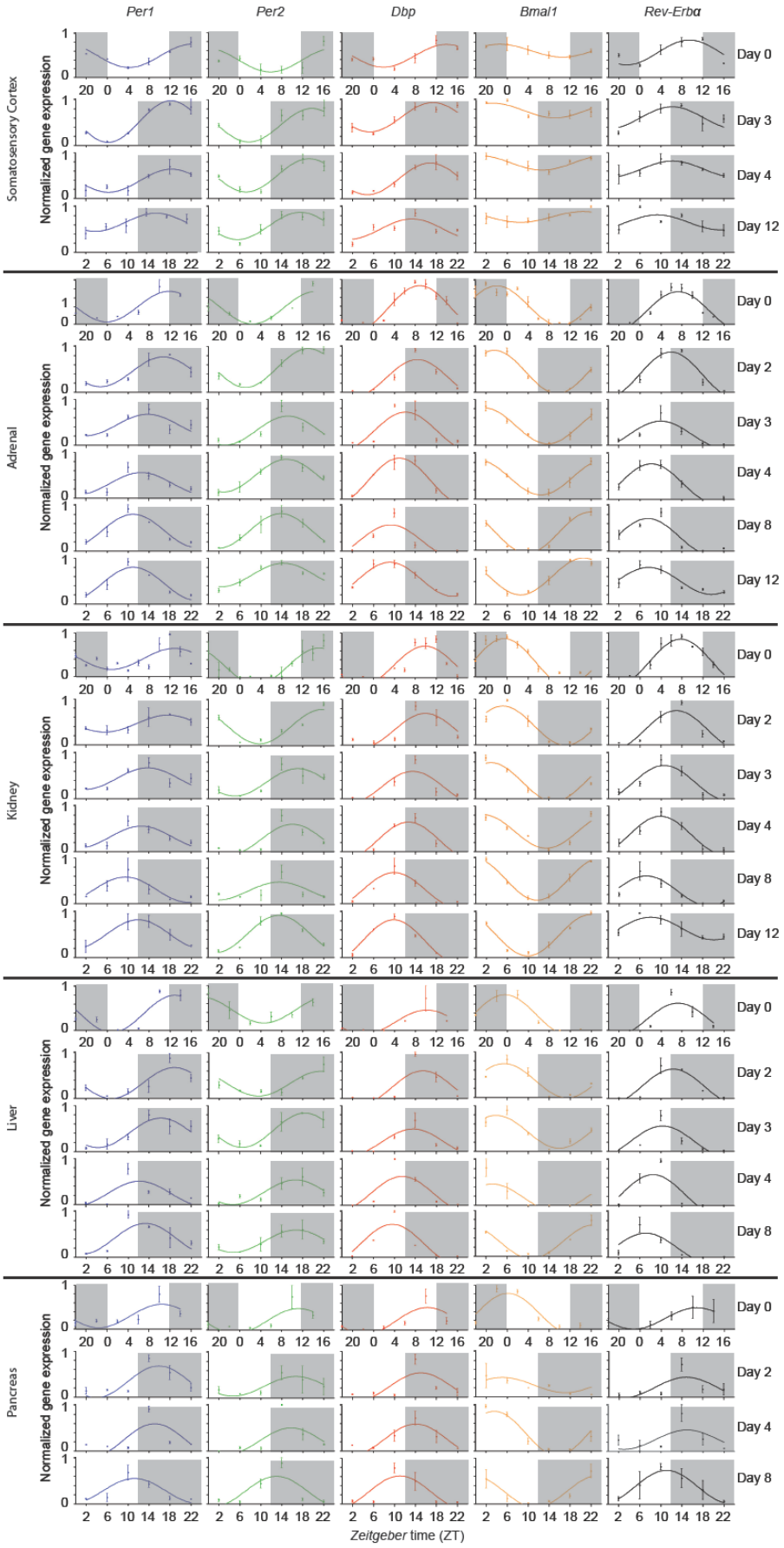
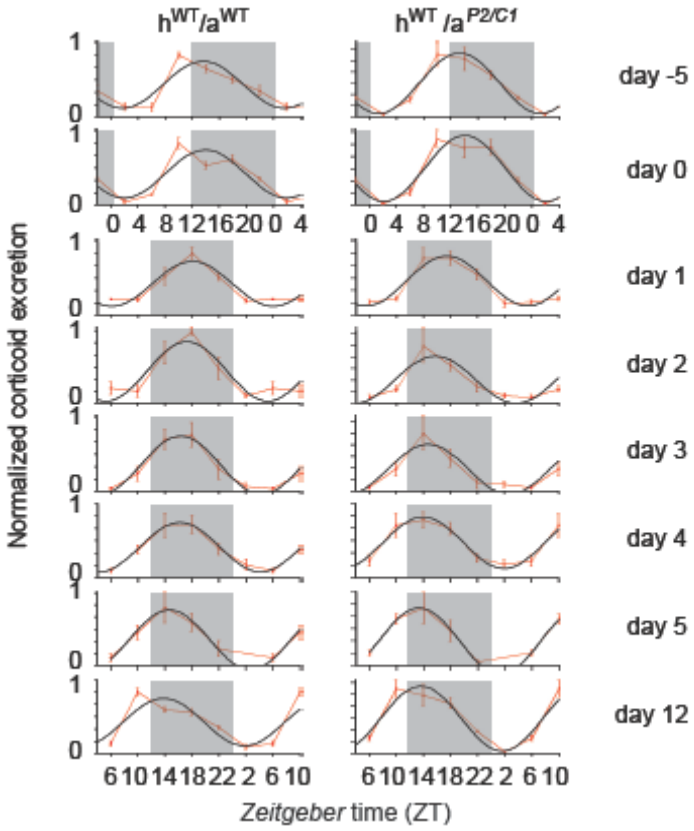


Figure 6. *Transient desynchronization within and between tissues during jet lag.*

Before jet lag all genes and organs are in synchrony with each other depicted by the black clock hand (top). Organotypic and gene specific spreading of expression phases occur during jet lag (middle), which resynchronize after overcoming jet lag (bottom). Color code represents different normalized maxima phase shifts of different clock genes examined; *Per1 (*blue), *Per2* (green), *Dbp* (red), *Bmal1* (yellow), *Rev-Erbα* (grey) during jet lag.



Supplemental Figure 1. *Resetting of Clock Genes during Jet Lag in Peripheral Oscillators.* Diurnal *mRNA* profiles of different clock genes (3 animals per time point) at days 0, 2, 3, 4, 8 and 12 after the LD shift were fitted with sine waves. Light and dark phases are marked by white or grey shadings. *Zeitgeber* times are depicted at the bottom of days 0, 8 or 12.



Supplemental Figure 2. *Adrenal control of Resetting of Corticosterone Excretion during Jet Lag* Diurnal normalized corticosterone profiles of wild-type mice (5 animals per time point) at days -5, 0 to 5 and 12 after the LD shift and the fitted sine waves. Light and dark phases are marked by white or grey shadings. *Zeitgeber* times are depicted at the bottom of day 0 and day 12.

2.3. Additional Data

2.3.1. Surgery and age related effects on jet lag

To analyze the long-term consequences of adrenal gland transplantation on the animal's circadian system we compared behavioral resetting at different times after surgery. One month after surgery auto-grafted wild-type animals reset completely within 5 days (**Figure 24A**). One month later, the same animals needed around 2 days longer to entrain their activity behavior to the new LD cycle. After 3 months, the difference in time was even greater for the same animals. They now needed about 8 days to complete the shift. An end of this development was reached after 4 months, when the shift was completed in ca. 9 days. These differences were independent of the graft genotype, because similar prolonged behavioral adaptation times were found in wild-type mice with arrhythmic adrenals (**Figure 24B**). Importantly, when comparing PS_{50} values of wild-type with those of wild-type mice with disrupted adrenal clocks at different time points after surgery, significant differences were found after 4 months only (**Figure 24C**).

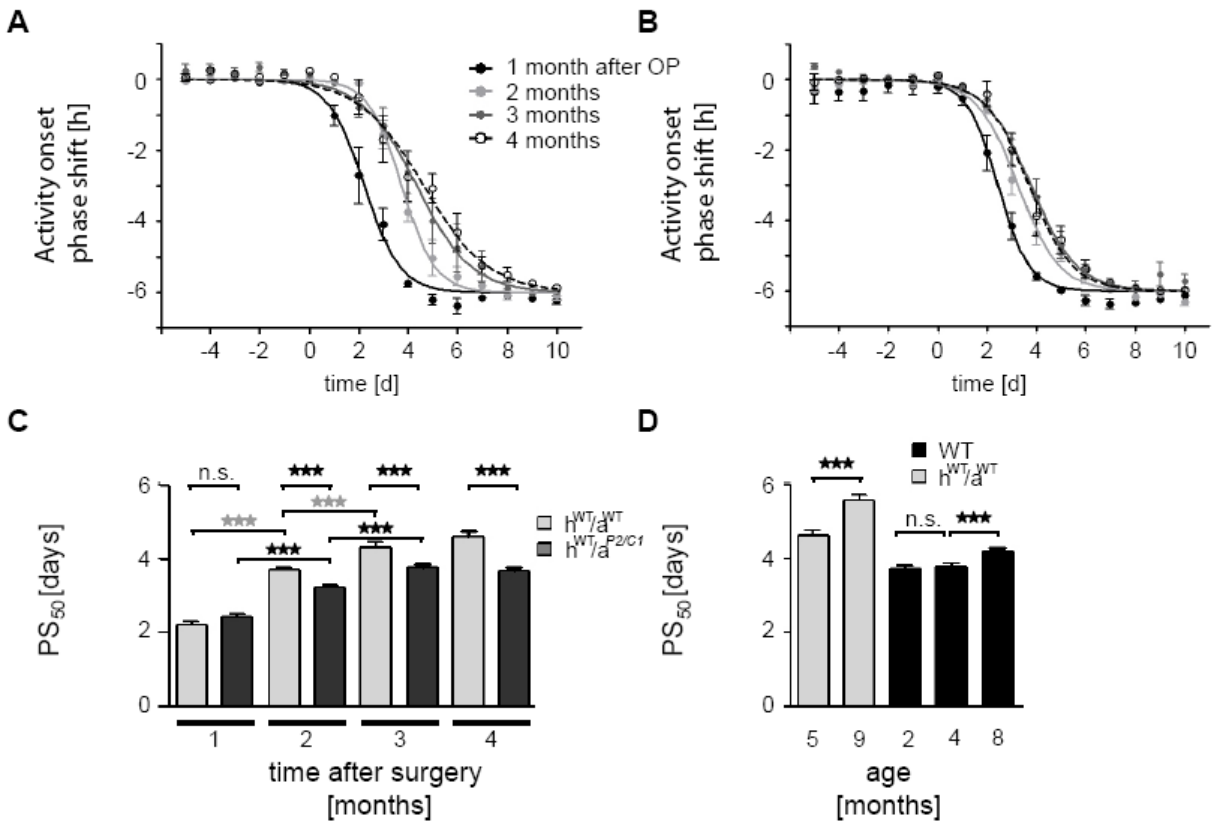


Figure 24: Surgery effects on behavioral resetting at different times after surgery. (A) auto-grafted control animals (h^{WT}/a^{WT}) and (B) wild-type mice carrying arrhythmic adrenals ($h^{WT}/a^{P2/C1}$). (C) Comparison of PS_{50} values of both h^{WT}/a^{WT} and $h^{WT}/a^{P2/C1}$ from the curves depicted in A and B. (D) PS_{50} values of resetting behavior of 5 and 9 month old $h^{WT}/a^{P2/C1}$ and 2, 4 and 8 month old non-operated wild-type animals (average ± SEM). OP, surgery.

Longer adaptation times were also observed in older auto-grafted wild-types (5 vs. 9 months) (4.6 ± 0.15 days for 5 month old wild-types vs. 5.6 ± 0.14 days in 9 month old animals, n = 4, p < 0.0001)(**Figure 24D**). To distinguish between surgery effects and those of ageing, the same experiment was repeated with non-operated wild-type mice. The PS_{50} values of these animals were significantly higher in 8 month old animals compared to younger ones, whereas no difference was found between animals of 2 and 4 months of age (3.7 ± 0.1 days for 4 month old wild-types, p < 0.0001, n = 9, vs. the same, but 8 months old animals; 3.8 ± 0.1 days in 5 months old animals vs. 4.2 ± 0.1 days the same, but 8 months old animals, each n = 9, p = 0,002).

In summary, the surgery influences the time needed to complete jet lag in all grafted animals. Additionally, longer resetting times were found in older mice.

2.3.2. Impact of adrenal clock function on period length at constant conditions

In addition to the wild-type animals carrying arrhythmic mutant adrenals described in the manuscript above, we generated *Per2/Cry1* mutant hosts with wild-type adrenal grafts. First, we examined whether the SCN pacemaker was affected in grafted mice by analyzing locomotor activity rhythms. After transplantation all mice were allowed to recover for at least 12 weeks (for details see *Materials and methods*) and then kept under LD 12:12 conditions (**Figure 25**). After 2 weeks in LD, mice were released into constant darkness (DD), followed by another period of constant light for 2 weeks and subsequent release into a second LD phase.

The wild-type activity pattern in LD with high running wheel activity during the dark phase was preserved in all animal groups independent of a functional adrenal and/or host clock (**Figure 25, Figure 27, Figure 28**). Independent of a dysfunction in the adrenal clock, $h^{WT}/a^{P2/C1}$ mice showed activity patterns similar to those seen in wild-type mice (**Figure 25A**). In DD periodograms of both groups show strong circadian rhythmicity of high amplitudes (**Figure 25C, D**) with a period of 23.3 ± 0.1 hrs for h^{WT}/a^{WT} (n = 5) and 23.5 ± 0.1 hrs for $h^{WT}/a^{P2/C1}$ (n = 5, mean ± SEM) (**Figure 26**, **DD**).

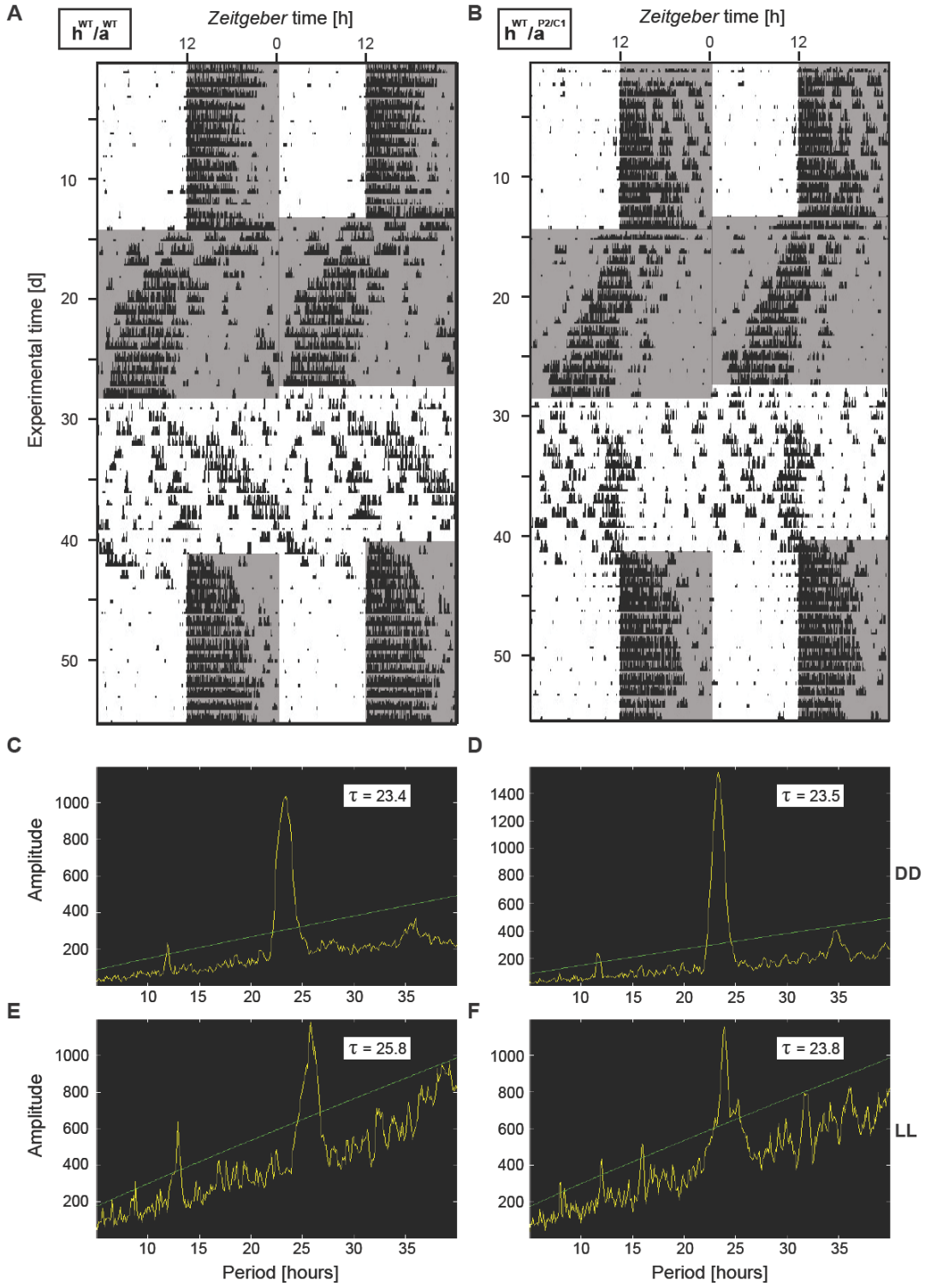


Figure 25: Activity profiles of auto-grafted wild-type animals (h^{WT}/a^{WT}) and wild-type hosts with *Per2/Cry1* mutant adrenals ($h^{WT}/a^{P2/C1}$). Upper panels show representative double-plotted actograms of h^{WT}/a^{WT} (A) and $h^{WT}/a^{P2/C1}$ (B) in LD, DD and LL (transition indicated by the line below LD). Shaded and non-shaded areas indicate light and dark phase. (C-F) Panels show periodograms of the same animals. (C) and (D) DD, (E) and (F) LL. The diagonal line depicts significance as given by the ClockLab program.

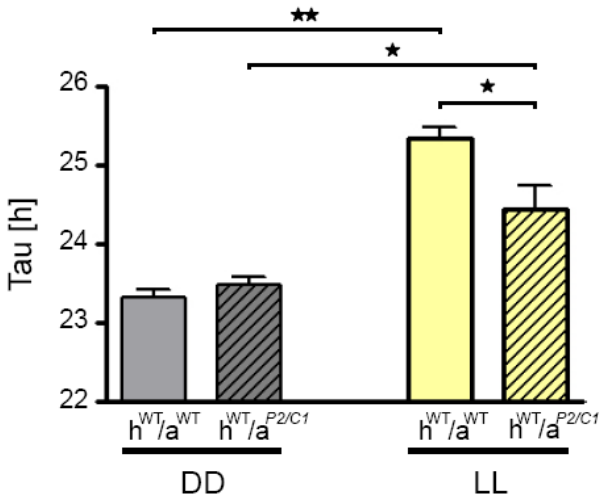


Figure 26: Free-running period of h^{WT}/a^{WT} and $h^{WT}/a^{P2/C1}$ mice under constant conditions. DD (n = 5) and LL (n = 9)(mean ± SEM).

Rhythmicity was also preserved in most of these animals under constant light conditions (**Figure 25 E, F**), but 2 wild-type and 4 $h^{WT}/a^{P2/C1}$ mice became arrhythmic (**Table 1 and 2**). This phenomenon has previously been described for wild-type animals (Ohta et al. 2005). The free-running period length of sham operated wild-type animals was 25.3 ± 0.1 hrs (mean ± SEM, n = 9), significantly longer than that seen in DD (p = 0.003, **Figure 26**). Interestingly, whereas wild-type animals carrying arrhythmic adrenals also showed significantly longer endogenous periods in LL than in DD (p = 0.027, **Figure 26**), period lengths in LL were significantly shorter than those of the auto-grafted controls (τ_{LL} = 24.4 ± 0.3 hrs, mean ± SEM, n = 9, p = 0.019; **Figure 25,** LL).

Table 1: τ in DD and LL of h^{WT}/a^{WT}.

	DD	LL
h^{WT}/a^{WT} 1	23.40	25.80
h^{WT}/a^{WT} 2	23.20	25.60
h^{WT}/a^{WT} 3	23.40	arr.
h^{WT}/a^{WT} 4	23.00	arr.
h^{WT}/a^{WT} 5	23.60	25.50
h^{WT}/a^{WT} 6	------	25.70
h^{WT}/a^{WT} 7	------	25.70
h^{WT}/a^{WT} 8	------	25.20
h^{WT}/a^{WT} 9	------	24.80
h^{WT}/a^{WT} 10	------	24.90
h^{WT}/a^{WT} 11	------	24.90

Table 2: τ in DD and LL of h^{WT}/a^{P2C1}.

	DD	LL
h^{WT}/a^{P2C1} 1	23.30	23.80
h^{WT}/a^{P2C1} 2	23.20	22.50
h^{WT}/a^{P2C1} 3	23.6	arr.
h^{WT}/a^{P2C1} 4	23.8	arr.
h^{WT}/a^{P2C1} 5	23.51	23.90
h^{WT}/a^{P2C1} 6	------	25.3
h^{WT}/a^{P2C1} 7	------	24.6
h^{WT}/a^{P2C1} 8	------	arr.
h^{WT}/a^{P2C1} 9	------	25.0
h^{WT}/a^{P2C1} 10	------	25.2
h^{WT}/a^{P2C1} 11	------	24.7
h^{WT}/a^{P2C1} 12	------	25.0
h^{WT}/a^{P2C1} 13	------	arr.

When animals of the other cross-genotype combination ($h^{P2/C1}/a^{WT}$) were transferred from LL to the second LD schedule, in 3 animals small activity bouts were detectable during the light phase (**Figure 27,** indicated by red squares).

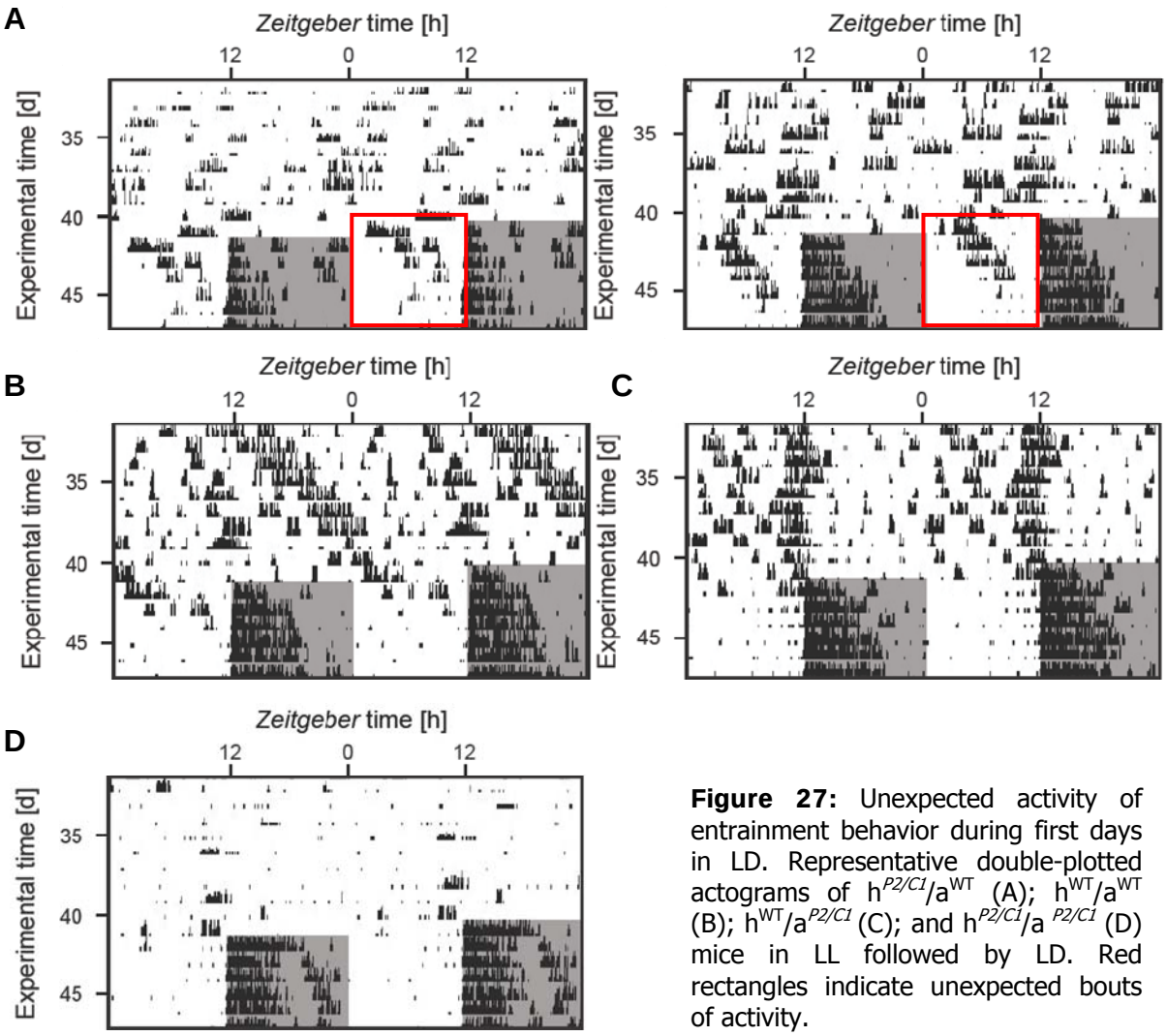


Figure 27: Unexpected activity of entrainment behavior during first days in LD. Representative double-plotted actograms of $h^{P2/C1}/a^{WT}$ (A); h^{WT}/a^{WT} (B); $h^{WT}/a^{P2/C1}$ (C); and $h^{P2/C1}/a^{P2/C1}$ (D) mice in LL followed by LD. Red rectangles indicate unexpected bouts of activity.

The onset of these activity bouts gradually approached the light/dark transition, resembling "re-entrainment" of onsets observed during jet lag or at free-running (DD or LL) to entrained (LD) transitions in wild-type animals. This finding suggests the presence of an endogenous circadian rhythm in the preceding LL phase, even though such a rhythm was not detected in the LL periodograms (**Figure 28**). Wild-types - carrying a disrupted adrenal or not - show clear circadian peaks (**Figure 28B** & **C**), whereas the *Per2/Cry1* deficient animals/hosts show no peak at all and were therefore classified as being arrhythmic (**Figure 28D**).

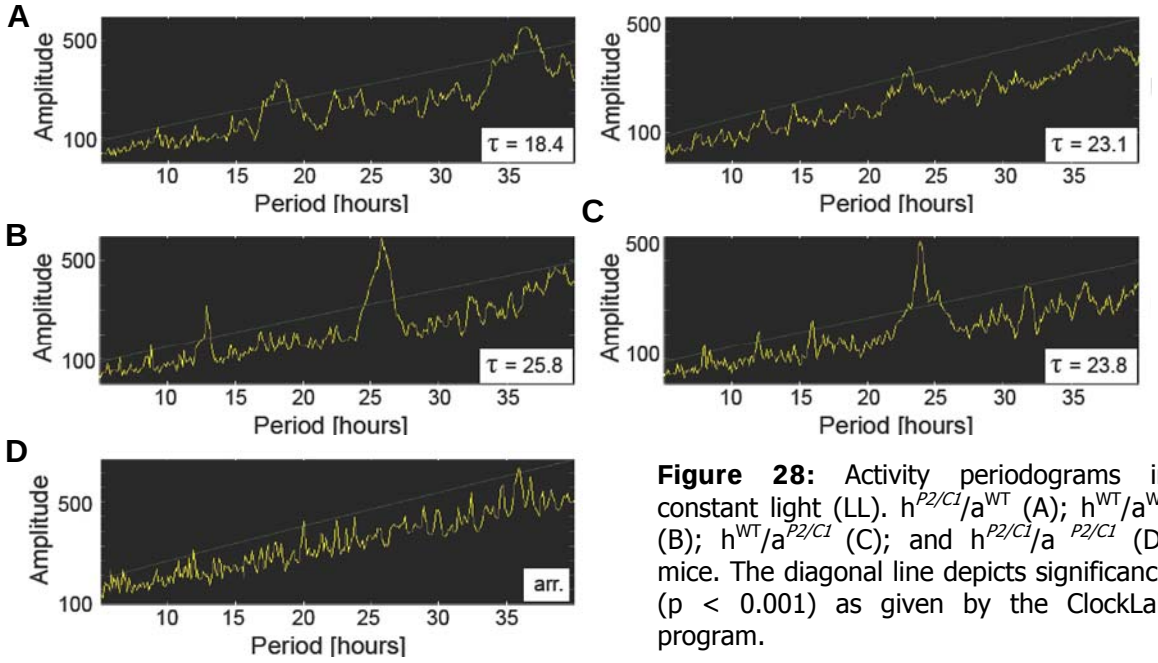


Figure 28: Activity periodograms in constant light (LL). $h^{P2/C1}/a^{WT}$ (A); h^{WT}/a^{WT} (B); $h^{WT}/a^{P2/C1}$ (C); and $h^{P2/C1}/a^{P2/C1}$ (D) mice. The diagonal line depicts significance ($p < 0.001$) as given by the ClockLab program.

Taken together, adrenal arrhythmicity leads to shortened rhythms in constant light in wild-type animals. Moreover, we found hints that wild-type adrenals can induce or stabilize residual rhythms in *Per2/Cry1* mutant hosts under LL conditions.

2.3.3. Impact of adrenal clock function on activity distribution in LD

At the LL to LD transition bouts of activity were observed during the light phase in $h^{P2/C1}/a^{WT}$ mice (**Figure 29)**. To analyze a possible general activity increase before light offset, we compared the activity profiles of the different cross-genotypes with their corresponding controls.

The LD activity profile of wild-type mice carrying mutant adrenals were very similar to the profile of auto-transplanted wild-type animals and of non-operated wild-type controls (**Figure 30**).

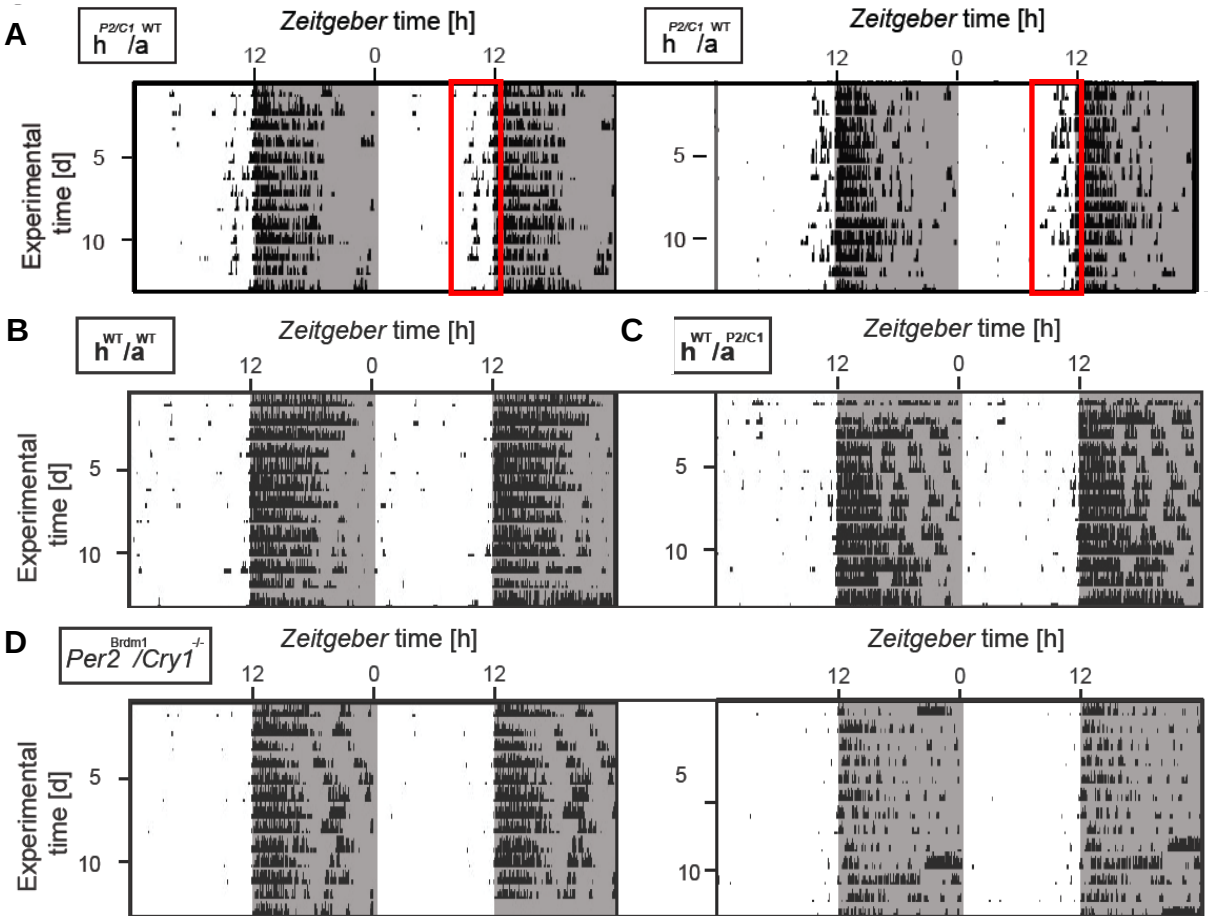


Figure 29: Activity patterns in LD of the different cross-genotypes. Representative double-plotted actograms of $h^{P2/C1}/a^{WT}$ (A), h^{WT}/a^{WT} (B), $h^{WT}/a^{P2/C1}$ (C), $h^{P2/C1}/a^{P2/C1}$ (*Per2*^{Brdm1}*/Cry1*$^{-/-}$, D), and $h^{P2/C1}/a^{WT}$ mice in LD. Red rectangles indicate bouts of activity at th end of the light phase.

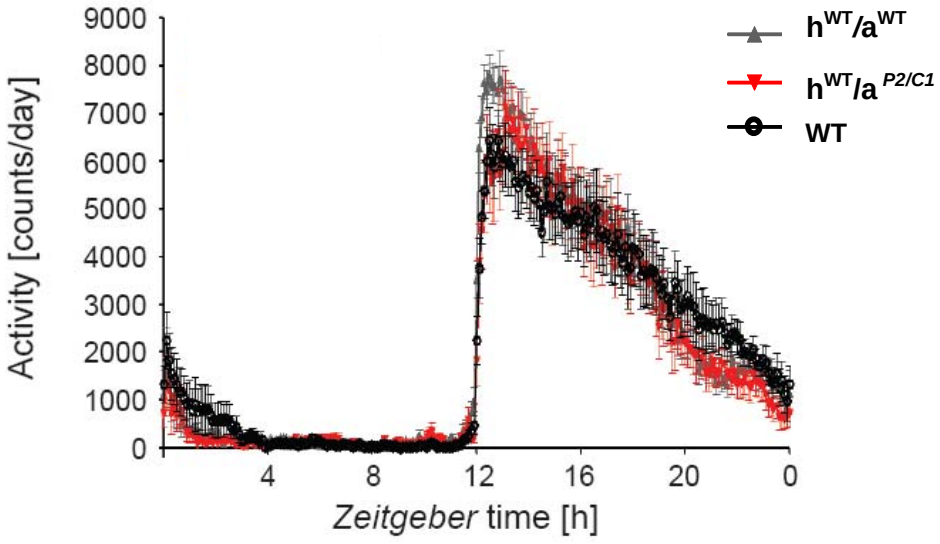


Figure 30: Activity profiles of the wild-type host cross-genotype and its controls in LD. WT (n = 15, black), h^{WT}/a^{WT} mice; (n = 13, grey) and $h^{WT}/a^{P2/C1}$ (n = 12, red). Values for each animal were average over 5 to 10 consecutive days (overall average ± SEM for all groups).

By contrast, *Per2/Cry1* double deficient hosts carrying wild-type adrenals showed different patterns than non-operated *Per2/Cry1* double deficient animals (**Figure 31**). Specifically, an increase in activity was seen several hrs before the light offset (starting around ZT 9) and in the second half of the night the amount of activity was decreased compared with complete mutants. Additionally, the running-wheel activity of both arrhythmic host genotypes reached the baseline level earlier than that of all wild-type host animals carrying either mutant adrenals or not (compare ZT 0-3 from **Figures 30** & **31**).

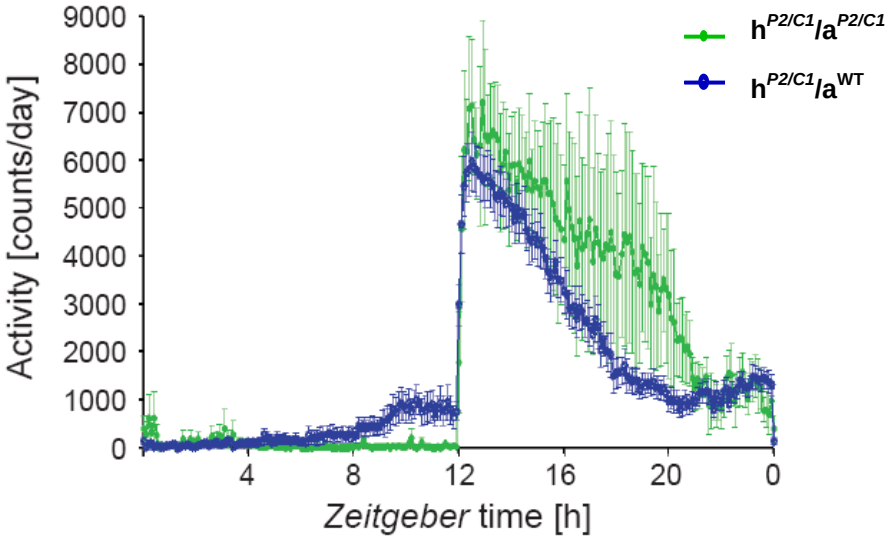


Figure 31: Activity profiles of the mutant host cross-genotype and its control group in LD. $h^{P2/C1}/a^{WT}$ (n = 13, blue) and $h^{P2/C1}/a^{P2/C1}$ mice (n = 3, green). Values for each animal were averaged over 5 to 10 consecutive days (overall average ± SEM for all groups).

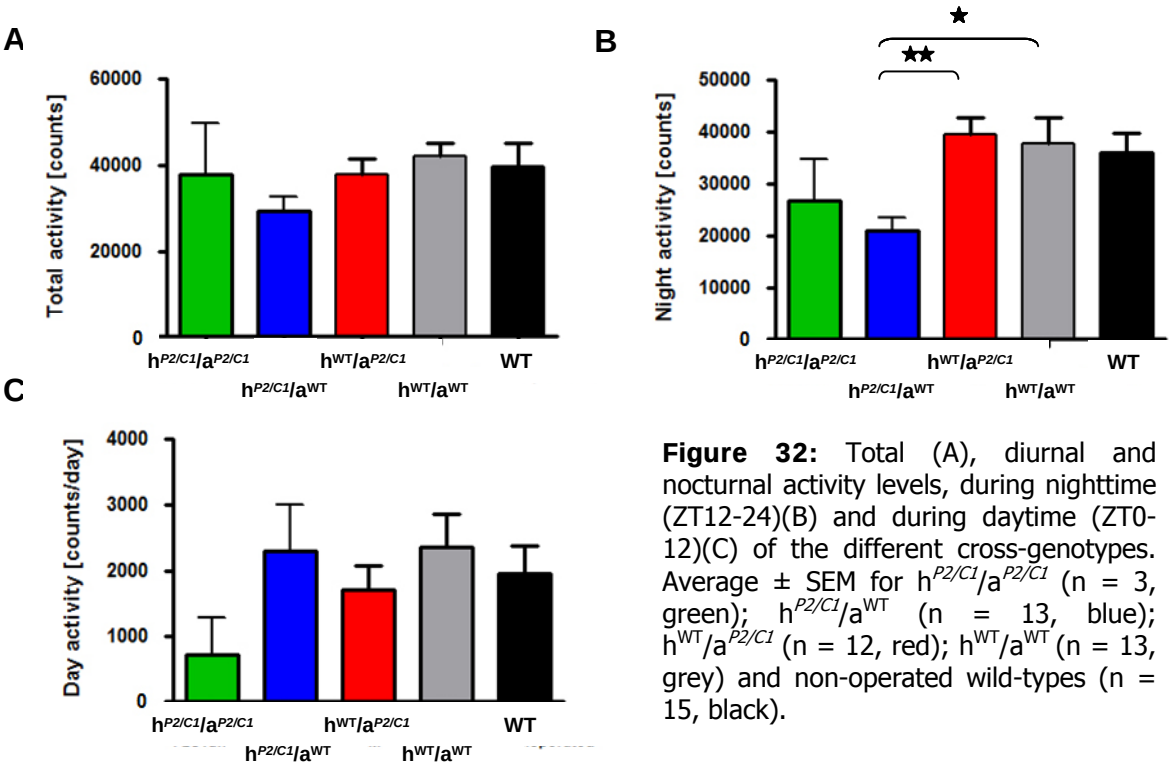


Figure 32: Total (A), diurnal and nocturnal activity levels, during nighttime (ZT12-24)(B) and during daytime (ZT0-12)(C) of the different cross-genotypes. Average ± SEM for $h^{P2/C1}/a^{P2/C1}$ (n = 3, green); $h^{P2/C1}/a^{WT}$ (n = 13, blue); $h^{WT}/a^{P2/C1}$ (n = 12, red); h^{WT}/a^{WT} (n = 13, grey) and non-operated wild-types (n = 15, black).

To address whether these differences affect the total amount of activity during the day, we calculated overall activity in all groups with either arrhythmic hosts and/or adrenals, auto-grafted and non-operated wild-types. We found no significant differences in total activity or daytime activity between all groups (**Figure 32**).

$h^{P2/C1}/a^{WT}$ run significantly less during the night in comparison to $h^{WT}/a^{P2/C1}$ or non-operated wild-types ($p = 0.003$). The daytime activity of $h^{P2/C1}/a^{WT}$ was only by trend higher than that of $h^{P2/C1}/a^{P2/C1}$, whereas all the other cross-combinations showed similar amounts of acitivity. For a more detailed analysis shorter intervals down to 3 hrs were calculated.

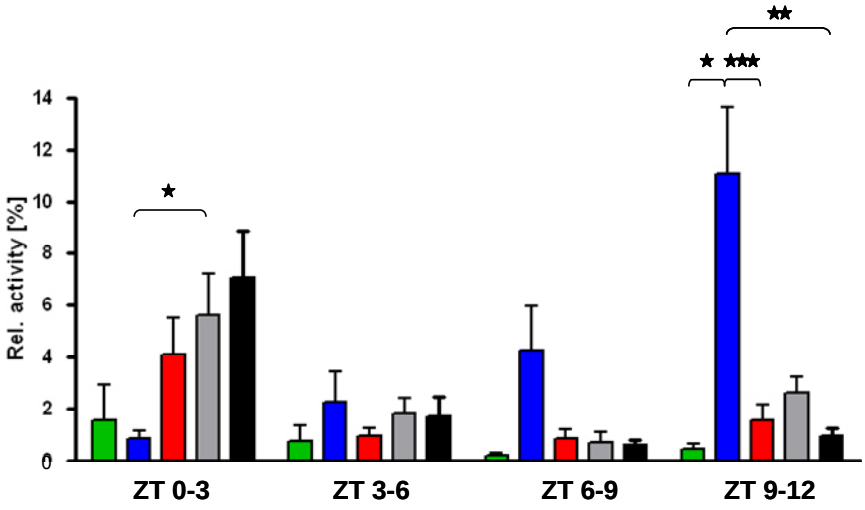


Figure 33: Diurnal distribution of running-wheel activity (in 3 hrs intervals) of the different cross-genotypes. Color code is the same as in Figure 32. Average ± SEM for $h^{P2/C1}/a^{P2/C1}$ (n = 3, green); $h^{P2/C1}/a^{WT}$ (n = 13, blue); $h^{WT}/a^{P2/C1}$ (n = 12, red); h^{WT}/a^{WT} (n = 13, grey) and non-operated wild-types (n = 15, black).

Differences in the relative amount of running wheel activity were detected between $h^{P2/C1}/a^{WT}$ and h^{WT}/a^{WT} during the first interval (ZT 0-3), but in the middle of the day (between ZT 3 and ZT 9) no differences were seen. The daytime activity of $h^{P2/C1}/a^{WT}$ already rose from ZT 3 to 6, but was significantly higher from ZT 9 to 12 only (vs. $h^{P2/C1}/a^{P2/C1}$, $p < 0.5$; vs $h^{WT}/a^{P2/C1}$ $p < 0.001$; non-treated wild-types $p < 0.01$) (**Figure 33**).

Taken together, we found marked differences in the amount of running wheel activity at the end of the day in $h^{P2/C1}/a^{WT}$ and a minor decrease in activity at the beginning of the day, which might suggest the "feeling" of an advanced activity profile in these mice when in LD.

2.3.4. Impact of adrenal clock function on resetting during jet lag

2.3.4.1. Activity monitoring

GCs have the potential to change the phase of clocks in the periphery (Balsalobre, Brown et al. 2000) and adrenalectomized rats show accelerated phase shifting behavior under jet lag conditions (Sage, Ganem et al. 2004). We showed that the adrenal circadian clock and the SCN adapt to the new light schedule at comparable rates (Kiessling et al., above). Disruption of adrenal clock function accelerates entrainment during jet lag (Kiessling et al., above), indicating that adrenal clock function is necessary for normal adaptation. Next, we analyzed re-entrainment behavior in mutant hosts with wild-type adrenal grafts to test if adrenal clock function is also sufficient for this behavior.

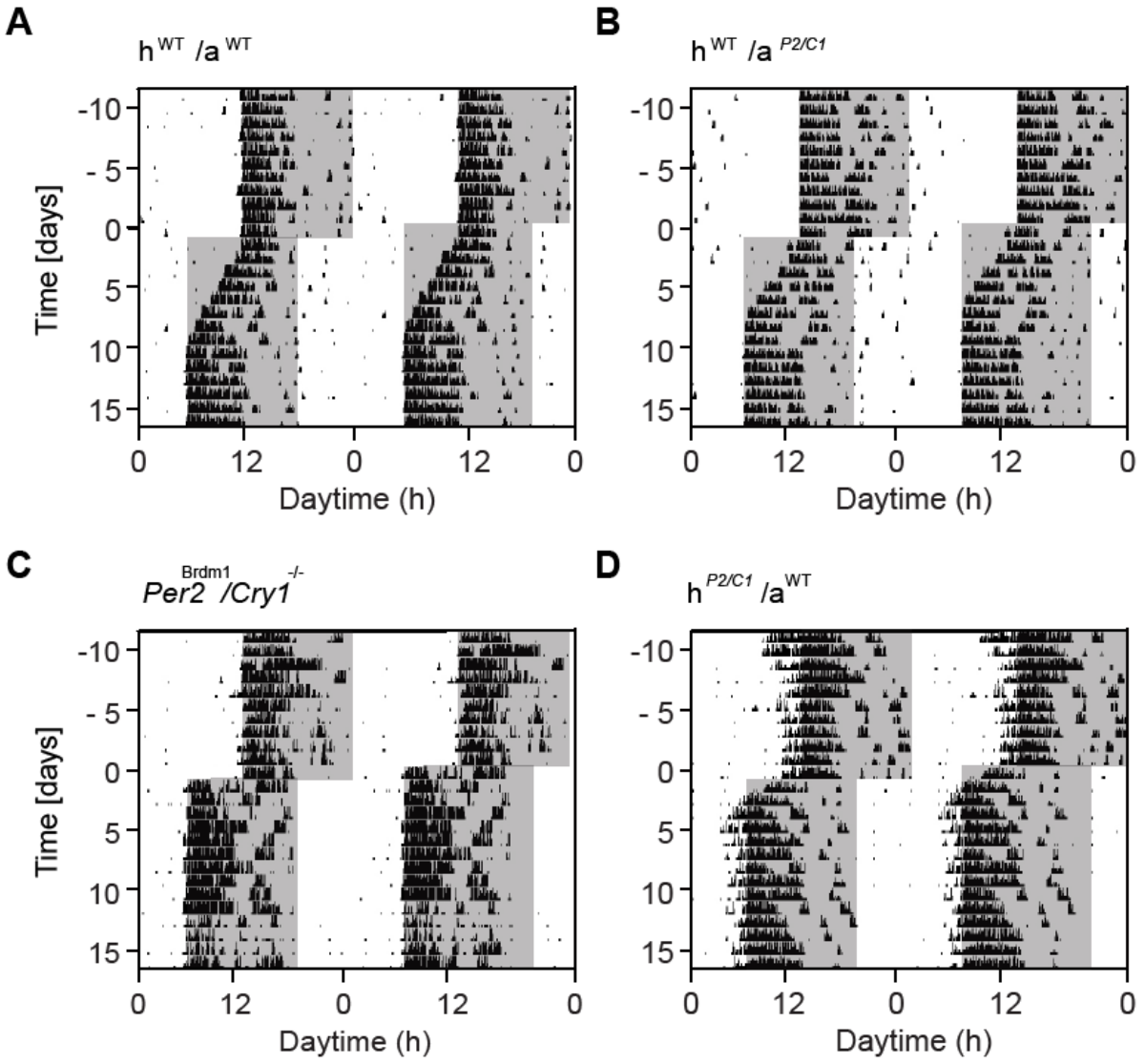


Figure 34: Representative double-plotted actograms of the different cross-genotypes before, during and after a 6h phase advance of the LD cycle. (A) h^{WT}/a^{WT} , (B) $h^{WT}/a^{P2/C1}$, (C) $h^{P2/C1}/a^{P2/C1}$, (D) $h^{P2/C1}/a^{WT}$.

Animals of different cross genotypes were subjected to a 6 hrs phase advance and the re-entrainment of locomotor activity was compared to that of auto-grafted wild-type controls (**Figure 34**). As has been shown above (Kiessling et al.) in control animals, running-wheel activity took about 9 days to adapt to the new light dark regimen. $h^{WT}/a^{P2/C1}$ mice, however, adapted significantly faster, reaching full entrainment after about 7 days (**Figure 34**). The speed of entrainment of *Per2|Cry1* double mutant animals ($h^{P2/C1}/a^{P2/C1}$) and arrhythmic hosts carrying wild-type adrenals ($h^{P2/C1}/a^{WT}$) in comparison to $h^{WT}/a^{P2/C1}$ and h^{WT}/a^{WT} is depicted in **Figure 35**. Activity onsets of *Per2|Cry1* double mutant animals took about 1 day to fully entrain to the 6 hrs advanced LD cycle (**Figure 34 & 35**). By contrast, $h^{P2/C1}/a^{WT}$ animals did not reach the new phase of entrainment until the second day after the shift (**Figure 34 & 35**). In other words, they entrain faster than animals with an intact SCN clock and mutant adrenals, but slower than completely arrhythmic mice, which fully reset during the first day after the LD shift.

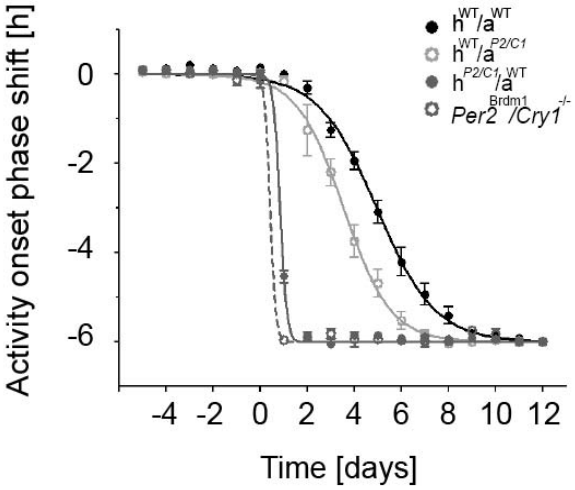


Figure 35: Resetting kinetics of activity onsets during jet lag (average ± SEM). PS_{50} values of activity resetting were reduced by 82.9 % in $h^{P2/C1}/a^{WT}$ (4.95 ± 0.07 days for h^{WT}/a^{WT} and 0.85 ± 0.11 days for $h^{P2/C1}/a^{WT}$; $p < 0.0001$; $n = 5$ for $h^{P2/C1}/a^{WT}$ and $n = 6$ for h^{WT}/a^{WT}.

Together, these data indicate that adrenal clock function modulates re-entrainment, but is not sufficient to control normal adaptation to jet lag.

2.3.4.2. Corticosterone excretion rhythms

As shown above, corticosterone production is closely related to a functional adrenal clock, as the latter gates the response of the steroidogenic machinery to ACTH (see Oster et al. 2006, above). In addition, we demonstrated that the re-entrainment time for corticosterone excretion rhythms is accelerated in animals lacking a functional adrenal clock. Here we analyze the relationship between ACTH in the blood, plasma corticosterone levels and the corticosterone excretion in the feces of wild-type animals at different days during jet lag.

Under entrained conditions (day 0) ACTH levels in the blood showed a strong peak around ZT 16 (**Figure 36**). At the same ZT plasma corticosterone concentration was high (**Figure 37**). By contrast, corticoid excretion peaked about 4 hrs later (**Figure 38**). On the second day after shifting the LD phase 6 hrs forward, we found two ACTH maxima, one around ZT 6 and a second at ZT 15 (as accessed by sine curve fitting) (**Figure 36**). Similarly, we also detected a twin peak in plasma corticosterone at ZT 10 & 18 (**Figure 37**). The second maximum of plasma corticosterone around ZT 18 had not shifted in comparison to the original one seen at day 0.

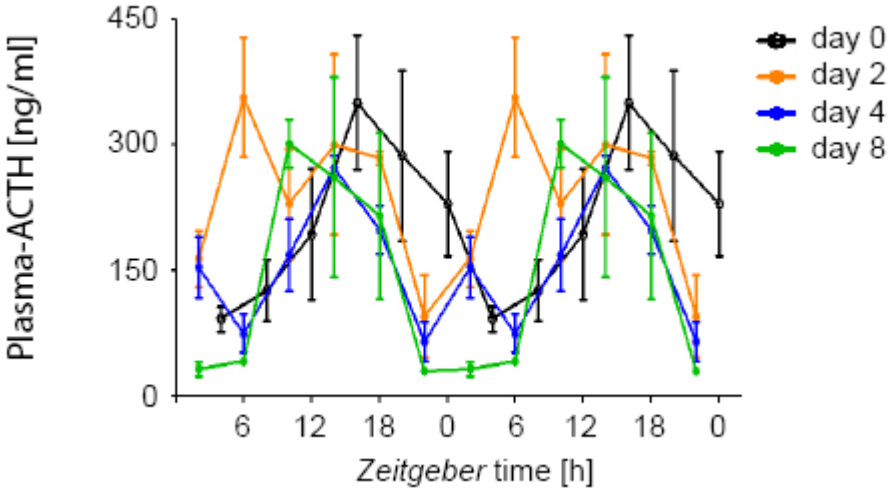


Figure 36: Wild-type plasma ACTH concentrations at different days during jet lag.

At the fourth day during jet lag the ACTH and plasma corticosterone maxima had shifted to ZT 14 while corticoid excretion peaked about 2 hrs later at around ZT 16. ACTH, plasma corticosterone and corticoid excretion rhythms had shifted almost completely after 8 days into the new LD regimen. Peaks of ACTH and plasma corticosterone were found at ZT 10-12, whereas corticoid excretion retained its maximum at ZT 16.

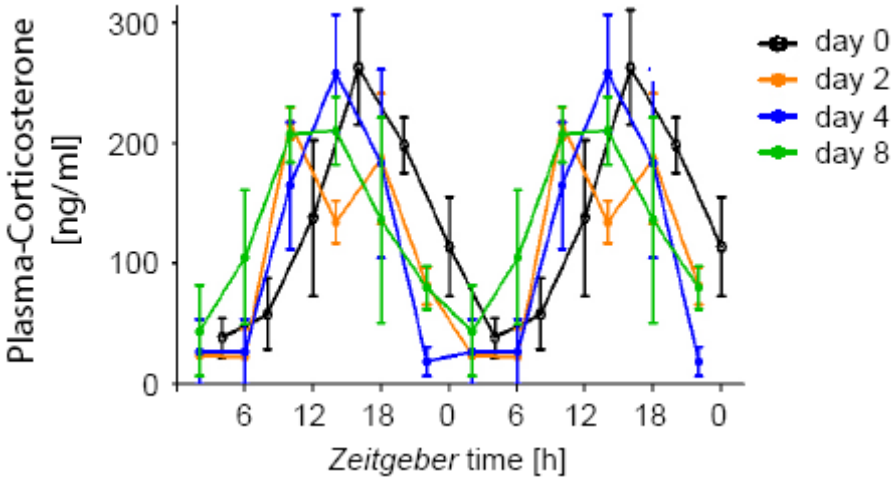


Figure 37: Wild-type plasma corticosterone concentrations at different days during jet lag.

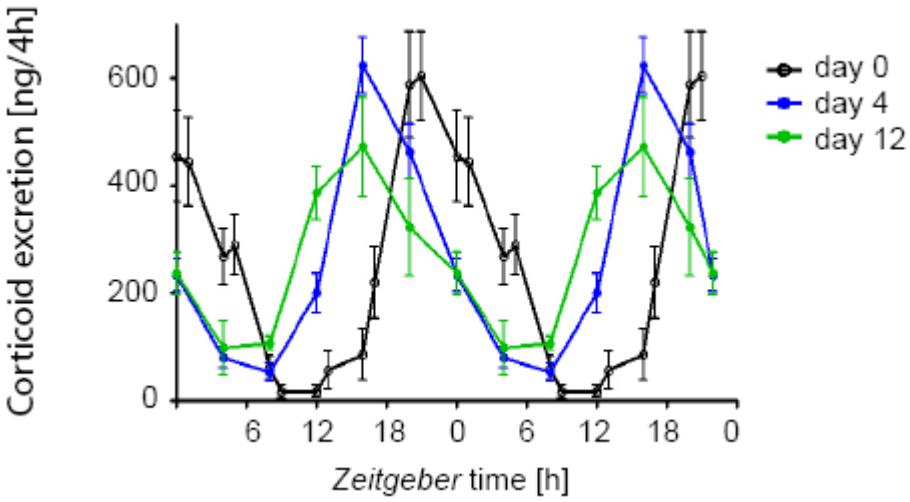


Figure 38: Wild-type fecal corticosterone excretion rhythms at different days during jet lag.

These results indicate a strong relationship between plasma ACTH, corticosterone levels in the blood and corticoid excretion in the feces. Both plasma values rise at similar times, fecal excretion parallels these rhythms, also under jet lag conditions, but with a phase delay of several hours. In conclusion, the corticoid excretion rhythm during jet lag seems to be a good output parameter for the adaptation rate of the adrenal physiology. These data is in agreement with previous findings from our group (Abraham et al. 2005).

Corticoid excretion rhythms were also measured in the auto-grafted and in all of the cross-transplanted animals during jet lag conditions (**Figure 39**).

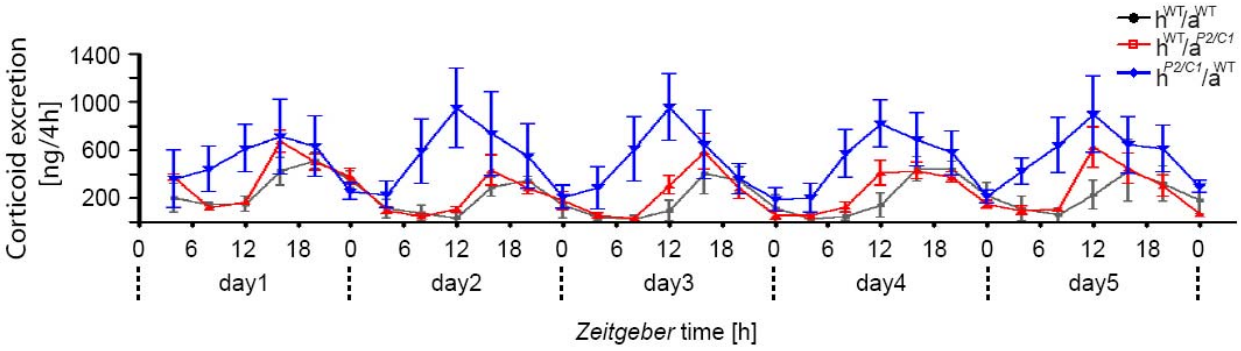


Figure 39: Fecal corticoid excretion rhythms in the different cross-genotypes at different days during jet lag.

As we showed before, wild-type animals containing mutant adrenals entrain faster to the shifted light-dark cycle (Kiessling et al., above). In animals lacking the SCN clock, but harboring a functional adrenal clock, the corticosterone peak was advanced from day 1 on during jet lag. Moreover, the excretion rhythm shifted much faster than in auto-grafted controls (by ~ 69%) (**Figure 40**).

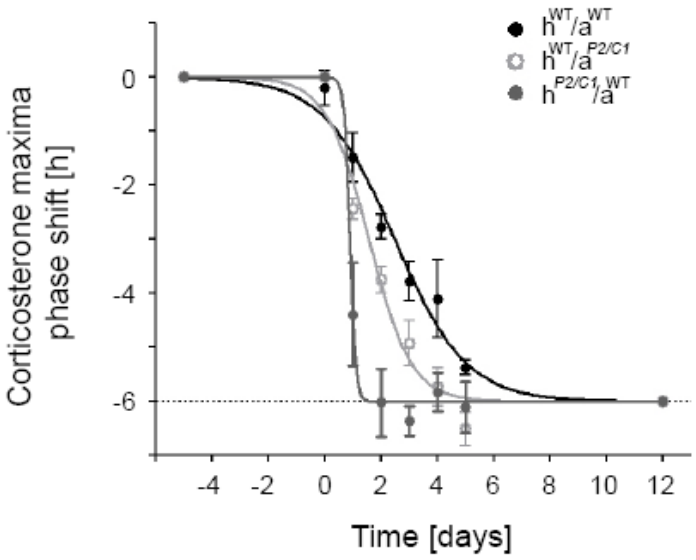


Figure 40: Resetting kinetics of corticosterone excretion maxima peak times during jet lag (average ± SEM). On average, PS_{50} values of corticosterone resetting were reduced by 63.8 % in $h^{P2/C1}/a^{WT}$ (2.49 ± 0.17 days for h^{WT}/a^{WT} and 0.90 ± 0.10 days; for $h^{P2/C1}/a^{WT}$; $p < 0.0001$; n = 5 for h^{WT}/a^{WT} and n = 7 for $h^{P2/C1}/a^{WT}$.

In contrast to $h^{WT}/a^{P2/C1}$ and h^{WT}/a^{WT} animals (Kiessling et al., above) corticosterone excretion phase shifts in $h^{P2/C1}/a^{WT}$ closely mimic those of activity onsets in these mice (compare **Figure 35** & **Figure 40**).

Chapter 3

Conclusion & Perspectives

In summary, our transplantation studies reveal that the adrenal clock can be light-entrained even in the absence of a functional SCN pacemaker. It modulates circadian corticosterone rhythms by gating the sensitivity of the adrenal to ACTH. We show in mice subjected to jet lag that the adrenal circadian clock influences the kinetics of re-entrainment via the rhythmic release of glucocorticoids (GC). Consistent with this critical role of GCs, a pre-jet lag administration of a GC synthesis blocker reset the phase of the GC rhythm and reduced the duration of the jet lag.

Most of the interpretations that arise from the work presented here have been addressed in the publications above. Therefore, in this part I will focus on some conclusions regarding the SCN-adrenal axis with a special regard to phenotypical data derived from the *Per2/Cry1* double mutants with wild-type adrenals and vice versa.

3.1. The role of the adrenal clock in the generation of GC rhythms

The question remains open, to which extend peripheral clocks contribute to the regulation of physiological circadian rhythms; whether they – in a semi-autonomous manner – control physiology of the tissue, or whether they merely translate a rhythmic signal originating from the SCN. Given the fact that circadian regulation is achieved mainly by transcriptional regulation of CCGs, it is interesting to look at the regulation of clock gene rhythms in the periphery itself. It was reported that transcriptional rhythms in transplanted *Clock* mutant fibroblasts cannot be restored by a wild-type SCN pacemaker (Pando, Morse et al. 2002). Interestingly, in the same paper the authors also showed that the period length phenotype of *Per1* mutant fibroblasts is rescued by systemic cues, indicating a dominance of the SCN over the periphery, but within certain limits. In this work we show that gene expression rhythms in *Per2/Cry1* deficient adrenals cannot be restored by the wild-type host SCN. Nevertheless, these animals still produce rhythmic (though dampened) corticosterone signals (**Figure 41, b**), illustrating that cues originating from the SCN are sufficient to drive circadian corticosterone excretion alone. Kornmann and colleagues have shown that in the liver a small number of CCGs – as well as, notably, the core clock gene *Per2* – retain rhythmicity after ablation of liver clock function. Nevertheless, the large majority of rhythmic transcripts is locally controlled and, thus, ceases to cycle when the liver clock is disrupted. (Kornmann, Schaad et al. 2007). However, after ablation of the SCN no circadian rhythm can

be detected in peripheral organs (Hara, Wan et al. 2001; Akhtar, Reddy et al. 2002), indicating that local clocks are necessary, but not sufficient to maintain transcriptional rhythms in the absence of a regular input from the SCN (**Figure 41, d**).

For the adrenal and its secretion of GCs this view is supported by a study by Son et al. (2008) that show that in a transgenic mouse that over-expresses a *Bmal1* antisense transcript in the adrenal cortex gene expression and GC production rhythms are lost (Son, Chung et al. 2008). This finding is not supported by our own experiments where GC rhythms persist in the absence of an adrenal clock (**Figure 41, b**), though with a dampened amplitude. Interestingly, Son et al also reported that CRH and ACTH secretion was not rhythmic in their mice, which was not seen in our model. It remains open why a stronger

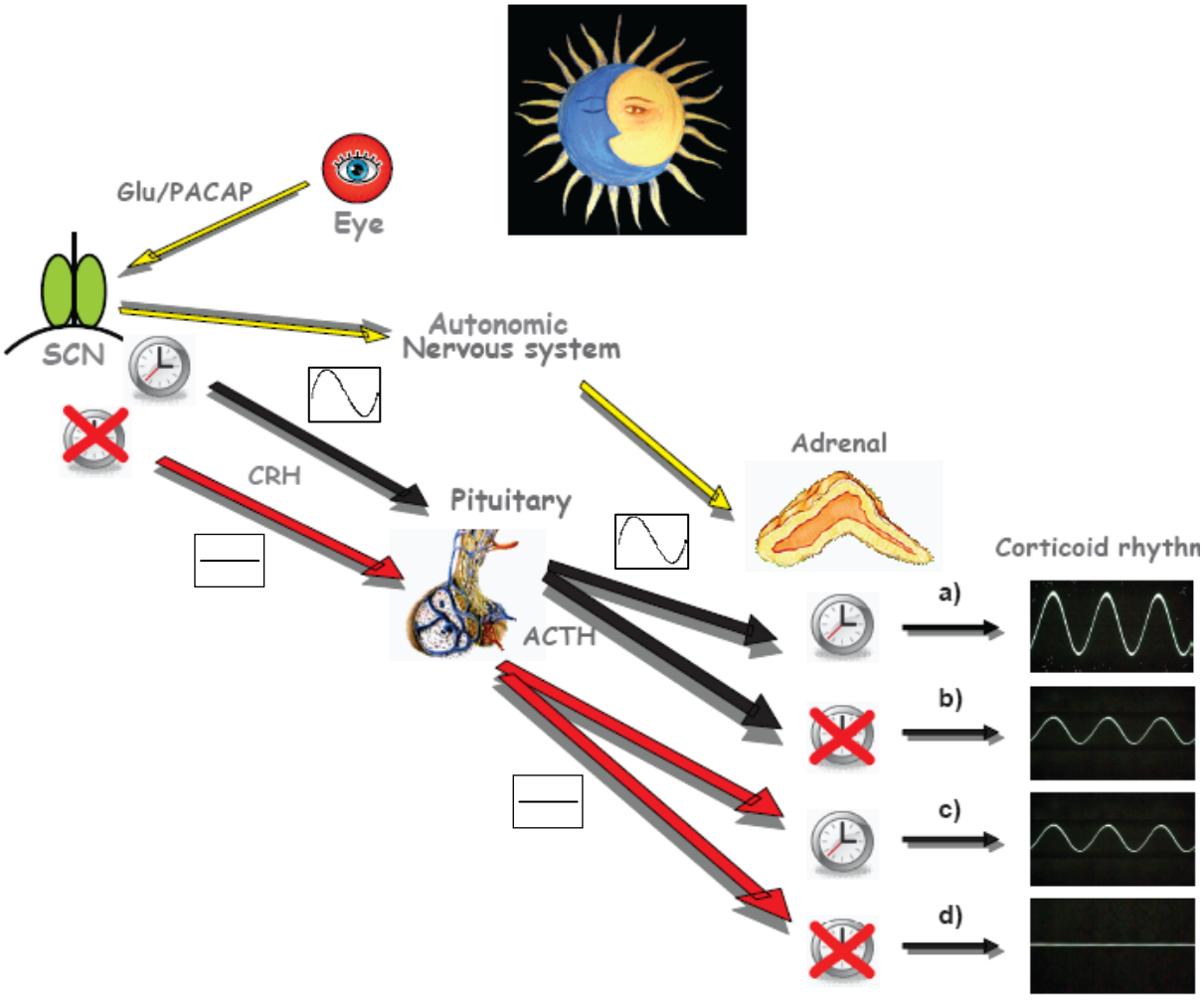


Figure 41: Role of the adrenal clock in circadian glucocorticoid release. The light information reaches the SCN via PACAP and glutamate (Glu), activates rhythmic release of CRH that evokes circadian ACTH secretion from the pituitary. ACTH in turn, regulates circadian corticoid release from the adrenal. In addition, neuronal signals generated by the SCN propagate through the autonomic nervous system to the adrenal to contribute to the circadian regulation of corticoid production. The different integrative pathways are illustrated including disrupted clocks marked by red crosses. Abbreviations: Glu, glutamate: SCN, suprachiasmatic nuclei; CRH, corticotrophin-releasing hormone; ACTH, adrenocorticotropin hormone. a) both clocks, b) the adrenal clock, c) the SCN clock or d) no clock is/are functional. For more details see text.

impact of adrenal clock function is seen after knock down of *Bmal1* than after deletion of *Per2* and *Cry1*, but more detailed studies on the physiological consequences of both manipulations will be needed to solve this puzzle.

Unfortunately, Son et al. did not perform the opposite experiment of analyzing adrenal function after disruption of the SCN clock. We demonstrated that the peripheral clock in the adrenal is sufficient to regulate corticoid rhythms in LD (**Figure 41, c**) (see Oster et al., (Oster, Damerow et al. 2006) above), but gene expression as well as corticoid excretion rhythms were completely abolished when animals were released into constant conditions (DD). This indicates that in vivo the adrenal clock can sustain its rhythm only in the presence of a *Zeitgeber* signal, which, like light, does not need to originate from the SCN itself. Without this input a wild-type graft cannot sustain rhythmicity. We do not know if this mechanism of entrainment is special for adrenal oscillators and corticoid production, or if this finding can be extended to other peripheral oscillators. Some data suggest that the neuronal light input to this gland (Ishida, Mutoh et al. 2005) plays an important role in this context.

3.2. Photic entrainment of peripheral oscillators

In contrast to our findings in the transplantation model, explant cultures of different peripheral tissues show rhythmic clock gene expression for several weeks in culture (Welsh, Yoo et al. 2004). Persistent rhythmicity in GC secretion was also found in adrenal explants (Andrews and Folk 1964; Andrews 1971). What could be the reasons for the rapid deterioration of peripheral rhythms after withdrawal of an external *Zeitgeber* in vivo? The current theory states that in vitro a lack of coupling between different cells in the culture (e.g. different adrenocortical cells) leads to a gradual loss of rhythmicity due to an increasing averaging of phases, resulting from small individual differences in periodicity between cells. In the intact organism, however, cells are never uncoupled, but have to respond to various endocrine, metabolic and neuronal stimuli, many of which have the potential to reset clock rhythms. In *Per2/Cry1* deficient hosts the cells of a wild-type adrenal graft will be exposed to a plethora of such – non-circadian – signals, released by the other tissues and likely counteracting with rhythm generation in the transplant. Only by the help of the strong photic *Zeitgeber* under LD conditions, adrenal clocks become resistant to this kind of noise. In constant darkness, this dominance is lost and the adrenal clock succumbs to these irregular or ultradian influences. In explant cultures such disrupting factors are excluded, hence rhythm sustainability is more stable than in vivo.

Ishida et al. (Ishida, Mutoh et al. 2005) showed that *Per* gene expression and corticosterone release from the adrenal can be directly activated by a short nocturnal light pulse. This effect is similar, though less efficient, to what is observed at the level of the SCN

and hence may explain why the adrenal can be directly entrained by light. Photic input requires functional nerve connections from the brain, via the SCN. Also, the sensitivity of the adrenal to ACTH stimulation, and hence its circadian gating function, is known to be regulated via the splanchnic nerve (Jasper and Engeland 1994; Jasper and Engeland 1997; Ulrich-Lai, Arnhold et al. 2005). Therefore, it is essential that in the adrenal graft neuronal connections are restored after transplantation. We have shown that neuronal markers are expressed after some time in adrenal grafts and re-innervation of adrenal grafts has also been reported in rats (Ulrich-Lai and Engeland 2000). Our data indicate that graft re-innervation is sufficient to light-entrain transplant clocks, even if the functionality of the connection was not tested.

Interestingly, when measuring re-entrainment rates of grafted wild-type mice at different times after surgery, only after 2 months of recovery the genotype of the graft showed a significant effect. This might indicate that functional re-innervation – and, thus, normal re-entrainment of (wild-type) graft clocks – would need a longer time to complete than was previously estimated (Ulrich-Lai and Engeland 2000; Oster, Damerow et al. 2006). To prove this, it would be important to better quantify the functionality of such innervations, e.g. by a light pulse protocol and measurement of either *Per* gene induction or GC responses and comparison with non-operated animals (Shigeyoshi, Taguchi et al. 1997; Ishida, Mutoh et al. 2005). Of note, age proofed to be an important factor regulating jetlag re-entrainment. In older mice, adaptation rates were slower than in younger animals of the same genotype. Because such age-related effects were observed in all genotypes, regardless of whether the animals underwent surgery or not, we cannot completely exclude an overlapping of age and recovery-related effects. More sophisticated tools would be needed to address this problem, such as an inducible tissue specific adrenal clock knockout in wild-type or an inducible rescue of adrenal clock function in arrhythmic mice.

3.3. Stabilizing feedback from the adrenal to the SCN

Some of the behavioral data of this work suggest a feedback from the adrenal (clock) to the SCN pacemaker, such as a shortened free-running period in LL after disruption of the adrenal clock, entrainment bouts in mutant animals with a functional adrenal clock. In line with this, Son et al. reported that an adrenal specific knock-down of *Bmal1* attenuates overall activity levels and circadian profiles in DD conditions (Son, Chung et al. 2008). However, we could not confirm this specific observation in our animal model: a *Per2/Cry1* deficient adrenal clock in wild-type hosts did not significantly affect the activity pattern in constant darkness. However, in the "rescue experiment" with the other cross-genotype combination (wild-type graft in a mutant host) we observe marked effects of adrenal

genotype on activity patterns. Unoperated *Per2/Cry1* double mutant animals show rhythmic activity in LD (a clock-independent, directly induced negative masking effect that has similarly been observed in various other "arrhythmic" clock gene mutants (Vitaterna, King et al. 1994; van der Horst, Muijtjens et al. 1999; Bunger, Wilsbacher et al. 2000; Bae, Jin et al. 2001; Zheng, Albrecht et al. 2001), whereas when released in constant darkness they become immediately arrhythmic (Oster, Yasui et al. 2002). By contrast, the activity distribution of mutant hosts carrying wild-type grafts was altered in LD. These mice show elevated activity at the end of their rest period and – at least in some animals – indications of a re-entrainment process after transition from LL to LD, which would not be expected from a mouse with a complete deletion of circadian clock function. The most likely explanation would be a feedback of adrenal clock rhythms on SCN function and, thus, the regulation of rest/activity rhythms (**Figure 42**) Supporting this, animals harboring a defective adrenal clock show a different regulation of period length under LL conditions. It seems possible that this might also be caused by an altered feedback from the adrenal clock.

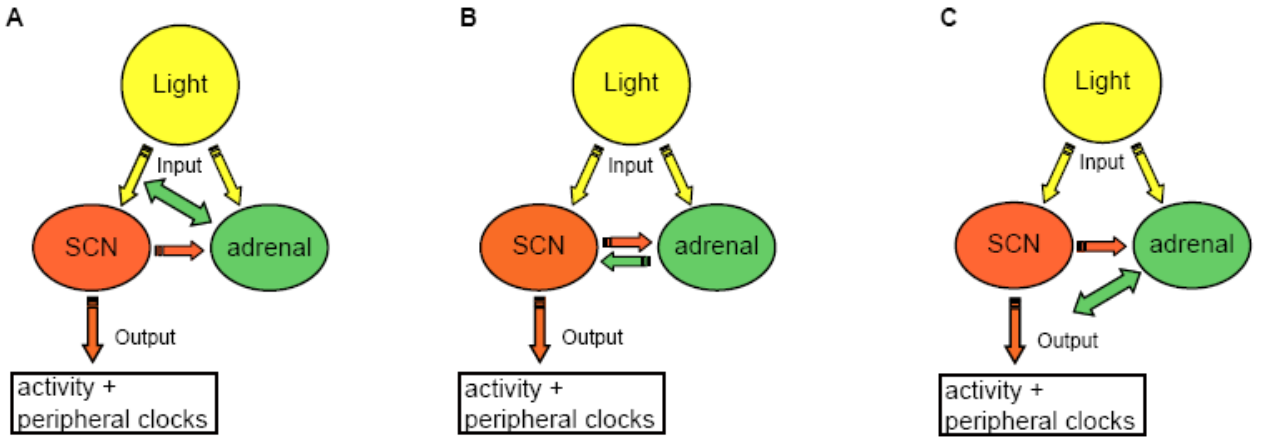


Figure 42: Possible pathways from the adrenal affecting SCN-controlled activity behavior. (A) The adrenal clock affects either the input pathway to the SCN (A), the SCN directly (B) or the output of the SCN (C).

However, circadian rhythmicity in *Per2/Cry1* deficient animals can be restored, if kept in constant light of high light intensities (from 200 Lux on upwards), which is also reflected in the corticosterone rhythm (Abraham, Dallmann et al. 2006). This indicates that in *Per2/Cry1* mice functional clockwork is still present, but instable and might be strengthened by the wild-type adrenal. Comparing the irradiation threshold of rhythmicity in unoperated *Per2/Cry1* and in *Per2/Cry1* hosts/wild-type adrenal graft animals would be a suitable experiment to quantify the "rhythmicising" influence of the adrenal clock.

If one assumes that such a feedback exists (**Figure 42**), it would be interesting to explore the CNS targets of adrenal timing signals. Schibler and co-workers (Schibler, Ripperger et al. 2003) have postulated that the SCN might not respond to GCs directly

(because of its lack of GCR expression)(**Figure 42B**). However, several other nuclei within the hypothalamus are sensitive to GCs and have neuronal connections to the SCN (see also discussion in Kiessling et al., above). Even though this work focuses on GCs, it should also be noted that mineral corticoid and catecholamine secretion from the adrenal also follow a circadian pattern and, hence, these hormones might also carry time information from the adrenal to the SCN pacemaker. Our transplantation approach is not selective enough to distinguish between these factors, but with the right *Cre*-driver lines it might in the future be possible to gentically dissect clock function in the adrenal and to separately analyze the role of the different endocrine factors released from this gland. What would be the function of an adrenal (peripheral) feedback on SCN function? This will be clearer when adrenal-to-SCN communication is viewed in the context of jetlag.

3.4. The adrenal clock has an impact on resetting

A resetting phenotype was observed in wild-type mice lacking a functional adrenal clock. These animals show faster re-entrainment of running wheel behavior during jet lag, suggesting that the wild-type adrenal stabilizes the phase of the circadian system against external time cues (in this case, light). Of note, although the adrenal clock seems important for the regulation of re-entrainment, it is itself not sufficient to drive normal resetting and rhythmicity under stable state conditions. Thus, even though this work argues for a clear importance of the adrenal clock (e.g. in comparison with other peripheral oscillators), it cannot be put on the same level as the SCN pacemaker.

Accelerated entrainment was also observed in mutant hosts carrying wild-type adrenals. Interestingly, these animals entrain slower than unoperated mutants. Since we have shown that entrainment speed depends on GC rhythms, one might argue that the residual GC rhythm of these animals (in LD) might suffice to upkeep a certain amount of SCN feedback. A significant phase advance of the GC rhythm could not be confirmed in these animals (maybe also due to masking), but GC excretion levels are reduced at the end of the night, closely mimicking the activity profiles. In comparison with auto-grafted wild-type controls, advanced GC and activity rhythms were strongly enhanced during transient days of jet lag (see Additional data), supporting the hypothesis that GC rhythms and those of activity are functionally coupled.

A feedback loop between the adrenal and the SCN clock along multiple pathways, such as the HPA axis, the autonomic nervous system, endocrine parameters (e.g. GCs) and additional levels of regulation, enable integration of the metabolic status of the organ with environmental cues. Simultaneously, the timing system becomes more resistant to noise, like rapid changing environmental cues, but at the same time allows responding to important

environmental signals, such as stressors. In our view, peripheral clocks might contact hypothalamic centres, including the SCN, to maintain and stabilize a balance between body and brain. We favor a model, where the ability to adjust to new LD cycles is regulated by both, the central clock and the adrenal clock. If one of these oscillators is disrupted, resetting is accelerated. This does not necessarily mean that both oscillators have the same impact on resetting speed. As indicated by our transplantation model, the SCN seems to drive the resetting, while the adrenal rather modulates it.

3.5. Outlook: adrenal corticoids and other clocks

From our data it seems clear that adrenal GC rhythms affect those of activity, e.g. during jet lag. It remains to be shown, however, how exactly this impact is mediated and whether it involves other circadian clocks - in the periphery or in the brain. Earlier work using GC analogue injections in mice have clearly shown that GCs have the potential to reset other clocks in vivo (Balsalobre, Brown et al. 2000), but is the adrenal able to drive circadian oscillation in other peripheral oscillators? Clock gene expression in the kidney of arrhythmic mice carrying wild-type adrenals was not restored by the graft. This would suggest that rhythmic corticosterone per se is not sufficient to re-establish rhythmicity in a mutant kidney clock. This, however, could also be a threshold effect based on a not fully functional GC rhythm in grafted adrenal glands. Moreover, other tissues were not tested. As discussed in the beginning, the transplantation model has strong advantages over in-vitro studies, but is possibly limited by the functionality of the graft after re-innervation. Modern techniques allow genetic disruption and rescue of one or more clocks at the same time or even in an inducible fashion. For example, a tissue specific rescue of the adrenal and the liver clock in an otherwise arrhythmic host would allow to directly test for a link between two peripheral clocks, but also to look at the impact of the SCN on such peripheral connections. On the other hand the question still remains which CNS areas are involved in a putative adrenal feedback pathway. Using mini-pumps, releasing rhythmic but inverted corticosterone in adrenal-ectomized animals might allow detection of these specific brain areas which should then express inverted target gene transcription rhythms. Tissue specific brain ablation, e.g. by lesion, would allow to illustrate the pathways involved in the adrenal feedback. Altered behavioral resetting, complementary to our transplantation model, might indicate the specific impact of the destroyed area. Since disrupted GC feedback can contribute to neurophysiological disorders, such as major suppression (which is also an important hallmark of jet lag), the identification of GC rhythm targets in the brain might offer new therapeutic approaches to jet lag associated syndroms (Arendt and Marks 1982).

Chapter 4

Material and methods

4.1 Animal handling and sample collection

All animal experiments were done with prior permission by the Office of Consumer Protection and Food Safety of the State of Lower Saxony and in accordance with the German Law of Animal Welfare.

4.1.1. Mouse strains

For all experiments male wild-type (C57BL/6J x 129/SvHsD mixed background) and homozygous *Per2/Cry1* double (*Per2*^{tm1Brd} (Zheng, Larkin et al. 1999) and *Cry1*^{tm1Jhjh} (van der Horst, Muijtjens et al. 1999), Figure 43) mutant mice of 2-3 months of age were used as described (Oster, Yasui et al. 2002).

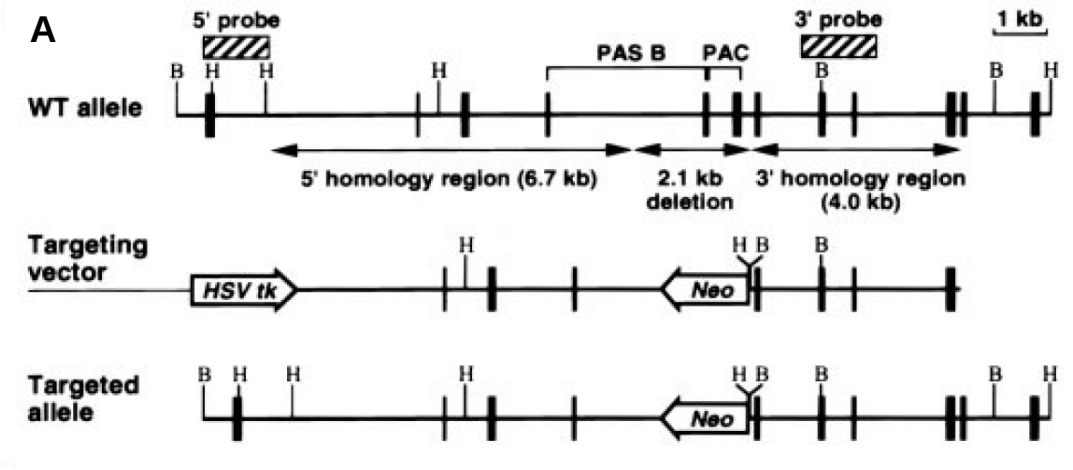


Figure 43: Targeted disruption of the *Per2* and *Cry1* genes in the mouse. **(A)** Genomic structure of a small part of the mouse *mPer2* gene, the targeting vector and the predicted structure of the targeted allele (from (Zheng, Larkin et al. 1999)). H, *Hin*dIII; B, *Bam* HI; *Neo*, neomycin resistance gene. **(B)** Targeted disruption of the C*ry1* gene and generation of *Cry*-deficient mice. Physical map of the wild-type *Cry1* locus, the targeting construct and the disrupted *Cry1* locus (from (van der Horst, Muijtjens et al. 1999)). Exons are indicated by black filled boxes. Gray boxes represent the probes used for screening of homologous recombinants and genotyping mice, localized external to the construct. Primers used for RNA analysis by RT- PCR are depicted as black arrowheads.

4.2. Animal experiments

4.2.1. Activity monitoring

During experiments animals were housed individually in transparent plastic cages (Tecniplast 1155M) that were equipped with a steel running wheel of 115 mm in diameter (Trixie 6083, Trixie GmbH, Germany). The axis of the wheel was equipped with a plastic disc holding a small magnet (article number 34.6401300702, Fehrenkemper Magnetsysteme, Germany). The magnet opens and closes a magnetic switch (Reed-Relais 60, Conrad Electronic, Germany) upon rotation of the running wheel. The switch was connected to a computer that counts the revolutions of the wheel (using the Activity Counting System Program, ClockLab, Actimetrics, Austin St. Evanston, USA). 12 cages of this type were placed in one isolation cabinet (custom made, length = 180cm; height = 54cm; depth = 69cm). Mice used for activity monitoring were generally 8 to 14 weeks old males (except stated otherwise). A number of wild-type controls were included in each isolation chamber. Animals were provided food and water *ad libitum* at all times. Cages were changed every two weeks in the middle of the activity phase to minimize phase shifts induced by a new environment (Mrosovsky, 1996). After cage changes animals were left alone for at least 4 days before new experiments were started. During constant conditions cages were never changed in phases used for period calculations. Running data analysis was performed using the ClockLab analysis software plug-in (Actimetrics) for MatLab (The Mathworks).

4.2.1.1. Light/dark (LD) cycle entrainment

Animals were kept in a 12h light (340-360 Lux bright white light)/ 12h dark cycle ("Lights on" = ZT0 at 7:00 in summer and 6:00 in winter; "Lights off" = ZT12 at 19:00 and 18:00 respectively) for 2 to 3 weeks (LD12:12). After 1 week of training activity profiles were taken for characterizing the entrainment of the animals.

We studied the following criteria:

i) Activity onsets: A minimum of 100 wheel revolutions per 5min bin after a minimum of 240min of rest. The average was taken for 14 consecutive days after 2 weeks of adaptation.

ii) Overall activity: average number of wheel evolutions and approximate covered distance in 24h

iii) Night time activity: like ii), but only during the dark phase (ZT12 – ZT 24)

iv) Day time activity: like ii), but only during the light phase (ZT0 – ZT 12)

a. 6h - Daytime activity: like ii), but only during the light phase (ZT6 – 12)
b. 3h - Daytime activity: like ii), but only during the light phase (ZT0 – 12)

4.2.1.2. Free-running in constant darkness

After entrainment to an LD12:12 cycle for at least 14 days lights were not turned on again at the following morning and animals were kept in constant darkness (DD) for two weeks. We studied the internal period (τ), the length of a subjective day determined by the time points of activity onset on two consecutive days. The average was taken from at least 4 consecutive days of stable rhythmicity in DD. Wheel-running activity was recorded, displayed and analyzed using ClockLab software (Actimetrics). Chi-square periodogram analysis of overall (and in addition individual defined-day segments of) running-wheel activity was used to determine behavioral rhythmicity. Animals were judged to be arrhythmic when the p-value for an individual segment of activity was below the confidence-interval, set to 0.001. When they exhibited multiple period peaks that undershot the p-value threshold, these animals were judged to be rhythmic, even if not predominantly circadian (defined as a rhythm with a period length between 20 and 30 hrs).

4.2.1.3. Free-running in constant light

Activity monitoring in constant light (LL) was performed like described for DD. In these experiments light intensities of 340-360 Lux were applied. The isolation boxes were equipped with dimmable halogen lamps (four for each cage). Light intensity was measured with a Luxmeter (Testo, Germany) and averaged for all cages (deviations were less than 5% in all cases).

4.2.1.4. Shifted LD cycles – jet lag

After entrainment to an LD12:12 cycle for at least 14 days lights were not turned off at 19:00/18:00 the next day, but 6h earlier (13:00 / 12:00). From there a shifted light cycle was presented to the animals ("Lights on" = ZT0 at 01:00 in summer and 00:00 in winter; "Lights off" = ZT12 at 07:00 and 06:00 respectively). We measured the number of days needed by an animal to adapt to the new LD cycle by determining the onsets of activity after the LD shift. After at least another 14 days the experiment was repeated. Shifts were always done with a short light phase at the transition day, e.g. "lights on" at 06:00 / 07:00 and "new lights off" at 12:00 / 13:00 at the same day. Individual activity onsets before and after the shift were determined by using the ClockLab software (Actimetrics, minimum of 100 wheel revolutions per 5min bin after a minimum of 120min of rest), corrected by visual inspection and averaged over the whole cohort to asses adaptation rates.

4.2.2. Adrenal transplantation

Transplantations of adrenal fragments were performed as described (Musholt, Klebs et al. 2002; Oster, Damerow et al. 2006) with minimal changes. Male wild-type and *Per2/Cry1* double mutant mice at the age of 6-8 weeks were anesthetized by i.p. injection of 100/10mg/kg Ketamin/Xylazin. Blood vessel connections were cut and closed by electrocoagulation and both adrenal glands were removed following median laparotomy. After removal of adjacent fat, each gland was fragmented into four pieces. Fragmented adrenal glands of *Per2/Cry1* double mutant mice were transplanted underneath the kidney capsule of adrenalectomized wild-type host animals and *vice versa*. The capsule of the host's kidney was lifted with forceps and incised. The fragmented adrenal gland was pushed underneath the capsule up to the pole of the kidney, and the tissue fragments of the second gland of the donor animal were brought to the upper pole of the other kidney. Both adrenals were transplanted into a single host. The incision was closed by electro-coagulation. To control for the transplantation procedure, wild-type animals received their own wild-type adrenal transplants following adrenal-ectomy. After the surgery, animals recovered for twelve weeks under standard LD conditions to ensure complete re-innervations of the transplanted tissues (Ulrich-Lai and Engeland 2000).

4.2.3. Hormone measurements and pharmacological treatments

Fecal samples were collected at 4 hrs intervals for at least 24 hrs in LD and a second cohort in DD, starting time at ZT/CT10. For analysis of corticosterone excretion during jet lag, samples were collected before (day -5 and day 0) and following the jet lag treatment (day 1 – 5 and day 12). For all experiments wheel cages equipped with wire grid floors were used (1cm mesh size, custom made). To rule out stress induced effects, animals were transferred to the collection cages 3 days prior to the first sampling interval. Paper towels were placed under the cage of each mouse and removed every 4 hrs. The fecal samples were then immediately stored at -80° C in 2ml reaction tubes (Eppendorf, Hamburg, Germany).

4.2.3.1. Corticosterone extraction from feces

Fecal samples were dried overnight in a vacuum centrifuge (Concentrator 5301, Eppendorf) for at least 16 hrs, weighed and powderized using the Precellys homogenizator system (Precellys 24 lysis & homogenizator, peQLAB, Erlangen, Germany). Dried fecal samples were kept in special reinforced tubes prefilled with ceramic beads with a diameter of 2.8 mm (Precellys Ceramic Kit 2.8 mm, reinforced, peQLAB). Theses tubes were shaken 4 times under the hood with 6800 rpm shaking speed for 30 sec sections with 30 sec breaks

between each session. The powder of each sample was rinsed with 100% ethanol and transferred to 15ml polypropylene tubes (to avoid spillage during boiling) with plug seal caps (430052, Corning, NY, USA). 100% ethanol was added to 10ml and the suspension was boiled in a water bath for 20 minutes. Solids were spun down for 15 minutes at 900rpm x g at room temperature (RT) (Labofuge 400R, Heraeus Instruments, Hanau, Germany). Supernatants were transferred to another 15ml tube (Corning) while the pellets were re-suspended with 100% ethanol (5ml), vortexed for exactly 1 minute and spun a second time at the same conditions. The pellet was discarded and both supernatants were combined, dried in a Petri dish (Ø 8 cm) while stored in an incubator at 55° C for about 2h, until all liquid has evaporated. By usage of a cell lifter (costar 3008, Corning, NY, USA) the dried fecal extracts were solved in 1ml 100% methanol and stored at -80° C before use.

4.2.3.2. Corticosterone and ACTH extraction from plasma

For blood corticosterone and ACTH determination, animals were sacrificed by cervical dislocation and plasma extraction was performed using Microvette CB300 LH tubes (Sarstedt, Nürnbrecht, Germany). The capillary was filled with 300µl blood taken from the trunk and then centrifuged for 5–10 minutes at max. 1000rpm x g. Supernatant plasma was kept at -80° C until usage.

4.2.3.3. Quantification of hormone metabolites

Fecal and blood corticoid metabolite quantification was done using the ImmuChem™ Double Antibody- Corticosterone-^{125}I Radioimmunoassay Kit (MP Biomedicals, Orangeburg, NY) as described below (4.5.1.1.). Quantification of blood ACTH was done by immunoradiometric assay (ACTH IRMA; CatNo. 13002352 130-Kit, DiaSorin, Kiel-Wellsee, Germany) as described below (4.5.1.2.).

4.2.4. Pharmacological treatments

Administration of metyrapone (MET, Alliance UK) to wild-type mice was done for 16 days prior to the jet lag procedure (4.2.1.4.). One group of animals (MET_i) was injected i.p. at ZT2 - 4 with MET dissolved in water (200 mg/kg body weight per day). Injections were stopped one day before the LD shift (day -1). A second group of animals (MET_d) received MET with their drinking water for 16 days before and during the whole jet lag period. Four groups of 3 to 4 MET_d animals each received different MET dosages (0.6g/l, 1.0g/l, 1.4g/l and 1.8g/l per day). At day 16 of the treatment (that is one day before the jet lag) and at day 4 thereafter fecal samples were collected to analyze the phase shift of corticoid excretion rhythms (see

Figure 5, Kiessling et al., above). Untreated sham wild-type littermates were used as controls for all experiments

4.2.5. Tissue collection

Animals were sacrificed at indicated time points by cervical dislocation. Eyes were removed prior to tissue dissection under a 15 W safety red light to prevent acute light effects on clock gene expression (Albrecht, Sun et al. 1997). Tissue samples were dissected at the fifth day before jet lag (day -5), immediately before jet lag (day 0), during jet lag (day2, 3, 4, 8) and after completion (day 12). Tissue collection was performed every 4 hrs, starting 2 hrs before light offset (ZT10) and finishing after 24 hrs. Tissues samples were transferred into RNAlater (Ambion, Foster City, USA; ~1ml per 0.25g of tissue) and stored on ice for immediate usage, or at -80° C for later RNA extraction.

4.3. Molecular biological methods

4.3.1. Genotyping

Tail tips (~1cm) taken at the age of weaning (3-4 weeks) were used as samples. Tips were digested in a 2ml (Eppendorf) tube overnight at 55° C in 500µl of 100mM Tris/HCl, 5mM EDTA, 200mM NaCl, 0.2% SDS, 100mg/ml Proteinase K (pH 8.5).

4.3.1.1. DNA extraction

Genomic DNA was precipitated with 100% Ethanol. The DNA cloud was transferred to a new tube using a pipette tip and washed once with 70% Ethanol and 100% Ethanol before drying. The pellet was then dissolved in 50ml TE buffer (10mM Tris/ HCl, 1mM EDTA, pH 7.5) and stored at 4° C.

4.3.1.2. Polymerase chain reaction (PCR) protocols

Per2: Reaction components for 25µl total volume:

- 2,5 µl 10 x Ammonium Buffer (Ampliqon, Hamburg, Germany)
- 1 µl dNTPs [10 mM]
- 2 µl Primer1 [10 pM/µl]
- 1 µl Primer2 [10 pM/µl]
- 1 µl Primer3 [10 pM/µl]
- 15 µl DEPC H_2O
- 1 µl MgCl2 [25 mM/µl]
- 0,5 µl Taq (Ampliqon, Hamburg, Germany)
- 1 µl tail DNA (80-100ng)

Cycling conditions:
- 94° C 3min
- 94° C 30sec
- 61° C 30sec -0.5° C per cycle
- 72° C 1min Go to step 2, 38 times
- 72° C 10min 4° C
- Break

Primer sequences:
Primer1 (WT-rev): 5´- GAA CAC ATC CTC ATT CAA AGG -3´
Primer2 (WT-fw): 5´- GCT GGT CCA GCT TCA TCA ACC -3´
Primer3: (mut-NEO) 5´- CGC ATG CTC CAG ACT GCC TTG -3´ (neo-generic)
wt +/+ 381 bp Primer: 1 and 2 & mut -/- 120 bp Primer: 1 and 3

Cry1: Reaction components for 25µl total volume:

Cry1 wt:
- 2,5 µl 10x Ammonium buffer
- 1 µl dNTP [10 mM]
- 1 µl Primer1 [10 pM/µl]:
- 1 µl Primer2 [10 pM/µl]:
- 1 µl MgCl2 [25 mM/µl]
- 17 µl DEPC H_2O
- 0,5 µl Taq (Ampliqon)
- 1 µl tail DNA (100ng)

Cry1 mut:
- 2,5 µl 10x Ammonium buffer
- 1 µl dNTP [10 mM]
- 1 µl Primer1 [10 pM /µl]
- 1 µl Primer3 [10 pM /µl]
- 1 µl MgCl2 [25 mM /µl]
- 17 µl DEPC H_2O
- 0,5 µl Taq (Ampliqon)
- 1 µl tail DNA (100ng)

Cycling conditions:
- 95° C 3min
- 95° C 30sec
- 62° C 30sec -0.5° C per cycle
- 72° C 4min Go to step 2, 38 times
- 72° C 7min4° C break

Primer sequences:
Primer1 (WT-fw): 5´- GCA TGA CCC CTC TGT CTG AT -3´
Primer2 (WT-rev): 5´- TTC TTG TCC CAA GGG ATC TG -3´
Primer3 (mut-NEO): 5´- CGC ATC GCC TTC TAT CGC CT -3´ (neo-generic)
wt +/+ 2.2 kp Primer: 1 and 2 & mut -/- 2.2 kp Primer: 1 and 3

4.3.2. RNA extraction and RNA purification

Collected frozen tissues (as described in *4.2.5.*) were thawed and homogenized using reinforced tubes prefilled with ceramic beads with a diameter of 2.8 mm (Precellys Ceramic Kit 2.8 mm, reinforced, peQLAB) for the Precellys system (Precellys 24 lysis & homogenizator, peQLAB). The homogenizer was used with the following settings: 6800 rpm shaking speed, 3 times repetition of 30 sec sections with 30 sec breaks between each session. Afterwards suspensions were centrifuged (15 min, 13k rpm, 4° C) and supernatants

were transferred to fresh tubes and stored at -80° C until RNA purification. RNA isolation was performed with RNeasy Micro Kit (Qiagen) for SCN and adrenals and with the RNeasy Mini Kit (Qiagen) for liver, kidney and pancreas according to the manufacturer's protocols. Total RNA pellets were stored at –80° C.

4.3.3. cDNA synthesis

cDNA was synthesized from RNA samples (*4.3.2.*) using Thermoscript RT – PCR Kit (Gibco-Invitrogen, Paisley, United Kingdom). Reaction setup:

4.3.3.1. RNA denaturation:

- 1 µl Oligo (dT) [50 µM/µl]
- 2 µl Primer2 [1 µg/µl]
- 7 µl DEPC H_2O

Denature for 5 minutes at 65° C then place on ice.

4.3.3.2. Reverse transcription reaction-mix:

- 4 µl 10 x Buffer
- 1 µl DTT [0.1 M/µl]
- 2 µl DNTPs [10 mM/µl]
- 1 µl DEPC H_2O
- 1 µl RNaseOut
- 1 µl Thermoscript Enzyme

Add 10 µl cDNA synthesis mix for each 10 µl denaturation mix. Incubate at 53° C for 60 minutes, then 85° C for 5 minutes.

4.3.3.3. RNA template removal

Add 1 µl RNase H for each 20 µl reaction. Incubate for 20 minutes at 37° C. Keep on ice and use immediately or store at -80° C untill needed.

4.3.4. Quantitative real-time PCR (qPCR).

Quantitative real-time PCR (qPCR) was performed with a iCycler thermocycler ((Bio-Rad, Hercules, California) with iQ-SYBR Green Supermix (Bio-Rad) according to the manufacturer´s protocol. Primer sequences are detailed in Table 1. Standard curve efficiency estimation and relative quantification of expression levels were performed as described in section 4.3.4.2-4.

4.3.4.1. Primer sequences

Table 3: Primer pairs used for the quantification of clock gene mRNAs, product size and Entrez Gene ID.

Gene	DNA-Sequenz	length (bp) [Bp]	GenBank ID
Per1 RT fw	5'-TGGCTCAAGTGGCAATGAGTC-3'	247	NM_011065
Per1 RT rev	5'-GGCTCGAGCTGACTGTTCACT-3'		
Per2 RT fw	5'-GCCAAGTTTGTGGAGTTCCTG-3'	226	NM_011066
Per2 RT rev	5'-CTTGCACCTTGACCAGGTAGG-3'		
Bmal1 RT fw	5'-CCTAATTCTCAGGGCAGCAGAT-3'	239	NM_007489
Bmal1 RT rev	5'-TCCAGTCTTGGCATCAATGAGT-3'		

Rev-α RT fw	5'-AGCTCAACTCCCTGGCACTTAC-3'	266	NM_145434
Rev-α RT rev	5'-CTTCTCGGAATGCATGTTGTTC-3'		
Dbp RT fw	5'-AATGACCTTTGAACCTGATCCCGCT-3'	184	NM_016974
Dbp RT rev	5'-GCTCCAGTACTTCTCATCCTTCTGT-3'		

Primer pairs (**Table 3**) were designed with *Primer3* (*http://frodo.wi.mit.edu/cgibin/primer3/ primer3www.cgi*) using cDNA sequences from NCBI Entrez Gene (*www.ncbi.nlm.nih.gov*). Amplicon size was chosen around 200bp. To avoid possible cross reactions on homology sides, primer gene-specificity was checked with *BLASTn* (*www.ncbi.nlm.nih.gov/blast*). Primer pairs were synthesized by *MWG* and best annealing temperatures were tested by gradient PCR with a cDNA pool of all time points examined (ZT 2, 6, 10, 14, 18, 22).

4.3.4.2. Standard curve efficiency estimation

To determine the amplification efficiency for each primer set, a standard curve was generated with a cDNA dilution series of 1:10, 1:40, 1:160, 1:640 and 1:2560 and fitted by linear regression. Data analysis was performed with the iCycler software, assessing the amplification efficiency as the exponent of the base, indicating the amount of cycles needed for doubling the amount of cDNA synthesis (in the ideal case 1 = doubling the amount of cDNA each cycle: E = 100%). For direct comparison of each qPCR with each primer set, these efficiency values are needed.

4.3.4.3. Assay protocoll

Each well of the 96-well plate contained 10 µl DNA Master SYBR Green I mix (BioRad), 5 µl 1:40 diluted cDNA and 5 µl primer-mix (1.4 µM). Each 96-well plate was used for one gene and one standard-curve (dilution as detailed above) of this gene with triplicates for each well. For a daytime profile cDNAs of 3 different animals from 6 time points were measured (ZT2, 6, 10, 14, 18 and 22). Amplification program of the iCycler:

- 94° C for 15sec
- 60° C for 15sec
- 72° C for 20sec Go to step 1, 50 times

As control for specificity of each primer sets a melt curve was determined by heating up of the PCR products to 90° C with 0.5° C / 1 sec. *Ef1α* was used for normalization of the cDNA concentrations.

4.3.4.4. Data analysis

The iCycler software (BioRad) automatically analyzed the CT- values (threshold cycling numbers) for each well located above a pre-defined threshold line. These CT- values depict the cycle number at which the SYBR green fluorescence value reaches a specific threshold, set to a defined position above the background levels for each plate setup. Relative

quantification of expression levels by a modified ΔΔCT calculation was performed as described (Pfaffl 2001). Expression values of all days during jet lag were normalized to the averaged expression at day 0 before jet lag.

4.4. Histological methods

4.4.1. Radioactive in situ hybridization (ISH)

In situ hybridisation was performed as described by Albrecht and colleagues (Albrecht et al. 1998).

4.4.4.1. cDNA templates

The probes for *mPer1* and *mPer2* were as described (Albrecht, Sun et al. 1997). The *mPer1* probe corresponds to nucleotides 1 to 619 (GenBank accession number AF022992). The *mPer2* probe was made from a cDNA corresponding to nucleotides 229 to 768 of GenBank AF036893). The *mPer2* probe is located outside the region deleted in the mutant. The *Dbp* probe was as described (Oster, van der Horst et al. 2003), made from a cDNA corresponding to nucleotides 2 to 951 of GenBank NM016974. The *Bmal1* probe was as described (Oster, Yasui et al. 2002) corresponding to nucleotides 654 to 1290 (AP015953). The *Rev-Erbα* probe was made from a cDNA corresponding to nucleotides 1211 to 2151 of GeneBank NM145434. PCR products had already been cloned into pCR II Topo vector using TOPO TA Cloning Kit (Invitrogen) (Oster, Yasui et al. 2002; Oster, van der Horst et al. 2003). The vector containing *Per1, Per2*, *Dbp or Bmal1* was then used to propagate single clones in LB medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) sodium Chloride, 1mg/ml ampicillin). Maxi preparation of plasmid DNA was performed using QIAfilter Plasmid Maxi Kit (Qiagen). Linearization of vectors for in vitro transcription was done with *EcoR I* (for both *Per*s), *Not I* (for *Bmal1* & *Dbp*).

4.4.4.2. RNA probes

^{35}S-UTP (PerkinElmer, Monza, Italy) labelled RNA probes were made using RNA Transcription Kit (Maxi Script Labelling Kit, Ambion, Austin, USA) with T7, T3 or SP6 RNA polymerases. 1µg linearized plasmid template was used in a standard setup sufficient for about 24 slides. Transcription setup for ^{35}S-UTP labeled antisense RNA probes for clock gene transcripts were performed as described:

- 1 µg linearised cDNA template (or 500ng if PCR product is used)
- 8 µl DEPC H_2O

- 2 µl 10xTranscription buffer (supplied with the Kit)
- 1 µl each rATP, rCTP and rGTP (supplied with the Kit)
- 1 µl RNase inhibitor (supplied with the Kit)
- 5 µl α^{35} S-UTP [1M/µl]; 1Ci/M; 19µCi/µl
- 1 µl RNA Polymerase (supplied with the Kit)

Mix gently by pipetting up and down and incubate for 2 h at 37° C / 400 rpm in a Thermomixer (Eppendorf).

Template degradation:

- 19 µl DEPC-H_2O
- 1.7 µl $MgCl_2$ [0.3 M]
- 1 µl DNaseI [2 U]

Mix gently using a pipette tip and incubate for 15min at 37° C / 400 rpm in a Thermomixer (Eppendorf).

Probe Preparation:

- 100 µl DEPC- H_2O
- 100 µl yeast tRNA [1 mg/ml]
- 250 µl NH_4Ac [4 M]
- 1 ml EtOH [100%]

Vortex and incubate on ice for 10 min. Then spin down for 15 min at 4° C /14000 rpm (place tube lid hinges on the outside). Carefully remove supernatant and resuspend pellet in 200 µl DEPC- H_2O.

- 200 µl Add NH_4Ac [4 M]
- 800 µl Add EtOH [100 %]

Vortex and incubate on ice for 10 min. Spin down for 10 min at 4° C / 14000 rpm and discard the supernatant. Then dissolve the pellet in 100 µl ISH Hybridisation Buffer (HybMix, Ambion). Take 2 µl of the dissolved sample and measure activity in a beta-counter with 5 ml scintillator cocktail. Normal incorporation yields 1-6 M cpm in a 2 µl sample. Discard preparations with less than 300k cpm. Dilute the probe with HybMix (Ambion, USA) up to 100 µl and 2-6 M cpm per slide and add 1/100 vol. 1 M DTT (Biomol, Hamburg, Germany). Store probes on ice if used on the same day or at -80° C for up to 1 week.

4.4.4.3. Tissue preparation and paraffin sections

Either paraffin embedded or frozen tissue can be used. For paraffin embedding animals were sacrificed by cervical dislocation at the designated time points, tissues were removed and immersion fixed in 4 % paraformaldehyde (PFA) in PBS (pH 7.4) at 4° C for 12 - 18 h. Tissues were then dehydrated with ethanol (30, 50, 70 and 100 %, 3 h each at 4° C) and transferred to xylene. Xylene was changed once and replaced by 50 % xylene / 50 % paraffin and 3x paraffin at 60° C before pouring the samples into embedding forms to

solidify. Tissues were cut on the following day or later using a microtome (R. Jung, Hamburg, Germany) at 8 µm thickness and stored at RT before use.

De-waxing steps:

All de-waxing steps were performed with paraffin embedded sections in Tissue Tek II cuvette racks (Sakura Finetec).

o	10 min	2x X-Tra-Solve (Medite, Burgdorf, Germany)
o	2 min	2x 100 % Ethanol
o	30 sec	each 95/70/50/30 % Ethanol
o	5 min	0.9 % sodium chloride
o	5 min	PBS
o	20 min	4 % PFA (pH 7.4 in PBS)
o	5 min	PBS
o	5 min	Proteinase K (4 µg/µl in 50 mM Tris/ HCl, 5 mM EDTA pH 8.5)
o	5 min	0.2 N HCL
o	5 min	PBS
o	20 min	4 % PFA pH 7.4
o	stir 3 min	Acetylation in 0.1 M Triethanolamine/ HCl pH 8.0: add 750 µl acetic anhydride on 250 ml
o	stir 7 min	add another 750 µl acetic anhydride
o	5 min	PBS
o	5 min	0.9 % sodium chloride
o	30 sec	each 30/50/70/80/95 % Ethanol
o	2x 1min	100 % Ethanol

Air-dry slides at a RNase- free place

4.4.4.4. Hybridization

Hybridization was performed in humidified chambers (5x SSC, 50 % (v/v) formamide) in a hybridization oven over night (at least 16 hrs) at 55-58° C. Probe was put on the slides (100 µl per slide) and spread over the whole area using a pipette tip before covering with a cover slip.

4.4.4.5. Post-hybridization

Post-hybridization was performed in Tissue Tek II cuvettes (Sakura Finetec).

Removal Wash:

• 10 min at 64° C (shaking)	5x SSC/ 40 mM mercaptoethanol (700 µl 100%; beta-MeSH, Sigma-Aldrich, St. Louis, USA) cover slips were removed using forceps
• 20 min at 64° C	5x SSC/ 40 mM beta-MeSH (700 µl) (shaking)
• 30 min at 64° C	2x SSC/ 50 % formamide/ 40mM beta-MeSH
• 30 min at 37° C	2x NTE (50 mM sodium chloride, 10 mM Tris/ HCl, 5 mM EDTA, pH 8)/ 40 mM beta-MeSH
• 30 min at 37° C	RNase A [20 µg/ml] in NTE (add fresh 200 µl)
• 15 min at 37° C	NTE
• 30 min at 64° C	2x SSC/ 50 % formamide/ 40 mM beta-MeSH

- 15 min at RT 2x SSC
- 15 min at RT 0,1x SSC
- 30 sec at RT each 30/50/70 % EtOH/0.3 M ammoniumacetate
- 30 sec each 95/100/100 % EtOH

Air-dry slides before exposure to Biomax MS film (Kodak)
Expose over night or if necessary some days

4.4.4.6. Quantification

Exposed films were developed with the X-omat1000 (Kodak) and scanned with a flatbed scanner (Hewlett Packard, Palo Alto, USA). Relative quantification of expression levels was performed by densitometric analysis of autoradiograph films using the Scion Image 1.62 software (Scion Corporation, National Institutes of Health, USA). Three sections per brain were used and for each tissue background subtracted from adjacent hypothalamic areas on the same slide. Measurements from different animals/ experiments were combined for statistical analysis performed with GraphPad Prism software (GraphPad Software, San Diego, USA).

4.5. Immunological methods

4.5.1. Radio-immuno assay (RIA)

4.5.1.1. Corticosterone RIA

For quantification of corticoid metabolites in blood plasma or fecal samples, the *ImmuChemTM Double Antibody 125Iod-Radioimmunoassays* (MP Biomedicals, LLC) was used according to the manufactures protocol with some modifications. Plasma samples were diluted at 1:100. Fecal methanol extracts were diluted at 1:5. All samples were measured in duplicates. The standard curve and specific volumes for all solutions were prepared as illustrated in the pipetting schemee in **Table 4**.

Table 4: Step by step protocol and pipetting scheme to quantify fecal and plasma corticosterone concentrations. Standards (top, tube 1-16), all solutions included in the kit and for each sample (exemplarily tube 17- 18) as duplicates for the first sample feces extracted or plasma metabolites.

Tube Nr.		Ster Dil.[µl]	Cal/Spl [µl]	aCpB [µl]	125I [µl]	PrecSol [µl]
1,2	NSB	75	-	-	50	125
3,4	0	25	-	50	50	125
5,6	25	-	25	50	50	125
7,8	50	-	25	50	50	125
9,10	100	-	25	50	50	125
11,12	250	-	25	50	50	125
13,14	500	-	25	50	50	125
15,16	1000	-	25	50	50	125
17,18	Feces	5[µl] + 20[µl] (Ster Dil.)(1:5)		50	50	125
17,18	Plasma	0.25[µl] + 24.75[µl] (Ster Dil.)(1:100)		50	50	125
	1.	Distribute Ster.Dil/Cal/Sample-use standard 1,5ml tubes!				
	2.	Add aCpB				
	3.	Add CpB125I, vortex briefly, spin down				
	4.	Incubate for 2 hrs at RT				
	5.	Add prec.sol., vortex thoroughly				
	6.	Spin for 15 min, 10k at RT				
	7.	Aspirate SN				
	8.	Cut off lids and count pellets (1 min per sample)				

4.5.1.2. ACTH RIA

For quantification of ACTH from blood plasma an immunoradiometric assay (ACTH IRMA; CatNo. 13002352 130-Kit, DiaSorin, Kiel-Wellsee, Germany) was used. The prepared plasma samples were diluted at 1:4. All samples were measured in duplicates. The standard curve was prepared following the pipetting scheme illustrated in **Table 5**.

Table 5: Step by step protocol and pipetting scheme to quantify plasma ACTH concentrations. Standards (top, tube 1-12), all solutions included in the kit and for each sample (exemplarily tube 13-14) as duplicates for the first sample of plasma ACTH.

NR		Dil.[µl]	Cal/Spl [µl]	I125 Tracer [µl]	Ball
1,2	0		200	50	1
3,4	1		200	50	1
5,6	2		200	50	1
7,8	3		200	50	1
9,10	4		200	50	1
11, 12	5		200	50	1
13, 14	Samples	150 [µl](Dil.) + 50µl Plasma		50	1

1.	Distribute CalSample-use standard 2ml tubes!
2.	Add I125 Tracer, vortex without foam (add to bottom)
3.	Add one ball for each tube (coast ball in the tube)+ close
4.	Incubate for 20 hrs at 18-25°C
7.	Aspirate SN
8.	Wash balls (2x(1ml WashSol+aspire))x3 times
9.	Cut off lids and count pellets (per tube 1min)

4.6. Data analysis

4.6.1. Sine wave fitting

All real-time and ISH gene expression data analyses as well as corticosterone rhythm analyses were performed using the Prism software (GraphPad). To test for diurnal variation and rhythm peak time points a sine wave equation was fitted to the data.

$$y = BaseLine + Amplitude \times \sin\left(Frequency \times x + PhaseShift\right)$$

For gene expression and corticosterone data frequency was fixed to 24 hrs. Maxima were calculated by determining the axis section of the second derivative. To resolve the resetting

half point (PS_{50}) a sigmoid dose-response curve with variable slope was fitted to the sine wave maxima (corticosterone) or activity onsets (locomotor activity) for each group and day.

$$y = Bottom + (Top - Bottom) \div (1 + 10\text{^}((\log EC50 - x) \times HillSlope))$$

To test if the best-fit PS_{50} values differ between data sets, data were compared by extra sum-of-squares F test using p-values of less than 0.05 as a threshold. Maxima of gene expression or corticosterone at specific days were compared by Mann-Whitney rank sum test even if normality tests and equal variance tests were positive, reflecting the small sample sizes (3 to 9). A statistically significant difference was assumed with p-values less than 0.05.

4.6.2. Correlation analysis

Correlation between phase-shift activity onsets and corticosterone maxima as well as correlation between PS_{50} values of activity onset and hormone maxima shifts were performed using linear regression by Prism software (GraphPad). The quality of fit was estimated by R^2 determination. Departure from linearity was tested with Runs test. Normality and homoscedasticity tests were passed for all data sets.

4.6.3. Group comparison

Differences between groups during the different photoperiods, between activity totals, PS_{50}-values at different times after surgery and at various ages were compared by Mann-Whitney rank sum test or, if the same animals were compared, the Wilcoxon-Signed rang test or the Kruskal-Wallis ANOVA followed by Dunn's test, if more than 2 groups were compared. Rank tests were used, even if normality tests and equal variance tests were positive, reflecting the small sample sizes (3 vs. 23). A statistically significant difference was assumed with p-values less than 0.05.

Chapter 5

References

Abdel-Malek, Z. A. (2001). "Melanocortin receptors: their functions and regulation by physiological agonists and antagonists." Cell Mol Life Sci 58(3): 434-41.

Abe, H., S. Honma, et al. (2001). "Clock gene expressions in the suprachiasmatic nucleus and other areas of the brain during rhythm splitting in CS mice." Brain Res Mol Brain Res 87(1): 92-9.

Abe, K., J. Kroning, et al. (1979). "Effects of destruction of the suprachiasmatic nuclei on the circadian rhythms in plasma corticosterone, body temperature, feeding and plasma thyrotropin." Neuroendocrinology 29(2): 119-31.

Abraham, D., R. Dallmann, et al. (2006). "Restoration of circadian rhythmicity in circadian clock-deficient mice in constant light." J Biol Rhythms 21(3): 169-76.

Agostino, P. V., S. A. Plano, et al. (2007). "Sildenafil accelerates reentrainment of circadian rhythms after advancing light schedules." Proc Natl Acad Sci U S A 104(23): 9834-9.

Akashi, M. and T. Takumi (2005). "The orphan nuclear receptor RORalpha regulates circadian transcription of the mammalian core-clock Bmal1." Nat Struct Mol Biol 12(5): 441-8.

Akhtar, R. A., A. B. Reddy, et al. (2002). "Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus." Curr Biol 12(7): 540-50.

Albrecht, U. and G. Eichele (2003). "The mammalian circadian clock." Curr Opin Genet Dev 13(3): 271-7.

Albrecht, U., Z. S. Sun, et al. (1997). "A differential response of two putative mammalian circadian regulators, mper1 and mper2, to light." Cell 91(7): 1055-64.

Albrecht, U., B. Zheng, et al. (2001). "MPer1 and mper2 are essential for normal resetting of the circadian clock." J Biol Rhythms 16(2): 100-4.

Alfarez, D. N., O. Wiegert, et al. (2002). "Corticosterone and stress reduce synaptic potentiation in mouse hippocampal slices with mild stimulation." Neuroscience 115(4): 1119-26.

Allen, G., J. Rappe, et al. (2001). "Oscillating on borrowed time: diffusible signals from immortalized suprachiasmatic nucleus cells regulate circadian rhythmicity in cultured fibroblasts." J Neurosci 21(20): 7937-43.

Andrews, R. V. (1971). "Circadian rhythms in adrenal organ cultures." Gegenbaurs Morphol Jahrb 117(1): 89-98.

Andrews, R. V. and G. E. Folk, Jr. (1964). "Circadian Metabolic Patterns in Cultured Hamster Adrenal Glands." Comp Biochem Physiol 11: 393-409.

Antle, M. C. and R. Silver (2005). "Orchestrating time: arrangements of the brain circadian clock." Trends Neurosci 28(3): 145-51.

Arendt, J. (2009). "Managing jet lag: Some of the problems and possible new solutions." Sleep Med Rev.

Arendt, J. (2009). "Managing jet lag: Some of the problems and possible new solutions." Sleep Med Rev 13(4): 249-256.

Arendt, J. and V. Marks (1982). "Physiological changes underlying jet lag." Br Med J (Clin Res Ed) 284(6310): 144-6.

Aschoff, J. and H. Pohl (1978). "Phase relations between a circadian rhythm and its zeitgeber within the range of entrainment." Naturwissenschaften 65(2): 80-4.

Atkinson, H. C., S. A. Wood, et al. (2006). "Diurnal variation in the responsiveness of the hypothalamic-pituitary-adrenal axis of the male rat to noise stress." J Neuroendocrinol 18(7): 526-33.

Bae, K., X. Jin, et al. (2001). "Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock." Neuron 30(2): 525-36.

Balsalobre, A. (2002). "Clock genes in mammalian peripheral tissues." Cell Tissue Res 309(1): 193-9.

Balsalobre, A., S. A. Brown, et al. (2000). "Resetting of circadian time in peripheral tissues by glucocorticoid signaling." Science 289(5488): 2344-7.

Balsalobre, A., S. A. Brown, et al. (2000). "Resetting of circadian time in peripheral tissues by glucocorticoid signaling." Science 289(5488): 2344-7.

Balsalobre, A., F. Damiola, et al. (1998). "A serum shock induces circadian gene expression in mammalian tissue culture cells." Cell 93(6): 929-37.

Barrett, K. E., S. M. Barman, et al. (2009). Ganong's Review of Medical Physiology. Stamford McGraw-Hill Medical.

Berson, D. M. (2007). "Phototransduction in ganglion-cell photoreceptors." Pflugers Arch 454(5): 849-55.

Berson, D. M., F. A. Dunn, et al. (2002). "Phototransduction by retinal ganglion cells that set the circadian clock." Science 295(5557): 1070-3.

Biello, S. M., D. Janik, et al. (1994). "Neuropeptide Y and behaviorally induced phase shifts." Neuroscience 62(1): 273-9.

Bittman, E. L., L. Doherty, et al. (2003). "*Period* gene expression in mouse endocrine tissues." Am J Physiol Regul Integr Comp Physiol 285(3): R561-9.

Bosler, O. and A. Beaudet (1985). "VIP neurons as prime synaptic targets for serotonin afferents in rat suprachiasmatic nucleus: a combined radioautographic and immunocytochemical study." J Neurocytol 14(5): 749-63.

Brown, S. A., G. Zumbrunn, et al. (2002). "Rhythms of mammalian body temperature can sustain peripheral circadian clocks." Curr Biol 12(18): 1574-83.

Buijs, R. M. and A. Kalsbeek (2001). "Hypothalamic integration of central and peripheral clocks." Nat Rev Neurosci 2(7): 521-6.

Buijs, R. M., A. Kalsbeek, et al. (1993). "Suprachiasmatic nucleus lesion increases corticosterone secretion." Am J Physiol 264(6 Pt 2): R1186-92.

Buijs, R. M., C. G. van Eden, et al. (2003). "The biological clock tunes the organs of the body: timing by hormones and the autonomic nervous system." J Endocrinol 177(1): 17-26.

Buijs, R. M., J. Wortel, et al. (1999). "Anatomical and functional demonstration of a multisynaptic suprachiasmatic nucleus adrenal (cortex) pathway." Eur J Neurosci 11(5): 1535-44.

Bunger, M. K., L. D. Wilsbacher, et al. (2000). "*Mop3* is an essential component of the master circadian pacemaker in mammals." Cell 103(7): 1009-17.

Cardone, L., J. Hirayama, et al. (2005). "Circadian clock control by SUMOylation of BMAL1." Science 309(5739): 1390-4.

Casanueva, F. F. and C. Dieguez (1999). "Neuroendocrine regulation and actions of leptin." Front Neuroendocrinol 20(4): 317-63.

Cascio, C. S., J. Shinsako, et al. (1987). "The suprachiasmatic nuclei stimulate evening ACTH secretion in the rat." Brain Res 423(1-2): 173-8.

Cavigelli, S. A., S. L. Monfort, et al. (2005). "Frequent serial fecal corticoid measures from rats reflect circadian and ovarian corticosterone rhythms." J Endocrinol 184(1): 153-63.

Cermakian, N., L. Monaco, et al. (2001). "Altered behavioral rhythms and clock gene expression in mice with a targeted mutation in the Period1 gene." EMBO J 20(15): 3967-74.

Challet, E., L. C. Solberg, et al. (1998). "Entrainment in calorie-restricted mice: conflicting zeitgebers and free-running conditions." Am J Physiol 274(6 Pt 2): R1751-61.
Cheifetz, P. N. (1971). "The daily rhythm of the secretion of corticotrophin and corticosterone in rats and mice." J Endocrinol 49(3): xi-xii.
Cheng, M. Y., C. M. Bullock, et al. (2002). "Prokineticin 2 transmits the behavioural circadian rhythm of the suprachiasmatic nucleus." Nature 417(6887): 405-10.
Cho, K. (2001). "Chronic 'jet lag' produces temporal lobe atrophy and spatial cognitive deficits." Nat Neurosci 4(6): 567-8.
Cho, K., A. Ennaceur, et al. (2000). "Chronic jet lag produces cognitive deficits." J Neurosci 20(6): RC66.
Clark, B. J., J. Wells, et al. (1994). "The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR)." J Biol Chem 269(45): 28314-22.
Colwell, C. S., S. Michel, et al. (2003). "Disrupted circadian rhythms in VIP- and PHI-deficient mice." Am J Physiol Regul Integr Comp Physiol 285(5): R939-49.
Comperatore, C. A. and G. P. Krueger (1990). "Circadian rhythm desynchronosis, jet lag, shift lag, and coping strategies." Occup Med 5(2): 323-41.
Coupland, R. E., T. L. Parker, et al. (1989). "The innervation of the adrenal gland. III. Vagal innervation." J Anat 163: 173-81.
Cuninkova, L. and S. A. Brown (2008). "Peripheral circadian oscillators: interesting mechanisms and powerful tools." Ann N Y Acad Sci 1129: 358-70.
Cutler, D. J., M. Haraura, et al. (2003). "The mouse VPAC2 receptor confers suprachiasmatic nuclei cellular rhythmicity and responsiveness to vasoactive intestinal polypeptide in vitro." Eur J Neurosci 17(2): 197-204.
Daan, S. and C. S. Pittendrigh (1976). "A functional analysis of circadian pacemakers in nocturnal rodents " J Comp Physiol 106: 223-355.
Dallman, M. F., W. C. Engeland, et al. (1978). "Nycthemeral rhythm in adrenal responsiveness to ACTH." Am J Physiol 235(5): R210-8.
Damiola, F., N. Le Minh, et al. (2000). "Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus." Genes Dev 14(23): 2950-61.
Davidson, A. J., O. Castanon-Cervantes, et al. (2009). "Visualizing jet lag in the mouse suprachiasmatic nucleus and peripheral circadian timing system." Eur J Neurosci 29(1): 171-80.
Davidson, A. J., M. T. Sellix, et al. (2006). "Chronic jet-lag increases mortality in aged mice." Curr Biol 16(21): R914-6.
Davidson, A. J., S. Yamazaki, et al. (2003). "SCN: ringmaster of the circadian circus or conductor of the circadian orchestra?" Novartis Found Symp 253: 110-21; discussion 121-5, 281-4.
Delarue, C., V. Contesse, et al. (2001). "Role of neurotransmitters and neuropeptides in the regulation of the adrenal cortex." Rev Endocr Metab Disord 2(3): 253-67.
Devlin, P. F. and S. A. Kay (2001). "Circadian photoperception." Annu Rev Physiol 63: 677-94.
Dickmeis, T. (2009). "Glucocorticoids and the circadian clock." J Endocrinol 200(1): 3-22.
Dijkstra, I., R. Binnekade, et al. (1996). "Diurnal variation in resting levels of corticosterone is not mediated by variation in adrenal responsiveness to adrenocorticotropin but involves splanchnic nerve integrity." Endocrinology 137(2): 540-7.
Dioum, E. M., J. Rutter, et al. (2002). "NPAS2: a gas-responsive transcription factor." Science 298(5602): 2385-7.
Dunlap, J. C. (1999). "Molecular bases for circadian clocks." Cell 96(2): 271-90.
Dunlap, J. C., J. J. Loros, et al. (1999). "Eukaryotic circadian systems: cycles in common." Genes Cells 4(1): 1-10.

Durgan, D. J., N. A. Trexler, et al. (2006). "The circadian clock within the cardiomyocyte is essential for responsiveness of the heart to fatty acids." J Biol Chem 281(34): 24254-69.

Dwivedi, Y. and G. N. Pandey (2000). "Adrenal glucocorticoids modulate [3H]cyclic AMP binding to protein kinase A (PKA), cyclic AMP-dependent PKA activity, and protein levels of selective regulatory and catalytic subunit isoforms of PKA in rat brain." J Pharmacol Exp Ther 294(1): 103-16.

Edery, I. (2000). "Circadian rhythms in a nutshell." Physiol Genomics 3(2): 59-74.

Edgar, D. M., T. S. Kilduff, et al. (1991). "Influence of running wheel activity on free-running sleep/wake and drinking circadian rhythms in mice." Physiol Behav 50(2): 373-8.

Edwards, A. V., C. T. Jones, et al. (1986). "Reduced adrenal cortical sensitivity to ACTH in lambs with cut splanchnic nerves." J Endocrinol 110(1): 81-5.

Ehrhart-Bornstein, M., J. P. Hinson, et al. (1998). "Intraadrenal interactions in the regulation of adrenocortical steroidogenesis." Endocr Rev 19(2): 101-43.

Engeland, W. C. and M. M. Arnhold (2005). "Neural circuitry in the regulation of adrenal corticosterone rhythmicity." Endocrine 28(3): 325-32.

Englund, A., L. Kovanen, et al. (2009). "NPAS2 and PER2 are linked to risk factors of the metabolic syndrome." J Circadian Rhythms 7: 5.

Fakunding, J. L., R. Chow, et al. (1979). "The role of calcium in the stimulation of aldosterone production by adrenocorticotropin, angiotensin II, and potassium in isolated glomerulosa cells." Endocrinology 105(2): 327-33.

Filipski, E., F. Delaunay, et al. (2004). "Effects of chronic jet lag on tumor progression in mice." Cancer Res 64(21): 7879-85.

Foster, R. H. (2004). "Reciprocal influences between the signalling pathways regulating proliferation and steroidogenesis in adrenal glomerulosa cells." J Mol Endocrinol 32(3): 893-902.

Francois-Bellan, A. M. and O. Bosler (1992). "Convergent serotonin and GABA innervation of VIP neurons in the suprachiasmatic nucleus demonstrated by triple labeling in the rat." Brain Res 595(1): 149-53.

Freedman, M. S., R. J. Lucas, et al. (1999). "Regulation of mammalian circadian behavior by non-rod, non-cone, ocular photoreceptors." Science 284(5413): 502-4.

Gallo-Payet, N. and M. D. Payet (2003). "Mechanism of action of ACTH: beyond cAMP." Microsc Res Tech 61(3): 275-87.

Gau, D., T. Lemberger, et al. (2002). "Phosphorylation of CREB Ser142 regulates light-induced phase shifts of the circadian clock." Neuron 34(2): 245-53.

Gillette, M. U. and S. A. Tischkau (1999). "Suprachiasmatic nucleus: the brain's circadian clock." Recent Prog Horm Res 54: 33-58; discussion 58-9.

Gofflot, F., N. Chartoire, et al. (2007). "Systematic gene expression mapping clusters nuclear receptors according to their function in the brain." Cell 131(2): 405-18.

Griefahn, B., C. Kunemund, et al. (2002). "Effects of electromagnetic radiation (bright light, extremely low-frequency magnetic fields, infrared radiation) on the circadian rhythm of melatonin synthesis, rectal temperature, and heart rate." Ind Health 40(4): 320-7.

Guler, A. D., J. L. Ecker, et al. (2008). "Melanopsin cells are the principal conduits for rod-cone input to non-image-forming vision." Nature 453(7191): 102-5.

Haimov, I. and J. Arendt (1999). "The prevention and treatment of jet lag." Sleep Med Rev 3(3): 229-40.

Hankins, M. W., S. N. Peirson, et al. (2008). "Melanopsin: an exciting photopigment." Trends Neurosci 31(1): 27-36.

Hara, R., K. Wan, et al. (2001). "Restricted feeding entrains liver clock without participation of the suprachiasmatic nucleus." Genes Cells 6(3): 269-78.

Harmer, S. L., S. Panda, et al. (2001). "Molecular bases of circadian rhythms." Annu Rev Cell Dev Biol 17: 215-53.

Hastings, M., J. S. O'Neill, et al. (2007). "Circadian clocks: regulators of endocrine and metabolic rhythms." J Endocrinol 195(2): 187-98.
Hastings, M. H. (2000). "Circadian clockwork: two loops are better than one." Nat Rev Neurosci 1(2): 143-6.
Hattar, S., R. J. Lucas, et al. (2003). "Melanopsin and rod-cone photoreceptive systems account for all major accessory visual functions in mice." Nature 424(6944): 76-81.
Haus, E. (2007). "Chronobiology in the endocrine system." Adv Drug Deliv Rev 59(9-10): 985-1014.
Heikkila, P., A. I. Kahri, et al. (1989). "The effect of low- and high-density lipoprotein cholesterol on steroid hormone production and ACTH-induced differentiation of rat adrenocortical cells in primary culture." Cell Tissue Res 256(3): 487-94.
Herzog, E. D. and G. Tosini (2001). "The mammalian circadian clock shop." Semin Cell Dev Biol 12(4): 295-303.
Hirota, T. and Y. Fukada (2004). "Resetting mechanism of central and peripheral circadian clocks in mammals." Zoolog Sci 21(4): 359-68.
Ishida, A., T. Mutoh, et al. (2005). "Light activates the adrenal gland: timing of gene expression and glucocorticoid release." Cell Metab 2(5): 297-307.
Jakubcakova, V., H. Oster, et al. (2007). "Light entrainment of the mammalian circadian clock by a PRKCA-dependent posttranslational mechanism." Neuron 54(5): 831-43.
Janik, D. and N. Mrosovsky (1994). "Intergeniculate leaflet lesions and behaviorally-induced shifts of circadian rhythms." Brain Res 651(1-2): 174-82.
Jasper, M. S. and W. C. Engeland (1994). "Splanchnic neural activity modulates ultradian and circadian rhythms in adrenocortical secretion in awake rats." Neuroendocrinology 59(2): 97-109.
Jasper, M. S. and W. C. Engeland (1997). "Splanchnicotomy increases adrenal sensitivity to ACTH in nonstressed rats." Am J Physiol 273(2 Pt 1): E363-8.
Jin, X., L. P. Shearman, et al. (1999). "A molecular mechanism regulating rhythmic output from the suprachiasmatic circadian clock." Cell 96(1): 57-68.
Johnson, R. F., L. P. Morin, et al. (1988). "Retinohypothalamic projections in the hamster and rat demonstrated using cholera toxin." Brain Res 462(2): 301-12.
Jud, C., I. Schmutz, et al. (2005). "A guideline for analyzing circadian wheel-running behavior in rodents under different lighting conditions." Biol Proced Online 7: 101-16.
Juhl, F., J. Hannibal, et al. (2007). "Photic induction of c-Fos in enkephalin neurons of the rat intergeniculate leaflet innervated by retinal PACAP fibres." Cell Tissue Res 329(3): 491-502.
Kalsbeek, A., F. Kreier, et al. (2007). "Minireview: Circadian control of metabolism by the suprachiasmatic nuclei." Endocrinology 148(12): 5635-9.
Kalsbeek, A., I. F. Palm, et al. (2006). "SCN outputs and the hypothalamic balance of life." J Biol Rhythms 21(6): 458-69.
Kalsbeek, A., M. Ruiter, et al. (2003). "The diurnal modulation of hormonal responses in the rat varies with different stimuli." J Neuroendocrinol 15(12): 1144-55.
Kaneko, M., N. Hernandez-Borsetti, et al. (2006). "Diversity of zebrafish peripheral oscillators revealed by luciferase reporting." Proc Natl Acad Sci U S A 103(39): 14614-9.
Kaneko, M., K. Kaneko, et al. (1981). "Adrenal sensitivity to adrenocorticotropin varies diurnally." Endocrinology 109(1): 70-5.
Kawasaki, T., K. Uezono, et al. (1983). "Comparison of circadian rhythms of the renin-angiotensin-aldosterone system and electrolytes in clinically healthy young women in Fukuoka (Japan) and Minnesota (USA)." Acta Endocrinol (Copenh) 102(2): 246-51.
Keidel, W.-D. (1985). Kurzgefaßtes Lehrbuch der Physiologie. Stuttgart Thieme.
Kemppainen, R. J. and E. N. Behrend (1997). "Adrenal physiology." Vet Clin North Am Small Anim Pract 27(2): 173-86.
Kennaway, D. J. (2005). "The role of circadian rhythmicity in reproduction." Hum Reprod Update 11(1): 91-101.

King, D. P. and J. S. Takahashi (2000). "Molecular genetics of circadian rhythms in mammals." Annu Rev Neurosci 23: 713-42.
Klein, D., R. Moore, et al. (1991). The Suprachiasmatic Nucleus: The Mind.s Clock. . New York, Oxford Univ Press.
Ko, C. H. and J. S. Takahashi (2006). "Molecular components of the mammalian circadian clock." Hum Mol Genet 15 Spec No 2: R271-7.
Konopka, R. J. and S. Benzer (1971). "Clock mutants of Drosophila melanogaster." Proc Natl Acad Sci U S A 68(9): 2112-6.
Kornmann, B., O. Schaad, et al. (2007). "Regulation of circadian gene expression in liver by systemic signals and hepatocyte oscillators." Cold Spring Harb Symp Quant Biol 72: 319-30.
Kovac, J., J. Husse, et al. (2009). "A time to fast, a time to feast: the crosstalk between metabolism and the circadian clock." Mol Cells 28(2): 75-80.
Kramer, A., F. C. Yang, et al. (2001). "Regulation of daily locomotor activity and sleep by hypothalamic EGF receptor signaling." Science 294(5551): 2511-5.
Kraves, S. and C. J. Weitz (2006). "A role for cardiotrophin-like cytokine in the circadian control of mammalian locomotor activity." Nat Neurosci 9(2): 212-9.
Kume, K., M. J. Zylka, et al. (1999). "mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop." Cell 98(2): 193-205.
Le Minh, N., F. Damiola, et al. (2001). "Glucocorticoid hormones inhibit food-induced phase-shifting of peripheral circadian oscillators." EMBO J 20(24): 7128-36.
Lee, C., J. P. Etchegaray, et al. (2001). "Posttranslational mechanisms regulate the mammalian circadian clock." Cell 107(7): 855-67.
Lemmer, B. (2007). "The sleep-wake cycle and sleeping pills." Physiol Behav 90(2-3): 285-93.
Lemos, D. R., J. L. Downs, et al. (2006). "Twenty-four hour rhythmic gene expression in the rhesus macaque adrenal gland." Mol Endocrinol.
LeSauter, J., N. Hoque, et al. (2009). "Stomach ghrelin-secreting cells as food-entrainable circadian clocks." Proc Natl Acad Sci U S A 106(32): 13582-7.
Levine, J. D., P. Funes, et al. (2002). "Resetting the circadian clock by social experience in Drosophila melanogaster." Science 298(5600): 2010-2.
Li, C. and W. H. Wong (2001). "Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection." Proc Natl Acad Sci U S A 98(1): 31-6.
Lin, D., T. Sugawara, et al. (1995). "Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis." Science 267(5205): 1828-31.
Lindhe, O., B. O. Lund, et al. (2001). "Irreversible binding and adrenocorticolytic activity of the DDT metabolite 3-methylsulfonyl-DDE examined in tissue-slice culture." Environ Health Perspect 109(2): 105-10.
Liu, S., Y. Cai, et al. (2007). "Chronobiological analysis of circadian patterns in transcription of seven key clock genes in six peripheral tissues in mice." Chronobiol Int 24(5): 793-820.
Low-Zeddies, S. S. and J. S. Takahashi (2001). "Chimera analysis of the Clock mutation in mice shows that complex cellular integration determines circadian behavior." Cell 105(1): 25-42.
Manna, P. R. and D. M. Stocco (2005). "Regulation of the steroidogenic acute regulatory protein expression: functional and physiological consequences." Curr Drug Targets Immune Endocr Metabol Disord 5(1): 93-108.
Maywood, E. S., J. S. O'Neill, et al. (2007). "Genetic and molecular analysis of the central and peripheral circadian clockwork of mice." Cold Spring Harb Symp Quant Biol 72: 85-94.
Maywood, E. S., E. Smith, et al. (1997). "A thalamic contribution to arousal-induced, non-photic entrainment of the circadian clock of the Syrian hamster." Eur J Neurosci 9(8): 1739-47.

McDearmon, E. L., K. N. Patel, et al. (2006). "Dissecting the functions of the mammalian clock protein BMAL1 by tissue-specific rescue in mice." Science 314(5803): 1304-8.
McNamara, P., S. B. Seo, et al. (2001). "Regulation of CLOCK and MOP4 by nuclear hormone receptors in the vasculature: a humoral mechanism to reset a peripheral clock." Cell 105(7): 877-89.
Menaker, M. and A. Eskin (1966). "Entrainment of circadian rhythms by sound in Passer domesticus." Science 154(756): 1579-81.
Mendoza, J., D. Clesse, et al. (2008). "Serotonergic potentiation of dark pulse-induced phase-shifting effects at midday in hamsters." J Neurochem 106(3): 1404-14.
Meng, Q. J., A. McMaster, et al. (2008). "Ligand modulation of REV-ERBalpha function resets the peripheral circadian clock in a phasic manner." J Cell Sci 121(Pt 21): 3629-35.
Meyer-Bernstein, E. L. and L. P. Morin (1999). "Electrical stimulation of the median or dorsal raphe nuclei reduces light-induced FOS protein in the suprachiasmatic nucleus and causes circadian activity rhythm phase shifts." Neuroscience 92(1): 267-79.
Miller, W. L. (2007). "Steroidogenic acute regulatory protein (StAR), a novel mitochondrial cholesterol transporter." Biochim Biophys Acta 1771(6): 663-76.
Mistlberger, R. E. (1994). "Circadian food-anticipatory activity: formal models and physiological mechanisms." Neurosci Biobehav Rev 18(2): 171-95.
Miyazaki, K., M. Mesaki, et al. (2001). "Nuclear entry mechanism of rat PER2 (rPER2): role of rPER2 in nuclear localization of CRY protein." Mol Cell Biol 21(19): 6651-9.
Mizoguchi, K., R. Ikeda, et al. (2008). "Suppression of glucocorticoid secretion induces a behaviorally depressive state in rotarod performance in rat." Pharmacol Biochem Behav 90(4): 730-4.
Moga, M. M. and R. Y. Moore (1997). "Organization of neural inputs to the suprachiasmatic nucleus in the rat." J Comp Neurol 389(3): 508-34.
Mohawk, J. A., K. Cashen, et al. (2005). "Inhibiting cortisol response accelerates recovery from a photic phase shift." Am J Physiol Regul Integr Comp Physiol 288(1): R221-8.
Moore-Ede, M. C., D. A. Kass, et al. (1977). "Transient circadian internal desynchronization after light-dark phase shift in monkeys." Am J Physiol 232(1): R31-7.
Moore, R. Y. and V. B. Eichler (1972). "Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat." Brain Res 42(1): 201-6.
Moore, R. Y. and N. J. Lenn (1972). "A retinohypothalamic projection in the rat." J Comp Neurol 146(1): 1-14.
Muhlbauer, E., S. Wolgast, et al. (2004). "Indication of circadian oscillations in the rat pancreas." FEBS Lett 564(1-2): 91-6.
Muller, J. (1998). "Regulation of aldosterone biosynthesis: the end of the road?" Clin Exp Pharmacol Physiol Suppl 25: S79-85.
Musholt, T. J., S. H. Klebs, et al. (2002). "Transplantation of adrenal tissue fragments in a murine model: functional capacities of syngeneic and allogeneic grafts." World J Surg 26(8): 950-7.
Nagano, M., A. Adachi, et al. (2003). "An abrupt shift in the day/night cycle causes desynchrony in the mammalian circadian center." J Neurosci 23(14): 6141-51.
Nicholson, W. E., J. H. Levine, et al. (1976). "Hormonal regulation of renal ornithine decarboxylase activity in the rat." Endocrinology 98(1): 123-8.
Niijima, A. (1992). "Electrophysiological study on the vagal innervation of the adrenal gland in the rat." J Auton Nerv Syst 41(1-2): 87-92.
Niijima, A., K. Nagai, et al. (1993). "Effects of light stimulation on the activity of the autonomic nerves in anesthetized rats." Physiol Behav 54(3): 555-61.
Nonaka, H., N. Emoto, et al. (2001). "Angiotensin II induces circadian gene expression of clock genes in cultured vascular smooth muscle cells." Circulation 104(15): 1746-8.
Obrietan, K., S. Impey, et al. (1998). "Light and circadian rhythmicity regulate MAP kinase activation in the suprachiasmatic nuclei." Nat Neurosci 1(8): 693-700.

Oishi, K., N. Amagai, et al. (2005). "Genome-wide Expression Analysis Reveals 100 Adrenal Gland-dependent Circadian Genes in the Mouse Liver." DNA Res 12(3): 191-202.
Oishi, K., H. Fukui, et al. (2000). "Rhythmic expression of BMAL1 mRNA is altered in Clock mutant mice: differential regulation in the suprachiasmatic nucleus and peripheral tissues." Biochem Biophys Res Commun 268(1): 164-71.
Oishi, K., I. Murai, et al. (2000). "The pineal gland is not essential for circadian expression of rat period homologue (rper2) mRNA in the suprachiasmatic nucleus and peripheral tissues." Brain Res 885(2): 298-302.
Oishi, K., K. Sakamoto, et al. (1998). "Humoral signals mediate the circadian expression of rat period homologue (rPer2) mRNA in peripheral tissues." Neurosci Lett 256(2): 117-9.
Oster, H. (2006). "The genetic basis of circadian behavior." Genes Brain Behav 5 Suppl 2: 73-9.
Oster, H., S. Baeriswyl, et al. (2003). "Loss of circadian rhythmicity in aging *mPer1*$^{-/-}$*mCry2*$^{-/-}$ mutant mice." Genes Dev 17(11): 1366-79.
Oster, H., S. Damerow, et al. (2006). "Transcriptional profiling in the adrenal gland reveals circadian regulation of hormone biosynthesis genes and nucleosome assembly genes." J Biol Rhythms 21(5): 350-61.
Oster, H., S. Damerow, et al. (2006). "The circadian rhythm of glucocorticoids is regulated by a gating mechanism residing in the adrenal cortical clock." Cell Metab 4(2): 163-73.
Oster, H., G. T. van der Horst, et al. (2003). "Daily variation of clock output gene activation in behaviorally arrhythmic mPer/mCry triple mutant mice." Chronobiol Int 20(4): 683-95.
Oster, H., C. Werner, et al. (2003). "cGMP-dependent protein kinase II modulates mPer1 and mPer2 gene induction and influences phase shifts of the circadian clock." Curr Biol 13(9): 725-33.
Oster, H., A. Yasui, et al. (2002). "Disruption of mCry2 restores circadian rhythmicity in mPer2 mutant mice." Genes Dev 16(20): 2633-8.
Panda, S., M. P. Antoch, et al. (2002). "Coordinated transcription of key pathways in the mouse by the circadian clock." Cell 109(3): 307-20.
Panda, S. and J. B. Hogenesch (2004). "It's all in the timing: many clocks, many outputs." J Biol Rhythms 19(5): 374-87.
Pando, M. P., D. Morse, et al. (2002). "Phenotypic rescue of a peripheral clock genetic defect via SCN hierarchical dominance." Cell 110(1): 107-17.
Pando, M. P. and P. Sassone-Corsi (2001). "Molecular clocks. A vivid loop of light." Nature 410(6826): 311-3.
Papadimitriou, A. and K. N. Priftis (2009). "Regulation of the hypothalamic-pituitary-adrenal axis." Neuroimmunomodulation 16(5): 265-71.
Parker, K. L. and B. P. Schimmer (1997). "Steroidogenic factor 1: a key determinant of endocrine development and function." Endocr Rev 18(3): 361-77.
Parker, K. L. and B. P. Schimmer (2001). "Genetics of the development and function of the adrenal cortex." Rev Endocr Metab Disord 2(3): 245-52.
Parker, T. L., A. A. Mohamed, et al. (1990). "The innervation of the adrenal gland. IV. The source of pre- and postganglionic nerve fibres to the guinea-pig adrenal gland." J Anat 172: 17-24.
Payne, A. H. and D. B. Hales (2004). "Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones." Endocr Rev 25(6): 947-70.
Penev, P. D., D. E. Kolker, et al. (1998). "Chronic circadian desynchronization decreases the survival of animals with cardiomyopathic heart disease." Am J Physiol 275(6 Pt 2): H2334-7.
Perreau-Lenz, S., P. Pevet, et al. (2004). "The biological clock: the bodyguard of temporal homeostasis." Chronobiol Int 21(1): 1-25.

Peschke, E. and D. Peschke (1998). "Evidence for a circadian rhythm of insulin release from perifused rat pancreatic islets." Diabetologia 41(9): 1085-92.
Pfaffl, M. W. (2001). "A new mathematical model for relative quantification in real-time RT-PCR." Nucleic Acids Res 29(9): e45.
Pittendrigh, C. S. (1993). "Temporal organization: reflections of a Darwinian clock-watcher." Annu Rev Physiol 55: 16-54.
Preitner, N., F. Damiola, et al. (2002). "The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator." Cell 110(2): 251-60.
Prosser, H. M., A. Bradley, et al. (2007). "Prokineticin receptor 2 (Prokr2) is essential for the regulation of circadian behavior by the suprachiasmatic nuclei." Proc Natl Acad Sci U S A 104(2): 648-53.
Provencio, I., M. D. Rollag, et al. (2002). "Photoreceptive net in the mammalian retina. This mesh of cells may explain how some blind mice can still tell day from night." Nature 415(6871): 493.
Pu, Z., H. J. Krugers, et al. (2007). "Corticosterone time-dependently modulates beta-adrenergic effects on long-term potentiation in the hippocampal dentate gyrus." Learn Mem 14(5): 359-67.
Raghuram, S., K. R. Stayrook, et al. (2007). "Identification of heme as the ligand for the orphan nuclear receptors REV-ERBalpha and REV-ERBbeta." Nat Struct Mol Biol 14(12): 1207-13.
Ralph, M. R., R. G. Foster, et al. (1990). "Transplanted suprachiasmatic nucleus determines circadian period." Science 247(4945): 975-8.
Ramakers, C., J. M. Ruijter, et al. (2003). "Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data." Neurosci Lett 339(1): 62-6.
Ramakrishnan, S. N. and G. E. Muscat (2006). "The orphan Rev-erb nuclear receptors: a link between metabolism, circadian rhythm and inflammation?" Nucl Recept Signal 4: e009.
Reddy, A. B., M. D. Field, et al. (2002). "Differential resynchronisation of circadian clock gene expression within the suprachiasmatic nuclei of mice subjected to experimental jet lag." J Neurosci 22(17): 7326-30.
Rehfeld, J. F. (1998). "Accurate measurement of cholecystokinin in plasma." Clin Chem 44(5): 991-1001.
Reinberg, A., Y. Touitou, et al. (1982). "[Circadian and circannual rhythms in results of functional tests in endocrinology]." Ann Endocrinol (Paris) 43(5): 309-35.
Reppert, S. M. and D. R. Weaver (2001). "Molecular analysis of mammalian circadian rhythms." Annu Rev Physiol 63: 647-76.
Reppert, S. M. and D. R. Weaver (2002). "Coordination of circadian timing in mammals." Nature 418(6901): 935-41.
Reyes, B. A., J. S. Pendergast, et al. (2008). "Mammalian peripheral circadian oscillators are temperature compensated." J Biol Rhythms 23(1): 95-8.
Richards, J. S. (2001). "New signaling pathways for hormones and cyclic adenosine 3',5'-monophosphate action in endocrine cells." Mol Endocrinol 15(2): 209-18.
Ripperger, J. A., L. P. Shearman, et al. (2000). "CLOCK, an essential pacemaker component, controls expression of the circadian transcription factor DBP." Genes Dev 14(6): 679-89.
Roenneberg, T. and M. Merrow (2002). ""What watch?...such much!" Complexity and evolution of circadian clocks." Cell Tissue Res 309(1): 3-9.
Rosbash, M. (2009). "The implications of multiple circadian clock origins." PLoS Biol 7(3): e62.
Rosenfeld, P., J. A. Van Eekelen, et al. (1988). "Ontogeny of the type 2 glucocorticoid receptor in discrete rat brain regions: an immunocytochemical study." Brain Res 470(1): 119-27.

Rudic, R. D., P. McNamara, et al. (2004). "BMAL1 and CLOCK, two essential components of the circadian clock, are involved in glucose homeostasis." PLoS Biol 2(11): e377.

Rudney, H. and R. C. Sexton (1986). "Regulation of cholesterol biosynthesis." Annu Rev Nutr 6: 245-72.

Rusak, B. and I. Zucker (1979). "Neural regulation of circadian rhythms." Physiol Rev 59(3): 449-526.

Rutter, J., M. Reick, et al. (2001). "Regulation of clock and NPAS2 DNA binding by the redox state of NAD cofactors." Science 293(5529): 510-4.

Sage, D., J. Ganem, et al. (2004). "Influence of the corticosterone rhythm on photic entrainment of locomotor activity in rats." J Biol Rhythms 19(2): 144-56.

Sage, D., D. Maurel, et al. (2001). "Involvement of the suprachiasmatic nucleus in diurnal ACTH and corticosterone responsiveness to stress." Am J Physiol Endocrinol Metab 280(2): E260-9.

Sage, D., D. Maurel, et al. (2002). "Corticosterone-dependent driving influence of the suprachiasmatic nucleus on adrenal sensitivity to ACTH." Am J Physiol Endocrinol Metab 282(2): E458-65.

Saper, C. B., J. Lu, et al. (2005). "The hypothalamic integrator for circadian rhythms." Trends Neurosci 28(3): 152-7.

Schibler, U. and F. Naef (2005). "Cellular oscillators: rhythmic gene expression and metabolism." Curr Opin Cell Biol 17(2): 223-9.

Schibler, U., J. Ripperger, et al. (2003). "Peripheral circadian oscillators in mammals: time and food." J Biol Rhythms 18(3): 250-60.

Schibler, U. and P. Sassone-Corsi (2002). "A web of circadian pacemakers." Cell 111(7): 919-22.

Sewer, M. B. and M. R. Waterman (2001). "Insights into the transcriptional regulation of steroidogenic enzymes and StAR." Rev Endocr Metab Disord 2(3): 269-74.

Shibata, S. and R. Y. Moore (1993). "Neuropeptide Y and optic chiasm stimulation affect suprachiasmatic nucleus circadian function in vitro." Brain Res 615(1): 95-100.

Shigeyoshi, Y., K. Taguchi, et al. (1997). "Light-induced resetting of a mammalian circadian clock is associated with rapid induction of the mPer1 transcript." Cell 91(7): 1043-53.

Silver, R., J. LeSauter, et al. (1996). "A diffusible coupling signal from the transplanted suprachiasmatic nucleus controlling circadian locomotor rhythms." Nature 382(6594): 810-3.

Simpson, E. R. and M. R. Waterman (1988). "Regulation of the synthesis of steroidogenic enzymes in adrenal cortical cells by ACTH." Annu Rev Physiol 50: 427-40.

So, A. Y., T. U. Bernal, et al. (2009). "Glucocorticoid regulation of the circadian clock modulates glucose homeostasis." Proc Natl Acad Sci U S A.

Sollars, P. J., D. P. Kimble, et al. (1995). "Restoration of circadian behavior by anterior hypothalamic heterografts." J Neurosci 15(3 Pt 2): 2109-22.

Son, G. H., S. Chung, et al. (2008). "Adrenal peripheral clock controls the autonomous circadian rhythm of glucocorticoid by causing rhythmic steroid production." Proc Natl Acad Sci U S A 105(52): 20970-5.

Srinivasan, V., D. W. Spence, et al. (2008). "Jet lag: therapeutic use of melatonin and possible application of melatonin analogs." Travel Med Infect Dis 6(1-2): 17-28.

Stephan, F. K. and I. Zucker (1972). "Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions." Proc Natl Acad Sci U S A 69(6): 1583-6.

Stocco, D. M. (1999). "Steroidogenic acute regulatory protein." Vitam Horm 55: 399-441.

Stocco, D. M. (2001). "StAR protein and the regulation of steroid hormone biosynthesis." Annu Rev Physiol 63: 193-213.

Stocco, D. M., X. Wang, et al. (2005). "Multiple signaling pathways regulating steroidogenesis and steroidogenic acute regulatory protein expression: more complicated than we thought." Mol Endocrinol 19(11): 2647-59.

Storch, K. F., O. Lipan, et al. (2002). "Extensive and divergent circadian gene expression in liver and heart." Nature 417(6884): 78-83.

Storch, K. F., C. Paz, et al. (2007). "Physiological importance of a circadian clock outside the suprachiasmatic nucleus." Cold Spring Harb Symp Quant Biol 72: 307-18.

Straume, M. (2004). "DNA microarray time series analysis: automated statistical assessment of circadian rhythms in gene expression patterning." Methods Enzymol 383: 149-66.

Strubbe, J. H. and G. van Dijk (2002). "The temporal organization of ingestive behaviour and its interaction with regulation of energy balance." Neurosci Biobehav Rev 26(4): 485-98.

Sujino, M., K. H. Masumoto, et al. (2003). "Suprachiasmatic nucleus grafts restore circadian behavioral rhythms of genetically arrhythmic mice." Curr Biol 13(8): 664-8.

Szafarczyk, A., G. Ixart, et al. (1979). "Effects of lesions of the suprachiasmatic nuclei and of p-chlorophenylalanine on the circadian rhythms of adrenocorticotrophic hormone and corticosterone in the plasma, and on locomotor activity of rats." J Endocrinol 83(1): 1-16.

Tapp, W. N. and B. H. Natelson (1989). "Circadian rhythms and patterns of performance before and after simulated jet lag." Am J Physiol 257(4 Pt 2): R796-803.

Tharp, G. D. and G. E. Folk, Jr. (1965). "Rhythmic Changes in Rate of the Mammalian Heart and Heart Cells during Prolonged Isolation." Comp Biochem Physiol 14: 255-73.

Torres-Farfan, C., M. Seron-Ferre, et al. (2006). "Immunocytochemical demonstration of day/night changes of clock gene protein levels in the murine adrenal gland: differences between melatonin-proficient (C3H) and melatonin-deficient (C57BL) mice." J Pineal Res 40(1): 64-70.

Tosini, G. and M. Menaker (1996). "Circadian rhythms in cultured mammalian retina." Science 272(5260): 419-21.

Turek, F. W., C. Joshu, et al. (2005). "Obesity and metabolic syndrome in circadian Clock mutant mice." Science 308(5724): 1043-5.

Ulrich-Lai, Y. M., M. M. Arnhold, et al. (2005). "Adrenal splanchnic innervation contributes to the diurnal rhythm of plasma corticosterone in rats by modulating adrenal sensitivity to ACTH." Am J Physiol Regul Integr Comp Physiol.

Ulrich-Lai, Y. M. and W. C. Engeland (2000). "Rat adrenal transplants are reinnervated: an invalid model of denervated adrenal cortical tissue." J Neuroendocrinol 12(9): 881-93.

van der Horst, G. T., M. Muijtjens, et al. (1999). "Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms." Nature 398(6728): 627-30.

Vielhaber, E. L., D. Duricka, et al. (2001). "Nuclear export of mammalian PERIOD proteins." J Biol Chem 276(49): 45921-7.

Vitaterna, M. H., D. P. King, et al. (1994). "Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior." Science 264(5159): 719-25.

Wassle, H. (2004). "Parallel processing in the mammalian retina." Nat Rev Neurosci 5(10): 747-57.

Waterhouse, J., T. Reilly, et al. (2007). "Jet lag: trends and coping strategies." Lancet 369(9567): 1117-29.

Watts (1991). The efferent projections of the suprachiasmatic nucleus: anatomical insights into the control of circadian rhythms. New York, Oxford University Press.

Welsh, D. K., D. E. Logothetis, et al. (1995). "Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms." Neuron 14(4): 697-706.

Welsh, D. K., S. H. Yoo, et al. (2004). "Bioluminescence imaging of individual fibroblasts reveals persistent, independently phased circadian rhythms of clock gene expression." Curr Biol 14(24): 2289-95.

Whitmore, D., N. S. Foulkes, et al. (1998). "Zebrafish Clock rhythmic expression reveals independent peripheral circadian oscillators." Nat Neurosci 1(8): 701-7.

Wiegert, O., M. Joels, et al. (2006). "Timing is essential for rapid effects of corticosterone on synaptic potentiation in the mouse hippocampus." Learn Mem 13(2): 110-3.

Wittmann, M., J. Dinich, et al. (2006). "Social jetlag: misalignment of biological and social time." Chronobiol Int 23(1-2): 497-509.

Xia, Z., H. Dudek, et al. (1996). "Calcium influx via the NMDA receptor induces immediate early gene transcription by a MAP kinase/ERK-dependent mechanism." J Neurosci 16(17): 5425-36.

Yagita, K., F. Tamanini, et al. (2002). "Nucleocytoplasmic shuttling and mCRY-dependent inhibition of ubiquitylation of the mPER2 clock protein." EMBO J 21(6): 1301-14.

Yamaguchi, S., H. Isejima, et al. (2003). "Synchronization of cellular clocks in the suprachiasmatic nucleus." Science 302(5649): 1408-12.

Yamazaki, S., R. Numano, et al. (2000). "Resetting central and peripheral circadian oscillators in transgenic rats." Science 288(5466): 682-5.

Yan, L., S. Miyake, et al. (2000). "Distribution and circadian expression of dbp in SCN and extra-SCN areas in the mouse brain." J Neurosci Res 59(2): 291-5.

Yaylaoglu, M. B., A. Titmus, et al. (2005). "Comprehensive expression atlas of fibroblast growth factors and their receptors generated by a novel robotic *in situ* hybridization platform." Dev Dyn 234(2): 371-86.

Yin, L., J. Wang, et al. (2006). "Nuclear receptor Rev-erbalpha is a critical lithium-sensitive component of the circadian clock." Science 311(5763): 1002-5.

Yoo, S. H., S. Yamazaki, et al. (2004). "PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues." Proc Natl Acad Sci U S A 101(15): 5339-46.

Young, M. E. and M. S. Bray (2007). "Potential role for peripheral circadian clock dyssynchrony in the pathogenesis of cardiovascular dysfunction." Sleep Med 8(6): 656-67.

Yu, W., M. Nomura, et al. (2002). "Interactivating feedback loops within the mammalian clock: BMAL1 is negatively autoregulated and upregulated by CRY1, CRY2, and PER2." Biochem Biophys Res Commun 290(3): 933-41.

Zheng, B., U. Albrecht, et al. (2001). "Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock." Cell 105(5): 683-94.

Zheng, B., D. W. Larkin, et al. (1999). "The mPer2 gene encodes a functional component of the mammalian circadian clock." Nature 400(6740): 169-73.

Zimmermann, E. and V. Critchlow (1967). "Effects of diurnal variation in plasma corticosterone levels on adrenocortical response to stress." Proc Soc Exp Biol Med 125(2): 658-63.

Zylka, M. J., L. P. Shearman, et al. (1998). "Molecular analysis of mammalian timeless." Neuron 21(5): 1115-22.

Chapter 6

Curriculum vitae

Silke Kießling

* 22.07.1980	in Bremen/ Germany
06/00	"Abitur"from German secondary school qualifying for university admission, Europaschule Johannes-Kepler-Gymnasium, Garbsen, Germany
10/00- 10/02	Basic studies of biology, University of Hanover, Germany
10/02	"Vordiplom" in biology, University of Hanover
10/04	Diploma exams at the University of Hanover, Germany; main subjects: zoology / physiology, behavioral biology and biophysics,
03/04-06/04	Project work in the group of Prof. Dr. Angelika Anders, Institute of Biophysics, University of Hanover on: "In Vivo Absorption Spectra of Human Skin in the Ultradian Wavelength Range as Measured by Optoacoustics"
10/04 - 06/05	Diploma project on: "Characterisation of *mPer1* and *mPer2* mutant mice", Institute of Zoology, Department for Ecophysiology and Chronobiology, University of Veterinary Medicine in Hanover, Germany
Since 09/05	Ph.D. studentship in the group of Prof. G. Eichele at the Max Planck Institute for Biophysical Chemistry, Department of Genes and Behavior in Goettingen/Germany on: "Functional analysis of the adrenal circadian clock"

Publications:

Kiessling S., Eichele G. and Oster H., 2009. A Role for Adrenal Glucocorticoids in the Circadian Resynchronization during Jet Lag. reviewed by *The Journal of Clinical Investigation*

Oster, H., Damerow, S., Kiessling, S., Jakubcakova, V., Abraham, D., Tian, J., Hoffmann, M.W., and Eichele, G. 2006. The circadian rhythm of glucocorticoids is regulated by a gating mechanism residing in the adrenal cortical clock. *Cell Metab* 4:163-173.

Chapter 7

Acknowledgements

First of all I would like to thank Gregor Eichele for supervision and the advice he gave me throughout the laborious years I spent in his group in Hannover and in Göttingen. I appreciate that he gave me the opportunity to realize this project and the room he gave me to try my own solutions with generous equipment.

I would like to thank Stephan Steinlechner for all the help and advice he gave me since I finished my diploma thesis in his group and for accepting reference of my thesis, too.
Hansjörg Küster I would like to thank for accepting to examine my thesis.

Most of all I would like to thank Henrik Oster for his support, his patience and all the brilliant ideas, even in his absence. His doors and ears were always open for the problems I had during the last four years.

Thanks to all the people at the Max Planck Institute in Hannover and Göttingen who helped me a lot in finishing this work, especially Jana Husse for the inspiring comments she made and Ana Martinez-Hernandez who did an excellent job in revising this thesis.
And I want to thank the whole Circadian Rhythm Group, as they are Dominic Landgraf, Nadine Naujokat, Judit Kovac, Brid Bode and Anton Shostak. They helped me to take my work with humor, even if it was the collection and processing of feces ☺.

Zu allerletzt möchte ich mich herzlichst bei meiner Familie, meiner Mutter Monika und meinem Vater Reiner ebenso wie bei meinem Bruder Dirk und meinem Freund Jan für ihre Unterstützung, moralischen Beistand und ihre Geduld bedanken, die mir durch die ganzen letzten Jahre geholfen und mich meinen Zielen ein großes Stück näher gebracht haben.
I am very grateful that you allowed me to do what I love to do.

Without all this people this thesis could never have been written. Thanks a lot.

Silke.

Hannover, the 21.10.2009

Erklärung zur Dissertation

gemäß §6(1) der Promotionsordnung der Naturwissenschaftlichen Fakultät der Gottfried Wilhelm Leibniz Universität Hannover

für die Promotion zum Dr. rer. nat.

Hierdurch erkläre ich, dass ich meine Dissertation mit dem Titel

Functional Analysis of the Adrenal Circadian Clock

selbständig verfasst und die benutzten Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfeleistungen herangezogenen Institutionen vollständig angegeben habe.

Die Dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere Prüfungsarbeit verwendet.

_____

(Unterschrift)

Name: Silke Kießling