Genetic interaction of *Per*- and *Dec*-genes in the mammalian circadian clock

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Brid Bode

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Thesis Committee:

Dr. Henrik Oster, Circadian Rhythms Group, Max Planck Institute for Biophysical Chemistry (Reviewer)

Prof. Dr. Julia Fischer, Cognitive Ethology, German Primate Center (Reviewer)

Prof. Dr. Walter Paulus, Dept. of Clinical Neurophysiology, Georg-August-University Göttingen

Date of the oral examination: 31.05.2011

Brid Bode
Gartenstraße 3
37073 Göttingen

Göttingen, 20.04.2011

Affidavit that the thesis with the title ‘genetic interaction of *Per*- and *Dec*-genes in the mammalian circadian clock’ submitted by me, Brid Bode, was written independently and with no other sources and aids than quoted.

Brid Bode

I Summary

Many behavioural and physiological processes in mammals display circadian (24 hour) rhythms controlled by an internal timekeeping system – the circadian clock. The pacemaker of the circadian clock of mammals is located in the suprachiasmatic nuclei (SCN) of the hypothalamus and synchronizes peripheral oscillators in a hierarchical manner to the external light/dark (LD) cycles by humoral and neuronal pathways. The molecular timekeeping machinery consists of a network of transcriptional translational feedback loops (TTL). The mammalian core TTL includes CLOCK/(NPAS2) and BMAL1/(ARNTL) which together activate the transcription of E-box controlled clock genes such as *Period (Per1-3)*, *Cryptochrome (Cry1,2)* and *Dec (1,2)*. PER (1,2) and CRY (1,2) heterodimerize and translocate back to the nucleus where they repress CLOCK/BMAL1 mediated transcription. The basic helix-loop-helix transcription factors DEC1 (BHLHE40) and DEC2 (BHLHE41) can interact with the core TTL by binding to CLOCK/BMAL1 complexes or to E-box elements, forming an accessory feedback mechanism. In *Drosophila*, the DEC ortholog CWO shows synergistic interaction to PER. This promoted, us to analyze PER(1,2)-DEC interactions in the murine circadian system. We generated *Per(1,2)/Dec* double and triple mutant mice and measured circadian locomotor behaviour and clock gene expression in the SCN. Our wheel-running data suggest synergistic *Per(1,2)-Dec* interactions in photic entrainment with an advanced activity onset indicative of impaired sleep behaviour. Under free-run, the *Per1-Dec* interactions remain synergistic whereas the *Per2-Dec* interaction becomes antagonistic together with a partial rescue of the *Per2* phenotype. This rescue is seen at multiple levels including period length and rhythmicity of behaviour as well as clock gene expression in the SCN. The molecular data suggest a bimodular regulatory function of *Per(1,2)-Dec* on E-box controlled clock genes in the SCN, moreover *Per1-Dec* bimodularity is time-of–day dependent. For the first time, we show that DECs together with PER1 activate the transcription of *Bmal1* in the SCN. Investigations of photic phase delay response suggest a model for phase delay resetting with an essential role of *Per2*/PER2 and a minor role of *Per1*/PER1. Together, our results show interactions of *Per(1,2)* and *Dec(1,2)* feedback in the mammalian pacemaker with different modes of interactivities under entrained and free-run conditions together with a compensation effect. These data postulate a conservation of *Per-Dec (Cwo)* interactions between vertebrate and invertebrate circadian clocks.

II Zusammenfassung

Viele Verhaltens- und physiologische Prozesse zeigen zirkadiane (~24 Stunden; von lat. *circa dies*: „ungefähr ein Tag“) Rhythmen, welche durch interne Uhren reguliert werden. In Säugetieren sitzt der zentrale zirkadiane Schrittmacher im *Nucleus suprachiasmaticus* (SCN) des Hypothalamus. Von dort werden untergeordnete Uhren in den Organen des Körpers kontrolliert, die dann das Zeitsignal in physiologische Befehle umsetzen. Auf molekularer Ebene basieren zirkadiane Uhren auf einem Netzwerk von transkriptionell-translatorischen Rückkopplungsschleifen (TTL, engl. *transcriptional translational feedback loops*) aus sog. Uhrengenen, darunter *Period (Per1-3)*, *Cryptochrome (Cry1,2)*, *Bmal1* (*Arntl*) und *Clock* (*Npas2*). CLOCK und BMAL1 sind zwei Transkriptionsfaktoren, die über cis-regulatorische E-Box-Promotorelemente die Expression der *Period*- und *Cryptochrome*-Gene aktivieren. PER- und CRY-Proteine heterodimerisieren im Zytoplasma und werden in den Zellkern zurückgeführt. Dort inhibieren sie den CLOCK/BMAL1-Komplex – und damit ihre eigene Produktion.

Die Helix-Loop-Helix-Transkriptionsfaktoren DEC1 (BHLHE40) und DEC2 (BHLHE41) interagieren über Bindung an den CLOCK/BMAL1-Komplex oder an E-Box- Sequenzen sowohl inhibitorisch als auch aktivatorisch mit dem zirkadianen TTL. Für *Drosophila* wurde eine synergistische Interaktion zwischen dem DEC-Ortholog CWO und PER postuliert, welches eine ähnliche *Per(1,2)-Dec*-Interaktion in der Säugetieruhr vermuten lässt. Um dies zu untersuchen, wurden *Per(1,2)/Dec*-doppel- und tripel-mutante Mäuse (*Mus musculus*) generiert, die im Laufrad hinsichtlich ihres zirkadianen Verhaltens als auch auf molekularer Ebene per *In Situ*-Hybridisierung analysiert wurden. Es zeigte sich, dass synergistische *Per(1,2)-Dec*-Interaktionen während des Entrainments vorliegen. *Per-Dec*-Doppel- und Tripelmutanten zeigen einen vorgezogenen Aktivitätsbeginn, ähnlich zu Menschen mit „Vorgelagertem Schlafphasensyndrom“ (ASPS, engl. *advanced sleep phase syndrome*). Unter konstanten Umweltbedingungen ist der *Per1-Dec*-Synergismus beibehalten. Allerdings zeigen *Per2* und *Dec1/2* nun antagonistische Interaktionen. In *Per2/Dec*-Doppelmutanten zeigt sich zudem ein Rekonstitutionseffekt hinsichtlich Periodenlänge und Rhythmik im Verhalten als auch auf molekularer Ebene im SCN. Weiterhin zeigten die *In situ*-Daten, dass im SCN eine bimodale Funktion der *Per(1,2)-Dec*-Interaktion auf die Uhrengenexpression vorliegt, wobei die *Per1-Dec* Interaktion tageszeitabhängig variiert. Erstmalig konnten wir zudem nachweisen, dass die

DECs zusammen mit PER1 die *Bmal1*-Transkription im SCN regulieren. Zusammenfassend lässt sich sagen, dass Gen-spezifische, tageszeit- und lichtabhängige funktionelle Interaktionen zwischen den *Per(1,2)*- und *Dec(1,2)*-Rückkopplungsschleifen im zirkadianen Uhrensystem der Säugetiere vorliegen. Die PER-CWO-Interaktion in der Uhr der Fliege scheint im TTL der Säugetiere weitestgehend konserviert.

Abbreviations

°C	degree Celsius
µm	micrometer
^{35}S-UTP	sulphur-35 labeled uridinetriphosphate
3V	third ventricle
5-HT	serotonin
A	adenine
ACTH	adrenocorticotropic hormone
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ASPS	advanced sleep phase syndrome
AVP	arginine vasopressin
bHLH	basic heix-loop-helix
Bmal1	Brain-and-muscle-arntl-like protein 1
BPD	bipolar disorder
C	cytosine
Calb	calbindin
cAMP	cyclic adenosine monophosphate
CCG	clock-controlled gene
cDNA	complementary DNA
CK1	casein kinase 1
Clock	circadian locomotor output cycles kaput
CMV	human cytomegalovirus
CNS	central nervous system
CO	carbon monoxide
CRE	cAMP response element
CREB	cAMP response element binding protein
Cry	Cryptochrome
CT	Circadian time
cwo	clockwork orange
Dbp	D-box binding protein
DD	constant darkness
DFG	German Research Foundation
dm	dorsomedial
DMH	dorsomedial nucleus of the hypothalamus
DNA	deoxyribonucleic acid
DRN	dorsal raphe nucleus
DSPS	delayed sleep phase syndrom
e.g.	for example
E4bp4	E4 promoter-binding protein 4

engl.	English
F1 or F2	generation after the parent generation
FAA	food anticipatory activity
FASPS	familial advanced sleep phase syndrome
FBXL3	F-box and leucine-rich repeated protein 3
FEO	food-entrainable oscillator
Fig.	Figure
G	guanine
GABA	gamma-aminobutyric acid
GH	growth hormone
GHT	geniculate-hypothalamic tract
Glu	glutamate
GRP	gastin-releasing peptide
h	hour
HA	human influenza hemagglutinin
HEK293	Human Embryonic Kidney 293 cells
i.e.	id est
IGL	intergeniculate leaflet
ipRGCs	intrinsically photosensitive retinal ganglion cells
ISH	*in situ* hybridisation
lat.	Latin
LD	light/dark
LL	constant light
LP	light pulse
luc	luciferase
MAP	mitogen-activated protein
MDD	major depressive disorder
mEnk	met-Enkephalin
min	minute
MPO	medial preoptic region
MRN	median raphe nucleus
n.a.	not analysed
n.i.	no genetic interaction
NAD	nicotinamide adenine dinucleotide
NAMPT	nicotinamide phosphorybosyltransferase
NMDAR	N-Methyl-D-Aspartate receptor
NO	nitric oxide
Npas2	neuronal PAS domain protein 2
NYP	neuropeptide Y
OP	optic chiasm
P	parent generation

p.m.	post meridiem (engl. after midday)
PACAP	pituitary adenylate cyclase activating polypeptide
PAS	Period-Arnt-Single-minded
PCR	polymerase chain reaction
Per	*Period*
PFA	paraformaldehyde
PK2	prokineticin 2
PKA	protein kinase A
PKCα	protein kinase C α
PLM	periodic limb movements
PRC	phase response curve
PVN	paraventricular nucleus
RHT	retino-hypothalamic tract
RLS	restless legs syndrome
RNA	ribonucleic acid
RORE	retinoic acid-related orphan receptor response element
RORα	retinoid-related orphan receptor α
S	supplement
SAD	seasonal affective disorder
SEM	standard error of the mean
siRNA	small interfering RNA
T	thymine
TGFα	transforming growth factor α
TTL	transcriptional/translational feedback loop
VIP	vasoactive intestinal polypeptide
vl	ventrolateral
vol.	volume
WT	wild-type
ZT	*Zeitgeber* time
τ	tau, period lenght

1. Chapter 1: Introduction

Most organisms living on the earth are under the steady influence of daily changes resulting from the rotation of the planet around its axis. During evolution, organisms have adapted their physiological and behavioural parameters to these periodic environmental changes by developing internal mechanisms to determine the time of day - circadian clocks (from *circa* (lat.) – approximately, *dies* (lat.) – day) (Ouyang, Andersson et al. 1998).

1.1. Circadian rhythms

In 1729 the French astronomer Jean Jacques Ortous de Mairan wrote the first scientific report on endogenously driven biological rhythms with periods of approximately 24 hours (h). He observed the daily leaf movements of *Mimosa pudica* when the plants were kept in a closed cupboard. Since then circadian rhythms have been observed at all levels of biological organisation, from behavioural changes in mammals, flies and fish to the activity of enzymes and the transcription of specific genes (Pittendrigh 1993).

Endogenous circadian clocks share certain properties including self-sustainment, temperature compensation and the ability of entrainment (or synchronisation) in a time-dependent manner by environmental stimuli – termed *Zeitgebers* (‘time givers’ in German). Circadian clocks maintain a stable endogenous period of approximately 24 h, even in absence of external *Zeitgebers*. Because of a deviation of the internal period from 24 h (see below) the internal timing system has to be reset (shifted) every day by a few minutes to stay in synchrony with the environmental time. The main *Zeitgeber* for the mammalian clock is light, but there are other, non-photic, *Zeitgebers* like food, drugs and social interactions. In the absence of external time cues, i.e. under constant lighting conditions (constant darkness (DD) or constant light (LL)), behavioural and physiological rhythms begin to free-run with a period slightly different to 24 h. In mice, a well-established readout for circadian rhythms is locomotor activity, which can be measured by counting running-wheel revolutions. Behavioural activity can be visualized as an actogram (Figure 1) in which wheel revolutions (represented in black dots) are

plotted against time. A double-plotted actogram shows two days per horizontal line. Day 2 is repeated on the next horizontal line together with day 3 (and so on). Under alternating light/dark (LD) cycles, the locomotor activity of a mouse shows an alteration of 12 h : 12 h rest (called *rho*) and active (*alpha*) periods. If the internal period (*τ, tau*) is exactly 24 h the animal is entrained to the external LD cycle. The LD cycle is measured in *Zeitgeber* times (ZT) based on the period of the *Zeitgeber* (in this case the light regime). ZT0 is defined as the time of 'lights on' and ZT12 as time of 'lights off'.

In the absence of a *Zeitgeber*, the circadian day is portioned into two phases: subjective day and subjective night, which correspond to the rest and active phase, respectively, for nocturnal species. The circadian day is scaled in 24 circadian hours (Circadian time; CT). Under constant conditions, the beginning of the subjective day is defined as CT0 (= CT24) and the beginning of the subjective night is CT12 (the onset of activity in nocturnal mice).

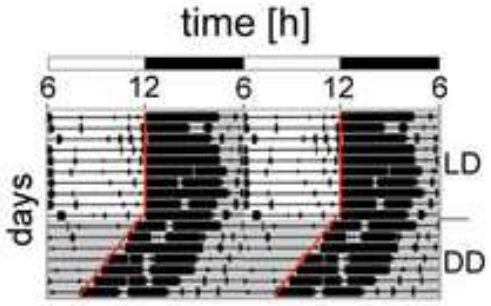

Figure 1: Actogram. Double plotted actogram of mouse locomotor activity (represented in black vertical bars). The mouse is entrained to a 12 h : 12 h LD cycle and after 10 days released into constant dark conditions (DD, dark phases marked in grey). In DD, the activity rhythm starts to free-run with a period of 23.5 h. ZT0 in LD corresponds to 6:00 p.m. The red lines indicate the onset of locomotor activity. Modified from (Jud, Schmutz et al. 2005)

1.1.1. Physiological circadian rhythms

The circadian oscillator controls diverse physiological processes such as the sleep/wake cycle, daily rhythms in energy metabolism, body temperature and hormone release (Saper, Cano et al. 2005). Blood concentrations of many hormones show circadian rhythms (Hastings, O'Neill et al. 2007). For instance, the hypothalamus sends circadian signals to the pituitary gland that releases hormones in a circadian fashion such as prolactin, adrenocorticotropic hormone (ACTH) and growth hormone (GH) that, in turn, stimulate peripheral endocrine glands like the thyroid, gonads, and the adrenal gland which themselves rhythmically release hormones. For example, ACTH stimulates the circadian synthesis of glucocorticoids in the adrenal glands (Reinberg and Smolensky 1982). The peak of glucocorticoid release correlates with the beginning of the activity phase, i.e. in the early morning in diurnal and in the early night in nocturnal animals

(Kalsbeek, Kreier et al. 2007). Glucocorticoids 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). Furthermore, melatonin synthesis in the pineal gland is under circadian control. Plasma melatonin levels are high during the night and low during the day. Melatonin is involved in sleep/wake cycle regulation (Cajochen, Krauchi et al. 2003) and also modulates cortisol secretion from the adrenal gland.

Various enzymes of metabolic pathways such as glycolysis, gluconeogenesis, cholesterol and lipid metabolism are under circadian control (Panda, Antoch et al. 2002), suggesting a strong link between the circadian system and metabolism. The circadian timekeeping system drives rhythmic metabolic processes and, vice versa, various metabolic parameters affect the clock (reviewed by Green, Takahashi et al. 2008). Thus, it is not surprising that deregulation of the circadian system can contribute to metabolic diseases (see below).

A further physiological aspect regulated by the circadian clock is the immune system, which consists of innate components and adaptive components (T and B lymphocytes). It was shown that the circadian system regulates immune responses in humans (Levi, Canon et al. 1988). The proliferative activity of circulating T- and B-cells in the peripheral blood varies throughout the day (Levi, Canon et al. 1988). T-cell response and proliferation is most efficient in the morning; B-cells, however, show higher reactivity in the evening (Deshmukh 2006).

1.2. The mammalian clock system

The circadian clock system consists of three main parts: 1) a central pacemaker which generates clock rhythms, 2) an input pathway which resets the clock to the outside environment by photic as well as non-photic *Zeitgebers*, and 3) clock output pathways to efferent systems.

1.2.1. The suprachiasmatic nucleus (SCN)

In 1972, two independent groups identified the pacemaker of the mammalian circadian clock in the ventrolateral hypothalamus - the suprachiasmatic nuclei (SCN). Lesion of

these structures results in a loss of corticosterone secretion (Moore and Eichler 1972) and of drinking and locomotor activity rhythms (Stephan and Zucker 1972). Further SCN lesions in rats show disrupted circadian rhythms of melatonin secretion (Klein and Moore 1979) suggesting a SCN-mediated regulation of melatonin production in the pineal gland.

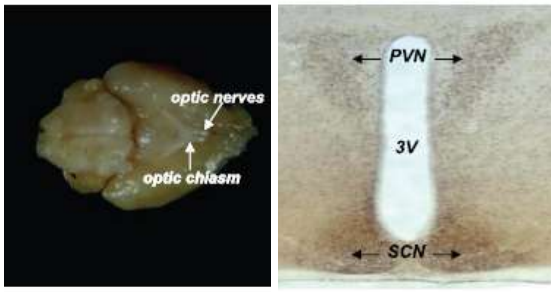

Figure 2: Anatomical view of the SCN. Left picture shows a ventral view on the mouse brain. Right picture shows light-induced *c-Fos* expression in the ventromedial hypothalamus. Modified from (Golombek and Rosenstein 2010)

The bilateral SCN consist of approximately 10,000 heterogeneous neurons. They are located dorsal to the optic chiasm on either side of the third ventricle (Figure 2). The two main subdivisions of the SCN are the ventrolateral area (vlSCN or 'core') and the dorsomedial region (dmSCN or 'shell') (Moore, Speh et al. 2002). The core SCN sits adjacent to the optic chiasm and contains neurons that synthesize vasoactive intestinal polypeptide (VIP) and gastrin-releasing peptide (GRP). The dorsomedial SCN expresses arginine vasopressin (AVP), calbindin (Calb) and met-Enkephalin (mEnk) (Golombek and Rosenstein 2010; Figure 3).

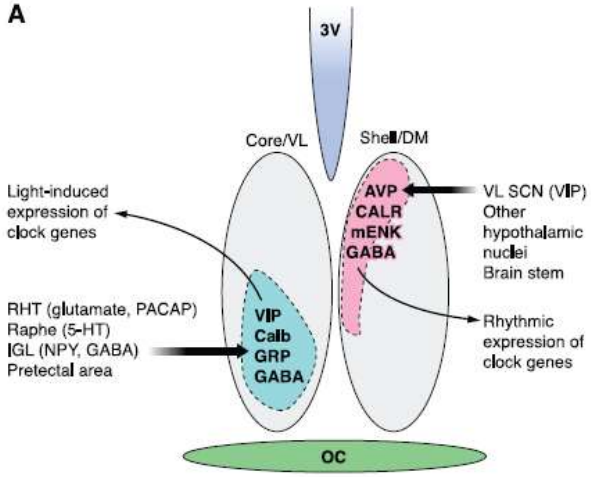

Figure 3: Subdivisions of SCN. The left SCN represents the neurochemical subdivision of the ventrolateral SCN, which is connected to the RHT, the raphe nuclei and the IGL. The right SCN shows the neurotransmitter subdivision of the dorsomedial SCN, which receives innervation from the ventrolateral SCN and other hypothalamic areas.
RHT, retino-hypothalamic tract; 3V, third ventricle; OC, optic chiasm; IGL, intergeniculate leaflet; VL, ventrolateral; DM, dorsomedial; VIP, vasoactive intestinal polypeptide; Calb, calbindin; GRP, gastrin-releasing peptide; AVP, arginine vasopressin; CALR, calretinin; mENK, met-Enkephalin. Modified from (Golombek and Rosenstein 2010)

In 1979, Inouve et al. demonstrated that SCN neurons are able to maintain circadian rhythms of spontaneous electrical activity even in isolation from other brain structures (Inouye and Kawamura 1979). Moreover, these rhythms are maintained *ex vivo* such as in

SCN slices and in dissociated SCN cells (Green and Gillette 1982; Shibata, Oomura et al. 1982; Welsh, Logothetis et al. 1995; Yamaguchi, Isejima et al. 2003). Neuropeptide signaling including VIP (through VPAC receptors) and GRP-mediated communication is necessary for neuronal synchronization within the SCN (Maywood, Reddy et al. 2006; Maywood, O'Neill et al. 2007; Hughes, Guilding et al. 2008). Others proposed that intra-SCN synchronizing mechanisms include nitric oxide (NO) and gamma-aminobutyric acid (GABA) neurotransmission (Liu and Reppert 2000; Albus, Vansteensel et al. 2005; Aton, Colwell et al. 2005; Vosko, Schroeder et al. 2007).

Taken together, the SCN contain autonomous cellular oscillators, which are synchronized by neuronal and neuropeptide signalling. The oscillatory coupling of the SCN neurons is essential for the nuclei to act as a pacemaker (Liu, Welsh et al. 2007).

1.2.2. Clock input

The SCN clock can be reset via three different pathways: 1) the retino-hypothalamic tract (RHT), 2) the geniculate-hypothalamic tract (GHT) and 3) serotonergic (5-HT) input from the dorsal raphe nucleus (DRN) and median raphe nucleus (MRN) (Dibner, Schibler et al. 2010). The RHT pathway transmits photic information from the eye (Moore and Lenn 1972), whereas the GHT and the raphe provide non-photic input to the SCN (Figure 4).

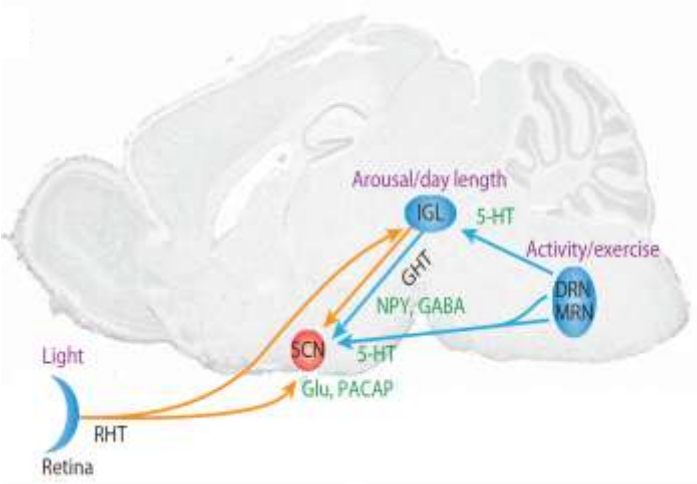

Figure 4: Afferent pathways to the SCN in rat brain. Photic input (orange arrows) and non-photic input (blue arrows) to the SCN. 5-HT, serotonin; DRN, dorsal raphe nucleus; IGL, intergeniculate leaflet; GABA, gamma-aminobutyric acid; GHT, geniculohypothalamic tract; Glu, glutamate; MRN, median raphe nucleus; NPY, neuropeptide Y; PACAP, pituitary adenylate cyclase activating polypeptide; RHT, retino-hypothalamic tract; SCN, suprachiasmatic nucleus; Modified from (Dibner, Schibler et al. 2010)

The classical retinal photoreceptors are not critical for circadian synchronization as mice with ablation of rods and cones still entrain to light (Freedman, Lucas et al. 1999; Lucas, Freedman et al. 2001). However, in the additional absence of the retinal photopigment melanopsin which is expressed in a subset of directly light sensitive ganglion cells (ipRGCs, intrinsically photosensitive retinal ganglion cells) (Provencio,

Rollag et al. 2002) mice fail to entrain to light/dark cycles and show abolished masking responses to light (Hattar, Lucas et al. 2003). Thus, melanopsin containing ipRGCs act as photic time sensors for the SCN clock.

The RHT transmits the light information from the ipRGCs via monosynaptic projections (Berson 2007) to the ventrolateral part of the SCN (SCN core), signalling via the neurotransmitters glutamate (Glu) and pituitary adenylate cyclase activating polypeptide (PACAP) (Figure 5; Ebling 1996; Hannibal, Ding et al. 1997; Hannibal 2002; Hirota and Fukada 2004; Hannibal 2006). Cells in the SCN express different glutamate (AMPAR and NMDAR) (Gannon and Rea 1994; Mick, Yoshimura et al. 1995; Ebling 1996; Mizoro, Yamaguchi et al. 2010) and PACAP receptors (Hannibal 2002). Neuronal stimulation of the SCN results in activation of intracellular signalling cascades including calcium-dependent kinases and proteases (calmodulin, MAP kinases and PKA) (Obrietan, Impey et al. 1998; Yokota, Yamamoto et al. 2001; Hirota and Fukada 2004; Antle, Smith et al. 2009). Ultimately the transcription factor cAMP response element binding protein (CREB) is phosphorylated (Ginty, Kornhauser et al. 1993; Gau, Lemberger et al. 2002) and induces the expression of clock genes, e.g. *Per1* (Shigeyoshi, Taguchi et al. 1997; Yan and Silver 2002), *Per2* (Shearman, Zylka et al. 1997; Yan and Silver 2002) and *Dec1* (Honma, Kawamoto et al. 2002) via binding to cAMP response element (CRE) sequences in the respective promoters (Gillette and Tischkau 1999; Obrietan, Impey et al. 1999; Hirota and Fukada 2004; Antle and Silver 2005).

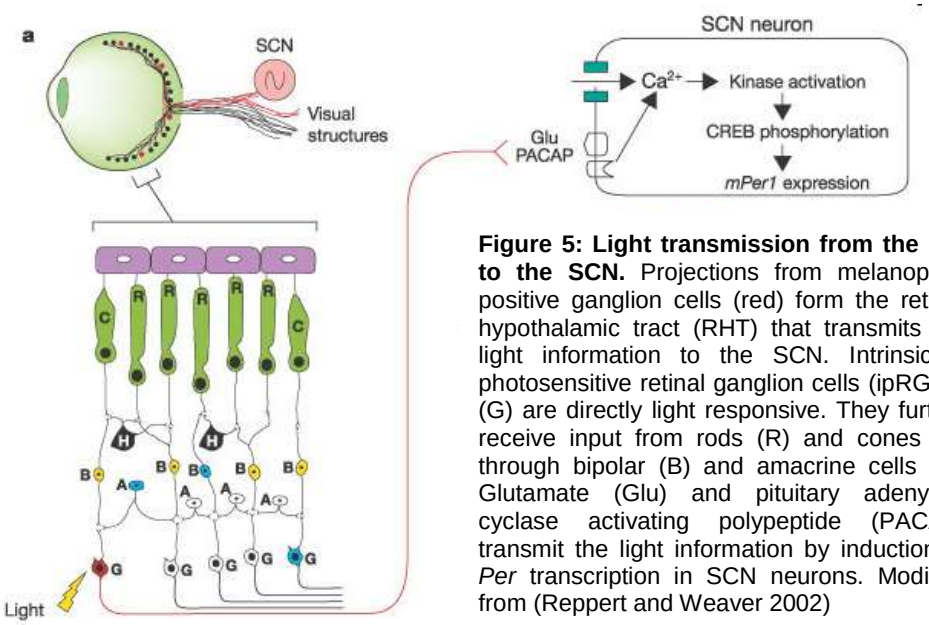

Figure 5: Light transmission from the eye to the SCN. Projections from melanopsin-positive ganglion cells (red) form the retino-hypothalamic tract (RHT) that transmits the light information to the SCN. Intrinsically photosensitive retinal ganglion cells (ipRGCs) (G) are directly light responsive. They further receive input from rods (R) and cones (C) through bipolar (B) and amacrine cells (A). Glutamate (Glu) and pituitary adenylate cyclase activating polypeptide (PACAP) transmit the light information by induction of *Per* transcription in SCN neurons. Modified from (Reppert and Weaver 2002)

Interestingly, the RHT projects not only to the SCN but also to the intergeniculate leaflet (IGL). The IGL relays photic signals to the SCN via the GHT based on neuropeptide Y (NPY) and GABA neurotransmission (Harrington, Nance et al. 1985; Moore and Card 1994; Jacob, Vuillez et al. 1999; Juhl, Hannibal et al. 2007).

In addition to light so called non-photic *Zeitgebers* exist (Hastings, Duffield et al. 1997) such as behavioural arousal (e.g. dark pulses during the light phase, novel wheel access; Reebs and Mrosovsky 1989; Van Reeth and Turek 1989), timed food availability (Mistlberger 1994; Stephan 2002; Feillet, Albrecht et al. 2006; Mendoza 2007), social contacts (Mrosovsky 1988; Mrosovsky, Reebs et al. 1989) and certain drugs (e.g. benzodiazepine, morphine; Van Reeth and Turek 1989; Marchant and Mistlberger 1995; Mrosovsky 1996). Figure 4 shows afferent projections to the SCN from different brain regions, such as the NPY system from the IGL and 5-HT input from the midbrain raphe which together mediates non-photic entrainment by behavioural arousal (Mrosovsky 1996; Hastings, Duffield et al. 1998). Further, mice can also entrain to daily food restriction, showing food anticipatory activity (FAA) prior to expected mealtime. FAA is represented by increase locomotion, corticosterone secretion and body temperature (Mistlberger 1994). The localisation and the molecular mechanism of this food-entrainable oscillator (FEO) are still unclear. Importantly, FAA is preserved in SCN-ablated animals (Stephan 2002).

1.2.3. Clock output

The central clock uses a dual control to coordinate daily rhythmicity in the periphery - neuronal and humoral pathways (Buijs and Kalsbeek 2001). SCN transplantation experiments show that the SCN generate the rhythmicity for the organism. The group of Menaker found that SCN transplantation restores circadian rhythms to arrhythmic animals with period of the donor genotype (Ralph, Foster et al. 1990). Other experiments show that the neurotransmitters GABA and glutamate are also crucial for transmission of SCN outputs (Hermes, Coderre et al. 1996). SCN neurons send efferent projections to other brain regions, such as the paraventricular nucleus (PVN), the medial preoptic region (MPO) and the dorsomedial nucleus of the hypothalamus (DMH) (Figure 6; see above) (Kalsbeek, Palm et al. 2006; Dibner, Schibler et al. 2010; Nader, Chrousos et al. 2010).

Placing SCN transplants encapsulated in a semipermeable membrane into the third ventricle restores behavioural rhythms in SCN-lesioned rats (Silver, LeSauter et al. 1996), postulating that diffusible molecules released by the transplant might be sufficient to restore rhythmicity without the need for synaptic connections. The current evidence suggests that the SCN also uses several humoral mediators as output signals such as argenine vasopressin (AVP) (Kalsbeek, Buijs et al. 1992), prokineticin 2 (PK2) (Cheng, Bullock et al. 2002), vasoactive intestinal polypeptide (VIP) (Kalsbeek and Buijs 1992) and transforming growth factor α (TGFα) (Kramer, Yang et al. 2001; Li, Sankrithi et al. 2002).

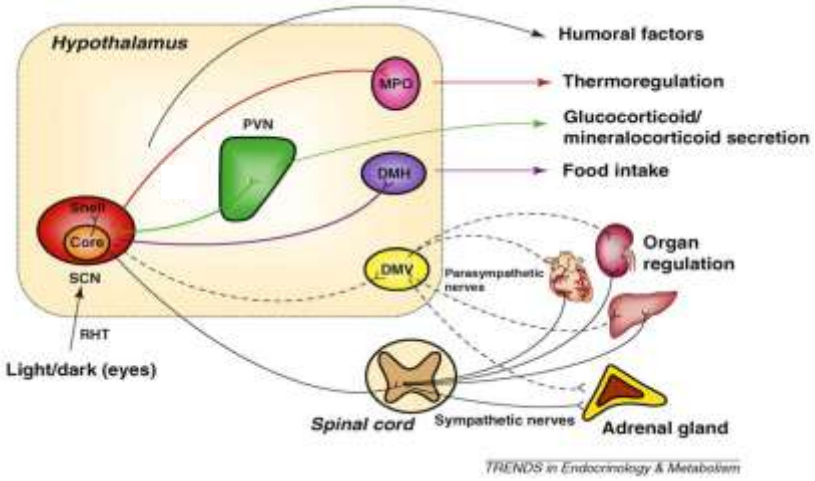

Figure 6: Neuronal and humoral pathways mediate the synchronization of peripheral rhythms. The SCN receives light information from the retina through the RHT. This time information is transmitted via efferent neurons from the SCN to other brain regions such as PVN, DMH and MPO which regulate glucocorticoid secretion, thermoregulation and food intake. PVN, paraventricular nucleus; DMH, dorsomedial nucleus of the hypothalamus; MPO, medial preoptic region; modified from (Nader, Chrousos et al. 2010)

1.2.4. The molecular clockwork

1.2.4.1. Transcriptional/translational feedback loops

The molecular basis of the circadian clock are interconnected transcriptional/translational feedback loops (TTL) that drive rhythmic expression of core clock components (Figure 7). These are defined as genes/proteins that are necessary for the generation and regulation of circadian rhythms within individual cells throughout the organism. In 1971, the first clock gene, *Period*, was identified in a mutagenesis screen in *Drosophila melanogaster* (Konopka and Benzer 1971). Since then other clock genes have been found in different model organisms such as cyanobacteria, fungi, flies, hamsters and mice as well as humans. These observations revealed that the molecular structure of

circadian clocks is evolutionary highly conserved (Rosbash 2009; Zhang and Kay 2010). Below, I will focus on the components of the mammalian TTL.

The positive limb of the mammalian core feedback loop includes members of the basic helix-loop-helix (bHLH)-Period-Arnt-Single-minded (PAS) transcription factor family: circadian locomotor output cycles kaput (CLOCK), its paralog neuronal PAS domain protein 2 (NPAS2) and their partner brain-and-muscle-Arnt-like protein 1 (BMAL1). CLOCK(NPAS2)/BMAL1 heterodimers activate the transcription of *cis*-regulatory E-box containing target genes including *Period* (*Per1-3*) and *Cryptochrome* (*Cry1*,*2*) genes (King, Zhao et al. 1997; Gekakis, Staknis et al. 1998; Hogenesch, Gu et al. 1998; Kume, Zylka et al. 1999; Bunger, Wilsbacher et al. 2000; Zheng, Albrecht et al. 2001). Negative feedback is achieved by the heterodimerization of PER and CRY proteins which together are translocated back to the nucleus and repress CLOCK(NPAS2)/BMAL1 mediated transcription (Figure 7) (Griffin, Staknis et al. 1999; Kume, Zylka et al. 1999; Okamura, Miyake et al. 1999; Shearman, Sriram et al. 2000; Lee, Etchegaray et al. 2001; Sato, Yamada et al. 2006), thereby shutting down their own expression.

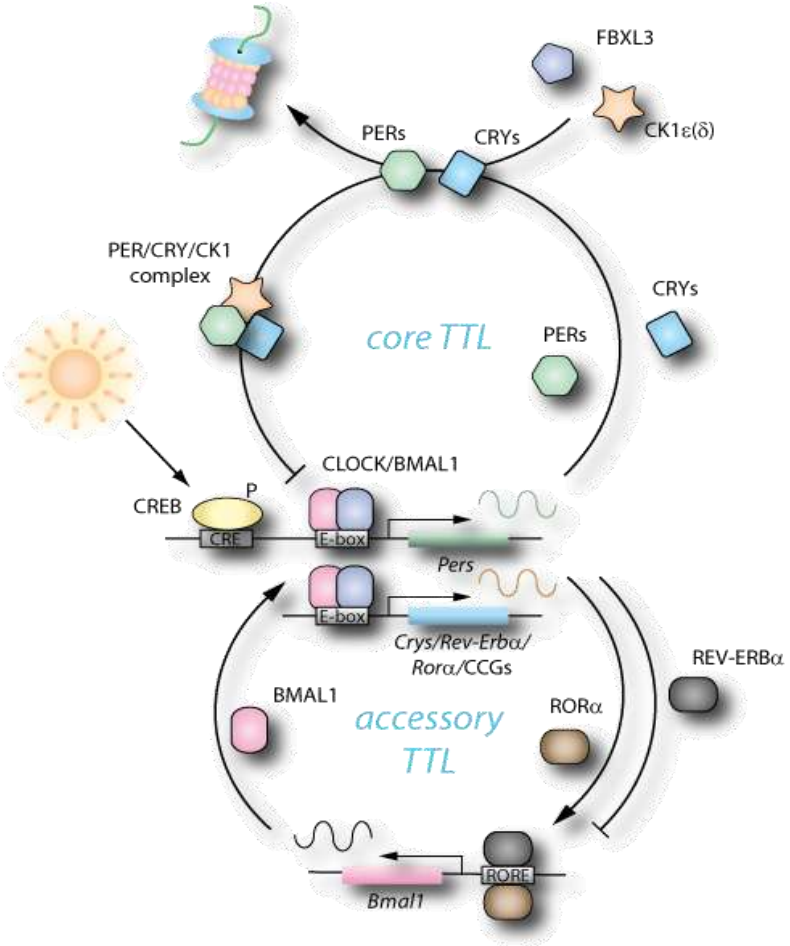

Figure 7: Transcriptional/translational feedback loops (TTLs) of the mammalian circadian clock. The CLOCK/BMAL1 complex activates the transcription of E-box containing clock (such as *Per*, *Cry*, *Rev-Erbα* and *RORα*) and clock-controlled genes (CCGs). PER and CRY heterodimerize and inhibit CLOCK/BMAL1-activated transcription. REV-ERBα and RORα form negative and positive feedbacks on *Bmal1* expression, respectively. Casein kinase 1 ε(δ) (CK1ε(δ)) and FBXL3 regulate the degradation of the PER/CRY complex. RORE, retinoic acid-related orphan receptor response element. Modified from (Oster 2006)

This autoregulatory feedback loop takes approximately 24 h to complete. Additionally, post-translational modifications such as phosphorylations and ubiquitinations regulate the period of the clock oscillator. Casein kinase (CK) 1ε and 1δ phosphorylate the clock proteins PER1, PER2, CRY1 and CRY2 (Akashi, Tsuchiya et al. 2002; Eide, Vielhaber et al. 2002) while F-box and leucine-rich repeat protein 3 (FBXL3) ubiquitinates CRY1 and CRY2 (Busino, Bassermann et al. 2007; Godinho, Maywood et al. 2007; Siepka, Yoo et al. 2007), negatively regulating PER1/2 and CRY1/2 accumulation by marking them for proteasomal degradation (Figure 7).

In addition to the core loop there are several accessory loops, one of which involves transcription of the orphan nuclear receptors *Rev-Erbα* and *Rorα* (Preitner, Damiola et al. 2002; Sato, Panda et al. 2004; Triqueneaux, Thenot et al. 2004; Akashi and Takumi 2005). REV-ERBα and RORα proteins compete for binding to retinoic acid-related orphan receptor response elements (ROREs) on the *Bmal1* promoter and repress or activate *Bmal1* transcription, respectively (Figure 7) (Preitner, Damiola et al. 2002; Ueda, Chen et al. 2002; Sato, Panda et al. 2004; Triqueneaux, Thenot et al. 2004; Akashi and Takumi 2005; Guillaumond, Dardente et al. 2005). An additional accessory loop is mediated via D-boxes, which are present in promoters of the *Per* genes. D-box-binding protein (DBP) and E4 promoter-binding protein 4 (E4BP4) act as activators or repressors, respectively, of the transcription of D-box containing genes. *Dbp* transcription itself is E-box regulated, whereas transcription of *E4bp4* is regulated by a RORE in its promoter.

Hundreds of rhythmic clock controlled genes (CCGs) are regulated by E-boxes, ROREs or D-boxes in their promoter regions. The phase of regulation depends on the combination of these elements on specific promoters and is highly tissue-specific, reflecting the physiological function of the tissue. In most tissues up to 10% of the whole transcriptome is rhythmically expressed (Panda, Antoch et al. 2002; Storch, Lipan et al. 2002; Panda and Hogenesch 2004).

Additional genes were discovered that influence the molecular machinery of the circadian system. A recent genome-wide small interfering RNA (siRNA) screen showed that more than 200 genes might play a role in the clock biology regulating amplitude and period of the cellular clock (Zhang, Liu et al. 2009). Thus, the circadian system appears more complex than presented in Figure 7. Further, it seems that clock genes are also affected by the metabolic state of a cell (reviewed by Kovac, Husse et al. 2009). Carbon monoxide (CO) inhibits DNA binding of NPAS2 and REV-ERBα (Dioum, Rutter et al. 2002) whereas REV-ERBα acts as sensor for heme which controls their DNA binding

activity (Kaasik and Lee 2004; Yin, Wu et al. 2007). Further, the DNA binding activity of CLOCK/BMAL1 heterodimers is dependent on the redox state of the cell (Rutter, Reick et al. 2001). The inhibition of the NAD biosynthesis enzyme nicotinamide phosphorybosyltransferase (NAMPT) promotes oscillation of *Per2* transcription by releasing CLOCK/BMAL1 from suppression by SIRT1 (Nakahata, Sahar et al. 2009; Ramsey, Yoshino et al. 2009).

1.2.4.2. *Dec1* and *Dec2*

Dec1 (*Bhlh40, Sharp2, Stra13, Clast5*) and *Dec2* (*Bhlh41, Sharp1*) were identified as cAMP-inducible genes in a culture system of human embryonic chondrocytes and by searching a data base for new bHLH transcription factor family members (Shen, Kawamoto et al. 1997; Fujimoto, Shen et al. 2001). Both DEC proteins are closely related (Fujimoto, Shen et al. 2001), but human DEC1 (hDEC1, 412 amino acids) is shorter than hDEC2 (482 amino acids). The mouse *Dec* genes are located on the same chromosome (*Dec1*: chr. 6 E-F1; *Dec2*: chr. 6 G2-G3).

DEC1 and DEC2 are regulators of different biological processes including embryonic development, cell differentiation, cell growth, immune function and hormone expression (Yamada and Miyamoto 2005). Moreover, the expression of *Dec1* and *Dec2* genes shows circadian rhythms in the SCN, several other brain regions and in the periphery (Honma, Kawamoto et al. 2002; Noshiro, Furukawa et al. 2005). *Dec* transcription is activated by CLOCK(NPAS2)/BMAL1 heterodimers binding to E-boxes in the *Dec* promoters and light exposure acutely induces *Dec1* transcription in SCN neurons (Honma, Kawamoto et al. 2002; Butler, Honma et al. 2004; Rossner, Oster et al. 2008). These data indicate that the *Decs*/DECs are involved in the circadian TTL. It was shown that DEC1 as well as DEC2 act as transcriptional regulators by competition with CLOCK/BMAL1 complexes for the *cis*-regulatory E-box elements, or by direct protein-protein interaction with BMAL1 (Honma, Kawamoto et al. 2002; Sato, Kawamoto et al. 2004) (Figure 8). Both DECs negatively regulate their own transcription *in vitro* (Honma, Kawamoto et al. 2002; Azmi, Sun et al. 2003; Li, Xie et al. 2003; Hamaguchi, Fujimoto et al. 2004; Kawamoto, Noshiro et al. 2004; Li, Song et al. 2004) and have tissue-dependent regulatory functions as co-activators or co-repressors at E-box sites (Rossner, Oster et al. 2008). *In vitro*, ovine DEC1 has bidirectional transcriptional function as an activator or repressor of *Per1* and *Rev-Erbα* transcription, respectively (Dardente, Fustin et al. 2009). *In vivo* analyses

suggest a role of DEC1 in circadian output regulation in the periphery (Grechez-Cassiau, Panda et al. 2004) where it modulates the phase of clock gene expression (Nakashima, Kawamoto et al. 2008). Further, studies on *Dec1/2* mutant mice suggest that the *Dec*s have redundant functions in the mammalian circadian clock (see below) (Rossner, Oster et al. 2008). In *Drosophila*, the *Dec* ortholog *clockwork orange* (*cwo*) shows similar transcriptional regulatory functions, and synergistic interactions with PER in the timekeeping mechanism were postulated (Kadener, Stoleru et al. 2007; Lim, Chung et al. 2007; Matsumoto, Ukai-Tadenuma et al. 2007; Richier, Michard-Vanhee et al. 2008), indicative of an analogue PER-DEC synergistic interaction in the mammalian circadian system.

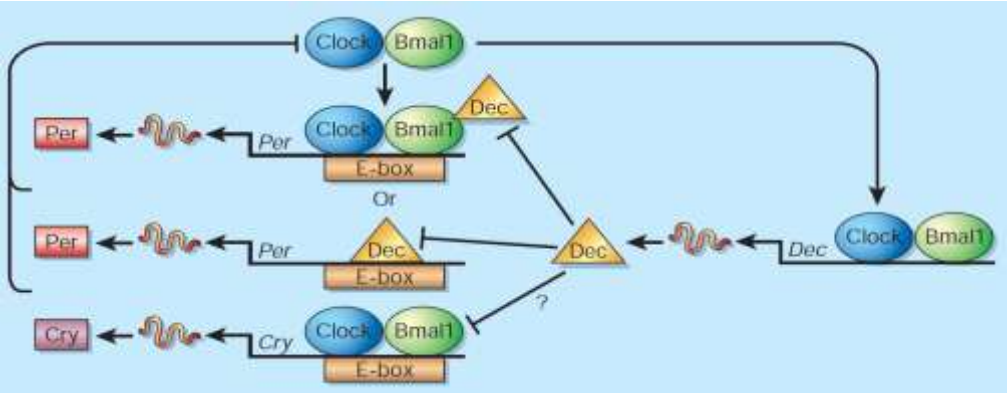

Figure 8: DEC regulation. Transcription of *Dec* genes is activated by CLOCK/BMAL1 heterodimer complexes. The DEC proteins repress *Per* transcription by interfering with CLOCK/BMAL1 activity or by binding to E-box. (Alvarez and Sehgal 2002)

1.2.5. Peripheral clocks

The circadian clock is not only present in the SCN, but also in peripheral cells. Microarray studies identified oscillating genes in many mammalian organs and tissues, e.g. liver, skeletal muscle and brown and white adipose tissues (Akhtar, Reddy et al. 2002; Kita, Shiozawa et al. 2002; Panda, Antoch et al. 2002; Storch, Lipan et al. 2002; Ueda, Chen et al. 2002; Reddy, Karp et al. 2006; Zvonic, Ptitsyn et al. 2006; McCarthy, Andrews et al. 2007), suggesting that the circadian system influences biosynthetic and metabolic processes such as cholesterol and lipid metabolism, glycolysis and gluconeogenesis, oxidative phosphorylation and detoxification pathways. The molecular clock of peripheral cells is based on a phase-delayed (4-12 h relative to the circadian oscillation in the SCN) TTL involving the same or homolog clock components. Cultured

cells and tissue explants from liver, lung, kidney, spleen etc. exhibit robust circadian oscillations in gene expression and *Per2::Luc* explants from SCN and tissues of the periphery show also robust rhythms (Balsalobre, Damiola et al. 1998; Yamazaki, Numano et al. 2000; Yoo, Yamazaki et al. 2004; Yamazaki, Yoshikawa et al. 2009). This indicates a hierarchical timekeeping system where time information is sent from the SCN to semi-autonomous oscillators in the periphery to synchronize the physiology of peripheral organs. Each individual cell possesses its own self-sustained circadian oscillator and the damping clock rhythms observed in cell culture (e.g. fibroblasts) are due to desynchronization between individual oscillators, rather than due to the loss of rhythmicity in individual cells (Nagoshi, Saini et al. 2004; Welsh, Yoo et al. 2004) (Figure 9).

Figure 9: Luminescence recording of mPER2::LUC primary fibroblasts. Normalized luminescence from cultured *Per2::Luc* primary fibroblasts after serum shock is illustrated. Averaged period length of the fibroblast clock is 25.65 h. The dampened rhythm is due to the desynchronization of individual cellular oscillators (Welsh, Yoo et al. 2004).

1.3. Clock mutants

To understand the role of each clock gene, mutant or *knock out* animals of clock genes have been generated and investigated. *Clock*$^{\Delta 19/\ \Delta 19}$ mutant mice – the *Clock*$^{\Delta 19}$ mutant allele encodes a dominant negative version of CLOCK that binds BMAL1, but lacks transcriptional activity – display a lengthened period and become arrhythmic in constant darkness (Vitaterna, King et al. 1994). In contrast, the phenotype of *Clock*$^{-/-}$ mice is milder (Debruyne, Noton et al. 2006) which might be explained by a certain redundancy with the CLOCK paralog NPAS2 (Reick, Garcia et al. 2001; DeBruyne, Weaver et al. 2007). *Bmal1*$^{-/-}$ mice show impaired entrainment to LD cycles and, upon release into constant darkness, behavioural and molecular rhythms are lost indicating a complete disruption of the circadian clock (Bunger, Wilsbacher et al. 2000). These observations suggest that CLOCK/NPAS2 and BMAL1 are critical components of the molecular circadian clock.

The clock continues to oscillate in *Per1*, *Per2*, *Cry1* or *Cry2* single mutant mice (van der Horst, Muijtjens et al. 1999; Vitaterna, Selby et al. 1999; Zheng, Larkin et al. 1999; Bae, Jin et al. 2001; Cermakian, Monaco et al. 2001; Zheng, Albrecht et al. 2001). The DD period length of *Per1* and *Per2* mutant mice is shortened compared to wild-type mice and *Per2* mice gradually become arrhythmic under these conditions (Zheng, Larkin et al. 1999; Zheng, Albrecht et al. 2001). *Cry1*$^{-/-}$ mice have a shorter and *Cry2*$^{-/-}$ mice a longer period length in constant darkness compared to wild-type animals (van der Horst, Muijtjens et al. 1999; Vitaterna, Selby et al. 1999). The simultaneous disruption of *Per1* and *Per2* or of *Cry1* and *Cry2* causes behavioural and molecular arrhythmicity (van der Horst, Muijtjens et al. 1999; Vitaterna, Selby et al. 1999; Bae, Jin et al. 2001; Zheng, Albrecht et al. 2001) highlighting an essential role for PER and CRY in the circadian system. *Per2/Cry1* double mutants show disrupted activity and clock gene expression rhythms in the SCN and several tissues. In contrast, *Per2/Cry2* mutant mice display normal circadian rhythmicity indicating that PER1/CRY1, but not PER1/CRY2, complexes are sufficient to drive the circadian clock (Oster, Yasui et al. 2002). Additionally *Per1/Cry1* and *Per1/Cry2* mutant mice display rhythmic activity and circadian clock gene expression, but *Per1/Cry2* animals loose rhythmicity in an age-dependent manner. Thus, the PER/CRY complexes have different potentials to regulate the circadian clock and not all interactions between PER and CRY are equal *in vivo* (Oster, Yasui et al. 2002; Oster, Baeriswyl et al. 2003). Nevertheless, PER and CRY are necessary for a functional clock.

Dec1 (*Bhlhe40*^{tm1Tan}) and *Dec2* (*Bhlhe41*^{tm1Mjro}) mutants entrain readily to LD cycles and have stable rhythms under constant darkness conditions with normal period lengths (*Dec* single mutants) or with a lengthened period (*Dec1/2* double mutants) compared to wild-type mice (Rossner, Oster et al. 2008). In contrast, a study by Nakashima and colleagues showed a lengthened DD period for *Dec1*$^{-/-}$ mice (Nakashima, Kawamoto et al. 2008). Photic phase resetting is preserved in *Dec* single mutants, but in the absence of both *Dec* genes clock resetting is impaired with a reduced phase delay response (Rossner, Oster et al. 2008). In a jet lag paradigm, *Dec1* single and *Dec1/2* double mutants re-entrain slower to delayed LD cycles while *Dec* single mutant mice re-entrain faster to an advanced LD cycle (Nakashima, Kawamoto et al. 2008; Rossner, Oster et al. 2008). Gene expression of *Per1,2* and *Bmal1* in the cerebral cortex and the liver is elevated in *Dec1/2* mutant mice, but *Bmal1* mRNA accumulation is unchanged and *Per2* transcription is blunted in the SCN of these animals. In contrast, *Dec* single mutants show normal *Per2* and *Bmal1* transcription in the SCN (Rossner, Oster et al. 2008).

1.4. Photic resetting of the circadian system

In a natural environment, circadian rhythms in animals have to be frequently reset to stay entrained to the day-night cycle because the endogenous clock period is not exactly 24 h. Photic resetting can be studied by short exposure to light during the night, which resets circadian activity rhythms. Pittendrigh showed that early-night light exposure delays the circadian clock, such that the animal awakens later (Pittendrigh 1976). A light pulse during late night, on the other hand, advances the circadian clock and the animal awakens earlier. An overview of the daily variation in the ability of the murine clock to shift its phase in response to photic stimuli can be represented by a phase response curve (PRC) as shown in Figure 10. Light exposure does not affect the circadian phase when applied during the subjective day (dead zone).

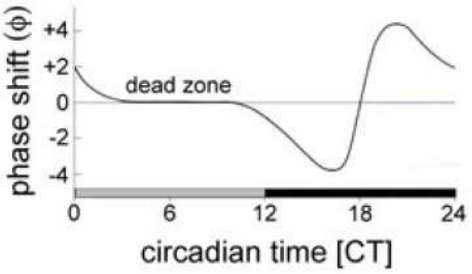

Figure 10: Photic phase response curve (PRC). The PRC was determined using an Aschoff type I protocol. Light pulse exposure between CT11 and CT18 induces phase delays (negative values). Light pulses between CT19 and CT3 generate phase advance responses (positive values). Between CT4 and CT10 no phase shift can be observed (dead zone). The grey and black bars represent subjective day and night, respectively. Modified from (Jud, Schmutz et al. 2005)

To reset the circadian clock, light must alter the phase of the TTL in the SCN. Photic phase resetting of the SCN clock is believed to depend on acute photic *Per1* and *Per2* (and possibly *Dec1*) induction in SCN neurons after light exposure during the night (Albrecht, Sun et al. 1997; Shearman, Zylka et al. 1997; Shigeyoshi, Taguchi et al. 1997; Miyake, Sumi et al. 2000; Honma, Kawamoto et al. 2002; Yan and Silver 2002; Rossner, Oster et al. 2008). Via photic activation of *Per* expression, PER protein levels increase in the SCN neurons with a delay (Yan and Silver 2004). This may shift the phase of the TTL, although the mechanism is still unknown. In line, *Per2* and *Per1* single mutant mice show decreased behavioural responses to nocturnal light exposure in the first or second half of the dark phase, respectively (Albrecht, Zheng et al. 2001; Spoelstra, Albrecht et al. 2004). *Dec1/2* double mutants show reduced resetting which is restricted to phase delays (Rossner, Oster et al. 2008).

Of note, other resetting mechanisms have been reported at the posttranscriptional level. Photic activation of, and consecutive interaction with protein kinase C alpha (PKCα)

causes a temporary stabilization and cytoplasmatic retention of PER2 protein. This leads to a prolongation of the PER/CRY negative feedback on CLOCK/BMAL1, and a phase delay of the TTL (Jakubcakova, Oster et al. 2007).

1.5. Clinical aspects

In mammals, the circadian clock controls a large number of physiological variables. Therefore, it is not surprising that circadian disruption is associated with various diseases and physiological disorders. Sleep disorder is a medical disorder of sleep patterns including delayed sleep phase syndrome (DSPS) and advanced sleep phase syndrome (ASPS). DSPS patients prefer to go to bed very late at night and sleep late in the morning, resulting in a shift of peak alertness to midnight. In ASPS, the opposite situation occurs. DSPS and ASPS can be hereditary. In one form of familial advanced sleep phase syndrome (FASPS), a mutation in the human *PER2* gene was identified leading to decreased PER2 phosphorylation by CK1 and reduced protein turnover (Jones, Campbell et al. 1999; Toh, Jones et al. 2001).

Restless legs syndrome (RLS) is a neurological sleep disorder characterized by an urge to move the limbs, accompanied by periodic limb movements (PLM) during sleep, leading to severe insomnia (Walters 1995; Allen, Picchietti et al. 2003). The symptoms are generally present during periods of rest and more frequent in the evening or night. In RLS circadian rhythms appear not to be altered, as shown by normal 24 h profiles of physiological markers such as core body temperature, cortisol and melatonin secretion (Wetter, Collado-Seidel et al. 2002; Tribl, Waldhauser et al. 2003; Michaud, Dumont et al. 2004). However, because of the diurnality of RLS symptoms an implication of the circadian clock in RLS etiology has been proposed. Michaud et al. suggest that melatonin might be involved in the worsening of RLS symptoms in the evening and at night. Further, a therapeutic effect of melatonin in patients with PLM but without RLS has been reported (Kunz and Bes 2001). The pathophysiological basis of RLS remains unclear, but pharmacological data indicate the dopaminergic system plays an essential role (Hening, Allen et al. 1999; Kraus, Schuld et al. 1999).

Jet lag is a consequence of desynchrony between the circadian system and external time as a result of crossing several time zones. Depending on the extent of the phase shift, the circadian system requires several days to fully adapt to the destination time zone (Srinivasan, Singh et al. ; Kiessling, Eichele et al. 2010). The adaptation after westbound

flights is faster than after eastbound flights due to the fact that the clock has a better capacity for phase delays than phase advances (see also PRC, Figure 10) (Khalsa, Jewett et al. 2003). Jet lag affects sleep-wake cycles as well peripheral physiology, e.g. in the gastrointestinal tract, liver, pancreas and kidney (Waterhouse, Reilly et al. 2007).

A circadian mood disorder is seasonal affective disorder (SAD) – also called winter depression. SAD is characterized by recurrent depression that occurs annually, usually at the same time each year, for several years (Rosenthal, Sack et al. 1984). SAD is predominantly found in northern countries during wintertime correlating with very short daylight periods. Lewy et al. postulated a phase shift hypothesis of SAD which suggests that seasonal depression occurs when intrinsic circadian rhythms, such as melatonin and temperature rhythms, are phase delayed relative to external time and/or the sleep/wake cycle (Lewy and Sack 1988). This phase shift can be alleviated by light therapy (exposure to artificial bright light) (Lewy, Kern et al. 1982; Rosenthal, Sack et al. 1984; Wirz-Justice, Bucheli et al. 1986) and/or administration of melatonin.

It was suggested that the circadian system also plays a role in mental disorders such as major depressive disorder (MDD; unipolar disorder) and bipolar disorder (BPD). Circadian symptoms of MDD include insomnia, elevated core body temperature (Persaud 2000) and cortisol (Gold, Drevets et al. 2002; Keller, Flores et al. 2006) and lower melatonin secretion (Paparrigopoulos 2002). BPD is frequently associated with insomnia or hypersomnia, early-morning awakenings, reduced sleep efficiency and reduced sleep latency. Phase advances in the diurnal rhythms of plasma cortisol (Linkowski, Mendlewicz et al. 1985) implicate circadian disturbances in BPD. *Clock* mutant mice show a mania-like phenotype with increased reward values of appetitive stimuli and reduced depressive and anxiety-like behavior (Roybal, Theobold et al. 2007).

Circadian parameters are disrupted in a wide spectrum of further central nervous system (CNS) disorders such as Parkinson's disease, Alzheimer's disease and dementia (Barnard and Nolan 2008). It is, however, difficult to define whether the disruption in circadian parameters is just a consequence of CNS disease, or whether it contributes to the development of the disorder.

A whole range of metabolic diseases are associated with misalignment of the circadian clock and metabolism (Kovac, Husse et al. 2009). Shift workers possess an elevated risk for the development of obesity, type II diabetes, metabolic syndrome and cardiovascular complications (Bray and Young 2007). Animal models further support a crucial role for the circadian clock in the regulation of metabolism. *Clock* mutant mice show increased food

intake and develop obesity and a diabetic phenotype (Turek, Joshu et al. 2005). In another mouse model of obesity (KK-A^y mice) clock gene rhythms in liver and white adipose tissues are severely attenuated (Ando, Yanagihara et al. 2005).

Circadian clocks were shown to be involved in cell cycle regulation and thus might play a role in cancer development. Clock gene mutations (e.g. in *Per* genes) increase the incidence of cancer. Strongly dysregulated *PER* expression is found in Taiwanese woman with breast cancer (Chen, Choo et al. 2005) and *Per2* mutant mice spontaneously develop tumours, e.g. lymphomas (Fu, Pelicano et al. 2002; Lee 2006). It was shown that light exposure at night might promote cancer development (Travis, Allen et al. 2004; Schernhammer, Berrino et al. 2008), increasing the risk of colorectal cancer in night-shift workers (Schernhammer, Laden et al. 2003).

1.6. Aim

Synergistic function of the circadian transcription factors PER and CWO was postulated for the *Drosophila* clock (Kadener, Stoleru et al. 2007). Based on the high conservation between the fruit fly and the mammalian circadian system, we hypothesized a similar synergism between *Per(1,2)* and *Dec(1,2)* in the murine circadian clock.

In vitro, it is very difficult to mimic spatial and temporal clock depending interactions. Some of the proposed components of the mammalian oscillators are important *in vitro*, but appear not relevant for clock function *in vivo* such as *Per3* (Shearman, Jin et al. 2000). Therefore, we chose to study the *Per(1,2)-Dec* interaction in the living animal. We generated homozygous *Per1/Dec(1,2)* or *Per2/Dec(1,2)* double mutant mice by crossing *Per* and *Dec* mutant animals. It was postulated that *Dec1* and *Dec2* have redundant functions (Rossner, Oster et al. 2008). Therefore, we also generated and investigated *Per/Dec* triple mutant mice. These mutants allowed us to assess *Per* and *Dec* functional interaction in behaviour using locomotor activity measurements under different conditions, i.e. under entrained conditions and free-run, to analyze the endogenous *Per-Dec* interaction in the circadian system *in vivo*.

In vitro, the transcription factors DEC1 and DEC2 were shown to act as bidirectional transcriptional regulators, i.e. co-activators and repressors (Rossner, Oster et al. 2008). Thus, the *in vivo* regulatory function of the *Per-Dec* interactions in the circadian pacemaker, the SCN, was also studied in these mutants.

2. Chapter 2: Material and Methods

2.1. Animals

Per2$^{m/m}$ (*Per2*^{Brdm1}; Zheng, Larkin et al. 1999), *Per1*$^{-/-}$ (*Per1*^{Brdm1}; Zheng, Albrecht et al. 2001), *Dec1*$^{-/-}$ (*Bhlhe40*^{tm1Tan}) and *Dec2*$^{-/-}$ (*Bhlhe41*^{tm1Mjro}; Sun, Lu et al. 2001; Rossner, Oster et al. 2008) mice were backcrossed to a C57BL/6J background (N10). Homozygous single mutants were then mated to obtain homozygous *Per1*$^{-/-}$*Dec1*$^{-/-}$, *Per1*$^{-/-}$*Dec2*$^{-/-}$, *Per2*$^{m/m}$*Dec1*$^{-/-}$ and *Per2*$^{m/m}$*Dec2*$^{-/-}$ double mutants as well *Per1*$^{-/-}$*Dec1/2*$^{-/-}$ and *Per2*$^{m/m}$*Dec1/2*$^{-/-}$ triple mutant mice. Figure 11 shows the breeding strategy for the generation of these mutants. The genotypes of the offspring were determined by PCR as described previously (Zheng, Larkin et al. 1999; Zheng, Albrecht et al. 2001; Rossner, Oster et al. 2008). The behavioural paradigms were performed on the homozygous double and triple mutants.

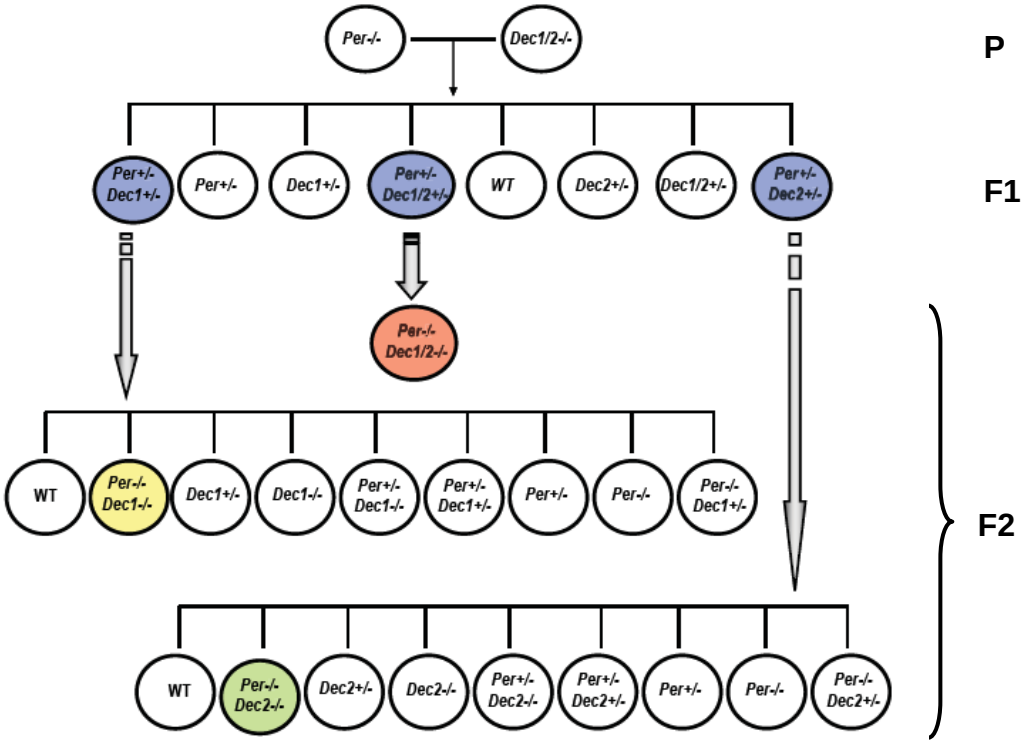

Figure 11. Breeding scheme for the generation of *Per/Dec* double and triple mutant mice. Bl6/J-backcrossed homozygous *Per1* or *Per2* single mutant mice were mated with Bl6/J-backcrossed homozygous *Dec* single mutant mice to generate within two generations (F2) homozygous *Per/Dec1* (yellow), *Per/Dec2* (green) double and *Per/Dec1/2* (red) triple mutant mice.

2.2. Behavioural paradigms

2.2.1. Running-wheel assays

Locomotor activity was analyzed using running wheels. All experiments were performed on male adult animals (2-10 months) and congenic C57Bl/6J mice were used as controls. Figure 12 shows a comparable installation of the isolated cabinets used, holding 12 wheel-running cages. The light conditions were regulated by computer-controlled fluorescent lights in the ceiling of each cabinet: light intensity variations between cages were less than ± 10 lux at 250 lux. Temperature was 20°C ± 0.5°C and humidity was 60% ± 5% throughout all experiments. Each cabinet was individually ventilated at 20x vol. per hour. Under normal conditions, the light was set to a 12 h light : 12 hours dark (LD) cycle with a light intensity of 250 lux during the light phase. Mice were individually housed in running wheel-equipped cages (Figure 12A) with water and food *ad libitum*. Running-wheel activity was individually recorded using ClockLab software on a computer running Windows XP.

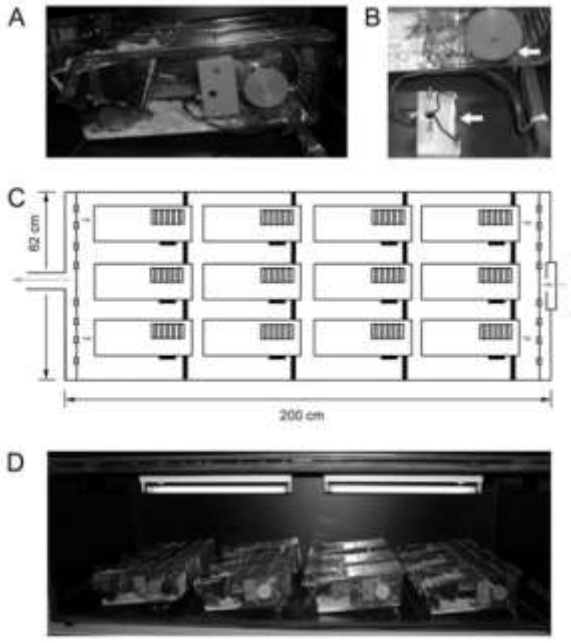

Figure 12. Overview of the cabinet with 12 wheel-running cages. (A) Wheel-running cage with an individually housed mouse. The running-wheel is connected via a magnetic switch for recording. With each rotation of the running wheel, the magnetic switch is opened and closed once. (B) The magnet (upper arrow) rotates simultaneously with wheel-revolution and the magnetic switch (lower arrow) transmits the wheel-revolutions to the computer. (C) Schematic representation of a cabinet with 12 wheel-running cages. (D) Picture of cabinet with two light bulbs (our setup held 4). From (Jud, Schmutz et al. 2005)

2.2.2. Masking

To test for photic masking capacities, animals were entrained to a 12 h : 12 h LD cycle for 10 days. On day 10, a light pulse of 100 lux was applied between ZT14 and ZT15. Before light administration, chow was removed from the hopper at ZT12 and some chow pellets were put directly into the cage to ensure comparable light exposures. Figure 13A depicts an exemplary actogram of wild-type mice during experimental days 7-10. Masking capacity was calculated as the running-wheel activity during the light exposure period (ZT14-15) on day 10 divided by the average running-wheel activity without light treatment on days 7-9 for the same animal at ZT14-15 (represented in Figure 13B as light phase).

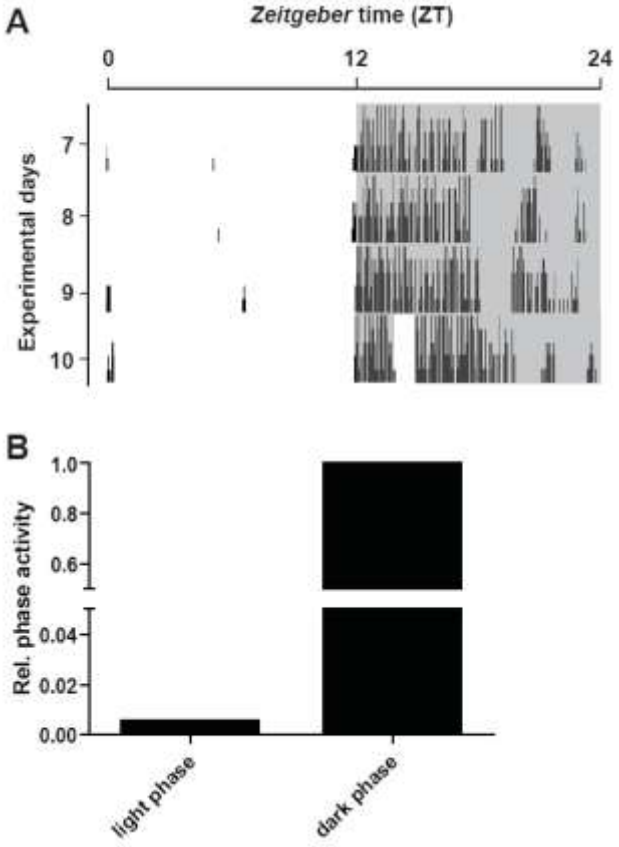

Figure 13. Masking experiment of an individual WT mouse. A. Locomotor activity of an individual WT mouse during a masking experiment. The mouse was for 9 days entrained to a 12 h : 12 h LD cycle. On day 10, a 100 lux light pulse was given for 1 h between ZT14-15. The grey shading indicates the dark phase. On the left side, the experimental days are indicated. The actogram shows day 7-10 of the experiment. B. Represents relative phase activity of this individual WT mouse (from A) during ZT14-15. Light phase represents the relative activity during the 1 h light exposure (ZT14-15) at day 10 and dark phase the averaged relative phase activity between ZT15-14 from experimental days 7-9.

2.2.3. Phase delay resetting

Activity phase delay shifting by single nocturnal light pulses (LP) was performed using an Aschoff type II protocol (LD → LP → DD) because *Per2/Dec* mutant animals become arrhythmic under constant darkness which prevents stable determination of activity onsets over extended times in DD. All animals were entrained to a 12 h : 12 h LD cycle with a

light intensity of 250 lux for two weeks. A 15 min light pulse (250 lux light intensity) was applied during the first half of the dark phase (ZT15) before animals were released into DD. Alternatively the LP was applied on the first day in DD at CT14. The phase shift response of each animal was determined as the difference between the regression lines through onsets before and after the light pulse on the first day after the light exposure (Figure 14 lower panel). This was compared to animals of the same genotype, which were released into DD without prior light exposure (Figure 14 upper panel) to extract the phase shift which was mediated by the light exposure from that produced by the mere release into DD.

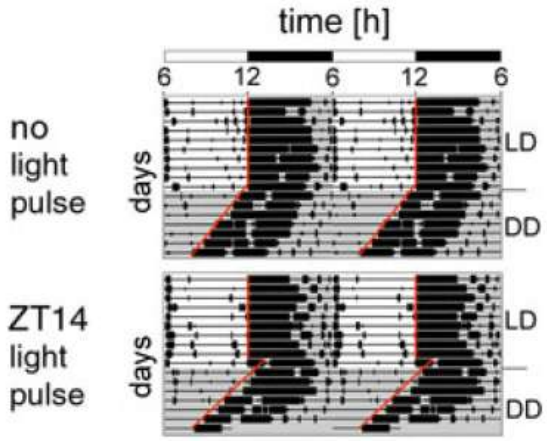

Figure 14. Typical actograms of WT animals in an Aschoff type II resetting protocol. Upper panel shows no light pulse treatment. Mice were entrained to a 12 h : 12 h LD cycle for 10 days and then released into DD. Lower panel shows a light pulse application for 15 min to the entrained mouse at ZT14. The phase shift is calculated by comparing the regression lines (red line) drawn through the onsets of wheel-running activity on the last days in LD and the first days in DD. The grey background represents darkness. Modified from (Jud, Schmutz et al. 2005)

3. Chapter 3: Results

3.1. ‘Genetic interaction of *Per1* and *Dec1/2* in the regulation of circadian locomotor activity’ (submitted to *Journal of Biological Rhythms*)

Brid Bode, Ali Shahmoradi, Moritz J. Rossner, Henrik Oster

ABSTRACT

In mammals circadian rhythms of behavior and physiology are controlled by a hierarchical system of endogenous clocks, with a circadian pacemaker located in the suprachiasmatic nuclei (SCN) of the hypothalamus that synchronizes peripheral oscillators found in most other tissues. The molecular clock machinery is regulated by interlocked transcriptional translational feedback loops (TTLs). The mammalian core TTL includes the transcriptional modulators PER (1-3) and CRY (1/2) that feedback on their own expression by interaction with CLOCK/BMAL1. An accessory loop involving the transcription factors DEC1 and DEC2 has been described that also impinges on CLOCK/BMAL1-mediated transactivation. In *Drosophila* the DEC ortholog CWO shows synergistic activity to PER. This prompted us to analyze PER1-DEC interaction in the mammalian SCN pacemaker. We generated *Per1/Dec* double and triple mutant mice to monitor circadian wheel-running behavior under entrained and free-running conditions. Further, we analyzed circadian expression profiles of the core clock genes *Per2*, *Rev-Erbα* and *Bmal1* in wild-type and *Per1/Dec* mutant SCN by *in situ* hybridization. The behavioral experiments revealed a critical role for *Per1-Dec* interaction in the synchronization of activity phase under entrained conditions. In constant darkness a synergistic function for *Per1* and *Dec1/2* in period regulation was found, correlating with disrupted clock gene transcription rhythms in the SCN. Together, our results suggest a partially redundant and bidirectional regulatory function for the two *Dec* genes in the TTL and a conservation of *Per-Dec (Cwo)* synergism between vertebrate and invertebrate clocks.

***Key words*:** *Per1, Dec1, Dec2,* photic entrainment, free-run, SCN, mice

INTRODUCTION

In most species 24 h rhythms of physiology and activity are regulated by endogenous circadian clocks. In mammals behavioral rhythms are controlled by a circadian pacemaker located in the suprachiasmatic nuclei (SCN) of the hypothalamus (Moore and Eichler 1972; Schibler and Sassone-Corsi 2002; Stephan and Zucker 1972). The SCN shows electrical activity rhythms of only approximately 24 h under free-running conditions (Inouye and Kawamura 1979). Therefore, to keep in synchrony with external time, the internal clock has to be reset each day by environmental *Zeitgebers* in a process termed *entrainment*. The predominant *Zeitgeber* of the mammalian system is light which reaches the SCN via the retino-hypothalamic tract (Moore and Lenn 1972). From the SCN, time information is transferred by various means to peripheral circadian oscillators found in all tissues and controlling local physiological rhythms (Buijs and Kalsbeek 2001; Oster 2006; Reppert and Weaver 2002).

At the molecular level SCN and peripheral clocks are based on interlocked transcriptional/translational feedback loops (TTLs) comprised from a set of clock genes that are highly preserved between vertebrate and invertebrate species (Zhang and Kay 2010). In the mammalian core TTL the transcription factors circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT-like 1 (BMAL1, ARNTL) heterodimerize and activate the transcription of *cis*-regulatory E-box containing target genes including *Period* (*Per1-3*) and *Cryptochrome* (*Cry1-2*). PER/CRY protein complexes accumulate during the course of the day and in the night relocate back into the nucleus where they inhibit their own transcription by interaction with CLOCK/BMAL1 (Griffin et al. 1999; Kume et al. 1999; Reppert and Weaver 2002). Recently two other transcription factors, DEC1 (BHLHE40) and DEC2 (BHLHE41), have been described to interact with the components of the circadian core TTL in the SCN (Honma et al. 2002; Noshiro et al. 2005). *Dec* mutant mice show moderate defects in light entrainment and free-running periodicity (Rossner et al. 2008). *Dec1* and *Dec2* transcription is activated by CLOCK/BMAL1 and nocturnal light exposure acutely stimulates *Dec1* expression in the SCN (Butler et al. 2004; Honma et al. 2002). On the other hand DEC1 and DEC2 impinge on circadian transcription by competing with CLOCK/BMAL1 for E-box occupation and/or by direct physical interaction with BMAL1 (Honma et al. 2002; Sato et al. 2004). The mode of interaction between the DECs and the other CLOCK/BMAL1 regulators – PERs and CRYs – however, remains unclear. Recent results suggest that DEC function can be either activatory or inhibitory, depending on tissue type as well as on promoter of target genes (Azmi et al. 2003; Dardente et al. 2009; Hamaguchi et al. 2004; Honma et al. 2002; Kawamoto et al. 2004; Li et al. 2004; Li et al. 2003; Rossner et al. 2008).

Studies in flies have proposed that *clockwork orange* (*cwo*), the *Drosophila* ortholog of the *Decs*, has a synergistic function to the *Per1/2* ortholog *period* (*per*) (Kadener et al. 2007). To test if this mode of interaction is preserved in mammals we analyzed circadian activity rhythms and clock gene expression in the SCN of *Per1/Dec* double and triple deficient mice. Our results show a synergistic function of *Per1* and *Dec1* together with *Dec1/Dec2* redundancy in the regulation of photic activity entrainment and of period length under free-running conditions. Thus, it appears that *per-cwo* interaction in the *Drosophila* TTL is largely conserved in the mammalian pacemaker.

MATERIALS AND METHODS

Animals

Per1 mutant mice (*Per1*^{Brdm1}; Zheng et al. 2001) were back-crossed to a C57BL/6J background (N10) and mated with *Dec1*$^{-/-}$ and *Dec2*$^{-/-}$ mice (Rossner et al. 2008) to produce double mutant *Per1*$^{-/-}$*Dec1*$^{-/-}$ and *Per1*$^{-/-}$*Dec2*$^{-/-}$ as well as triple mutant *Per1*$^{-/-}$*Dec1/2*$^{-/-}$ mice. The genotype of the offspring was determined by PCR as described (Rossner et al. 2008; Zheng et al. 2001). All experiments were done on male adult animals (2-10 months). Congenic age-matched C57Bl/6J mice were used as controls. All animal experiments were performed with permission from the Office of Consumer Protection and Food Safety of the State of Lower Saxony and in accordance with the German Animal Welfare Act.

Behavioral analysis

Mouse housing and handling were performed as described (Albrecht and Oster 2001). Wheel-running activity was analyzed using ClockLab software (Actimetrics, Evanston, IL). Prior to the experiments, animals were entrained to a 12 hours light: 12 hours dark (LD) cycle for at least 10 days (light intensity 250 lux). For analyzing free-running locomotor activity under constant darkness conditions (DD), lights were turned off at the end of the light phase (*Zeitgeber* time ZT12) and not turned on again on the next day. Activity profiles in LD, DD and constant light (LL, light intensity 50 lux) were assessed over a time of 4-7 consecutive days. The onset phase angle was determined by fitting a straight line to 7-10 consecutive activity onsets using ClockLab software and manual correction. Onset variation depicts the mean deviation of real activity onsets from a regression line through 7-10 consecutive onsets under stably entrained or free-running conditions. Rhythmicity and period length (τ) in DD and LL were determined by χ^2 periodogram analysis over an interval of 7-10 consecutive days. Period differences were calculated by comparison of period lengths between two different DD intervals separated by at least 10 days in LD.

In situ hybridization

Animals were entrained to LD for 10 days, released into DD and sacrificed on the second day in DD at 6 h intervals. Brains were dissected under dim red light, fixed by immersion in 4 % PFA

in PBS, dehydrated and paraffin embedded. 8 µm sections were hybridized with ^{35}S-UTP-labeled antisense RNA probes for clock gene transcripts as described (Oster et al. 2003; Oster et al. 2002). The *Rev-Erbα* probe template was generated by PCR (forward primer: CCCTCTACAGTGACAGCTCCA, reverse primer: TCAGACACCGTTTGTACTGGA) from murine adipose tissue cDNA. Relative quantification of expression levels was performed by densitometric analysis of autoradiograph films using Quantity One software (Bio-Rad, Munich, Germany). Background correction was performed by subtracting the optical density measured in the lateral hypothalamus. For each experiment 3 animals per genotype were used and 3 adjacent SCN sections per animal were analyzed.

Reporter gene assays

Dual reporter gene assays in HEK293 cells were performed as previously described (Rossner et al. 2008; Wehr et al. 2006) using a firefly luciferase *Bmal1* reporter gene (*Bmal1::luc*; Nagoshi et al. 2004) and a *Renilla* luciferase reporter as normalizing transfection control. CMV-driven expression plasmids coding for HA-tagged rat DEC1, Flag-tagged mouse DEC2 and V5-tagged mouse PER1 were used (Rossner et al. 2008). All firefly luciferase data were normalized to the *Renilla* luciferase data for the same sample. DEC1, DEC2 and PER1 encoding constructs were assembled using Gateway-mediated recombination as described (Wehr et al. 2006). The PER1 expression plasmids were kindly provided by Dr. Pablo Szendro, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany.

Statistical analysis

Statistical evaluation was performed using GraphPad Prism software (GraphPad, La Jolla, CA). Groups were compared by One-way ANOVA followed by Tukey's posttest for multiple comparisons. A p-value < 0.05 was considered significant. The rhythmicity of clock gene expression was evaluated using sine curve fitting with CircWave v1.4 (Oster et al. 2006).

RESULTS

Synergistic interaction of *Per1* and *Dec1/2* under entrained conditions

Per1/Dec double and triple mutant mice were born at the expected Mendelian ratios – taking into account that both *Dec* genes are located relatively close together on the same chromosome (Fujimoto et al. 2001; Sun et al. 1999). Mutant mice of all genotypes were viable and fertile and did not show any gross anatomical or behavioral abnormalities (data not shown). To study *Per1-Dec* interaction in the regulation of circadian entrainment *Per1/Dec* double and triple mutant mice were housed individually and their running-wheel activity was monitored under a 12 h light: 12 h dark cycle (LD). Under these conditions all animals entrained readily to the light/dark cycle (Figure 1). However, significantly advanced activity onsets were observed in *Per1* single (-0.29 ± 0.02 h) as well as *Per1/Dec1* (-0.56 ± 0.05 h), *Per1/Dec2* (-0.28 ± 0.05 h) double and *Per1/Dec1/2* (-0.81 ± 0.04 h) triple mutant mice (Figure 2A and statistics in Table S1). *Per1*$^{-/-}$*Dec1*$^{-/-}$ as well as *Per1*$^{-/-}$*Dec1/2*$^{-/-}$ mice showed significantly earlier activity onsets in comparison to wild-type (WT) and to single *Per1* mutant animals. In addition, the activity onsets of *Per1/Dec1/2* triple mutant mice were significantly advanced compared to those of both double mutant strains, indicating a synergistic interaction of *Per1* and *Dec1/2* and a certain functional redundancy of *Dec1* and *Dec2* in photic activity entrainment. These observations were supported when we measured entrainment stability by quantifying the day-to-day variation of activity onsets under LD conditions. *Per1* single as well as *Per1/Dec* double and triple mutant mice displayed decreased onset stability when compared to WT animals (Figure 2B). While single mutants showed onset variations between 0.11 and 0.19 h (*Per1*$^{-/-}$ 0.19 ± 0.03 h, *Dec1*$^{-/-}$ 0.11 ± 0.02 h and *Dec2*$^{-/-}$ 0.14 ± 0.02 h), this value increased gradually with decreasing *Per1/Dec* gene dosage (*Per1*$^{-/-}$*Dec2*$^{-/-}$ 0.26 ± 0.03 h, *Per1*$^{-/-}$*Dec1*$^{-/-}$ 0.30 ± 0.03 h and *Per1*$^{-/-}$*Dec1/2*$^{-/-}$ 0.48 ± 0.04 h), suggesting that a fully functional *Per1-Dec* interaction is necessary for efficient synchronization of activity phase with the external LD cycle.

Functional redundancy of *Dec1* and *Dec2* in period regulation and *Per1* interaction under free-running conditions

We next asked whether the *Per1-Dec* synergism observed in LD is conserved under free-running conditions, i.e. in the absence of external *Zeitgeber* signaling. To test this, we released the animals into constant darkness (DD). In DD, all mutants retained rhythmic circadian activity

patterns (Figure 1), but marked changes in the period of these rhythms were observed. With the exception of *Dec2*$^{-/-}$ mice (23.56 ± 0.03 h), all single, double and triple mutant genotypes displayed a significantly shortened DD period length when compared to wild-type controls (*Per1*$^{-/-}$ 22.39 ± 0.07 h, *Dec1*$^{-/-}$ 23.07 ± 0.06 h, *Per1*$^{-/-}$*Dec1*$^{-/-}$ 22.28 ± 0.10 h, *Per1*$^{-/-}$*Dec2*$^{-/-}$ 22.23 ± 0.11 h, *Per1*$^{-/-}$*Dec1/2*$^{-/-}$ 21.68 ± 0.10 h; WT 23.48 ± 0.02 h; Figure 2C and statistics in Table S1). While in the double mutant mice period length appeared mainly to be determined by the absence of *Per1*, *Per1/Dec* triple mutants showed a significant reduction in period length when compared to all single and double mutant strains. Similar, though statistically less powerful, effects were observed under constant light (LL) conditions. In LL, a loss of *Per1* led to an increase in period length by about 1 h (WT 25.12 ± 0.08 h, *Per1*$^{-/-}$ 26.09 ± 0.12 h; Figure S1 and statistics in Table S1). While no period change was observed in either *Dec1* or *Dec2* single mutants, a combination of *Per1* and *Dec1/2* deficiency further increased LL free-running period in a *Per1/Dec* gene dosage dependent manner (*Dec1*$^{-/-}$ 25.10 ± 0.09 h, *Dec2*$^{-/-}$ 25.18 ± 0.06 h, *Per1*$^{-/-}$*Dec1*$^{-/-}$ 25.54 ± 0.14 h, *Per1*$^{-/-}$*Dec2*$^{-/-}$ 26.34 ± 0.10 h, *Per1*$^{-/-}$*Dec1/2*$^{-/-}$ 26.66 ± 0.40 h; Figure S1 and statistics in Table S1). While the DD/LL data suggest a redundancy for *Dec1* and *Dec2* in the regulation of free-running period, functional interaction between *Per1* and *Dec* was only observed in the absence of both *Dec* genes.

A previous study had shown that *Per1* deficiency results in a marked instability of free-running period length over extended periods of time (Zheng et al. 2001). To test whether the observed *Per1-Dec* interaction in LD entrainment extends to this phenomenon we re-entrained all mice to LD and then re-released them into DD to compare activity period lengths of individual mice between both free-running intervals. In WT animals, period length was very stable with an average period difference between both DD episodes of less than 0.2 h (WT 0.11 ± 0.02 h; Figure 2D). As expected, increased period variations were observed in *Per1* mutant mice (*Per1*$^{-/-}$ 0.41 ± 0.06 h; Figure 2D and statistics in Table S1), while no significant changes in period variability were seen in *Dec1* and *Dec2* single mutant animals (*Dec1*$^{-/-}$ 0.27 ± 0.05 h, *Dec2*$^{-/-}$ 0.16 ± 0.04 h; Figure 2D and statistics in Table S1). Though a trend towards a further increase in period destabilization was seen in double and triple *Per1/Dec* mutants (*Per1*$^{-/-}$*Dec1*$^{-/-}$ 0.56 ± 0.05 h, *Per1*$^{-/-}$*Dec2*$^{-/-}$ 0.57 ± 0.11 h, *Per1*$^{-/-}$*Dec1/2*$^{-/-}$ 0.58 ± 0.04 h; Figure 2D and statistics in Table S1), none of the data reached significance when compared to *Per1* single mutant mice, indicating that long-term period stability is predominantly dependent on *Per1* function. In summary, our free-running activity data suggest a dominant role for *Per1* in the regulation of period length and stability while, in contrast to LD, functional interaction between *Per1* and *Dec1/2* was only detectable under triple mutant conditions.

Synergistic effect of *Per1* and *Dec1/2* on *Bmal1* expression in the SCN

In situ hybridization analysis was performed to extend our investigations to the molecular level. Core clock gene expression profiles were determined on coronal SCN sections of animals sacrificed at four different time points on the second day in DD. For *Per2*, no significant changes in expression levels were found on a single *Per1* or *Dec* deficient background (Figure S2A). All genotypes showed rhythmic *Per2* mRNA levels with peak expression during the late subjective day and comparable amplitudes (Figure 3A). This situation was preserved in *Per1/Dec* double mutants. In *Per1/Dec1/2* triple mutant animals, *Per2* expression was significantly reduced at CT14, reflecting an accelerated breakdown of *Per2* transcripts during the early subjective night and correlating with the shortened activity period observed in these animals (Figure 2C).

The effect on *Bmal1* expression was even stronger in these animals. While only minor changes in *Bmal1* transcript levels were observed in *Per1/Dec* single and double mutants (Figure 3C and Figure S2B), *Bmal1* mRNA rhythms were completely abolished in the SCN of *Per1/Dec* triple mutant animals. In wild-type, single and double mutant mice, *Bmal1* showed moderate rhythmic expression with peak levels between CT14 and CT20. In contrast, triple mutant *Per1*$^{-/-}$*Dec1/2*$^{-/-}$ mice displayed medium level *Bmal1* transcript concentrations throughout the whole day (red line in Figure 3C). Thus, deficiency of, either, *Per1, Dec1/2* or a combination of *Per1* and one *Dec* gene is dispensable for rhythmic *Bmal1* expression, but at least one set of *Dec* alleles seems necessary for normal *Bmal1* transcriptional regulation in the SCN.

So far, it is thought that PER and DEC proteins affect transcriptional regulation of the circadian TTL mainly by interfering with CLOCK/BMAL1-activated transcription via E-box enhancer elements (Honma et al. 2002; Reppert and Weaver 2002; Sangoram et al. 1998; Sato et al. 2004; Shearman et al. 2000). To test if the observed strong interactive effect of *Per1* and *Dec1/2* on (non-E-box-regulated) *Bmal1* transcription is mediated via an intermediate E-box-controlled gene/protein other than *Per2*/PER2 we examined the circadian expression of *Rev-Erbα* (*Nr1d1*), a potent negative regulator of circadian *Bmal1* transcription (Preitner et al. 2002). In wild-type animals *Rev-Erbα* expression in the SCN showed robust rhythms with peak levels during the early subjective day (Figure 3B and Figure S2C). Interestingly, and unlike what had been observed for *Per2* and *Bmal1*, *Rev-Erbα* levels were markedly changed in *Per1*, *Dec1* and *Dec2* single mutant mice with elevated expression during the second half of the subjective day (CT8; Figure S2C). This effect was also seen in *Per1/Dec* double and triple mutant animals. In *Per1*$^{-/-}$*Dec1/2*$^{-/-}$ mice this increase at CT8 resulted in a shift in expression peak phase by 4 h from CT4 to CT8

(Figure 3B). In addition, *Rev-Erbα* expression at CT20 was reduced, indicating an altered phase-relationship between *Per2* and *Rev-Erbα* expression rhythms in these mice.

To clarify the role of *Per1* and *Dec*s in *Bmal1* transcriptional regulation, we performed luciferase reporter gene assays in HEK293 cells. In reporter cells transfected with *Per1* plasmid alone no changes in luciferase activity were observed. Transfection of one *Dec* plasmid moderately but significantly increased luciferase activity by a factor of 2-4.5, with a higher induction capacity of *Dec2* (Figure 3D and statistics in Table S2; Dardente et al. 2009). This induction was unchanged after co-transfection with *Per1* in the presence of one *Dec*. However, co-transfection of both *Dec*s together with *Per1* resulted in a strong further increase of luciferase activity (Figure 3D and statistics in Table S2). Thus, both DECs, have an activatory role on *Bmal1* expression *in vitro* that is further potentiated by addition of *Per1*.

Taken together, DD expression profiling of core clock genes in the SCN and reporter gene assays revealed synergistic effects of *Per1* and *Dec1/2* on *Per2*, *Rev-Erbα* and, most pronounced, on *Bmal1* expression together with full functional redundancy of *Dec1* and *Dec2* and correlating with behavioral changes observed under the same conditions.

DISCUSSION

In this study, we show by behavioral and molecular investigation of clock function in *Per1/Dec* double and triple mutant mice that the previously postulated synergistic function (Kadener et al. 2007) of *Per* and *Dec* genes in the regulation of the invertebrate circadian core TTL is conserved in mammals. Our findings reveal a functional specificity in the interaction of *Dec1* and *Dec2* with *Per1* in photic entrainment. Synergistic interactions of *Dec* genes with *Per1* are critical for the normal synchronization of activity phase to the external LD cycle. Under free-running conditions *Per1-Dec* synergy is preserved, but *Dec1* and *Dec2* show full functional redundancy in the modulation of rhythm period. In contrast, the stability of rhythm sustainment under constant darkness appears to be exclusively dependent on *Per1* function.

It has previously been shown that *Per1* and *Dec* single mutant mice entrain to LD cycles without any significant alterations in wheel-running activity when compared to wild-type littermates (Nakashima et al. 2008; Rossner et al. 2008; Spoelstra et al. 2004; Zheng et al. 2001). However, *Per1* mutant mice show a tendency towards advanced activity onsets under these conditions (Spoelstra et al. 2004). In our experiments, single, double and triple *Per1/Dec* mutant mice entrained readily to an LD cycle, but *Per1* single and *Per1/Dec* double and triple mutants exhibited strongly advanced activity onsets (Figure 2A) or *predark activity* (Mrosovsky 2001). The mechanism behind this phenomenon is still largely unknown, but seems to involve a disruption of the negative masking capacity of light towards the end of the day (Mrosovsky 2001). This masking effect was also suggested to underlie the normal entrainment behavior of the short period *Per1* mutants in bright LD conditions (Zheng et al. 2001). The observed *Dec* dosage dependent cumulative advance of activity onsets in *Per1/Dec1* and *Per1/Dec1/2* mutants indicates a complementary, but specific, role of both *Dec* genes in this process. Given the lack of significant changes in activity onsets in *Dec* single mutants (Figure 2A; Rossner et al. 2008) the functional interaction with *Per1* seems essential for a normal phase relationship between activity and the external LD cycle. In humans, equivalents of advanced activity onsets are observed in advanced sleep phase syndrome (ASPS) (Wulff et al. 2009). In ASPS patients, *Per1* gene polymorphisms are associated with extreme morning preference and abnormal sleep patterns (Carpen et al. 2006). Further, the ASPS phenotype is also associated with *Per2* polymorphisms while alterations in *Per3* are correlated to late chronotypes (Archer et al. 2003; Ebisawa et al. 2001; Viola et al. 2007), suggesting a high level of interaction of the three *Per*s in this process (Jones et al. 1999; Toh et al. 2001). In contrast, *Per2* as well as *Per1/2* mutant mice show a disruption of sleep consolidation (Kopp et al. 2002; Shiromani et al. 2004) while *Per1*$^{-/-}$ mice exhibit no abnormal sleep phenotype

(Kopp et al. 2002). In contrast, in our study *Per1Dec1/2* mutant mice show strongly advanced activity onsets (Figure 2A), suggesting that *Per1-Dec* interaction might be essential for these effects. Mutations in the human *Dec2* gene are associated with a short sleep phenotype. However, *Dec2* deficient mice exhibit only minor changes in sleep architecture (He et al. 2009). Consistent with this, neither *Dec1* nor *Dec2* single mutant animals showed changes in their activity onsets (Figure 2A, Table S1). In general, sleep is regulated by interplay of homeostatic and circadian processes (Dijk and Czeisler 1995). Therefore, the observed predark activity could also result from alterations in the regulation of sleep drive. Our LD data suggest a strong interaction of *Per1* and *Dec1/2* in the regulation of rest/activity cycles under entrained conditions. It would, therefore, be interesting to test if comparable genetic interactions can be observed in human sleep phase pathologies.

In constant conditions all *Per1/Dec* mutant strains retained stable rhythmic activity patterns. DD period length was shortened in *Dec1* and *Per1* single mutant mice (Rossner et al. 2008; Zheng et al. 2001). In *Per1/Dec* double mutants period length was comparable to that of *Per1*$^{-/-}$ mice, while in mice deficient for *Per1* and both *Dec* genes dramatically shortened circadian period lengths were observed (Figure 2C, Table S1), suggesting a full functional redundancy for both *Dec*s in interacting with *Per1* under free-run conditions. This is supported by the constant light data (Figure S1). Following Aschoff's rule (Aschoff 1960) period lengths were increased in all investigated genotypes (Figure S1). Of all single mutants this effect was most pronounced in *Per1*$^{-/-}$ animals (Figure S1; Steinlechner et al. 2002). While functional *Dec1/2* redundancy seems to mask *Per1-Dec* synergism in double mutant animals *Per1/Dec1/2* triple mutants showed a further increase in period length.

Our *in situ* hybridization data suggest that either *Per1*, *Dec1* or *Dec2* are dispensable for rhythmic *Per2* expression in the SCN in constant darkness (Figure 3, Figure S2; Rossner et al. 2008; Zheng et al. 2001). A moderate reduction in *Per2* mRNA was seen in *Per1/Dec1/2* triple mutants at CT14 (Figure 3A) supporting a certain level of redundancy of *Dec1* and *Dec2* function in *Per2* transcriptional regulation. Studies on hepatic clock function indicate that *Per2* rhythms – unlike most other core clock genes – are stabilized by systemic factors independent of local TTL function (Kornmann et al. 2007). In line with this, changes in gene activity were more pronounced for *Rev-Erbα* and *Bmal1* in the triple mutant animals. Rhythmic *Bmal1* transcription in the SCN seems to critically depend on the presence of at least one functional *Dec* gene (Figure 3C). Of note, dampened clock gene expression rhythms have been observed in *Dec1/2* double mutants (Rossner et al. 2008). Our reporter gene assay data postulate a synergistic activatory transcriptional role of PER1 and DEC1/2 on *Bmal1* transcription (Figure 3D). It remains to be shown if this activatory

function is direct – of note, the mouse *Bmal1* promoter contains three non-canonical E-boxes (nucleotides 7,082-7,087 bp, 7,674-7,679 bp and 7,711-7,716 bp of GenBank AB064982.1) – or if it depends on PER1/DEC-mediated activation/inhibition of other *Bmal1* regulators. Together, these data postulate a critical role for *Per1-Dec1/2* interaction for *Bmal1* rhythmicity. In contrast to *Per2* and *Bmal1*, the amplitude of *Rev-Erbα* mRNA rhythms in *Per1/Dec1/2* triple mutants was increased, indicating a primarily inhibitory function for *Per1* and *Dec* on *Rev-Erbα* transcription. A similar mode of action has been postulated for *Per1* (Preitner et al. 2002). In contrast, during the second half of the subjective night, *Rev-Erbα* expression was suppressed in *Per1/Dec1/2* mutants indicating an activating *Dec* function at this time (Figure 3B). Such a time of day-dependent transactivation functionality of *Per-Dec* is supported by a number of *in vitro* studies showing that DECs have a bidirectional regulatory function on the transcription of E-box containing genes (Azmi et al. 2003; Dardente et al. 2009; Hamaguchi et al. 2004; Honma et al. 2002; Kawamoto et al. 2004; Li et al. 2003; Rossner et al. 2008).

Of note, the strengthened rhythm in *Rev-Erbα* expression in the triple mutants does not result in a rhythmic *Bmal1* expression (Figure 3, Figure S2). A possible explanation for this effect would be that the altered phase-relationship between the positive *Bmal1* regulator *Per2* (Schmutz et al. 2009; Shearman et al. 2000) and the transcriptional repressor *Rev-Erbα* (Preitner et al. 2002) results in a blunting of *Bmal1* transcription. However, further transcriptional modulators such as RORs or other uncharacterized proteins as well as post-translational processes could also be involved. It is likely that the disrupted *Bmal1* transcription in *Per1/Dec* triple mutants results in a weakened oscillator which is reflected in the shortened activity period of these animals in DD (Figure 2C). A similar cumulative effect on free-running period length was observed in other double mutant lines such as *Per1/Rev-Erbα* and *Per2/Rev-Erbα* (Jud et al. 2010; Schmutz et al. 2009).

It was recently postulated that in *Drosophila* CWO functionally synergizes with PER and has both activatory and inhibitory functions on CLK/CYC-activated transcription (Kadener et al. 2007; Richier et al. 2008). In this study, we show an analog *Per (1)-Dec* interaction in the mammalian TTL. The molecular mechanism behind the bi-directionality of PER and DEC action on E-box-mediated and/or *Bmal1* transcription remains to be solved, but seems to depend not only on daytime and tissue-type, but also on lighting conditions and the absence or presence of other TTL proteins such as BMAL1 (Rossner et al. 2008) and PER1.

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FIGURE LEGENDS

Figure 1. Wheel-running behavior under entrained and free-running conditions. (A-G) Representative locomotor activity records of (A) wild-type (WT), (B) *Per1*$^{-/-}$, (C) *Dec1*$^{-/-}$, (D) *Dec2*$^{-/-}$, (E) *Per1*$^{-/-}$*Dec1*$^{-/-}$, (F) *Per1*$^{-/-}$*Dec2*$^{-/-}$ and (G) *Per1*$^{-/-}$*Dec1/2*$^{-/-}$ mice under light/dark (white and grey shaded, respectively) and constant darkness (grey shaded) conditions. Black bars indicate wheel-revolutions per 6 min bin. Actograms are double-plotted with the activity of the following day plotted to the right and below the previous one. Gray shadings indicate dark phases.

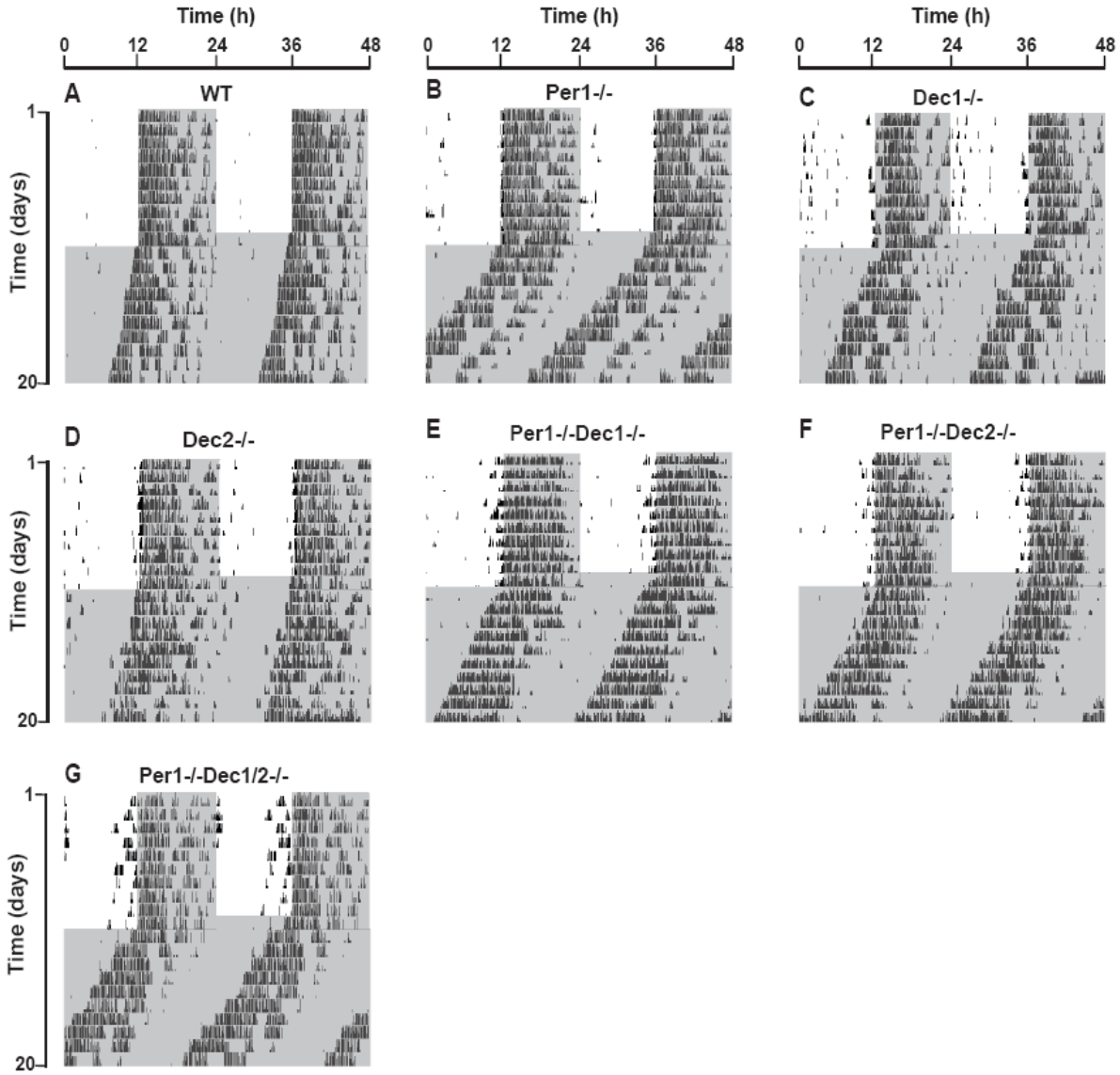
Figure 2. Analysis of locomotor behavior under LD and DD conditions. (A) Activity onset phase angles relative to ZT12 ("lights off") under LD conditions: wild-type (WT) -0.04±0.01 h (n = 20), *Per1*$^{-/-}$ -0.29±0.02 h (n = 20), *Dec1*$^{-/-}$ -0.19±0.02 h (n = 20), *Dec2*$^{-/-}$ 0.01±0.02 h (n = 12), *Per1*$^{-/-}$*Dec1*$^{-/-}$ -0.56±0.05 h (n = 20), *Per1*$^{-/-}$*Dec2*$^{-/-}$ -0.28±0.05 h (n = 17) and *Per1*$^{-/-}$*Dec1/2*$^{-/-}$ -0.81±0.04 h (n = 11). (B) Activity onset variations under LD conditions: WT 0.05±0.004 h (n = 20), *Per1*$^{-/-}$ 0.19±0.03 h (n = 20), *Dec1*$^{-/-}$ 0.11±0.02 h (n = 20), *Dec2*$^{-/-}$ 0.14±0.02 h (n = 12), *Per1*$^{-/-}$*Dec1*$^{-/-}$ 0.30±0.03 h (n = 19), *Per1*$^{-/-}$*Dec2*$^{-/-}$ 0.26±0.03 h (n = 17) and *Per1*$^{-/-}$*Dec1/2*$^{-/-}$ 0.48±0.04 h (n = 12). (C) Average free-running period length in constant darkness (DD): WT 23.48±0.02 h (n = 20), *Per1*$^{-/-}$ 22.39±0.07 h (n = 20), *Dec1*$^{-/-}$ 23.07±0.06 h (n = 20), *Dec2*$^{-/-}$ 23.56±0.03 h (n = 12), *Per1*$^{-/-}$*Dec1*$^{-/-}$ 22.28±0.10 h (n = 14), *Per1*$^{-/-}$*Dec2*$^{-/-}$ 22.23±0.11 h (n = 17), *Per1*$^{-/-}$*Dec1/2*$^{-/-}$ 21.68±0.10 h (n = 12). (D) Period variability between two DD intervals: WT 0.11±0.02 h (n = 10), *Per1*$^{-/-}$ 0.41±0.06 h (n = 10), *Dec1*$^{-/-}$ 0.27±0.05 h (n = 10), *Dec2*$^{-/-}$ 0.16±0.04 h (n = 10), *Per1*$^{-/-}$*Dec1*$^{-/-}$ 0.56±0.05 h (n = 10), *Per1*$^{-/-}$*Dec2*$^{-/-}$ 0.57±0.11 h (n = 10), *Per1*$^{-/-}$*Dec1/2*$^{-/-}$ 0.58±0.04 h (n = 12). Data are represented as mean ± SEM. *: $p < 0.05$ compared to WT, $^{\#}$: $p < 0.05$ compared to *Per1*$^{-/-}$, §: $p < 0.05$ compared to *Per1*$^{-/-}$*Dec1*$^{-/-}$ or *Per1*$^{-/-}$*Dec2*$^{-/-}$.

Figure 3. SCN clock gene expression and PER1/DEC influence on *Bmal1* transcription. DD expression profiles of (A) *Per2*, (B) *Rev-Erbα* and (C) *Bmal1* mRNA in the SCN of wild-type (WT; black line), *Per1*$^{-/-}$*Dec1*$^{-/-}$ (blue line), *Per1*$^{-/-}$*Dec2*$^{-/-}$ (green line) and *Per1*$^{-/-}$*Dec1/2*$^{-/-}$ (red line) mice determined by x-ray autoradiography of ^{35}S-labeled *in situ* hybridizations (ISH). Data are normalized to the average expression in wild-types and double plotted for visual clarity. All values are means ± SEM (n = 3). (D) Dual luciferase reporter gene assays using a firefly *Bmal1::luc* reporter construct, a *Renilla* luciferase reporter as transfection control and *Dec1*, *Dec2* and/or *Per1* expression plasmids in HEK293 cells. Data are normalized to *Bmal1::luc* control and represented as mean ± SEM (n = 12 replicates). *: $p < 0.05$ compared to *Baml1::luc* reporter (first column) or

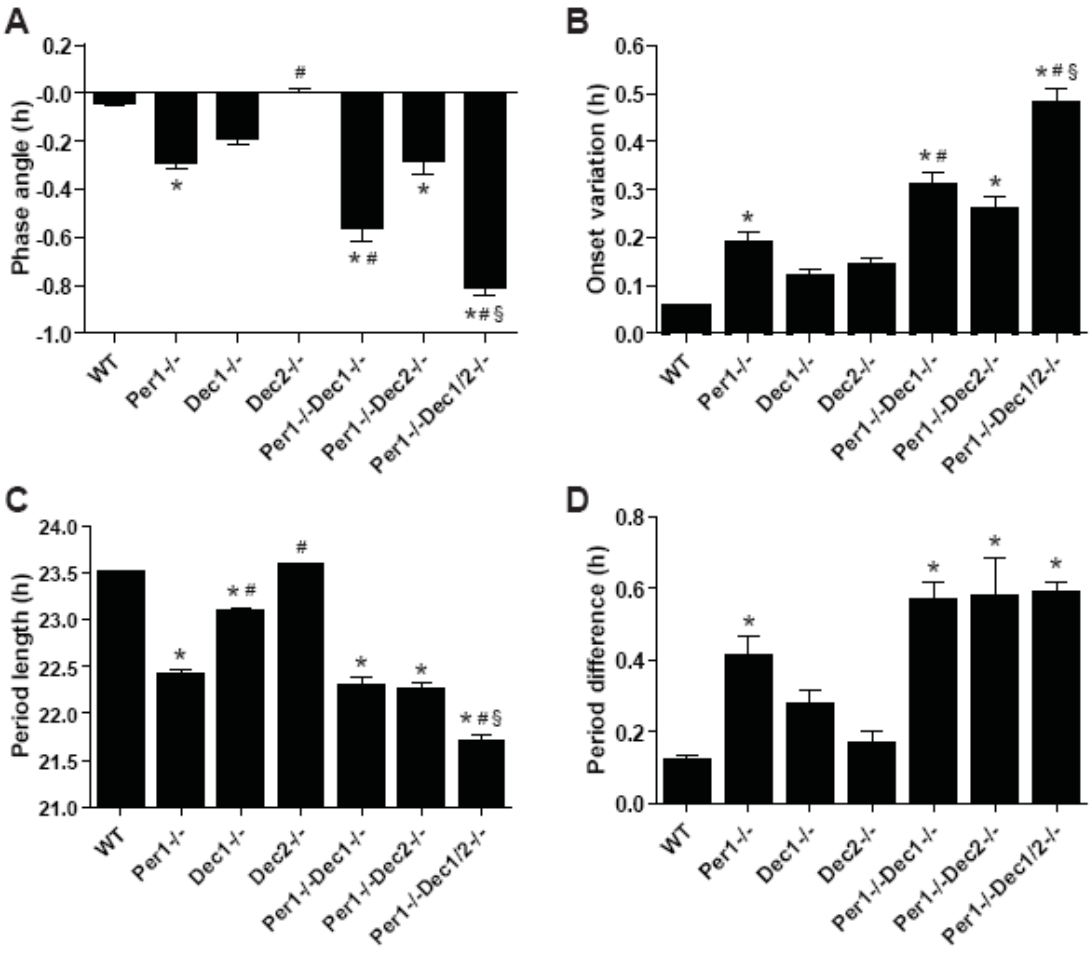
Bmal1::luc reporter and *Per1* expression plasmid (second column), §: $p < 0.05$ compared to *Bmal1::luc* reporter, *Per1* and one *Dec* expression plasmid (5th and 6th column). RLU: relative luminescence units.

Figure S1. Locomotor activity in LL. Average free-running period length in constant light ($LL_{50\ lux}$): WT 25.12±0.08 h (n = 12), *Per1*$^{-/-}$ 26.09±0.12 h (n = 15), *Dec1*$^{-/-}$ 25.10±0.09 h (n = 11), *Dec2*$^{-/-}$ 25.18±0.06 h (n = 12), *Per1*$^{-/-}$*Dec1*$^{-/-}$ 26.54±0.14 h (n = 11), *Per1*$^{-/-}$*Dec2*$^{-/-}$ 26.34±0.10 h (n = 11), *Per1*$^{-/-}$*Dec1/2*$^{-/-}$ 26.92±0.22 h (n = 11). Data are represented as mean ± SEM. *: $p < 0.05$ compared to WT, #: $p < 0.05$ compared to *Per1*$^{-/-}$, §: $p < 0.05$ compared to *Per1*$^{-/-}$*Dec1*$^{-/-}$ or *Per1*$^{-/-}$*Dec2*$^{-/-}$.

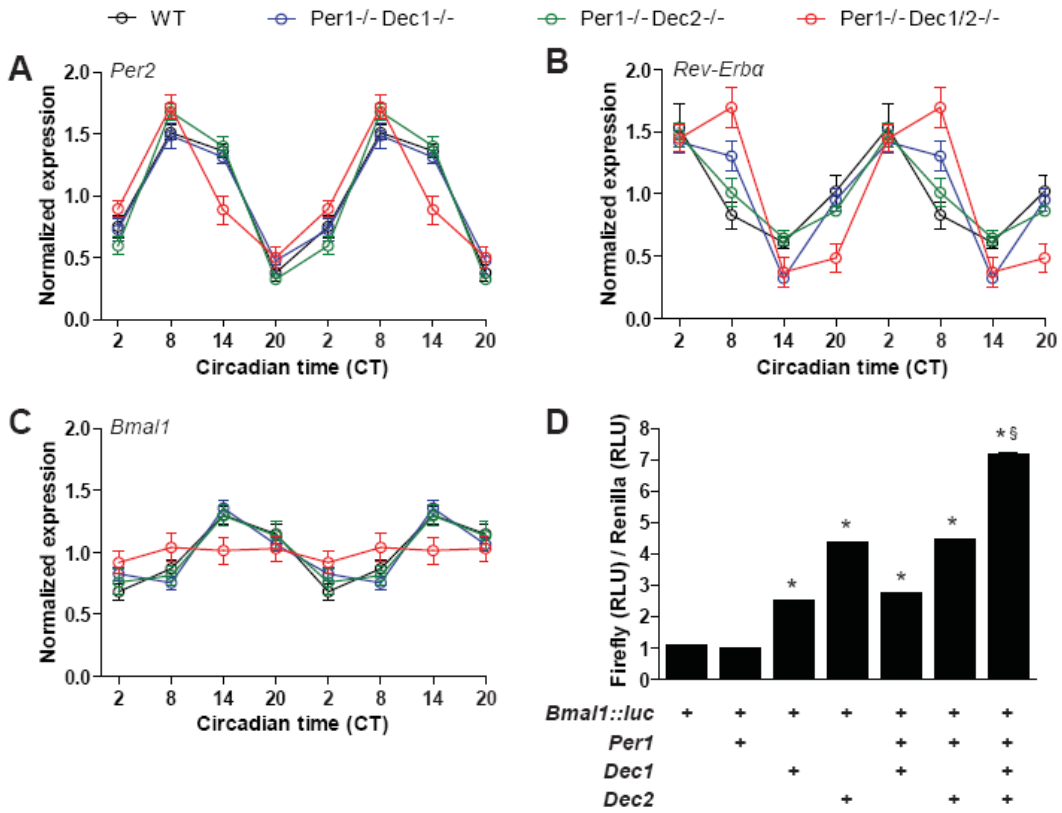
Figure S2. *In situ* hybridization (ISH) profiles of clock gene expression in the SCN. Normalized expression of (A) *Per2*, (B) *Bmal1* and (C) *Rev-Erbα* mRNA in the SCN in DD of wild-type (WT; black line), *Per1*$^{-/-}$ (red line), *Dec1*$^{-/-}$ (blue line) and *Dec2*$^{-/-}$ (green line) mice. Data are normalized to the average expression in wild-types and double plotted for visual clarity. All values are means ± SEM (n = 3).



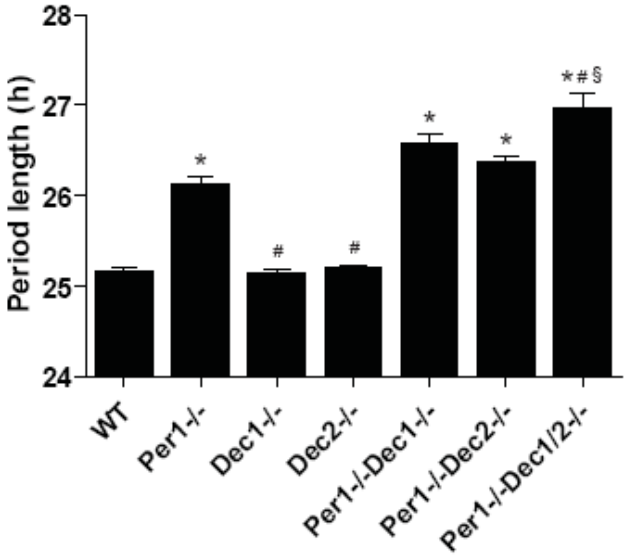
Bode et al. Figure 1



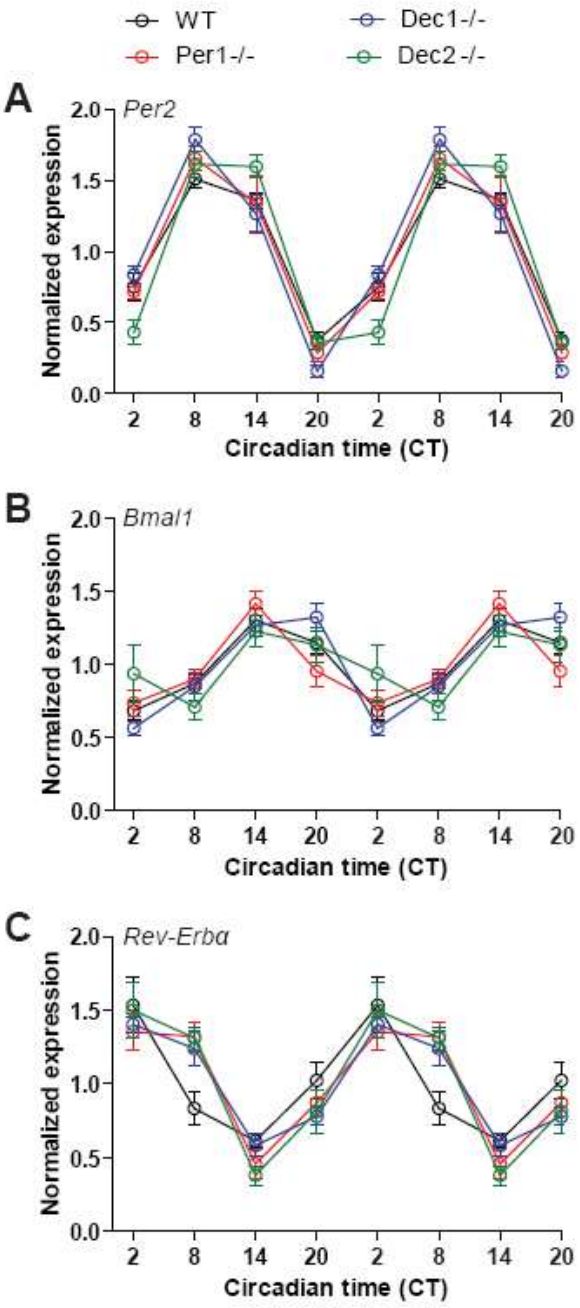
Bode et al. Figure 2



Bode et al. Figure 3



Bode et al. Figure S1



Bode et al. Figure S2

Table S1: Statistics of behavioral paradigms

Tested parameter	Genotype comparison ↓→	Per1-/-	Dec1-/-	Dec2-/-	Per1-/-Dec1-/-	Per1-/-Dec2-/-	Per1-/-Dec1/2-/-
Onset in LD	WT	***	ns	ns	***	***	***
	Per1-/-		ns	***	***	ns	***
	Dec1-/-			*	***		***
	Dec2-/-					***	***
	Per1-/-Dec1-/-					***	**
	Per1-/-Dec2-/-						***
Onset variation in LD	WT	**	ns	ns	***	***	***
	Per1-/-		ns	ns	*	ns	***
	Dec1-/-			ns	***		***
	Dec2-/-					ns	***
	Per1-/-Dec1-/-					ns	**
	Per1-/-Dec2-/-						***
Period length in DD	WT	***	***	ns	***	***	***
	Per1-/-		***	***	ns	ns	***
	Dec1-/-			***	***		***
	Dec2-/-					***	***
	Per1-/-Dec1-/-					ns	***
	Per1-/-Dec2-/-						***
Period length variation in DD	WT	*	ns	ns	***	***	***
	Per1-/-		ns	ns	ns	ns	ns
	Dec1-/-			ns	*		***
	Dec2-/-					***	***
	Per1-/-Dec1-/-					ns	ns
	Per1-/-Dec2-/-						ns
Period length in LL	WT	***	ns	ns	***	***	***
	Per1-/-		***	***	ns	ns	***
	Dec1-/-			ns	***		***
	Dec2-/-					***	***
	Per1-/-Dec1-/-					ns	ns
	Per1-/-Dec2-/-						*

ns = not significant * $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.001$

Bode et al. Table S1

Table S2: Statistics of reporter gene assay

Tested parameter	Expression plasmid comparison ↓ →	Bmal1::luc, Per1	Bmal1::luc, Dec1	Bmal1::luc, Dec2	Bmal1::luc, Per1, Dec1	Bmal1::luc, Per1, Dec2	Bmal1::luc, Per1, Dec1, Dec2
Reporter gene assay	Bmal1::luc	ns	***	***	***	***	***
	Bmal1::luc, Per1		***	***	***	***	***
	Bmal1::luc, Dec1			***	ns		***
	Bmal1::luc, Dec2					ns	***
	Bmal1::luc, Per1, Dec1					***	***
	Baml1::luc, Per1, Dec2						***

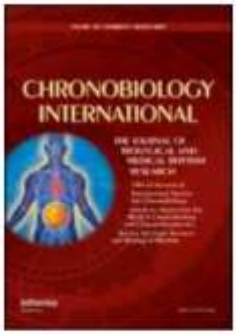
ns = not significant * $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.001$

Bode et al. Table S2

3.2. ‘Advanced Activity Phase Entrainment and Restored Free-Running SCN Rhythms in *Per2/Dec* Mutant Mice’ (submitted to *Chronobiology International*)

Brid Bode, Moritz J. Rossner, Henrik Oster

Chronobiology International



Advanced Activity Phase Entrainment and Restored Free-Running SCN Rhythms in Per2/Dec Mutant Mice

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Keywords:	Per2, Dec1, Dec2, entrainment, masking, SCN, mice

Advanced Activity Phase Entrainment and Restored Free-Running SCN Rhythms in *Per2/Dec* Mutant Mice

Brid Bode[1], Moritz J. Rossner[2], Henrik Oster[1,*]

[1]: Circadian Rhythms Group, Max Planck Institute for Biophysical Chemistry, 37077 Göttingen, Germany

[2]: Max Planck Institute for Experimental Medicine, 37075 Göttingen, Germany

*: corresponding author (henrik.oster@mpibpc.mpg.de; tel ++49-551-2012738; fax ++49-551-2012705)

ABSTRACT

Many behavioral and physiological processes display diurnal (24 hour) rhythms controlled by an internal timekeeping system – the circadian clock. In mammals, a circadian pacemaker is located in the suprachiasmatic nuclei (SCN) of the hypothalamus and synchronizes peripheral oscillators found in most other tissues with the external light-dark cycle. At the molecular level, circadian clocks are regulated by transcriptional translational feedback loops (TTLs) involving a set of clock genes. The mammalian core TTL includes the transcriptional modulators PER (1-3) and CRY (1/2) that inhibit their own expression by interaction with CLOCK/NPAS2 and BMAL1 (ARNTL). The basic helix-loop-helix transcription factors DEC1 (BHLHE40) and DEC2 (BHLHE41) can interact with this core TTL, forming an accessory feedback mechanism. We measured circadian locomotor behavior and clock gene expression in the SCN of *Per2/Dec* double and triple mutant mice to analyze the functional interaction of PER2 and DEC feedback on circadian pacemaker function in the SCN. Our wheel-running data suggest a synergistic function of *Per2* and *Dec1/2* in activity entrainment to a standard light/dark (LD) cycle, correlating with a cumulative deficiency in negative masking capacities in *Per2/Dec* double and triple mutant mice. In contrast, under constant darkness (DD) conditions, a deletion of either *Dec1* or *Dec2* partially rescued the *Per2* mutant short period/arrhythmicity phenotype, accompanied by a restoration of clock gene rhythms in the SCN. Together, our results show a strong interaction of *Per2* and *Dec1/2* feedback processes in the SCN with differential modes of interactivity under entrained and free-run conditions.

Key words: *Per2*, *Dec1*, *Dec2*, entrainment, masking, SCN, mice

INTRODUCTION

Environmental conditions on Earth are characterized by 24 h cycles brought about by our planet's rotation around its own axis. In most species internal timekeepers, so called circadian clocks, have evolved enabling an organism to anticipate and efficiently adapt behavior and physiology to these predictable changes (Green et al. 2008; Takahashi et al. 2008). In mammals these clocks are based on interlocked transcriptional translational feedback loops (TTLs) comprised of a set of clock genes including *Per*(*iod*) *1-3*, *Cry*(*ptochrome*) *1-2*, *Clock* and *Bmal1* (*Arntl*) (Ko and Takahashi 2006). The latter two transcription factors activate expression of *Per*s and *Cry*s via E-box promoter elements in the SCN during the day. PER/CRY protein complexes relocate back into the nucleus where they interfere with CLOCK/BMAL1, suppressing their own transcription during the course of the night (Griffin et al. 1999; Kume et al. 1999; Reppert and Weaver 2002). Recently two other transcriptional regulators, DEC1 (BHLHE40) and DEC2 (BHLHE41), have been shown to interfere with the circadian clock machinery (Alvarez and Sehgal 2002). Both genes are rhythmically expressed in the mammalian circadian pacemaker, the suprachiasmatic nucleus (SCN) (Honma et al. 2002; Noshiro et al. 2005). *Dec* gene transcription is activated by CLOCK/BMAL1 via *cis*-regulatory E-box elements (Hamaguchi et al. 2004; Kawamoto et al. 2004). Further, *Dec1* activity is highly light-responsive and has, thus, been implicated in light resetting of the SCN clock (Honma et al. 2002; Rossner et al. 2008). Both DEC proteins can modulate E-box-mediated transcription and directly interact with BMAL1 (Honma et al. 2002; Sato et al. 2004). *Dec1/2* single and double deficient mice show moderate alterations in clock period regulation and light resetting (Rossner et al. 2008). Interestingly, cell culture-based studies suggest that both DECs and PERs do not exclusively act as transcriptional repressors of E-box-controlled genes, depending on cell type and/or the presence of BMAL1 (Azmi et al. 2003; Dardente et al. 2009; Hamaguchi et al. 2004; Honma et al. 2002; Kawamoto et al. 2004; Li et al. 2004; Li et al. 2003; Rossner et al. 2008; Shearman et al. 2000; Zheng et al. 2001; Zheng et al. 1999). To better understand a potential interactivity between *Per2* and *Dec* in the regulation of the SCN pacemaker we have generated *Per2/Dec1/2* double and triple mutant mice to analyze behavioral and molecular effects of *Per2/Dec* deficiency on SCN clock function. Strong synergistic effects were observed between *Per2* and *Dec1/2* in activity phase entrainment, while free-running period length was determined by *Per2* function and partially rescued in the absence

of one *Dec* gene. Antagonistic interaction was observed in rhythm stabilization at behavioral and molecular levels under constant darkness conditions, suggesting that external environmental conditions (i.e. light) affect *Per2-Dec* cross-talk in the SCN.

MATERIALS AND METHODS

Animals

Per2$^{m/m}$ (*Per2*^{Brdm1}) mice (Zheng et al. 1999), *Dec1*$^{-/-}$ (*Bhlhe40*^{tm1Tan}) and *Dec2*$^{-/-}$ (*Bhlhe41*^{tm1Mjro}) mice (Rossner et al. 2008; Sun et al. 2001) were backcrossed to a C57BL/6J background and then mated to obtain double mutant *Per2*$^{m/m}$*Dec1*$^{-/-}$ and *Per2*$^{m/m}$*Dec2*$^{-/-}$ as well as triple mutant *Per2*$^{m/m}$*Dec1/2*$^{-/-}$ mice. The genotype of the offspring was determined by PCR as described (Rossner et al. 2008; Zheng et al. 1999). All experiments were performed on male adult animals (2-10 months). C57Bl/6J mice were used as controls. 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 Animal Welfare Act.

Behavioral analysis

Mouse housing and handling were performed as described (Albrecht and Oster 2001). Wheel-running activity was analyzed using ClockLab software (Actimetrics, Evanston, IL). Prior to the experiments, animals were entrained to a 12 hours light: 12 hours dark (LD) cycle for at least 10 days (light intensity 250 lux). For analyzing free-running locomotor activity under constant darkness conditions (DD), lights were turned off at the end of the light phase (*Zeitgeber* time, ZT12) and not turned on again on the next day. Activity profiles in LD and DD were assessed over a time of 4-7 consecutive days. Onset phase angles were determined by fitting a straight line to 7-10 consecutive onsets using ClockLab software and manual correction. Onset variation depicts the mean deviation of real activity onsets from a regression line through 7-10 consecutive onsets under stably entrained or free-running conditions. Rhythmicity and period length (τ) in DD were determined by χ^2 periodogram analysis over an interval of 7-10 consecutive days. To test for masking capacities animals were entrained to a LD cycle for 10 days. A 60 min light pulse (100 lux) was applied between ZT14 and ZT15 on day 10. The relative light phase activity was calculated as the wheel-running activity during the light exposure period on day 10 divided by the average wheel-running activity without light treatment on days 7-9 for the same animal at ZT14-15. To analyze acute phase shifting by single nocturnal light pulses animals were entrained to a LD cycle for at least two weeks. A 15 min light pulse (250 lux) was applied at ZT14 before animals were released into DD. Phase shifts were determined as the difference between the

regression lines through onsets before and after the light pulse on the first day after the light exposure as compared to animals of the same genotype which were released into DD without extra light treatment.

In situ hybridization

Animals were entrainted to LD for 10 days, released into DD and sacrificed on the second day at circadian times CT2, CT8, CT14 and CT20. Brains were dissected under dim red light, fixed, dehydrated and paraffin embedded. 8 µm sections were hybridized with ^{35}S-UTP-labeled antisense RNA probes for clock gene transcripts as described (Oster et al. 2003b; Oster et al. 2002). The *Rev-Erbα* probe template was generated by PCR (forward primer: CCCTCTACAGTGACAGCTCCA, reverse primer: TCAGACACCGTTTGTACTGGA) from murine adipose tissue cDNA. Relative quantification of expression levels was performed by densitometric analysis of autoradiograph films using Quantity One software (Bio-Rad, Munich, Germany). SCN data were adjusted to the optical density measured in the lateral hypothalamus next to the SCN. For each experiment 3 animals per genotype were used and 3 adjacent SCN sections per animal were analyzed.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (GraphPad, La Jolla, CA). Groups were compared by One-way ANOVA followed by Tukey's post test for multiple comparisons. A p-value < 0.05 was considered significant. The rhythmicity of clock gene expression was evaluated by CircWave v1.4 (Oster et al. 2006).

RESULTS

All strains were backcrossed for more than ten generations to a C57BL/6J background prior to this study. *Per2* mutant (*Per2*^{Brdml}), *Dec1* (*Bhlhe40*^{tm1Tan}) and *Dec2* (*Bhlhe41*^{tm1Mjro}) mutant mice were mated to yield *Per2/Dec* double and triple mutant animals. All genotypes were born at the expected Mendelian ratios, were viable and fertile and did not show any gross morphological and behavioral abnormalities (data not shown). Adult males (2-10 months) were singly housed in running-wheel equipped cages and analyzed for locomotor behavior under defined light conditions. Under 12 h: 12 h light dark (LD) conditions all animals entrained readily to the LD cycle with locomotor activity being predominantly confined to the dark phase (Fig. 1A-G). As reported before *Per2* mutant mice displayed a marked phase advance of activity onsets preceding the beginning of the dark phase by 0.6 h (Fig. 1H; Zheng et al. 1999). No significant phase advances were seen in *Dec* single mutants (Rossner et al. 2008), while activity onsets were dramatically advanced (1 h and more) in *Per2/Dec* double and triple mutant animals (Fig. 1H and statistics in supplemental Table 1). These phase angle advances were correlated to an increasing imprecision in activity onsets in double and triple mutant mice (Fig. 1I). While wild-type animals showed very predictable activity onsets in LD with day-to-day variations of less than 0.1 h, this variation was increased to 0.3 h in *Per2* single mutants and further to more than 0.4 h in *Per2/Dec* double and triple mutant animals (Fig. 1I and statistics in supplemental Table 1).

When released into constant darkness conditions (DD) all genotypes showed circadian rhythms of activity (Fig. 1A-G). Period length (τ) was 23.5 h in wild-type animals. As reported before period length was significantly shortened to 21.9 h in *Per2* mutant animals (Fig. 2A; Zheng et al. 1999). A moderate period reduction of 0.5 h was also seen in *Dec1* deficient mice while *Dec2* mutants did not show any significant period phenotype (Fig. 2A; Rossner et al. 2008). In *Per2/Dec* double and triple mutant animals period lengths fell between those seen in the single mutant mice, but were considerably closer to *Per2* mutants (Fig. 2A and statistics in supplemental Table 1). It had been reported that *Per2* mutant mice show unstable circadian rhythms and become completely arrhythmic upon extended time in DD (Zheng et al. 1999). To test if rhythm robustness was affected in *Per2/Dec* mutant animals we kept a subset of animals under DD conditions for more than three weeks. While the majority *Per2* mutants had lost their circadian activity rhythm by that time, *Per2/Dec1* and *Per2/Dec2* double mutants showed a

marked rescue in rhythm sustainability with more than 60 % (*Per2/Dec1*) and 90 % (*Per2/Dec2*) of animals retaining rhythmicity after 21 days in DD (Fig. 2B). This rescue effect seems to depend on the presence of at least one functional *Dec* gene as *Per2/Dec1/2* triple mutants showed rhythm sustainability similar to *Per2* mutant animals (Fig. 2B).

To test if this behavioral interaction was reflected at the molecular level we analyzed the circadian expression of the clock genes *Per1*, *Bmal1* and *Rev-Erbα* (*Nr1d1*) in the SCN of *Per2/Dec* mutant animals on the second day in DD using radioactive *in situ* hybridization on coronal brain sections. In wild-type animals *Per1*, *Bmal1* and *Rev-Erbα* mRNAs showed robust circadian rhythms of expression with peak levels at Circadian time (CT) 8, 14 and 2, respectively (Fig. 3A-C). As reported before, the expression rhythms of all three genes were severely blunted in *Per2* mutant mice (Zheng et al. 2001; Zheng et al. 1999). While only minor effects on expression rhythms were seen in *Dec* single mutant animals (Suppl. Fig. 1A-C), rescued transcription rhythms were observed for *Per1* and *Bmal1* in *Per2/Dec1* and *Per2/Dec2* double mutants and for *Rev-Erbα* in *Per2Dec2* mutants (Fig. 3A-C), correlating with the partial rescue of rhythm sustainment and period length in these animals (Fig. 2A, B). Of note, in comparison to wild-types, *Per1* and *Bmal1* peak activities were phase advanced in *Per2/Dec* double mutants (Fig. 3A, B) while *Rev-Erbα* expression was phase delayed in *Per2/Dec2* mutant mice (Fig. 3C). Together, these data indicate that the observed alterations in behavioral rhythm robustness are the direct consequence of changes in TTL transcription amplitudes in the SCN.

This finding prompted us to ask if the observed changes in activity onsets in *Per2/Dec* double and triple mutants (Fig. 1H) are related to alterations in the entrainment capacity of the SCN pacemaker to light. A short 15 min light pulse delivered at ZT14 on the last day in LD (Aschoff type II protocol) caused a 1.3 h delay in activity onsets in wild-type animals when compared to non light-exposed controls (Fig. 4A and statistics in supplemental Table 1). As previously reported this photic phase resetting was blunted in *Per2* mutant animals (0.5 h) (Albrecht et al. 2001). *Dec1* single, *Per2/Dec1* double and *Per2/Dec1/2* triple mutants showed a moderate reduction in phase delays (0.9-1 h) while a normal phase resetting capacity was observed in *Dec2* mutant animals (1.2 h) (Fig. 4A and statistics in supplemental Table 1). Of note, *Per2/Dec2* double mutant mice displayed even stronger photic phase delays than wild-type controls (1.8 *vs.* 1.3 h). In summary, the effects of *Per2/Dec* deficiency on light-mediated resetting of the circadian clock were less uniform and did not match the changes observed in LD onset behavior.

Therefore, we asked if the advanced activity onsets might reflect a decrease in acute behavioral responses to light exposure (so called *masking*) (Mrosovsky 1999). To test this we subjected stably entrained animals to a 1 h light pulse (100 lux) during the first half of the dark phase (ZT 14-15) and compared activity levels to those observed on previous days at the same time interval. While wild-type and single mutant mice showed a robust suppression of activity levels by 70-80 % under 100 lux illumination, masking was significantly impaired in *Per2/Dec* double mutants. The same trend was seen in *Per2Dec1/2* triple mutants (Fig. 4B and statistics in supplemental Table 1). These data closely correlate with the onset phenotypes observed in these animals (Fig. 1E-H). Hence, the activity onset regulation under LD conditions appears to primarily reflect changes in negative masking capacities in *Per2/Dec* mutant mice rather than changes in regulations of photic TTL resetting.

DISCUSSION

In this study we analyzed *Per2-Dec* interaction in the regulation of molecular and behavioral circadian rhythms in mice. We found that *Per2* and *Dec1/2* synergistically affect the regulation of activity entrainment, possibly via the regulation of negative masking. Under free-running conditions, however, deletion of *Dec1* or *Dec2* can partially rescue the *Per2* mutant phenotype for period length and rhythm stability. This effect is associated with changes in clock gene activity rhythms in the SCN pacemaker.

Activity phase angles under entrained conditions are often proportional to the difference between external and internal period length, thus depending on the daily phase shifting limits of the external light/dark cycle (Brown et al. 2008). Shortened periodicity in combination with reduced capacity for phase delays was also suggested to underlie the early activity onsets observed in *Per2* mutant mice (Spoelstra et al. 2004). While in this study in the single mutant animals masking was preserved (Fig. 4B) and, thus, seems not to affect predark activity, period length regulation and phase onset effects were clearly dissociated in *Per2/Dec* double and triple mutant mice (Fig. 1H, I, 2A). In these animals, negative masking, but not phase shifting capacity, correlates closely with onset phase angle alterations (Fig. 4). This suggests that the early onset observed under LD conditions might to a large part result from ineffective acute photic suppression of locomotor activity during the late day. Both *Per2* and the *Dec*s seem to be involved in this regulation. Interestingly, a close relationship between period length and activity phase in humans has so far only been reported for familiar advanced sleep phase syndrome (FASPS) patients that carry mutations directly or indirectly affecting PER2 stability and turnover (Jones et al. 1999; Toh et al. 2001). Other chronotypes affecting gene polymorphisms have been described that do not show significant alterations in free-running period (Archer et al. 2003; Ebisawa et al. 2001; Viola et al. 2007) and, thus, appear to rather reflect changes in photic input to the clock or to other arousal-regulating centers in the brain. An elegant cell-based study by Brown and colleagues shows that entrainment phase changes in humans can also result from changes in the molecular robustness of the cellular TTL machinery (Brown et al. 2008). In line with this, our *in situ* data suggest that in *Per2* single and *Per2/Dec* double mutants clock gene oscillations in the SCN are disrupted (Fig. 3). Interestingly, this results in an increased sensitivity of the SCN clock to acute nocturnal light pulses in *Per2Dec2* double mutant mice, but not in

Per2Dec1 mutants (Fig. 4A). Given the changes in base TTL function brought about by the functional deletion of single components it is possible that the integrated light response characteristics over the course of the day are altered in these mutants, resulting in a modified resetting of entrainment phase.

Photic phase resetting of the circadian clock is believed to depend on *Per1* and *Per2* (and possibly *Dec1*) induction in the SCN after light exposure during the subjective night (Albrecht et al. 1997; Honma et al. 2002; Miyake et al. 2000; Rossner et al. 2008; Shearman et al. 1997; Shigeyoshi et al. 1997; Yan and Silver 2002). In line with this *Per1* and *Per2* single mutant mice show decreased behavioral responses to nocturnal light exposure (Fig. 4A; Albrecht et al. 2001; Spoelstra et al. 2004). Phase resetting in *Dec1* single and *Per2/Dec1* double mutants is also affected, indicating that besides *Per2*, *Dec1* is more important for light resetting than *Dec2*, in line with the fact that only *Dec1* is acutely light-inducible in the SCN (Honma et al. 2002). However, the increased phase shifts of *Per2/Dec2* mutant mice suggest that an interaction of *Per2* and *Dec2* might be involved in the regulation of resetting.

Under free-running conditions period length and rhythm stability are strongly affected by a *Per2* mutation, resulting in gradual arrhythmicity under DD conditions (Zheng et al. 1999). This rhythm deterioration effect is partly rescued by an additional deletion of *Dec1* or *Dec2* in these mice, along with a partial restoration of period length and clock gene expression rhythms in the SCN (Fig. 2 and Fig. 3). In the absence of both *Decs* and *Per2*, however, the two effects are neutralized, indicating a certain functional redundancy of both *Decs* together with an antagonistic *Per2-Dec* interaction in free-run. Similar, though more dramatic, effects have been observed in *Per2-Cry* interaction. While *Per2/Cry2* double mutant animals show full period length and rhythmicity rescue (Oster et al. 2002), *Per2/Cry1/2* triple mutants are fully arrhythmic (Oster et al. 2003a). It was suggested that *Cry1/2* redundancy might be involved in this process in a way that PER1-CRY1 complexes could compensate for loss of PER2 and CRY2 in the corresponding mutants (Oster et al. 2002). In the same way, *Per1-Dec1* or *Per1-Dec2* interaction could compensate for the absence of *Per2* and one of the *Decs* in the double mutants. Whether this effect is based on physical interaction of the corresponding proteins or rather reflects a functional genetic interaction, e.g. via co-regulatory effects on target gene activation, remains to be shown.

Our expression data clearly show that the deletion of *Dec1* or *Dec2* function in a *Per2*-deficient background results in a restoration of clock gene expression rhythms together with a general up-

regulation of transcriptional activity (Fig. 3). This indicates that, unlike *Per2*, the *Dec* genes are primarily acting as transcriptional activators in the SCN. This finding is supported by previous data from *Dec1/2* double deficient animals (Rossner et al. 2008). While little consequences on clock gene activity are seen in *Dec* single mutants, effects are more pronounced in the double and triple mutant animals, indicating that the DEC proteins show a high grade of functional redundancy and – at least in the SCN – act primarily via interaction with PER(2). Transfection studies have suggested that the DECs might also have a function in transcriptional regulation of E-box genes independent of PER and CRY proteins (Azmi et al. 2003; Dardente et al. 2009; Hamaguchi et al. 2004; Honma et al. 2002; Kawamoto et al. 2004; Rossner et al. 2008). Our single mutant data, while not excluding this possibility for other tissues, clearly do not support such functionality for the SCN clock.

In the last years the chronobiological field has moved away from seeing the clock machinery as a single TTL comprised of a limited number of transcriptional regulators. More and more accessory loops – some of them going beyond the simple concept of transcriptional translational feedbacks – have been described (Zhang and Kay 2010). It will be an important task for the future to unravel the interactivity of this network of feedback loops to start to understand the mechanistics behind the generation and stabilization of molecular circadian rhythms. The characterization of *Per2-Dec* interaction as presented in this study is a first step into this direction, providing new insight into the genetic basis of entrainment and the regulation of free-run activity patterns in the living animal.

CONFLICT OF INTEREST

The authors report no conflicts of interest.

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FIGURE LEGENDS

Figure 1. Locomotor behavior under entrained (LD) conditions. (A-G) Representative double plotted locomotor activity records of (A) wild-type (WT), (B) $Per2^{m/m}$, (C) $Dec1^{-/-}$, (D) $Dec2^{-/-}$, (E) $Per2^{m/m}Dec1^{-/-}$, (F) $Per2^{m/m}Dec2^{-/-}$ and (G) $Per2^{m/m}Dec1/2^{-/-}$ mice under light/dark (white and grey shaded, respectively) and constant darkness (grey shaded) conditions. (H) Quantified phase angle under light/dark (LD) conditions: wild-type (WT) -0.04±0.01 h (n = 20), $Per2^{m/m}$ -0.62±0.06 h (n = 14), $Dec1^{-/-}$ -0.19±0.02 h (n = 20), $Dec2^{-/-}$ 0.01±0.02 h (n = 12), $Per2^{m/m}Dec1^{-/-}$ -1.15±0.07 h (n = 12), $Per2^{m/m}Dec2^{-/-}$ -1.26±0.08 h (n = 11) and $Per2^{m/m}Dec1/2^{-/-}$ -1.41±0.08 h (n = 6). (I) Quantification of onset variation under LD conditions: WT 0.05±0.004 h (n = 20), $Per2^{m/m}$ 0.31±0.02 h (n = 14), $Dec1^{-/-}$ 0.11±0.02 h (n = 20), $Dec2^{-/-}$ 0.14±0.02 h (n = 12), $Per2^{m/m}Dec1^{-/-}$ 0.43±0.02 h (n = 12), $Per2^{m/m}Dec2^{-/-}$ 0.46±0.03 h (n = 11) and $Per2^{m/m}Dec1/2^{-/-}$ 0.55±0.06 h (n = 6). Data are represented as mean ± SEM; *: $p < 0.05$ compared to WT, #: $p < 0.05$ compared to $Per2^{m/m}$, §: $p < 0.05$ compared to $Per2^{m/m}Dec1^{-/-}$ or $Per2^{m/m}Dec2^{-/-}$.

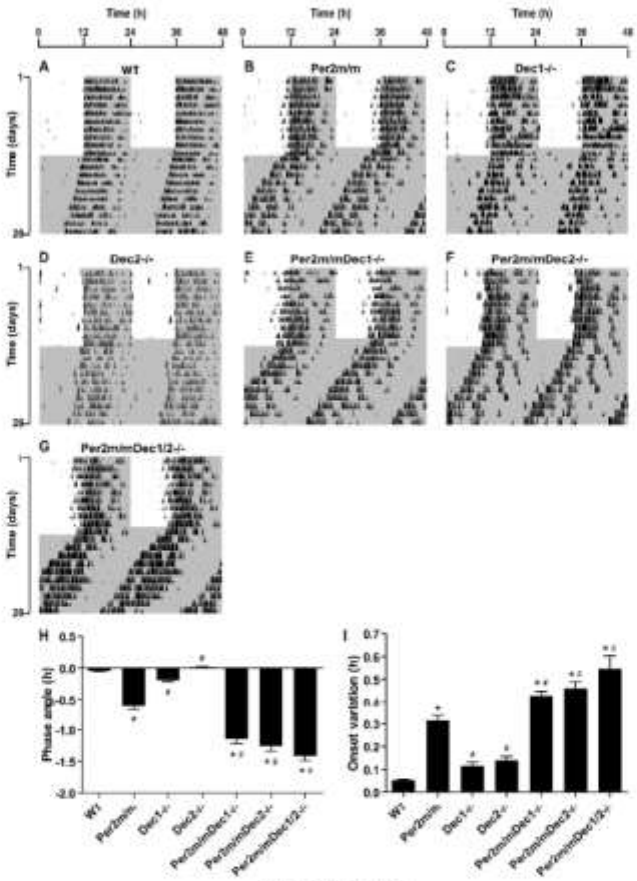
Figure 2. Locomotor behavior under free-running (DD) conditions. (A) Average free-running period length in constant darkness (DD): WT 23.48±0.02 h (n = 20), $Per2^{m/m}$ 21.96±0.06 h (n = 14), $Dec1^{-/-}$ 23.07±0.06 h (n = 20), $Dec2^{-/-}$ 23.56±0.03 h (n = 12), $Per2^{m/m}Dec1^{-/-}$ 22.54±0.08 h (n = 12), $Per2^{m/m}Dec2^{-/-}$ 22.30±0.05 h (n = 11), $Per2^{m/m}Dec1/2^{-/-}$ 22.17±0.19 h (n = 6). Data are represented as mean ± SEM; *: $p < 0.05$ compared to WT, #: $p < 0.05$ compared to $Per2^{m/m}$, §: $p < 0.05$ compared to $Per2^{m/m}Dec1^{-/-}$ or $Per2^{m/m}Dec2^{-/-}$. (B) Percentage of rhythmic animals during 21 days in DD of wild-type (WT), $Dec1^{-/-}$, $Dec2^{-/-}$ (grey line; per genotype n = 12), $Per2^{m/m}$ (red line; n = 9), $Per2^{m/m}Dec1^{-/-}$ (blue line; n = 12), $Per2^{m/m}Dec2^{-/-}$ (green line; n = 11) and $Per2^{m/m}Dec1/2^{-/-}$ mice (black line; n = 5).

Figure 3. *In situ* hybridization (ISH) profiles of clock genes in the SCN. Normalized mRNA expression levels of (A) *Per1*, (B) *Bmal1* and (C) *Rev-Erbα* in the SCN under constant darkness conditions of wild-type (WT; black line), $Per2^{m/m}$ (red line), $Per2^{m/m}Dec1^{-/-}$ (blue line) and $Per2^{m/m}Dec2^{-/-}$ (green line) mice (n = 3). Values are double plotted and represented as mean ± SEM.

Figure 4. Phase delay and masking responses to nocturnal light exposure. (A) Quantification of phase-delay responses after a 15 min light pulse at ZT14: WT -1.26±0.08 h (n = 8), $Per2^{m/m}$ -0.50±0.08 h (n = 8), $Dec1^{-/-}$ -0.86±0.08 h (n = 12), $Dec2^{-/-}$ -1.26±0.09 h (n = 11), $Per2^{m/m}Dec1^{-/-}$ -0.92±0.06 h (n = 11), $Per2^{m/m}Dec2^{-/-}$ -1.83±0.08 h (n = 11) and $Per2^{m/m}Dec1/2^{-/-}$ -1.07±0.03 h (n = 5). (B) Relative light phase activity during light exposure (100 lux) at ZT14-ZT15: WT 0.22±0.03 (n = 12), $Per2^{m/m}$ 0.28±0.02 (n = 10), $Dec1^{-/-}$ 0.34±0.02 (n = 6), $Dec2^{-/-}$ 0.19±0.03 (n = 6), $Per2^{m/m}Dec1^{-/-}$ 0.49±0.04 (n = 12), $Per2^{m/m}Dec2^{-/-}$ 0.58±0.05 (n = 11) and $Per2^{m/m}Dec1/2^{-/-}$ 0.39±0.05 (n = 5). Data are represented as mean ± SEM; *: $p < 0.05$ compared to WT, #: $p < 0.05$ compared to $Per2^{m/m}$, §: $p < 0.05$ compared to $Per2^{m/m}Dec1^{-/-}$ or $Per2^{m/m}Dec2^{-/-}$.

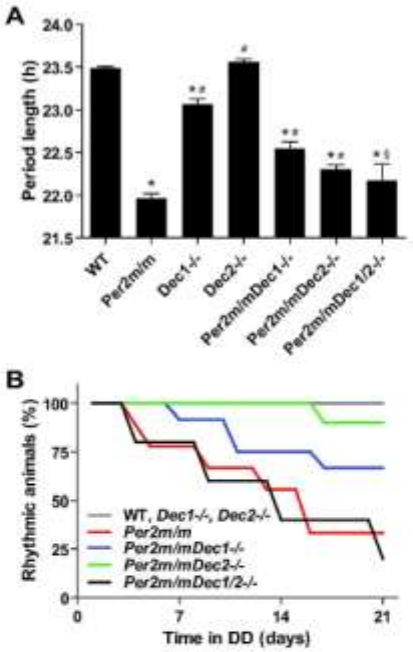
Supplemental figure 1. *In situ* hybridization (ISH) profiles of clock gene expression in the SCN. Normalized expression of (A) *Per1*, (B) *Bmal1* and (C) *Rev-Erbα* mRNA in the SCN under constant darkness of wild-type (WT; black line), $Dec1^{-/-}$ (blue line) and $Dec2^{-/-}$ (green line) mice (n = 3). Values are double plotted and represented as mean ± SEM.

Supplemental figure 2. Phase shift responses to a nocturnal light pulse. (A-G) Representative double plotted actograms of activity responses following a 15 min light pulse at ZT14 (arrow) of (A) WT, (B) $Per2^{m/m}$, (C) $Dec1^{-/-}$, (D) $Dec2^{-/-}$, (E) $Per2^{m/m}Dec1^{-/-}$, (F) $Per2^{m/m}Dec2^{-/-}$ and (G) $Per2^{m/m}Dec1/2^{-/-}$ mutant mice.



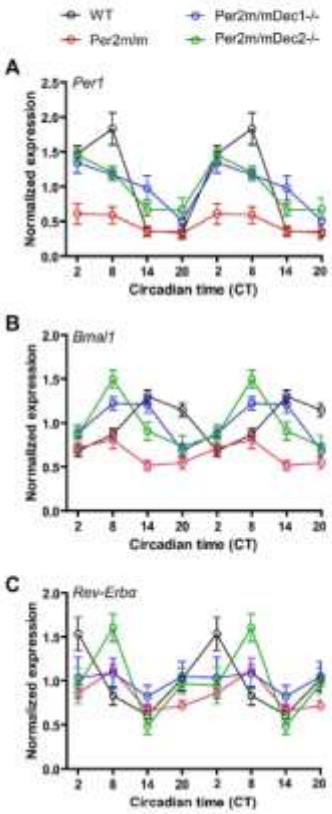
Bode et al. Figure 1

Locomotor behavior under entrained (LD) conditions. (A-G) Representative double plotted locomotor activity records of (A) wild-type (WT), (B) Per2m/m, (C) Dec1 / , (D) Dec2 / , (E) Per2m/mDec1 / , (F) Per2m/mDec2 / and (G) Per2m/mDec1/2 / mice under light/dark (white and grey shaded, respectively) and constant darkness (grey shaded) conditions. (H) Quantified phase angle under light/dark (LD) conditions: wild-type (WT) -0.04±0.01 h (n = 20), Per2m/m 0.62±0.06 h (n = 14), Dec1 / 0.19±0.02 h (n = 20), Dec2 / 0.01±0.02 h (n = 12), Per2m/mDec1 / 1.15±0.07 h (n = 12), Per2m/mDec2 / 1.26±0.08 h (n = 11) and Per2m/mDec1/2 / 1.41±0.08 h (n = 6). (I) Quantification of onset variation under LD conditions: WT 0.05±0.004 h (n = 20), Per2m/m 0.31±0.02 h (n = 14), Dec1 / 0.11±0.02 h (n = 20), Dec2 / 0.14±0.02 h (n = 12), Per2m/mDec1 / 0.43±0.02 h (n = 12), Per2m/mDec2 / 0.46±0.03 h (n = 11) and Per2m/mDec1/2 / 0.55±0.06 h (n = 6). Data are represented as mean ± SEM; *: $p < 0.05$ compared to WT, #: $p < 0.05$ compared to Per2m/m, §: $p < 0.05$ compared to Per2m/mDec1 / or Per2m/mDec2 / .
151x216mm (600 x 600 DPI)



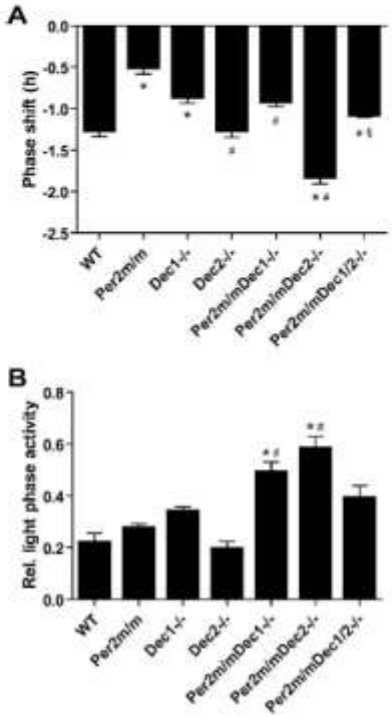
Bode et al. Figure 2

Locomotor behavior under free-running (DD) conditions. (A) Average free-running period length in constant darkness (DD): WT 23.48±0.02 h (n = 20), Per2m/m 21.96±0.06 h (n = 14), Dec1 / 23.07±0.06 h (n = 20), Dec2 / 23.56±0.03 h (n = 12), Per2m/mDec1 / 22.54±0.08 h (n = 12), Per2m/mDec2 / 22.30±0.05 h (n = 11), Per2m/mDec1/2 / 22.17±0.19 h (n = 6). Data are represented as mean ± SEM; *: $p < 0.05$ compared to WT, #: $p < 0.05$ compared to Per2m/m, §: $p < 0.05$ compared to Per2m/mDec1 / or Per2m/mDec2 / . (B) Percentage of rhythmic animals during 21 days in DD of wild-type (WT), Dec1 /- , Dec2 / (grey line; per genotype n = 12), Per2m/m (red line; n = 9), Per2m/mDec1 / (blue line; n = 12), Per2m/mDec2 / (green line; n = 11) and Per2m/mDec1/2 / mice (black line; n = 5).
81x182mm (600 x 600 DPI)



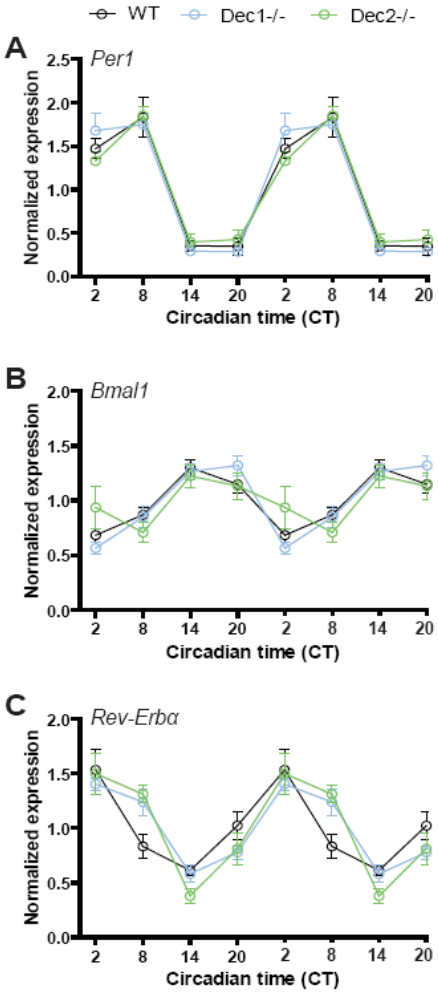
Bode et al. Figure 3

In situ hybridization (ISH) profiles of clock genes in the SCN. Normalized mRNA expression levels of (A) Per1, (B) Bmal1 and (C) Rev-Erbα in the SCN under constant darkness conditions of wild-type (WT; black line), Per2m/m (red line), Per2m/mDec1 / (blue line) and Per2m/mDec2 / (green line) mice (n = 3). Values are double plotted and represented as mean ± SEM.
107x211mm (600 x 600 DPI)

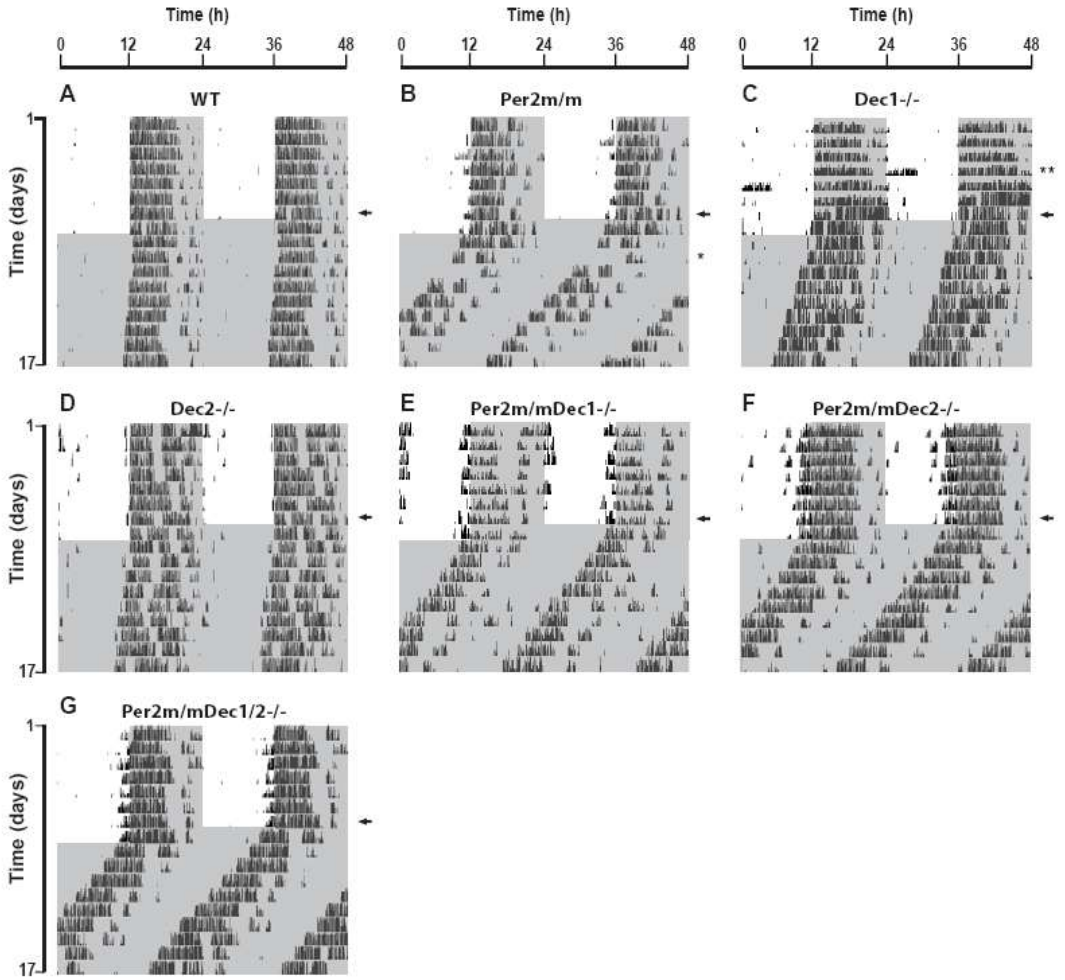


Bode et al. Figure 4

Phase delay and masking responses to nocturnal light exposure. (A) Quantification of phase-delay responses after a 15 min light pulse at ZT14: WT 1.26±0.08 h (n = 8), Per2m/m 0.50±0.08 h (n = 8), Dec1 / 0.86±0.08 h (n = 12), Dec2 / 1.26±0.09 h (n = 11), Per2m/mDec1 / 0.92±0.06 h (n = 11), Per2m/mDec2 / 1.83±0.08 h (n = 11) and Per2m/mDec1/2 / 1.07±0.03 h (n = 5). (B) Relative light phase activity during light exposure (100 lux) at ZT14-ZT15: WT 0.22±0.03 (n = 12), Per2m/m 0.28±0.02 (n = 10), Dec1 / 0.34±0.02 (n = 6), Dec2 / 0.19±0.03 (n = 6), Per2m/mDec1 / 0.49±0.04 (n = 12), Per2m/mDec2 / 0.58±0.05 (n = 11) and Per2m/mDec1/2 / 0.39±0.05 (n = 5). Data are represented as mean ± SEM; *: $p < 0.05$ compared to WT, #: $p < 0.05$ compared to Per2m/m, §: $p < 0.05$ compared to Per2m/mDec1 / or Per2m/mDec2 / .
76x179mm (600 x 600 DPI)



Bode et al. Suppl. Figure 1



Bode et al. Suppl. Figure 2

Supplemental Table 1: Statistics of behavioral parameters

Tested parameter	Genotype comparison	*Per2m/m*	*Dec1-/-*	*Dec2-/-*	*Per2m/mDec1-/-*	*Per2m/mDec2-/-*	*Per2m/mDec1/2-/-*
Onset in LD	WT	***	ns	ns	***	***	***
	Per2m/m		***	***	***	***	***
	Dec1-/-			ns	***		***
	Dec2-/-					***	***
	Per2m/mDec1-/-					ns	ns
	Per2m/mDec2-/-						ns
Onset variation in LD	WT	***	ns	ns	***	***	***
	Per2m/m		***	***	*	**	***
	Dec1-/-			ns	***		***
	Dec2-/-					***	***
	Per2m/mDec1-/-					ns	ns
	Per2m/mDec2-/-						ns
Period length in DD	WT	***	***	ns	***	***	***
	Per2m/m		***	***	***	**	ns
	Dec1-/-			***	***		***
	Dec2-/-					***	***
	Per2m/mDec1-/-					ns	*
	Per2m/mDec2-/-						ns
Photic resetting at ZT14	WT	***	*	ns	ns	***	ns
	Per2m/m		ns	***	*	***	**
	Dec1-/-			*	ns		ns
	Dec2-/-					***	ns
	Per2m/mDec1-/-					***	ns
	Per2m/mDec2-/-						***
Masking	WT	ns	ns	ns	***	***	ns
	Per2m/m		ns	ns	**	***	ns
	Dec1-/-			ns	ns		ns
	Dec2-/-					***	ns
	Per2m/mDec1-/-					ns	ns
	Per2m/mDec2-/-						ns

ns = not significant * $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.001$

3.3. Additional data

3.3.1. Phase delay responses and photic light suppression in *Per1/Dec* mutant mice

Advanced activity onsets of *Per2/Dec* mutant mice in LD were correlated with impaired masking behaviour (see Results 3.2.). *Per1/Dec* mutant mice also showed this pre-dark activity, but to a lesser extent. To test if the observed advanced onsets might stem from either impaired masking or defective phase shifting capacities, we first examined photic phase delays in response to exposure to a 15 min light pulse at ZT15 (Aschoff type II protocol; Figure 15A-H). Single mutant mice showed no significant difference in phase shifting behaviour compared to wild-type animals, but *Per1/Dec* double mutants displayed increased, while *Per1/Dec1/2* triple mutants showed decreased phase shifting responses (Figure 15H and statistics Table 1). These values did not correlate with the pre-dark activity phenotype of the different *Per1/Dec* mutant mice. Nevertheless, a synergistic interaction of *Per1* and *Dec1/2* in the regulation of phase delay resetting was observed.

We analyzed negative masking by applying a 100 lux light pulse for 1 h to stably entrained animals at ZT14-15. As expected, in wild-type mice the wheel-running activity was strongly suppressed during the 1 h light administration (Figure 15I). Single as well double mutants showed reduced activity comparable to that of wild-type controls (Figure 15I and statistics Table 1). In contrast, *Per1/Dec* triple mutant mice totally failed to suppress activity in response to light exposure (Figure 15I and statistics Table 1). Of note, the *Per1/Dec* triple mutant phenotype was not significantly different from that of *Dec1/2* double mutants (data not shown). In summary neither masking nor phase resetting behaviour correlate with pre-dark activity in *Per1/Dec* mutant mice. Functional interactions of *Per1* and *Dec1/2* were observed for phase resetting, but not for masking (Results 3.1.).

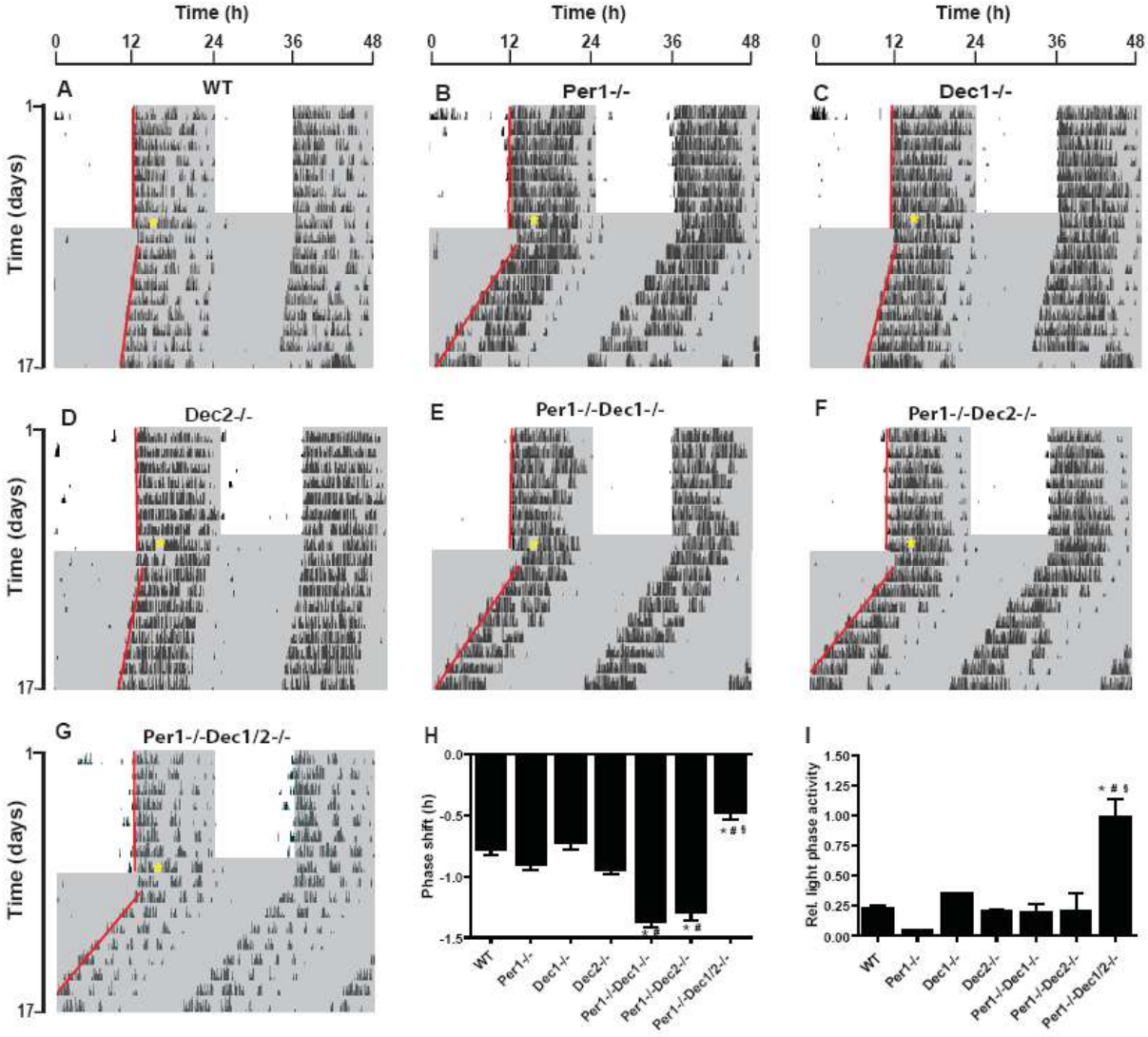


Figure 15. **Photic phase resetting and negative masking.** (A-G) Representative double plotted actograms of phase-delay response to 15 min light pulse treatments at ZT15 (yellow star) of (A) wild-type (WT), (B) *Per1*$^{-/-}$, (C) *Dec1*$^{-/-}$, (D) *Dec2*$^{-/-}$, (E) *Per1*$^{-/-}$*Dec1*$^{-/-}$, (F) *Per1*$^{-/-}$*Dec2*$^{-/-}$ and (G) *Per1*$^{-/-}$*Dec1/2*$^{-/-}$ mice. Red lines indicate regressions through the onsets of wheel-running activity for calculating activity phase shifts on the day after the light pulse administration. (H) Quantification of phase-delay responses after a light pulse at ZT15: WT -0.76±0.06 h (n = 12), *Per1*$^{-/-}$ -0.89±0.05 h (n = 12), *Dec1*$^{-/-}$ -0.71±0.07 h (n = 9), *Dec2*$^{-/-}$ -0.93±0.05 h (n = 10), *Per1*$^{-/-}$*Dec1*$^{-/-}$ -1.36±0.05 h (n = 10), *Per1*$^{-/-}$*Dec2*$^{-/-}$ -1.28±0.09 h (n = 10), *Per1*$^{-/-}$*Dec1/2*$^{-/-}$ -0.46±0.07 h (n = 11). (I) Relative light phase activity during light exposure (100 lux) at ZT14-ZT15: WT 0.22±0.03 (n = 12), *Per1*$^{-/-}$ 0.04±0.02 (n = 5), *Dec1*$^{-/-}$ 0.34±0.02 (n = 6), *Dec2*$^{-/-}$ 0.19±0.03 (n = 6), *Per1*$^{-/-}$*Dec1*$^{-/-}$ 0.18±0.09 (n = 9), *Per1*$^{-/-}$*Dec2*$^{-/-}$ 0.19±0.17 (n = 6) and *Per1*$^{-/-}$*Dec1/2*$^{-/-}$ 0.97±0.16 (n = 12). Data are represented as mean ± SEM; *: $p < 0.05$ compared to WT, $^{\#}$: $p < 0.05$ compared to *Per1*$^{-/-}$, §: $p < 0.05$ compared to *Per1*$^{-/-}$*Dec1*$^{-/-}$ or *Per1*$^{-/-}$*Dec2*$^{-/-}$.

Table 1: Statistics of behavioral tests

Tested parameter	Genotype comparison → / ↓	Per1-/-	Dec1-/-	Dec2-/-	Per1-/-Dec1-/-	Per1-/-Dec2-/-	Per1-/-Dec1/2-/-
Phase delay (ZT15)	**WT**	ns	ns	ns	***	***	*
	Per1-/-		ns	ns	***	**	***
	Dec1-/-			ns	***		ns
	Dec2-/-					*	***
	Per1-/-Dec1-/-					ns	***
	Per1-/-Dec2-/-						***
Masking	**WT**	ns	ns	ns	ns	ns	***
	Per1-/-		ns	ns	ns	ns	***
	Dec1-/-			ns	ns		**
	Dec2-/-					ns	***
	Per1-/-Dec1-/-					ns	***
	Per1-/-Dec2-/-						***

ns = not significant; * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001

3.3.2. Locomotor activity under constant light in *Per2/Dec* mutant mice

Concluding the study on *Per2-Dec* interaction, we examined activity period length in LL (50 lux light intensity) in *Per2/Dec* single, double and triple mutants. Entrained mice were released into LL and period length was determined by χ^2 periodogram analysis over an interval of 7-10 consecutive days using ClockLab software. Following Aschoff's rule, period lengths were increased in LL compared to DD (Aschoff 1960; Aschoff 1979). The LL period of *Per2* mutant mice was significantly shorter than that of wild-type animals. *Per2/Dec* double mutant mice did not show any significant period differences when compared to *Per2* single mutants (Figure 16 and statistics Table 2) indicating that no functional interaction of *Per2* and *Dec1* or *Dec2* was seen in LL. In contrast, *Per2/Dec* triple mutants showed a partial rescue of period length in LL compared to *Per2* single or *Per2/De*c double mutants (Figure 16 and statistics Table 2).

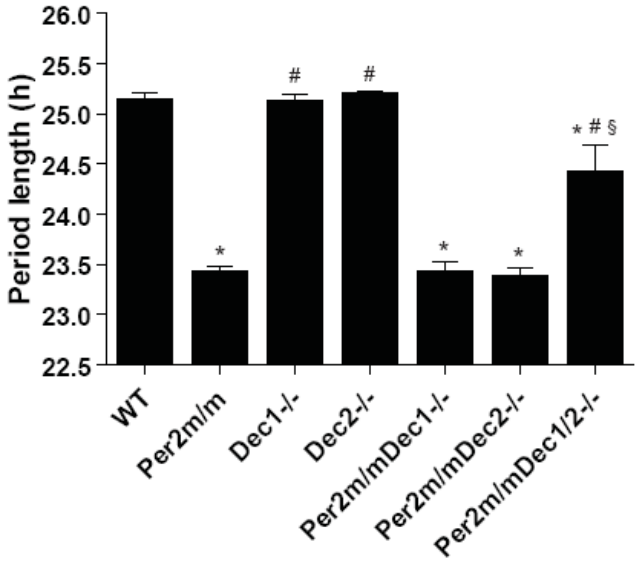


Figure 16. Locomotor activity in LL. Average free-running period length into constant light ($LL_{50\ lux}$): WT 25.12±0.08 h (n = 12), *Per2*$^{m/m}$ 23.40±0.09 h (n = 14), *Dec1*$^{-/-}$ 25.10±0.09 h (n = 11), *Dec2*$^{-/-}$ 25.18±0.06 h (n = 12), *Per2*$^{m/m}$*Dec1*$^{-/-}$ 23.41±0.12 h (n = 5), *Per2*$^{m/m}$*Dec2*$^{-/-}$ 23.36±0.10 h (n = 5), *Per2*$^{m/m}$*Dec1/2*$^{-/-}$ 24.41±0.29 h (n = 2). Data are represented as mean ± SEM. *: $p < 0.05$ compared to WT, $^{\#}$: $p < 0.05$ compared to *Per2*$^{m/m}$, §: $p < 0.05$ compared to *Per2*$^{m/m}$*Dec1*$^{-/-}$ or *Per2*$^{m/m}$*Dec2*$^{-/-}$.

Table 2: Statistics of for LL period

Paradigm	Genotype comparison	*Per2m/m*	*Dec1-/-*	*Dec2-/-*	*Per2m/m Dec1-/-*	*Per2m/m Dec2-/-*	*Per2m/m Dec1/2-/-*
Period length in LL	**WT**	***	ns	ns	***	***	*
	Per2m/m		***	***	ns	ns	***
	Dec1-/-			ns	***		*
	Dec2-/-					***	*
	Per2m/mDec1-/-					ns	**
	Per2m/mDec2-/-						**

ns = not significant; * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001

Of note, all behavioural experiments (from Results 3.1., 3.2., 3.3.1 and 3.3.2.) were also performed on *Dec1/2* double mutants to test the possibility that the observed *Per/Dec* triple mutant phenotype merely reflects the absence of both DECs. In each of the tests, triple mutants showed significant differences when compared to *Dec1/2* animals (data not shown), except for the *Per2Dec1/2* triple mutant masking phenotype. Phase delay shifting of triple mutants showed a trend towards a significant difference when compared to *Dec1/2* mutant mice.

Table 3 summarizes the findings from 3.1., 3.2., 3.3.1 and 3.3.2. with regard to *Per-Dec* interaction.

Table 3: Functional genetic interaction of *Per1* or *Per2* with *Dec1* and/or *Dec2*.

	Per1			***Per2***		
	Dec1	***Dec2***	***Dec1/2***	***Dec1***	***Dec2***	***Dec1/2***
Onset in LD	+	n.i.	+	+	+	+
Onset variation in LD	+	n.i.	+	+	+	+
Period length in DD	n.i.	n.i.	+	-	-	n.i.
Period length in LL	n.i.	n.i.	+	n.i.	n.i.	-
Photic resetting	+	+	n.i.	n.i.	-	n.i.
Masking	n.i.	n.i.	n.i.	+	+	n.i.

+: synergistic interaction; -: antagonistic interaction; n.i.: no genetic interaction

3.3.3. Photic phase delays in *Per* single mutant mice

The endogenous clock must be synchronized each day to stay in synchrony with the external light/dark cycle. This entrainment is based of daily resetting of the SCN clock by light. This photic phase resetting is hypothesized to depend on acute photic *Per1* and *Per2* induction in the SCN after light exposure during the night (see Introduction 1.4.). To understand the role of *Per* gene induction in clock resetting, we investigated phase delay resetting on the behavioural and molecular levels in *Per1* and *Per2* single mutants as well as in *Dec1/2* double mutant mice using an Aschoff type II protocol. As expected, *Per2* as well as *Dec1/2* mutant mice showed decreased, and *Per1* mutants normal phase shifting behaviour compared to wild-type mice (Figure 17 and statistics Table 4). This supports that PER2 and DECs have an essential function in phase delay resetting (Albrecht, Zheng et al. 2001; Rossner, Oster et al. 2008).

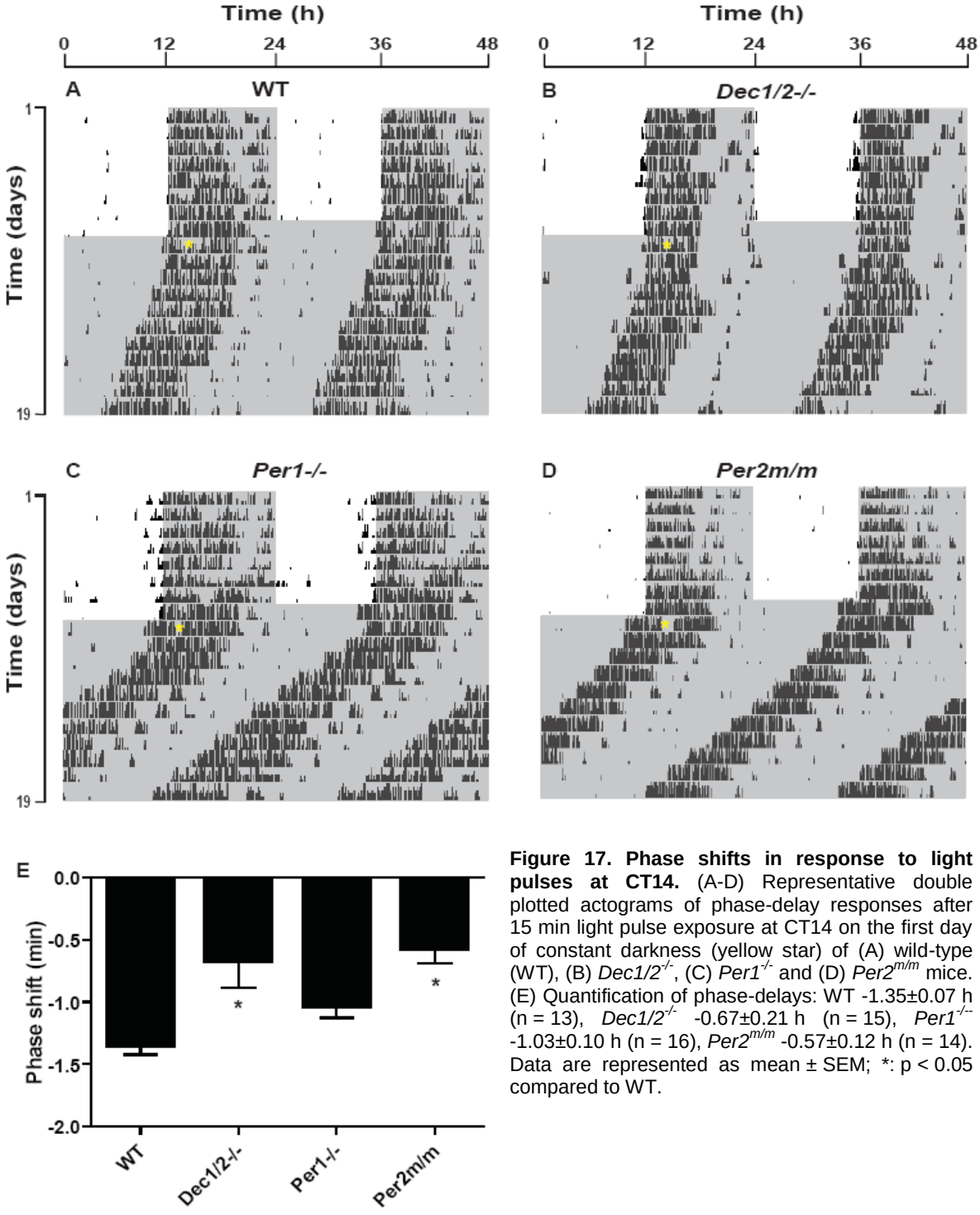


Figure 17. Phase shifts in response to light pulses at CT14. (A-D) Representative double plotted actograms of phase-delay responses after 15 min light pulse exposure at CT14 on the first day of constant darkness (yellow star) of (A) wild-type (WT), (B) *Dec1/2*$^{-/-}$, (C) *Per1*$^{-/-}$ and (D) *Per2*$^{m/m}$ mice. (E) Quantification of phase-delays: WT -1.35±0.07 h (n = 13), *Dec1/2*$^{-/-}$ -0.67±0.21 h (n = 15), *Per1*$^{-/--}$ -1.03±0.10 h (n = 16), *Per2*$^{m/m}$ -0.57±0.12 h (n = 14). Data are represented as mean ± SEM; *: $p < 0.05$ compared to WT.

Table 4: Statistics of phase delay resetting

Tested parameter	Genotype comparison → / ↓	Dec1/2-/-	Per1-/-	Per2-/-
Phase delay (CT14)	**WT**	*	ns	**
	Dec1/2-/-		ns	ns
	Per1-/-			ns

ns = not significant; * p ≤ 0.05 ;** p ≤ 0.01; *** p ≤ 0.001

To compare behavioural and molecular light responses, photic *Per1* and *Per2* induction was measured in the SCN after a 15 min light pulse administration at CT14 on the first day in DD using *in situ* hybridization. *Per* mRNA levels in the SCN were quantified at 15 min, 30 min, 60 min, 120 min and 240 min after the beginning of the light treatment (Figure 18). As expected, in wild-type mice light pulses at CT14 induced *Per1* as well *Per2* expression in the SCN peaking 60 min and 120 min, respectively, after the beginning of the light exposure (Figure 18 and Table 5) (Shigeyoshi, Taguchi et al. 1997; Yan and Silver 2002). Given that the *Per* mutants have non-functional PER1 or PER2 proteins, we did not investigate *Per* induction in the corresponding mutants. *Per1* induction was reduced in *Per2* mutant mice compared to wild-type mice (Figure 18; Table 5). We further investigated the *Per* induction in *Dec1/2* mutants which show reduced behavioural resetting similar to that seen in *Per2* mutant mutants (Figure 17E and statistics Table 4) (Rossner, Oster et al. 2008). In contrast to *Per2* mutant mice, *Dec1/2* mutant animals showed increased *Per1* induction compared to wild-type animals (Figure 18 and Table 5). Additionally, *Dec* deficiency had an influence on *Per2* induction as *Per2* peak expression was advanced by 90 min and overall induction was decreased in the *Dec1/2* mutants (Figure 18 and Table 5). *Per1* mutants showed increased *Per2* mRNA levels at the peak time compared to wild-type animals (Figure 18 and Table 5).

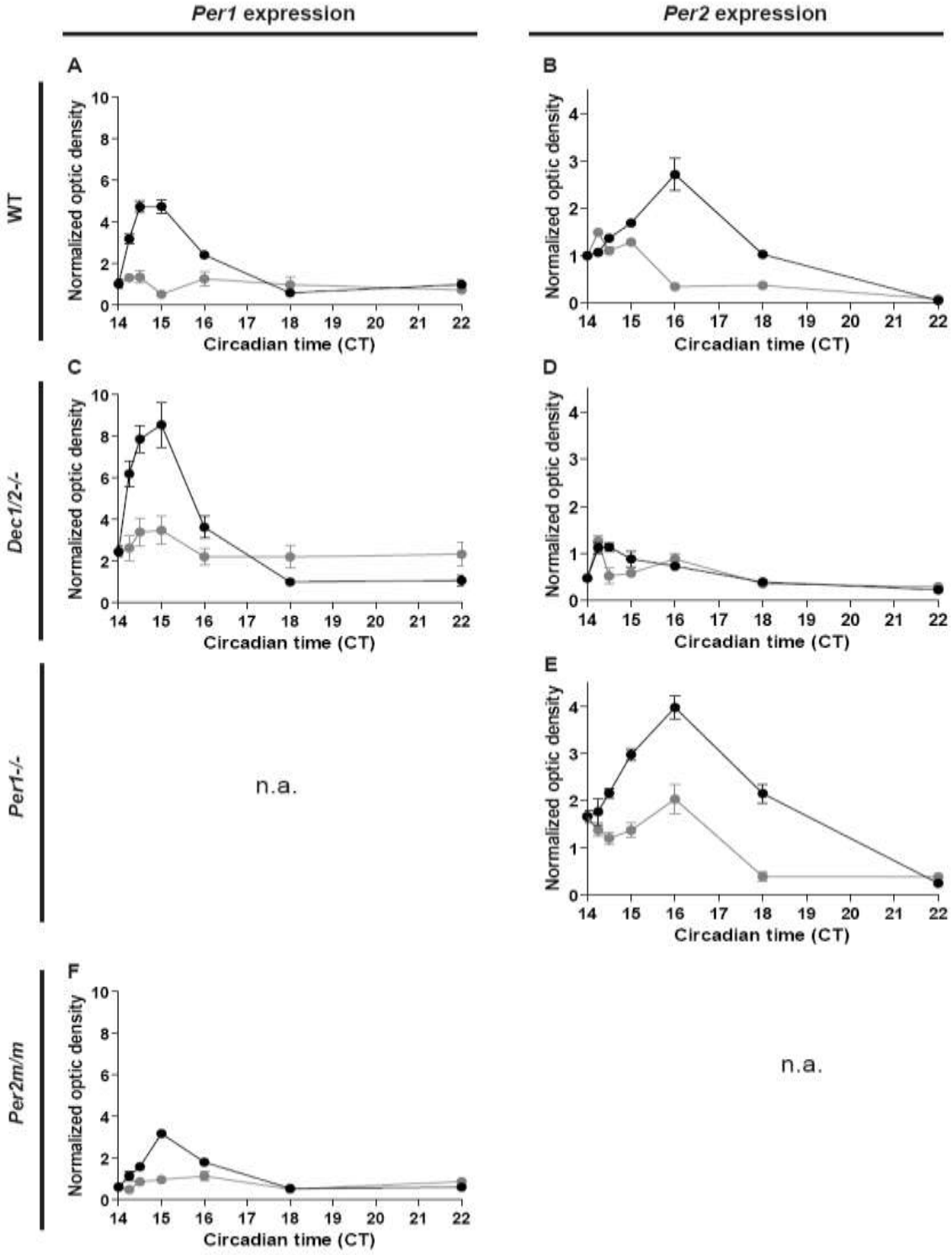


Figure 18. *In situ* hybridization (ISH) of photic *Per* gene inductions in the SCN. Normalized mRNA expression levels of *Per1* (left column; A, C and F) and *Per2* (right column; B, D and E) in the SCN under constant darkness (grey line) or with a 15 min light exposure at CT14 on the first day in DD (black line) in wild-type (WT; upper line), *Dec1/2*$^{-/-}$ (second line), Per1$^{-/-}$ (third line) and *Per2*$^{m/m}$ (lower line) mice (n = 3). Values are represented as mean ± SEM. n.a.: not analyzed

Table 5: Photic *Per* induction in the SCN after a phase delay light pulse

	photic *Per1* induction		photic *Per2* induction	
	peak after light pulse	**peak expression**	**peak after light pulse**	**peak expression**
WT	60 min	4.72±0.33	120 min	2.71±0.33
Dec1/2-/-	60 min	8.56±1.08 * #	30 min	1.14±0.09 * #
Per1-/-	n.a.	n.a.	120 min	3.97±0.23 *
Per2m/m	60 min	1.81±0.14 *	n.a.	n.a.

n.a. = not analyzed; * significant to wild-type # significant to *Per* mutant

4. Chapter 4: Discussion and Perspective

The molecular components of the *Drosophila* and the mammalian TTLs are largely conserved and their functions are very similar (Bell-Pedersen, Cassone et al. 2005; Looby and Loudon 2005). However, gene duplications resulted in multiple copies of some fly clock genes in mammals (e.g. *Per1-3*, *Dec1-2*). The two mammalian *Dec* genes have one ortholog in the *Drosophila* circadian clock – *clockwork orange* (*cwo*) – with similar transcriptional regulatory functions. For the fruit fly, it was postulated that PER and CWO have a synergistic function in the circadian timekeeping system (Kadener, Stoleru et al. 2007). Hence, we hypothesised a synergism of action for *Per(1,2)-Dec(1,2)* in the regulation of the mammalian circadian clock. To test this hypothesis, we analysed circadian behaviour and SCN clock gene expression rhythms of homozygous *Per1/Dec* and *Per2/Dec* double and triple mutant mice. *Per1-Dec* and *Per2-Dec* interactions are discussed separately in the attached manuscripts (Results 3.1. and 3.2.). Therefore, in this chapter I will focus on combining these findings.

4.1. Synergistic *Per1-Dec* and *Per2-Dec* interaction in photic entrainment

The pre-dark activities of *Per(1,2)/Dec* double and triple mutants show a *Dec* gene dependent increase compared to the corresponding *Per(1,2)* single mutant phenotype (*Per1/Dec1* 1.9 fold, *Per2/Dec1* 1.9 fold; *Per2/Dec2* 2.0 fold; *Per1/Dec1/2* 2.8 fold, *Per2/Dec1/2* 2.7 fold advance of onset activity to the corresponding *Per* single mutants). These data suggest that the strength of *Per1-Dec* interaction in photic entrainment is similar to that of *Per2* and *Dec1/2*. These interactions appear to be necessary for efficient synchronization of the activity phase to the external photic environment, and are synergistic (Results 3.1. and 3.2.). Whether they are based on physical association of the corresponding proteins, or rather reflect a functional genetic interaction, e.g. via co-regulatory effects on target gene activation, remains to be shown. Nevertheless, combined *Per-Dec* deficiency results not only in changes on the transcription level of clock genes in constant darkness (Results 3.1., 3.2.), but perhaps already in LD. Thus, analysis of clock gene expression under entrained conditions might provide evidence for

clock involvement in the pre-dark phenotype, especially during the second half of the light phase. In line with this, it was suggested for the pre-dark phenotype of *Per2* single mutants (Results 3.2.) that advanced *Bmal1* expression in the SCN might underlie the observed phase advance in activity in LD (Oster, Yasui et al. 2002). We show similarly advanced *Bmal1* expression in *Per2* and *Per2/Dec* double mutants in DD (Results 3.2.), supporting the view that an advanced *Bmal1* expression rhythm might cause pre-dark activity in the double and triple mutants.

For the *Per2-Dec1* and *Per2-Dec2* interactions correlations between onset activity and masking during the early dark phase (Figure 15I, Table 3) were shown. In contrast, the pre-dark activity of *Per1/Dec* mutants does not correlate neither to negative masking nor to phase delay phenotypes (Figure 15). Another possible reason might be disrupted sleep behaviour, as discussed in Results 3.1. Therefore, sleep behaviour in these double and triple mutant mice remains to be investigated.

4.2. Compensation effects cause partial rescue in free-run

Under photic entrainment conditions *Per-Dec* interactions are highly synergistic. In contrast, in the absence of the *Zeitgeber* light (i.e. DD or LL) *Per-Dec* interaction properties change (Table 3). The *Per1-Dec* interactions stay synergistic with functional *Dec* redundancy in free-run. The *Per2-Dec* interactions, however, change to antagonistic functionality and a partial rescue of the *Per2* phenotype occurs (period length, rhythmicity and clock gene expression in the SCN) in DD (Results 3.2.), suggesting a conserved *Dec* redundancy and postulating partial compensation by the corresponding PER1/DEC(1 or 2) protein complexes (Results 3.2.). Similar mechanisms were postulated by Oster et al. for *Per/Cry* interactions (Oster, Baeriswyl et al. 2003). Supporting this view, it was previously shown that the remaining *Dec* can compensate for deficiency of the corresponding other *Dec* gene *in vitro* and *in vivo* by increased expression levels (Grechez-Cassiau, Panda et al. 2004; Nakashima, Kawamoto et al. 2008; Liu, Sato et al. 2010). However, such compensation seems not to be sufficient to rescue the *Per1* phenotype under constant conditions (Results 3.1.). We suggest a model in which PER/DEC compensation shares some similarities (Figure 19) with the suggested PER/CRY compensation mechanism (Oster, Baeriswyl et al. 2003). According to this model, during the first days of darkness the compensation effect is strong, because the animals do not loose rhythmicity immediately (Results 3.2.). After several days, the

compensation capacity falls below a hypothetic threshold which is critical for normal clock function (Figure 19) – of note, in contrast to *Per2* and *Per2/Dec1/2* mutants only some *Per2/Dec* double mutants fall below the threshold and, hence, become arrhythmic. Efficient compensation of *Per/Dec* deficiency is restricted to PER1(CRY)/DEC(1 or 2) resulting in modified - but rescued - clock gene expression in the SCN (Results 3.2.) and perhaps also in peripheral tissues. Considering the behavioural phenotype of *Per2/Dec1/2* mutant mice, we would expect a non-rescued clock gene expression similar to that seen in *Per2* mutants, in case that PER1(CRY) would not be sufficient to compensate *Per2/Dec1/*2 deficiency *in vivo*.

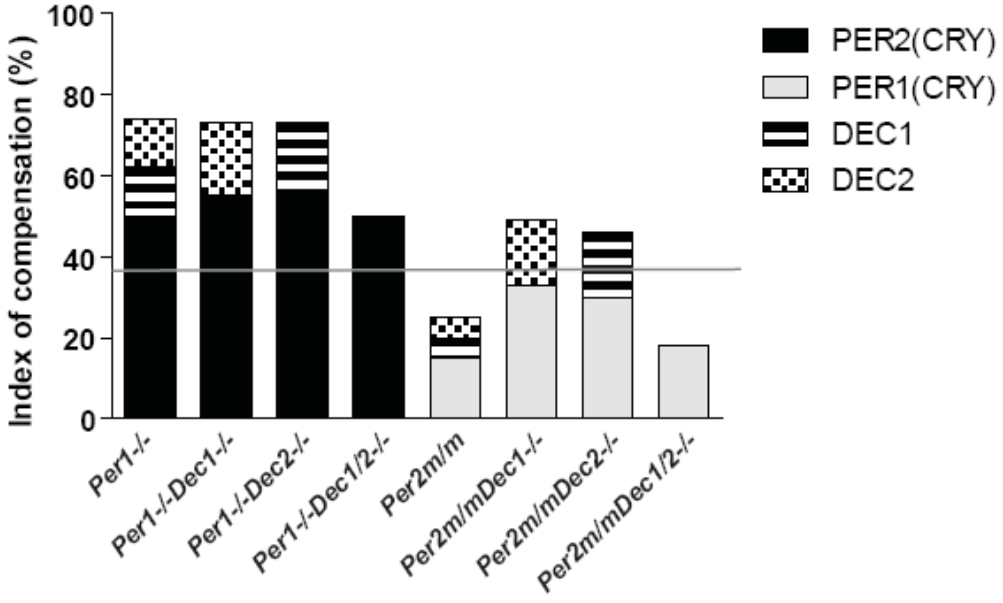


Figure 19. Compensation model. The model illustrates the compensation of *Per/Dec* deficiency with the indicated proteins after several days in DD. Black shows PER2(CRY), grey the PER1(CRY), black-white striped the DEC1 and black dotted the DEC2 proteins which mediate the corresponding compensation. The grey line represents the threshold for sufficient compensation to drive the rhythm of the circadian clock. The compensation effect for the *Per1/Dec* deficiency is not sufficient to increase the published *Per1* deficiency compensation. In contrast, the compensation of *Per2/Dec(1or2)* deficiency is sufficient by increasing the compensation effect compared to *Per2* deficiency accompanied with exceed of the threshold for sufficient clock function, but PER1(CRY) compensation of the *Per2/Dec1/2* deficiency falls below the threshold.

Of note, *Per2/Dec1/2* triple mutants show a partial rescue of period length in LL (Figure 16) similar to the *Per2/Dec* double mutant phenotype in DD (Results 3.2.). The restoration of period length in LL is most likely based on functional *Dec* redundancy. It would be interesting to test if the observed rescue in LL is also present at the molecular level, as it was shown for DD (Results 3.2.). Taken together, synergistic *Per1-Dec* interactions are present during photic entrainment and free-run, together with *Dec*

redundancy. In contrast, *Per2-Dec* synergism is restricted to photic entrainment and becomes antagonistic in free-run. This might be based on a disrupted compensation effect of PER1(CRY)/DEC (Figure 19). The stability of the clock depends on *Per1* and *Per2* function (Results 3.1., 3.2.) (Zheng, Larkin et al. 1999; Zheng, Albrecht et al. 2001) and is modified by the redundant *Dec*s based on their interaction with the *Per*s.

4.3. Bimodular transcriptional function of DEC in the mammalian pacemaker

On the molecular level, we observed DEC redundancy and interactivity with PER in the SCN. We cannot exclude the possibility that the DECs also have regulatory functions independent of the PERs on clock gene expression, e.g. in the periphery. For example, in a cell-based luciferase assay, DECs alone activate *Bmal1* expression, but PER1 potentiates their function as activators. We postulate a daytime dependent and bidirectional (activator and repressor) regulatory function of *Per1-Dec* interaction on E-box containing clock genes (see model in Figure 20), and a role as activators (direct or indirect) on *Bmal1* transcription in the circadian pacemaker (Results 3.1.). The model in Figure 20 illustrates the daytime dependent bimodular regulatory functionality of *Per1-Dec* interaction on E-box containing genes during entrained conditions in the SCN. During the day, the CLOCK/BMAL1 complex activates the transcription by binding to E-box containing promoters (Hogenesch, Gu et al. 1998; Hamilton and Kay 2008). The observed inhibitory effect of PER1/DEC1/2 on *Rev-Erbα* transcription might result from direct interaction with the CLOCK/BMAL1 complex. At the beginning of the night, the DECs might activate the expression of *Rev-Erbα* and *Per2* by direct binding to the E-box elements of their promoters. Limitations for this model are that PER1/2 as well as DEC1/2 proteins are rhythmically present in the SCN with a decreased level during the night and an increase in the light phase. Testing protein-protein interaction studies of PER1/DEC1/2 to BMAL1 (supporting the day function) and analyzing the protein interaction abilities on the promoter regions of *Rev-Erbα* and *Per2* by ChIP-Seq (supporting the night function) during the day might clarify this issue.

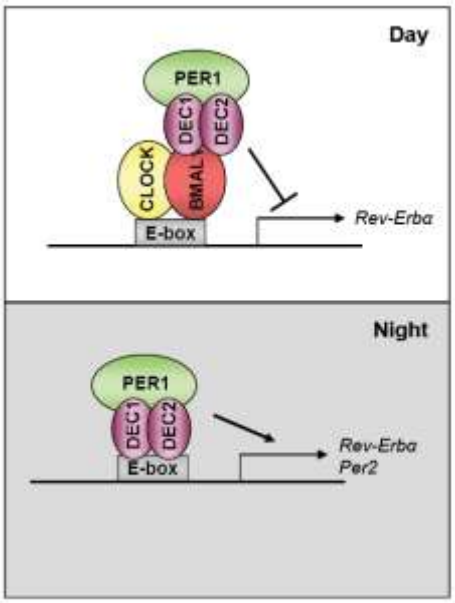


Figure 20. Daytime dependent bimodular PER1DEC1/2 function in the SCN. During the light phase (Day; upper part) the synergistic PER1 and DEC1/2 complexes inhibit CLOCK/BMAL1-mediated transcription of E-box regulated genes such as *Rev-Erbα* by direct interaction with CLOCK/BMAL1. During dark phase (Night; lower part) CLOCK/BMAL1 complexes are not present in the dark phase. Thus, PER1/DEC1/2 functions might be modified by the ability to direct binding to E-box elements resulting in activation of transcription of targets such as *Rev-Erbα* and *Per2*.

Of note, for our model we assume that PER and DEC proteins physically interact, but this has yet to be proven. Further, we cannot exclude that the functionality of the *Per-Dec* interaction in the periphery might be different to the SCN (Figure 20). Overall, the molecular changes on clock gene expression in the SCN are represented in the behavioural phenotypes under constant darkness (Results 3.1., 3.2.).

4.4. *Per2* expression is essential for phase delay resetting

The synchronization of the endogenous clock of the animal to the environmental light/dark cycle is based on a daily shift of the TTL in the SCN (see Introduction 1.4.). It was shown that the expression levels of *Per1* and *Per2* are induced in the SCN after a light pulse during the night (Shigeyoshi, Taguchi et al. 1997; Yan, Takekida et al. 1999; Yan and Silver 2002), and *Per1* and *Per2* single mutants display impaired phase shifting behaviour for phase advance and delay responses, respectively (Albrecht, Zheng et al. 2001). We focused on the phase delay response to determine the role of *Per* induction in photic resetting. The reduced behavioural resetting responses of *Per2* and *Dec1/2* mutant mice support the hypothesis that *Per2* and *Dec*s are involved in phase delay resetting (Figure 17) (Albrecht, Zheng et al. 2001; Rossner, Oster et al. 2008). Considering the reduced resetting behaviour of *Dec1/2* double mutants and the blunted *Per2* induction - but increased *Per1* induction - in the SCN of these animals, our data suggest that the

Per2 induction was too weak to mediate an efficient phase shift in the TTL. This proposes an essential *Per2*/PER2 function in phase delay resetting on the molecular and behavioural levels. Therefore, we suggest a model in which photic *Per2* induction efficiently delays the circadian system by exceeding a *Per2* mRNA threshold (Figure 21). In wild-type mice, sufficient additional PER2 protein is produced to extend the inhibition of the CLOCK/BMAL1 complex. This prolonged repression results in a delay of the molecular oscillator, resulting in the observed behavioural phase delay response on the next day.

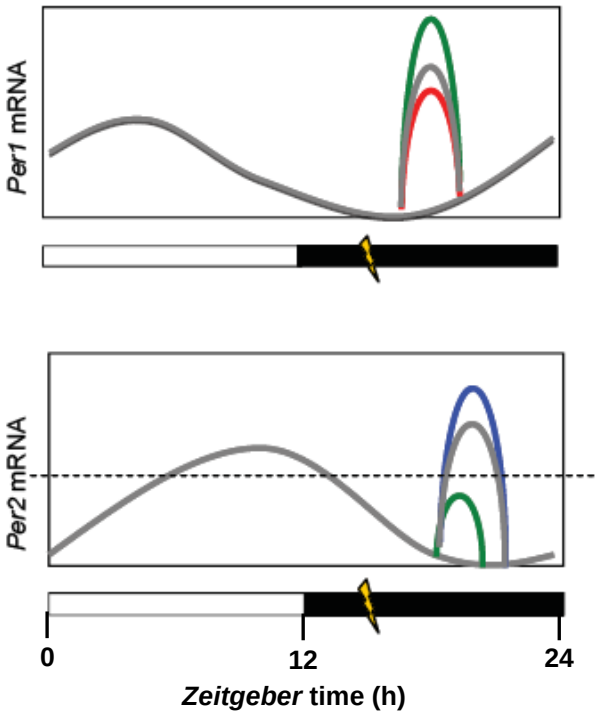


Figure 21. Model of photic *Per* induction in the SCN during phase delay resetting. The model shows the mRNA level of *Per1* (upper panel) and *Per2* (lower panel) after a light exposure in the first half of the night of wild-type (grey), *Dec1/2*$^{-/-}$ (green), *Per1*$^{-/-}$ (blue) and *Per2*$^{m/m}$ (red) mice. The white and black bars indicate the day and night, respectively, and the yellow flash the light pulse. The dashed line indicates the threshold of *Per2* induction sufficient to mediate a phase delay shift.

4.5. Potentiated PER1/DEC1 compensation elevates *Per1* expression efficiently for phase delay resetting

For phase delay responses we observed an antagonistic *Per2-Dec2* interaction, but no functional interaction between *Per2* and *Dec1* (Results 3.2.; Table 3). The strong phase delay response of *Per2/Dec2* mutant mice (Results 3.2.) might be explained by a similar PER1/DEC1 compensation mechanism as postulated for free-run. Of note, both of these genes are light inducible (Shigeyoshi, Taguchi et al. 1997; Yan, Takekida et al. 1999; Honma, Kawamoto et al. 2002; Yan and Silver 2002), which might potentiate the

compensation effect resulting in a strong phase delay response. This hypothesis could be tested by molecular analysis of *Per1* and *Dec1* expression following light exposure. If confirmed, it would suggest that photic *Per1* induction is potentiated by this compensation effect and is able to efficiently mediate a phase delay shift (Figure 22) by extended repression of the CLOCK/BMAL1 complex. According to this hypothesis, *Per2/Dec1* and *Per2/Dec1/2* mutants, which show weak phase delay responses (Results 3.2.), are not able to produce enough *Per1* mRNA following light exposure because the light-inducible *Dec1* gene is absent in these mutants.

Reduced phase delay resetting in *Per1/Dec1/2* mutants (Figure 15H) might reflect the reduced phase delay response already observed in *Dec1/2* mutants (data not shown) (Rossner, Oster et al. 2008). Alternatively, it may be caused by grossly impaired overall light reactivity (97% activity) as measured in negative masking (Figure 15I). In contrast, impaired light sensitivity in *Per2/Dec* mutants appears not to be strong enough to significantly influence resetting behaviour, suggesting that sufficient phase shifting requires a defined light input (intensity and/or duration), which is not reached in *Per1/Dec1/2*, but is reached in *Per2/Dec* mutants. Photic *Per2* induction in *Per1/Dec* double mutants might be stronger than in the *Per1* single mutants (Figure 21), causing the observed stronger phase delay effect (Figure 15H) and postulating that *Per1-Dec1* and *Per1-Dec2* synergisms are involved in the regulation of *Per2* induction by light. To test this hypothesis, photic *Per2* induction should be determined in the SCN. Figure 22 shows the suggested photic *Per* induction of these mutants.

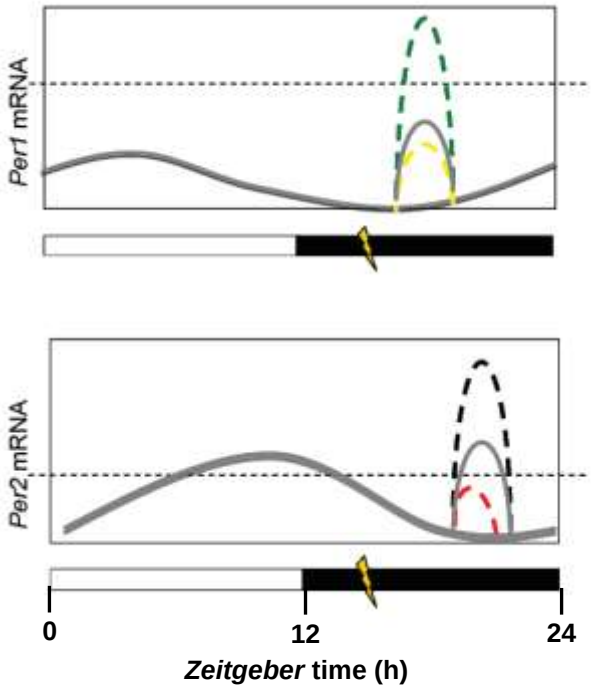


Figure 22. Model of photic *Per* induction in the SCN during phase delay resetting of the *Per/Dec* mutants. The model shows the hypothetical mRNA level (dashed line) of *Per1* (upper panel) and *Per2* (lower panel) induction after a light exposure in the first half of the night for wild-type (grey), $Per2^{m/m}Dec2^{-/-}$ (green, dashed), $Per2^{m/m}Dec1^{-/-}$, $Per2^{m/m}Dec1/2^{-/-}$ (yellow dashed), $Per1^{-/-}Dec1^{-/-}$, $Per1^{-/-}Dec2^{-/-}$ (black dashed) and $Per1^{-/-}Dec1/2^{-/-}$ (red dashed) mice. The white and black bars indicate day and night, respectively, and the yellow flashes represent the light pulse. The horizontal dashed line indicates the threshold of *Per* induction sufficient to mediate a phase delay shift.

In summary, phase delay responses are primarily based on *Per2* induction. It seems that the DECs, as well synergistic *Per1-Dec* interactions, are involved in regulating photic *Per2* induction in the SCN. Of note, if *Per1* induction is extended, e.g. by photic potentiation of a compensation effect as in *Per2/Dec2* mutants, *Per1*/PER1 levels become sufficient to mediate a phase delay shift. Overall, higher levels of PER1 are necessary compared to PER2 in order to efficiently delay the TTL. Therefore, PER2 is the rate-limiting factor for phase delays of the clock.

4.6. Conclusion and Perspective

It was postulated that PER and CWO synergize and inhibit CLK-mediated activation in the *Drosophila* TTL (Kadener, Stoleru et al. 2007), but it has also been shown that CWO can activate clock gene expression (Richier, Rodriguez-Lanetty et al. 2008). During evolution, the core molecular components of the circadian oscillator have been largely conserved between diverse organisms such as *Drosophila* and mice (Looby and Loudon 2005). Gene duplication has resulted in multiple copies of clock genes (e.g. *Per1-3*, *Cry1-2*, *Bmal1-2*, *Clock* and *NPAS2*) with functional compensation properties (e.g. PER/CRY compensation (Oster, Baeriswyl et al. 2003), functional redundancy (e.g. Cry (van der Horst, Muijtjens et al. 1999) and diversity of circadian regulatory mechanisms in multiple tissues (e.g. NPAS2 is the ortholog of CLOCK in peripheral tissues). These gene duplications should result in higher robustness and adjustment. Thus, gene duplications of core clock components have increased the fitness of the circadian system during evolution. In this study, we found for the fly's *per-cwo* synergism (Kadener, Stoleru et al. 2007) an analog *Per-Dec* interaction in mammals, indicating a conservation of these mechanisms during evolution. The fact that the *Dec* single and double mutants show mild or no phenotypes and only in the additional absence of *Per,* the role of the *Dec*s become unveiled, suggests that the DECs have fine-tuning roles in the circadian system.

The observed pre-dark activity indicates possible impaired sleep behaviour which might be used for modelling ASPS in mice, and offers the opportunity to investigate physiological interactions between sleep and the circadian system and to test treatments (e.g. melatonin administration) of ASPS to adjust sleep phase to the external environment.

In addition, the *Dec*s are functionally redundant in the circadian system *in vivo* – and we are the first group to show that the DECs together with PER1 positively regulate *Bmal1* transcription in the SCN. Furthermore, *Per1-Dec* interaction is time-dependent and bimodular with respect to its influence on E-box containing clock gene transcription (Figure 20), suggesting that other components of the TTL might also show bimodular functionality in the mammalian TTL in the SCN and in peripheral tissues. Further characterization of the *Per-Dec* interaction will provide new insights into the entrainment and stabilizing mechanism of the mammalian circadian system *in vivo*.

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Göttingen, 20.April 2011

Curriculum Vitae

Brid Bode

Geboren am 25. September 1980 in Wolfenbüttel, Deutschland

Gartenstraße 3, 37073 Göttingen
E-Mail brid.bode@mpibpc.de

Beruflicher Werdegang

seit 2007	Wissenschaftliche Mitarbeiterin am Max-Planck-Institut für biophysikalische Chemie, Göttingen, Deutschland, Abtl. Gene und Verhalten, Forschungsgruppe Zirkadiane Rhythmen Doktorarbeit: ‚Genetic interaction of *Per*- and *Dec*-genes in the mammalian circadian clock’
seit 2007	Doktorandenprogramm an der Göttinger Graduiertenschule für Neurowissenschaften und Molekulare Biowissenschaften (GGNB): Zentrum für Neurobiologie des Verhaltens (CSN)
2001- 2007	Studium der Biologie an der Georg-August Universität Göttingen, Deutschland; Schwerpunkte Entwicklungsbiologie, Mikrobiologie und Immunologie; Abschluss mit Diplom 05/2007
1993 - 2001	Gymnasium im Schloss Wolfenbüttel, Abitur 2001