Interaction of *Per* and *Cry* Genes in the Mammalian Circadian Clock

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Der Leiter der Doktorarbeit:    Der Dekan:

[Signature]

Prof. Urs Albrecht    Prof. Dionys Baeriswyl
“The mind is its own place, and in it self
Can make a Heav'n of Hell, a Hell of Heav'n.“

(John Milton, „Paradise Lost“)

Meiner Mutter und meinem Vater.
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Summary

To cope with the daily changes in their environment, in organisms, from cyanobacteria to humans, endogenous clocks have evolved that anticipate these changes and synchronize physiology and behavior accordingly. In mammals, the central circadian pacemaker is located in the suprachiasmatic nuclei (SCN) of the hypothalamus. From there subordinated cellular clocks in the peripheral organs are controlled to create the overall rhythm of the organism.

Circadian rhythms persist in the absence of external time information with near 24h periods, hence the term “circadian” from the Latin *circa dies* meaning “about one day” (Halberg, 1952). Time signals from the environment (so called Zeitgeber) like light, temperature variations, or food intake can phase shift the endogenous oscillator to synchronize the organism to geophysical time.

On the molecular level circadian clocks are built of cellular oscillators. Pacemaker genes like *Clock, Bmal1, mPer1* and *2, mCry1* and *2 or Casein kinase 1ε* are organized in a system of transcriptional/ translational feedback loops creating precise, stabilized 24h rhythms.

In this study we worked with two different model systems. We used transgenic mice (*Mus musculus*) with a targeted disruption of specific genes (*mPer1* and *2, mCry1* and *2*) or a combination of these genes to elucidate their function in the circadian clockwork. In a second project we examined the clock of the blind mole rat superspecies (*Spalax ehrenbergi*). Its isolated subterranean habitat, its adaptive visual and neuronal reorganization, and its polymorphic activity profile makes *Spalax* an extremely interesting model organism to study the evolution of the clock under special environmental conditions.

In mice we show that an additional deletion of the *mCry2* gene in *mPer2* mutants or a deletion of *mCry1* in *mPer1* mutants restores wild-type rhythmicity on the behavioral and the molecular level. This indicates that *mCry2* acts as a non-allelic suppressor of *mPer2* and *mCry1* of *mPer1* in the circadian oscillator. Young *mPer1/mCry2* double mutants display a very long free-running period and lose rhythmicity with progressing age while *mPer2/mCry1* mutants have no functional clock at all. This is accompanied by abnormal clock gene and protein regulation in the SCN and the eye.

Based on these findings we developed a model for the interaction of the *mPer* and *mCry* genes in the cellular oscillator providing new insights on how the clock is stabilized and integrates external time information at the molecular level. A multiunit complex of mPER and mCRY proteins forms the negative limb of the autoregulatory feedback loop controlling the periodic activation of clock genes. Specific preferences for homo and heteromeric protein
interactions between all mPER and mCRY proteins form a self-stabilizing oscillator at the transcriptional level. By deleting one or more of its components, the stability of the pacemaker is disturbed and the interaction preferences are altered. If this disturbance exceeds the compensation limits of the clock, the oscillation dampens and the animals become arrhythmic.

In *Spalax* we elucidated the molecular organization of the cellular clockwork by studying gene expression. We show that *Spalax* has a functional circadian clock on the molecular level with pacemaker genes like *Clock*, *Bmal1*, *Per1*, 2 and 3 as well as two *Crys*. The clockwork of *Spalax* is highly similar to other rodents although some properties show specific adaptation to its subterranean habitat.

Our findings suggest that the hypertrophic Harderian gland surrounding the rudimentary and visually blind eye of these animals plays a functional role in the circadian system probably stabilizing the SCN pacemaker during continuous absence of outside time information. *Spalax* can shift from a nocturnal to a diurnal activity pattern. We found that in nocturnal animals the central oscillator is uncoupled from the light driven input pathway indicating a specific control of clock entrainment pathways in both activity types.

Taken together this work provides new insights on redundant as well as distinct functions of the *Per* and *Cry* genes in the molecular mechanism of the circadian clockwork. It demonstrates the conservation of the system of autoregulatory feedback loops to stabilize circadian rhythmicity as well as highlights the evolutionary adaptation of external clock regulation to the ecotope of the organism.
Zusammenfassung

Um sich optimal an die periodischen Veränderungen ihres Lebensraums im Verlauf des Tages anzupassen, besitzen die Lebewesen, von den Purpurbakterien bis zum Menschen, eine innere Uhr, die diese Zyklen antizipiert und Stoffwechsel und Verhalten dazu synchronisiert. In Säugetieren sitzt der zentrale innere Schrittmacher im Nucleus suprachiasmaticus (SCN) des Hypothalamus. Von dort werden untergeordnete Uhren in den Organen des Körpers kontrolliert, die dann den Gesamtrhythmus des Organismus erzeugen. Auch wenn der Körper keine Zeitinformationen mehr von außen erhält, bleiben die zirkadianen Rhythmen stabil mit Periodenlängen von ungefähr 24h, daher der Ausdruck „zirkadian“ von dem lateinischen circa dies, was soviel heißt wie „ungefähr ein Tag“. Hinweise über die Tageszeit, so genannte Zeitgeber, wie Licht, Temperaturveränderungen oder Nahrungsaufnahme können die Phase des endogenen Oszillators verschieben, um so den Organismus ständig mit der geophysikalischen Zeit zu synchronisieren.

Auf molekularer Ebene besteht die zirkadiane Uhr aus einzelnen zellulären Oszillatoren. Uhrengene wie Clock, Bmal1, mPer1 und 2, mCry1 und 2 sowie Caseinkinase ε sind in einem System von Transkriptions-/Translations-Rückkopplungsschleifen organisiert, das einen präzisen und stabilen 24h-Rhythmus generiert.

In der vorliegenden Arbeit haben wir mit zwei unterschiedlichen Modellsystemen gearbeitet. Wir benutzten transgene Mäuse (Mus musculus), in deren Erbgut einzelne oder eine Kombination bestimmter Gene (mPer1 und 2 sowie mCry1 und 2) gezielt zerstört wurden, um deren Funktion im zirkadianen Uhrwerk zu bestimmen. In einem zweiten Projekt untersuchten wir die innere Uhr der Blindmaus (Spalax ehrenbergi). Sein isoliertes unterirdisches Habitat, eine adaptive visuelle und neuronale Reorganisation sowie sein polyphasisches Aktivitätsprofil machen Spalax zu einem äußerst interessanten Modellorganismus zum Studium der Entwicklung innerer Uhren unter besonderen Umweltbedingungen.

An Mäusen zeigen wir, dass eine zusätzliche Deletion des mCry2-Gens in mPer2-mutanten Tieren sowie eine Deletion von mCry1 in mPer1-Mutanten die wildtyp-артige Rhythmisität auf Verhaltens- sowie auf molekularer Ebene wiederherstellt. Dies deutet darauf hin, dass mCry2 als nicht-allelischer Suppressor von mPer2 und mCry1 als nicht-allelischer Suppressor von mPer1 im zirkadianen Oszillator fungiert. Junge mPer1/mCry2-Doppelmutanten zeigen eine extrem lange Periodenlänge unter Freilauflaufbedingungen. Sie verlieren ihre Rhythmisität jedoch mit fortschreitendem Alter. mPer2/mCry1-Mutanten dagegen besitzen von Geburt an
keine funktionierende innere Uhr. Dies geht einher mit veränderter Uhrenge- und –proteinregulation im SCN sowie im Auge.

Auf der Grundlage diese Beobachtungen haben wir ein Modell zur Interaktion der *mPer*- und *mCry*-Gene im zellulären Oszillator entwickelt. Ein multimerer Komplex aus mPER- und mCRY-Proteinen bildet den negativen Arm einer autoregulatorischen Rückkopplungsschleife, welche die periodische Aktivierung der Uhrengene kontrolliert. Spezifische Präferenzen für homo- und heteromere Interaktionen zwischen allen mPER- und mCRY-Proteinen generieren einen selbststabilisierenden Oszillator auf transkriptioneller Ebene. Entfernt man eine oder mehrere der Komponenten aus diesem System, wird dessen Stabilität beeinträchtigt und die Interaktionspräferenzen werden verändert. Überschreitet diese Störung die Kompensationsmöglichkeiten der Uhr, wird die Oszillation gedämpft und die Tiere werden arrhythmisch.

An *Spalax* untersuchten wir die molekulare Organisation des zellulären Uhrwerks anhand der Genexpression. Wir zeigen, dass *Spalax* eine funktionelle molekulare Uhr besitzt mit Uhrengenen wie *Clock, Bmal1, Per1, 2 und 3* sowie zwei *Crys*. Diese ist dem anderer Nagetiere sehr ähnlich, zeigt aber eine gewisse Anpassung an sein unterirdisches Habitat. Unser Ergebnisse deuten darauf hin, dass die hypertrophe Hardersche Drüse, die das rudimentäre und visuell blinde Auge dieser Tiere umgibt, eine funktionelle Rolle im zirkadianen System übernommen hat und den Schrittmacher im SCN während andauernder Abwesenheit äußerer Zeitinformation stabilisiert. *Spalax* kann von einem tagaktiven zu einem nachtaktiven Verhaltensmuster wechseln. Wir konnten zeigen, dass in nokturnalen Tieren der zentrale Oszillator der Uhr vom Lichteinfluss entkoppelt ist, was auf eine spezifische Kontrolle der Signalwege zur zeitlichen Synchronisierung der Uhr hindeutet.

Zusammengefasst gewährt diese Arbeit neue Einsichten in die redundanten sowie die spezifischen Funktionen der *Per*- und *Cry*-Gene im molekularen Mechanismus der zirkadianen Uhr. Sie betont die Konservierung des Systems autoregulatorischer Rückkopplungsschleifen zur Stabilisierung zirkadianer Rhythmik und zeigt ebenso die evolutionäre Adaption der externen Regulation der Uhr an die Lebensbedingungen des Organismus.
Abbreviations

α activity phase
αS-ATP α-thiophospho-adenosine-triphosphate
µg micrograms
µl microliters
τ internal period length
(v/v) volume per volume
(w/v) weight per volume
°C degrees Celsius
2n number of chromosomes
32P-dCTP phosphor-32-phosphate labeled deoxy -cytosinetriphosphate
35S-UTP sulphur-35-sulphate labeled uridinetriphosphate
5-HT serotonin
ACTH adrenocorticotropic hormone
AP accelerator potential
aPVN autonomic paraventricular nucleus
Art. article
AVP arginine vasopressin
Bmal1/2 brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1/2
BSA bovine serum albumine
Ca2+ calcium cation
CAL calretinin
cAMP cyclic adenosine monophosphate
CCG clock controlled gene
cDNA copy DNA
cGMP cyclic guanosine monophosphate
CK1δε casein kinase 1 δε
Clock circadian locomotor output cycles kaput
cm centimeters
CRE cAMP responsive elements
CREB cAMP responsive element binding protein
CRH corticotrophin-releasing hormone
CT circadian time
Dbp D-albumine binding protein
DD constant darkness
DMH dorsomedial hypothalamic nucleus
DNA deoxy -ribonucleic acid
EAP experimental accelerator potentials
EDTA ethylene-diamine-tetra-acetic acid
ER endoplasmatic reticulum
ES cells embryonic stem cells
et al. and co-workers
FASPS familial advanced sleep phase syndrome
Fig. figure
GABA γ-amino butyric acid
GH gonadotropic hormone
GHT geniculohypothalamic tract
GnRH gonadotrophin-releasing hormone
GRP gastrin-releasing peptide
h hours
HCl hydrochloric acid
Hprt hypoxanthine phosphoribosyltransferase gene
IGL intergeniculate leaflet
IML intermediolateral column of the spinal chord
ir immunoreactive
kb kilobases
LD light dark cycle
LGN lateral geniculate nucleus
LL constant light
Lx lux
<table>
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<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>M</td>
<td>mole per liter</td>
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<tr>
<td>MAP</td>
<td>mitogen activated protein</td>
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<tr>
<td>ME</td>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
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<tr>
<td>mm</td>
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<tr>
<td>mM</td>
<td>millimole per liter</td>
</tr>
<tr>
<td>MOP3/9</td>
<td>member of PAS superfamily 3/9</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-morpholino-propanesulfonic acid</td>
</tr>
<tr>
<td>MPN</td>
<td>medial preoptic nucleus</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
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<tr>
<td>NPAS2</td>
<td>neuronal PAS domain protein 2</td>
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<tr>
<td>NPY</td>
<td>neuropeptide Y</td>
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<tr>
<td>OAP</td>
<td>overall accelerator potential</td>
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<tr>
<td>ON</td>
<td>over night</td>
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<tr>
<td>PAS</td>
<td>period / aryl hydrocarbon receptor nuclear translocator / single-minded</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>paraformaldehyde</td>
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<td>phospho-histidine</td>
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<td>prokineticin 2</td>
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<td>protein kinase A</td>
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<td>protein kinase G</td>
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<td>PVN</td>
<td>paraventricular nuclei of the hypothalamus</td>
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<tr>
<td>RE</td>
<td>regression error</td>
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<td>RHT</td>
<td>retinohypothalamic tract</td>
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<td>superior cervical ganglion</td>
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<tr>
<td>TK</td>
<td><em>Herpes simplex</em> virus thymidine kinase gene</td>
</tr>
<tr>
<td>TNT</td>
<td>Tris normal saline Tween 20 buffer</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoylphorbol-13-acetate</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris-(hydroxymethyl)-aminomethane</td>
</tr>
<tr>
<td>TTL</td>
<td>transcriptional / translational feedback loop</td>
</tr>
<tr>
<td>UV</td>
<td>ultra-violet</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
</tr>
<tr>
<td>VIP</td>
<td>vasoactive intestinal peptide</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
<tr>
<td>ZT</td>
<td><em>Zeitgeber</em> time</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction

1.1. The Internal Clock

In 1729, the French astronomer Jean-Jaques Dortous de Mairan discovered that the diurnal closing and opening rhythm of *Mimosa* leaves continues with a nearly 24h (="circadian") period when these plants are kept isolated from outside time information (so called "Zeitgeber", in this case the sun) in a closed cupboard. This was the birth of modern chronobiology, the research on biological rhythms. Circadian rhythmicity has since then not only been observed in leaves but at all levels of organization, from the behavior of mammals, flies, and single cells down to the activity of enzymes and the transcription of specific genes (Pittendrigh, 1993).

Internal clocks have evolved throughout all *phyla* and species, from ancient cyanobacteria to the modern *Homo sapiens* (Dunlap, 1999). The purpose of these internal timekeepers is to anticipate regular changes in the environment and synchronize the status of the whole organism to maximally benefit from the temporal availability of natural resources. The most prominent environment-shaping factor of our world is the succession of day and night and the easiest way to keep track of it is to monitor the rising and setting of the sun. Although for most species – with the exception maybe of photosynthetic organisms – the mere presence or absence of light is not an important parameter itself, secondary phenomena like the diurnal or nocturnal occurrence of predators, prey, the temperature difference or the varying efficiency of certain sensual organs at different times have driven evolution to develop circadian clocks (Rensing et al., 2001).

But why does *Mimosa* keep on closing and opening its leaves in total darkness? Or: Why do we need a self-sustaining clock, when it would be enough to evolve a sensory system for the presence or absence of light? The first answer is anticipation. If our body would merely react to the presence of light we would lose a certain amount of time every morning when it is already bright outside but our physiology is still in transition from "night"-status to "day" or
from "inactive" to "active". This is especially true for most animals living at least temporally in environments isolated from outside time information, e.g. in caves or buildings (Nevo et al., 1982). By anticipating these transitions the organism saves energy because the physiology can be prepared in an economic way. Additionally the timed availability of internal resources serves to maximally benefit from temporally restricted resources granting an important advantage under evolutilonal pressure.

The second aspect of the autonomous internal pacemaker points to its role as a synchronizer of different physiological aspects of the organism. In order to work efficiently, e.g. using as little energy as possible, the activity of the organs responsible for physiological aspects of the body has to be temporally organized. The time information given by the circadian oscillator serves to harmoniously orchestrate the interplay of the different meta- and catabolic pathways by linking them to the central clock (Akhtar et al., 2002; Panda et al., 2002a; Storch et al., 2002).

For most organisms light is the prominent Zeitgeber transferring outside time information to the internal pacemaker because of its high reliability and easy way of detection. However, different Zeitgeber exist like temperature, humidity as well as social factors like stress and pheromonal communication and for some species these might be even dominant to the daily cycle of the sun (Hastings et al., 1995; see as well Oster et al., below).
1.2. Physiological Aspects

In mammals the central circadian clock is located in the *suprachiasmatic nuclei* of the hypothalamus (SCN). Many if not all major physiological processes of the body are under circadian control (Panda et al., 2002a; Storch et al., 2002). The clock coordinates the different pathways with regard to the solar cycle *via* two distinct mechanisms, the endocrine system and the direct regulation of the activity of pace setting enzymes (Buijs and Kalsbeek, 2001). The SCN directs the endocrine system partly by secretion of melatonin from the pineal and partly *via* the hypothalamus. Several pathways lead from the SCN to the pineal: There is innervation *via* the *paraventricular nuclei* (PVN), extracerebral pathways including the intermediolateral column of the spinal chord (IML) and the superior cervical ganglion (SCG) as well as direct signaling (reviewed in Schwartz et al., 2001).

From the pineal, feedback to the SCN is provided by melatonin reception as well as nervous signaling in the brain. Melatonin levels are high during the night. It is involved in the regulation of the sleep/wake cycle, influences the pituitary gland (see below) and controls the secretion of cortisols in the adrenal gland (Malpaux et al., 2001). The hypothalamus directs the daily variation of the body temperature and confers clock signaling to the pituitary gland. The pituitary gland releases hormones like GH, prolactin and ACTH and stimulates endocrine glands like the thyroid and the gonads which themselves produce a set of additional hormones (Goldman, 2001).

![Fig. 1: Regulation of the mammalian endocrine system by the circadian clock. Grey spheres show endocrine glands influenced by the central oscillator in the SCN. Red waves depict cycling hormonal levels and physiological parameters under circadian control (modified from http://www.rpi.edu/~hrushw/).](http://www.rpi.edu/~hrushw/)
Recent studies using gene expression profiling in the SCN and peripheral tissues demonstrate that the activation of a high number of rate-limiting enzymes of key physiological pathways is under circadian control. The set of these genes / enzymes varies from tissue to tissue creating an organ specific pattern orchestrated by the central pacemaker (Akhtar et al., 2002; Panda et al., 2002a; Storch et al., 2002).

Fig. 2: Global comparison of biological processes associated with the genes exhibiting circadian expression in liver and heart. The tree (gray dots and connecting paths) represents those categories (dots) in the gene ontology hierarchy for biological process that match genes expressed in liver (green) or heart (red) or both (yellow). Selected examples of biological process categories are indicated by the following numbers: 1, developmental process; 2, death; 3, cell growth and/or maintenance; 4, cell communication; 5, behavior; 6, transport; 7, stress response; 8, metabolism; 9, (metabolism) nucleobase/ nucleoside/ nucleotide; 10, (metabolism) amino-acid derivative (modified from Storch et al., 2002)
1.3. The Internal Clockwork

1.3.1. The Central Oscillator

The master circadian clock of the mammalian organism resides in the *suprachiasmatic nuclei* of the anterior hypothalamus (Klein et al., 1991). It is composed of a few thousand densely packed, parvocellular neurons. The pacemaker of the SCN maintains its rhythmicity even in the absence of outside time information with a period of nearly but not exactly 24h (hence the term "circadian" from lat. "circa" = approximately and "dies" = the day). Generally nocturnal species like mice have an internal period (τ) of less, diurnal species like man of more than 24h in constant darkness (DD) (see (Pittendrigh and Daan, 1976). Under natural conditions however, the clock is not free running but periodically synchronized to the external daycycle. Environmental parameters linked to daytime, so called "Zeitgeber", confer time information to the central clock and reset its phase to match the external conditions. From the central clock, time information is conferred to the body. Subordinated clocks in peripheral organs receive information from the SCN *via* the activation of clock controlled genes (‘CCGs’). Thereby the whole organism is synchronized with the solar cycle (reviewed in Herzog and Tosini, 2001 and Balsalobre, 2002).

1.3.2. Clock Input

Neurons in the ventrolateral part of the SCN receive glutamatergic input from the retina *via* the retinohypothalamic tract (RHT) (Moore and Lenn, 1972), neuropeptide Y (NPY) input from the intergeniculate leaflet (IGL) of the lateral geniculate nucleus (LGN) *via* the geniculohypothalamic tract (GHT) (Swanson and Cowan, 1975), and serotonergic (5-HT) input from the *Raphé nuclei* (Bosler and Beaudet, 1985; Francois-Bellan and Bosler, 1992). Neurons in the dorsomedial part of the SCN receive modest non-photic input from the cortex, basal forebrain and the hypothalamus (Moga and Moore, 1997).

The RHT conveys information about external lighting conditions to the SCN. Non-visual photoreceptors in the retina – the specific molecules are unknown, although good candidates are opsin based proteins like melanopsin located in the nuclear ganglion cell layer (Berson et al., 2002; Gooley et al., 2001; Hattar et al., 2002; Provencio et al., 2002) – activate monosynaptic projections to the SCN. Glutamate release at the synaptic terminals of the RHT
activate glutamate receptors resulting in a calcium influx in SCN neurons (reviewed in (Gillette and Mitchell, 2002)). This leads to an activation of calcium-dependent kinases, proteases and transcription factors (Xia et al., 1996). At the begin of the dark phase, light exposure causes phase delays (Pittendrigh and Daan, 1976). The downstream actions of calcium likely include activation of calmodulin, MAP kinases and PKA leading to a phosphorylation of the cAMP responsive element binding protein (CREB) which itself can activate mPer transcription via cAMP responsive elements (CRE) in the promoter (Gau et al., 2002; Gillette and Tischkau, 1999; Obrietan et al., 1998). In the late night light exposure in vivo (or glutamate receptor activation in vitro) causes phase advances via the activation of nitric oxide synthase (NOS) and cGMP dependent kinase (PKG) resulting in a phosphorylation of CREB (Ding et al., 1997; Gau et al., 2002).

A second pathway of photic signaling is formed by projections from the retina via NPY-reactive neurons in the IGL (Jacob et al., 1999). NPY levels in the ventrolateral SCN show a biphasic diurnal pattern with peaks at both light/dark and dark/light transitions. This rhythm however, is absent in DD suggesting a mere modulator function of the IGL in the responsiveness of the circadian system to light stimuli (Shinohara et al., 1993).

The serotonergic input from the Raphé nuclei seems to be tightly interlocked with the signaling from the retina (Cagampang and Inouye, 1994). Destruction of the 5-HT system increases light induced phase-shifts (Bradbury et al., 1997) while administration of the 5-HT precursor tryptophane inhibits photic phase shifts during subjective night (Glass et al., 1995).
Fig. 4: Calcium mediated signaling to the circadian clock. Elevated calcium levels after nocturnal light stimulation activate calcium dependent kinases leading to a phosphorylation of the transcription factor CREB which induces mPer gene expression.

Apart from light there exist so-called "non-photic" stimuli which are able to reset the circadian clock (Hastings et al., 1997). An increase of the activity level in nocturnal animals during the inactive period by a dark pulse, forced wheel-running or benzodiazepine injection result in large phase advances (Gannon and Rea, 1995; Van Reeth and Turek, 1989; Wickland and Turek, 1991). Non-photic phase shifts seem to be mediated by NPY via the IGL (Biello et al., 1994; Janik and Mrosovsky, 1994; Maywood et al., 1997). The cellular signal transduction cascade of these stimuli is largely unknown. It does not affect CREB phosphorylation, but a role for serum cortisol through arousal-induced adrenocortical activation has been suggested (Sumova et al., 1994).

1.3.3. Clock Output

The projection pattern from the SCN is predominantly ipsilateral consisting of mainly six anatomical components of intra- and extra-hypothalamic origin. First there are rostrally directed fibers to the preoptic area, namely the anteroventricular periventricular nucleus and the anteroventral periventricular nucleus. Secondly there is projection to the paraventricular nucleus of the hypothalamus, the dorsomedial nucleus and finally into the posterior hypothalamic area and the periaqueductal gray. A third line of efferent connections project
caudally to terminate in the region between the arcuate and the ventromedial nuclei. An additional projection is directed into the paraventricular nucleus of the thalamus and the paratenial nucleus. A small pathway leads to the intermediate lateral septal nucleus and a last one terminates in the intergeniculate leaflet of the lateral geniculate nucleus (Watts, 1991).

The individual SCN neurons contain different neuropeptides like AVP, VIP, GRP and somatostatin (reviewed in (Buijs and Kalsbeek, 2001)). Additionally about 30% of the SCN axons contain glutamate or GABA and a peptide transmitter (Castel and Morris, 2000; Hermes et al., 1996). The presence of these large number of transmitters in different combinations demonstrates the variety in SCN controlled signaling. GABA and AVP are essential for transmitting the daytime message of the SCN. GABA itself inhibits melatonin secretion from the pineal, which is the main signal of night time to the body.

The major target of efferent projections from the SCN is the paraventricular nucleus of the hypothalamus, an integrative center concerned with neuroendocrine, autonomic and behavioral processes (Swanson et al., 1987). It can influence the secretions of the anterior and posterior lobes of the pituitary gland as well as descending projections to the brain stem and the spinal cord. The PVN additionally controls the secretion of ACTH and thereby corticosterone. The extrahypothalamic projections are still poorly described functionally (Kalsbeek and Buijs, 2002).

Fig. 5: Projective targets of the SCN. The SCN uses four important means to organize hormonal secretion: first, by direct contact with neuroendocrine neurons containing gonadotrophin-releasing hormone (GnRH) or corticotrophin-releasing hormone (CRH); second, by contact with neuroendocrine neurons via intermediate neurons like those of the medial preoptic nucleus (MPN), the dorsomedial hypothalamic nucleus (DMH) or the sub-paraventricular nucleus (sPVN); third, by projections to the autonomic PVN (aPVN) to influence the autonomic nervous system, preparing the endocrine organs for the arrival of hormones; and fourth, by influencing its own feedback. (from Buijs and Kalsbeek, 2001).
1.3.4. SCN Architecture

The suprachiasmatic nuclei are bilaterally paired tiny nuclei located just above the optic chiasm (therefore the name). They consist of small neurons, neuropile and glial elements, particularly astrocytes (van den Pol et al., 1992). Most neurons of the SCN are GABAergic although a large variety of neuropeptides are synthesized throughout the whole nucleus (Okamura et al., 1990). So far four different types of SCN neurons have been identified: monopolar, radial, simple bipolar, and curly bipolar (Pennartz et al., 1997). The simple bipolar cells, which have the highest number of all types, form the efferent projections from the nuclei (Pennartz et al., 1998). Axon immunoreactivity for many compounds is higher in the nucleus than in adjacent areas of the hypothalamus (Van Den Pol, 1991). The main neuroactive substances found in the SCN are reviewed in Moore et al., 2002):

- **γ-Amino buteric acid**: GABA is the major neurotransmitter within the SCN and in SCN efferent projections. Therefore much of the local interaction within the SCN seems to be governed by inhibitory circuits. Stimulation of axons projecting into the periventricular region indeed results in a general depression of cells in the targeted area (Kow and Pfaff, 1984).

- **Gastrin-releasing Peptide**: GRP is involved in the hypothalamic control of thermoregulation and homeostasis (Brown and Vale, 1979). GRP immunoreactivity is mainly found ventral and lateral in the medial part of the nucleus. GRPir neurons are the smallest neurons in the SCN.

- **Vasoactive Intestinal Peptide**: VIP positive neurons are found in the same area as GRPir cells overlapping visual and Raphé afferents. VIPir neurons seem to be located slightly more ventral than the GRPir neurons. Together both cells comprise about 39% of all SCN neurons.

- **Vasopressin**: AVP-containing neurons are found throughout the whole body of the SCN with some higher concentration in the dorsomedial part. They comprise about 37% of all SCN neurons and co-localize in part with angiotensin II positive cells.

- **Calretinin**: CALir neurons are found mainly in the dorsolateral part and in adjacent hypothalamic areas of the SCN. Their number comes up to 14% of all SCN neurons.

- **Somatostatin**: SS is well expressed in the shell of the SCN near to the shell/core border. Somatostatin application modulates the firing activity of SCN neurons and depresses activity of neurons in the hypothalamic ventromedial nucleus (van den Pol, 1991).

While serotonin-mediated afferents do not terminate in a specific area of the SCN, photic signaling via glutamate and NPY mainly terminates in the ventrolateral part of the nuclei. Although there exist several neuronal connections between both nuclei of the SCN (Van Den Pol, 1980), both rhythms can be separated as seen in hamsters with a split locomotor activity.
In this case the nuclei oscillate in anti-phase (de la Iglesia et al., 2000). Ablation of one of the nuclei eliminates the split rhythm (Pickard and Turek, 1983).

SCN glial cells have mostly been omitted from models of cellular interactions underlying circadian rhythms. However, they make up a large part of the total cell number of the SCN. Astrocytes in the SCN seem to be metabolically active and show an increase in calcium levels on neurotransmitter release in the synaptic cleft indicating intraglial signaling probably mediated via GAP junctions upon neurotransmitter stimulation (van den Pol, 1991).

![Anatomical organization of the SCN](image_url)

Fig. 6: Anatomical organization of the SCN. Indicated are afferent and efferent neuronal projections and the major neuroactive substances detected in the core and the shell of the SCN (AVP: arginine vasopressin; GABA: \(\gamma\)-amino butyric acid; Glu: glutamate; GRP: gastrin-releasing peptide; GHT: geniculohypothalamic tract; 5HT: 5-hydroxytryptamine (serotonin); IGL: intergeniculate leaflet; NPY: neuropeptide Y; PHI: phospho-histidine; PVN: paraventricular nucleus of the hypothalamus; RHT: retinohypothalamic tract; SS: somatostatin; VIP: vasoactive intestinal protein; modified from www.aasmnet.org/MEDSleep/rhythmslides.htm).
1.4. The Cellular Clockwork

The dominant role of the SCN in the control of circadian rhythms has been identified by lesion experiments in the early seventies (Moore and Eichler, 1972; Stephan and Zucker, 1972). The origin of its circadian firing rate activity has been traced from small unit recordings (Groos and Hendriks, 1982) down to the rhythmicity of single neurons (Welsh et al., 1995). Therefore it seems that the rhythmic signal from the SCN is an integration of a large population of single cellular clocks connected to each other but each cell has a principally self-sustaining rhythm. Chimaeric mice with SCNs composed of two cell types of different internal period lengths indeed show a variety of circadian phenotypes with a period range depending on the relative number of each cell type in the SCN (Low-Zeddies and Takahashi, 2001). On the other hand in Clock mutant mice which become arrhythmic after some time in DD, it has been demonstrated that the isolated SCN neurons retain a rhythmic firing rate even when the animal has already lost its internal rhythm. Therefore the arrhythmicity in these mice seems to be a problem of cell communication rather than a breakdown of the cellular clockwork itself (Nakamura et al., 2002).

With the discovery of the first clock gene period in Drosophila melanogaster (Konopka and Benzer, 1971) research started on the molecular aspects of the circadian clock. Until now a growing number of clock and clock related genes have been discovered in species throughout all phyla. Most of these genes found in animals and fungi are listed in table 1 below. The molecular clockwork can be divided into three parts: Input, pacemaker and output. The pacemaker genes are important for the maintenance of rhythmicity under constant conditions. Their inactivation manifests as altered or disrupted rhythmicity in DD. Mutations or loss of input genes uncouples the internal clock from outside time information thereby desynchronizing the organism with respect to the solar cycle. Output genes transmit time information from the SCN to the body. A loss of an output gene results in the disruption of peripheral rhythms subordinated to the signaling pathway of that gene. Some of these genes have partially redundant functions in the internal clockwork. Therefore the disruption of one single gene can often be compensated by others making it difficult to define a clear phenotype in the single mutant organism.
Table 1: Clock genes in animals and fungi (from Cermakian and Sassone-Corsi, 2000)

<table>
<thead>
<tr>
<th>Input genes</th>
<th>Organism</th>
<th>Characteristics of protein product</th>
<th>Function (in the circadian system)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cry</td>
<td>Drosophila</td>
<td>Similar to DNA photolyases</td>
<td>Conveys photic information to PER–TIM</td>
</tr>
<tr>
<td>Immediate early genes (c-fos)?</td>
<td>Mammals, fish, amphibians</td>
<td>Transcription factors</td>
<td>Induced by light in the suprachiasmatic nucleus; important for photic entrainment</td>
</tr>
<tr>
<td>per¹, 2</td>
<td>Mammals</td>
<td>PAS domain</td>
<td>Induced by light in the SCN; Per1 important for photic entrainment</td>
</tr>
<tr>
<td>wc-1</td>
<td>Neurospora</td>
<td>PAS domain; GATA transcription factor</td>
<td>Required for light-induced τq expression</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pacemaker genes</th>
<th>Organism</th>
<th>Characteristics of protein product</th>
<th>Function (in the circadian system)</th>
</tr>
</thead>
<tbody>
<tr>
<td>clock</td>
<td>Mammals, birds, fish, amphibians</td>
<td>T(H)HIL–PAS factor</td>
<td>Dimerizes with BMAL1 and activates clock and clock-controlled gene expression</td>
</tr>
<tr>
<td>per</td>
<td>Mammals (3 genes), fish, insects</td>
<td>PAS domain</td>
<td>Negatively regulates CLOCK–BMAL1-driven transcription; positively regulates bmal1 (mammals) or clock (Drosophila) expression</td>
</tr>
<tr>
<td>bim</td>
<td>Mammals (1), fish Drosophila</td>
<td>T(H)HIL–PAS factor</td>
<td>In Drosophila, dimerizes with PER to repress CLOCK–BMAL1-driven transcription and activate clock expression</td>
</tr>
<tr>
<td>bmal1 (cycle)</td>
<td>Mammals (2 genes); Fish (2 genes)</td>
<td>T(H)HIL–PAS factor</td>
<td>Dimerizes with CLOCK and activates CLOCK and CLOCK-controlled gene expression (function of MOP3, a close human homologue, is not defined)</td>
</tr>
<tr>
<td>cry</td>
<td>Mammals (2 genes) Drosophila</td>
<td>Similar to DNA photolyases</td>
<td>In Drosophila, circadian; TIM and inhibits PER–TIM dimer</td>
</tr>
<tr>
<td>Cry (doubletime)</td>
<td>Mammals</td>
<td>Serine/threonine protein kinase</td>
<td>Phosphorylates and destabilizes PER (at least PER1 in mammals)</td>
</tr>
<tr>
<td>vila?</td>
<td>Drosophila</td>
<td>Similar to PAR leucine-zipper factors</td>
<td>Affects per and tim expression</td>
</tr>
<tr>
<td>dlc?</td>
<td>Mammals</td>
<td>PAR leucine-zipper transcription factor</td>
<td>May be involved in per1 expression</td>
</tr>
<tr>
<td>τq</td>
<td>Neurospora</td>
<td>PAR leucine-zipper transcription factor</td>
<td>Negatively regulates WC-1/WC-2-driven transcription of its own gene; positively regulates WC-1 expression</td>
</tr>
<tr>
<td>wc-1</td>
<td>Neurospora</td>
<td>PAS domain; GATA transcription factor</td>
<td>Dimerizes with WC-2 and activates τq expression</td>
</tr>
<tr>
<td>wc-2</td>
<td>Neurospora</td>
<td>PAS domain; GATA transcription factor</td>
<td>Dimerizes with WC-1 and activates τq expression</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Output genes</th>
<th>Organism</th>
<th>Characteristics of protein product</th>
<th>Function (in the circadian system)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pdf</td>
<td>Drosophila</td>
<td>Neuropeptide</td>
<td>Links the molecular clock to behaviour</td>
</tr>
<tr>
<td>avp</td>
<td>Mammals</td>
<td>Vasopressin peptide</td>
<td>Controls the activity of various output pathways</td>
</tr>
<tr>
<td>vila</td>
<td>Drosophila</td>
<td>Similar to PAR leucine-zipper factors</td>
<td>Indirectly controls PDF peptide oscillation</td>
</tr>
<tr>
<td>dlp</td>
<td>Mammals</td>
<td>PAR leucine-zipper transcription factor</td>
<td>Controls rhythmic expression of various genes; influences circadian behaviour</td>
</tr>
<tr>
<td>CREM</td>
<td>Mammals</td>
<td>bZIP transcription factor</td>
<td>The repressor isoform CER is involved in the rhythmic expression of NAC, the key enzyme in melatonin synthesis</td>
</tr>
<tr>
<td>τakeout</td>
<td>Drosophila</td>
<td>Similarity to ligand-binding proteins</td>
<td>Involved in output pathways that link the clock to feeding</td>
</tr>
</tbody>
</table>

The basic principle underlying all cellular clocks described so far is the transcriptional/translational feedback loop (reviewed in Panda et al., 2002b). In its simplest form it consists of an oscillator gene and its protein product (see Fig 7 below). The activation of the oscillator gene results in the production of the corresponding mRNA, which is translocated to the endoplasmatic reticulum where its message is translated into the cytoplasmic oscillator protein. When the concentration of the oscillator protein in the cytoplasm reaches a certain threshold, it re-translocates back into the nucleus where it can interfere with its own transcription machinery thereby inhibiting the activation of the oscillator gene. Subsequently the oscillator protein levels decrease due to constitutive degradation in the cytoplasm and in the nucleus. With the oscillator protein levels the inhibition of the oscillator gene transcription is reduced and the cycle starts again.
This basic principle seems to be preserved throughout all *phyla*. Some of the genes involved - like the *Cryptochromes* found in plants, insects, fishes, amphibia and mammals (reviewed in Sancar, 2000) - appear in many organisms studied so far. But the role these genes play in the clockwork underlies some variation. This has caused confusion in the beginning of molecular chronobiology and led to some likely misinterpretations due to inappropriate experimental setups (Cermakian et al., 2002; Sangoram et al., 1998; Yagita et al., 2000). In the following part I will therefore focus on the mammalian system. Although studies were done predominantly in nocturnal rodents like mice, rats and hamsters, the rodent clock believed to essentially follow the same organization as the human counterpart. As can be seen in the second part of this work, clock gene regulation is indeed highly similar in nocturnal and diurnal mammals (Avivi et al., 2001; Avivi et al., 2002). Additionally there are studies emerging on clock gene polymorphism and mutations in humans with phenotypes expected from the corresponding mouse mutants (Toh et al., 2001).

*Clock* was the first clock gene cloned in mammals (King et al., 1997). Clock mutants – identified in a chemical mutagenesis screen – show an abnormally long period in constant darkness (DD) finally causing arrhythmicity in homozygous animals (Vitaterna et al., 1994). CLOCK protein forms heterodimers with BMAL1 (or MOP3). Both proteins contain a PAS domain (from *per/ arnt/ sim*) important for protein/protein interactions, a motif conserved in many proteins involved in the generation of circadian rhythms (Gu et al., 2000; Kay, 1997;
Reppert, 1998). Additionally both have a basic helix-loop-helix motif (bHLH) for protein/DNA interaction. The CLOCK/ BMAL1 complex can bind to E-boxes containing the nucleotide sequence CACGTG found in the promoter regions of several other clock related genes like \textit{mPer1, mPer2, mCry1, mCry2, Dbp, AVP} among others (Darlington et al., 1998; Gekakis et al., 1998; Hogenesch et al., 1998; Jin et al., 1999; Kume et al., 1999). The mutated CLOCK protein can still bind BMAL1 but the transcriptional activation of other clock genes is deficient (Gekakis et al., 1998).

BMAL1 and the highly similar BMAL2 (also known as MOP9) can both form heterodimers with CLOCK. Together they can drive transcription from E-box elements and are co-expressed in neurons of the SCN (Gekakis et al., 1998; Hogenesch et al., 1998; Hogenesch et al., 2000; Honma et al., 1998; Ikeda et al., 2000; Jin et al., 1999; Ripperger et al., 2000). Mice with a targeted deletion in the \textit{Bmal1} gene show an impaired entrainment to an LD cycle with comparably high activity levels during the day and a variable onset of activity after “lights off”. Upon release into constant darkness (DD) the animals immediately become arrhythmic indicating a complete disruption of the circadian clockwork. Additionally it was shown that in these mutants \textit{mPer} and \textit{Dbp} gene expression is not cyclic anymore. Therefore CLOCK alone is not sufficient to activate E-box driven transcription nor can \textit{MOP9} compensate the loss of \textit{Bmal1} in these animals (Bunger et al., 2000).

If the transcriptional activation of clock genes by CLOCK/ BMAL1 is the positive arm of the transcriptional/ translational feedback loop the inhibition of CLOCK/ BMAL1 by the products of these clock genes constitutes its negative counterpart.

Both \textit{Cryptochromes} (mCRY1 and mCRY2) and \textit{Period} proteins have the ability to inhibit CLOCK/ BMAL1 with the CRYs having by far the biggest inhibitory effect \textit{in vitro}. \textit{mCry1} mRNA levels are cycling with a circadian period while \textit{mCry2} transcript shows only slight variations throughout the day. mCRY2 protein however, is prominently oscillating in the SCN. Both proteins are predominantly localized in the nucleus where they can interfere with the CLOCK/ BMAL1 heterodimer (Kume et al., 1999). Mice with a targeted disruption of the \textit{mCry1} gene show a shortened free-running period (\(\tau\)) in DD while a loss of \textit{mCry2} results in a prolonged \(\tau\) under constant conditions. A simultaneous deletion of both genes however, leads to a complete loss of rhythmicity in these animals indicating a complementary but essential role for both \textit{Cry} genes in the TTL of the central pacemaker (van der Horst et al., 1999; Vitaterna et al., 1999).

The transcripts of all three known \textit{mPer} genes oscillate with a 24h period in the SCN and most peripheral tissues (see below; Albrecht et al., 1997b; Shearman et al., 1997; Sun et al.,
1997; Takumi et al., 1998; Tei et al., 1997; Zylka et al., 1998b). All PER proteins contain PAS domains, which seem to play a role in PER/PER protein interactions (Yagita et al., 2000). Additionally in vitro studies suggest an interaction between both PER and CRY proteins (Griffin et al., 1999; Kume et al., 1999; Yagita et al., 2001). A partial deletion of the PAS domain of mPer2 results in a shortened free-running period in homozygous mice. Moreover these mice completely lose their rhythm after some time in constant darkness (Bae et al., 2001; Zheng et al., 1999). In contrast, mPer1 mutants do not get arrhythmic although their free-running period is reduced and destabilized (Bae et al., 2001; Cermakian et al., 2001; Zheng et al., 2001). mPer1/mPer2 double mutants do not show any circadian rhythm in DD indicating a complete disruption of the circadian pacemaker (Bae et al., 2001; Zheng et al., 2001). The deletion of mPer3 has only minor effects on the circadian phenotype in mice. Therefore mPer3 seems not to play a role in the central oscillator but may still be part of some clock regulated output pathway (Shearman et al., 2000a).

Low levels of clock gene transcripts in mPer mutant mice lead to the discovery of a second role of mPer2 in the TTL: It seems to have a positive influence on Bmal1 transcription (Shearman et al., 2000b). In vitro studies indicate that mPER2 as well as mCRY1 and mCRY2 can activate Bmal1 promoter driven transcription (Yu et al., 2002). Other studies suggest Rev-erba as a transcriptional inhibitor of Bmal1 that in turn can be inhibited by interaction with PER2 (Preitner et al., 2002). This second loop in the central oscillator is believed to stabilize the clock to ensure a precise and constant rhythmicity in the absence of regular Zeitgeber input (Hastings, 2000).

A third role of the two mPer genes lies in their transcriptional activation by light. While mPer1 expression can be induced throughout the night mPer2 is light sensitive only at the beginning of the dark phase (Albrecht et al., 1997b). Thus the mPers seem to form the link between the central oscillator and the input pathways to the clock. It has been demonstrated that mPer mutant mice show impaired resetting of their activity rhythms in response to nocturnal light pulses (Albrecht et al., 2001). While mPer2 mutants have deficiencies in clock delaying after a light pulse at the begin of the night, mPer1 mutants are not able to phase advance after light exposure before sunrise. This corresponds with the differential light inducibility of both mPer genes by light with mPer2 being light responsive only at the begin of the night (Albrecht et al., 1997b).

The mPer1 promoter contains CRE motifs capable of binding the phosphorylated form of the cAMP responsive element binding protein (CREB) (Shigeyoshi et al., 1997) activated by the Ca\(^{2+}\) mediated light signaling pathways in the SCN (see above). In addition, the hPer1
promoter has been shown to integrate signaling from other second messenger pathways like Protein kinase A, C, G and mitogen activated kinases (Akashi and Nishida, 2000; Lee et al., 1999; Motzkus et al., 2002; Motzkus et al., 2000; Prosser and Gillette, 1989; Sanada et al., 2000; Schak and Harrington, 1999; Tischkau et al., 2000). Thus the regulated transcription of the \textit{mPer} genes can act as a molecular integrator of cellular signaling to the circadian clockwork.

Some reviews still place \textit{mTim}, the mammalian homologue of \textit{Drosophila timeless}, in the central oscillator. Though \textit{mTim} is expressed at low levels in the SCN, it is not rhythmic nor does it respond to light as expected from \textit{Drosophila} (Field et al., 2000; Hastings et al., 1999). mTIM does not interact with any mPER protein \textit{in vivo} (Zylka et al., 1998a) although it co-immunoprecipitates with mCRY in over-expression studies (Field et al., 2000). Moreover, a targeted disruption of \textit{mTim} results in a defective kidney development resulting in the death of homozygous embryos before midgestation. Heterozygous mice show a normal circadian phenotype (Gotter et al., 2000). Thus \textit{mTim} is a developmental gene without substantial circadian function (Reppert and Weaver, 2001a).

Taken together the current understanding of the mammalian TTL works as follows:

CLOCK/ BMAL1 heterodimer binds to \textit{mPer} and \textit{mCry} promoters at the end of the night / begin of the day and activates their transcription. In the course of the day mPER and mCRY proteins accumulate in the cytoplasm and re-translocate back into the nucleus. There they form a multimeric complex that inhibits CLOCK/ BMAL1 activated transcription. Additionally mPER2 and/or mCRY1/2 activate \textit{Bmal1} transcription. During the night, while \textit{mPer/ mCry} transcription is low, mPER/ mCRY protein levels decrease due to degradation (see below) and BMAL1 levels rise. At dawn the critical relation between BMAL1 and mPER/ mCRY levels is reached, new active CLOCK/ BMAL1 heterodimers are formed and \textit{mPer/ mCry} transcription is initiated again.
Introduction

Fig. 8: Model of the circadian clockwork within an individual SCN neuron. The CLOCK/ BMAL1 heterodimer activates mPer and mCry transcription. mPER and mCRY proteins form a multimeric complex and inhibit CLOCK/ BMAL1. Both loops are linked by Reverb α, which is activated by CLOCK/ Bmal1 but inhibits Bmal1 transcription. Casein kinase 1ε phosphorylates the PER proteins which ultimately leads to their degradation. For further explanations see text above.

Additional aspects of the circadian clockwork with special respect to influences of photoperiod are discussed below.

Phosphorylation, proteolysis and the re-translocation into the nucleus of mPER and mCRY proteins are likely to be crucial for imparting a 24h time constant to the SCN clockwork (Lee et al., 2001; Young and Kay, 2001). One protein kinase which is able to phosphorylate both mPER and mCRY proteins and BMAL1 is Casein Kinase 1ε (CK1ε) (Eide et al., 2002; Keesler et al., 2000; Lowrey et al., 2000; Vielhaber et al., 2000). The “tau hamster” carrying a mutation in the CK1ε gene has a severely shortened free-running period of about 20h (Lowrey et al., 2000). Additionally, over-expression experiments suggest a role of CK1ε or CK1δ in the nuclear translocation of mPER proteins (Vielhaber et al., 2000). A recent study by Yagita and colleagues (Yagita et al., 2002) brings together all the three aspects of post-translational processing connected to the circadian machinery so far. In an elegant set of cell system based experiments the authors suggest a mechanism by which the timed interaction between mPER2, mCRY protein and CK1ε regulates the nuclear abundance of an mPER/ mCRY complex capable of interacting with the CLOCK/ BMAL1 transcription machinery.
They show that mPER2 contains functional nuclear import and export signals allowing the protein to shuttle freely between the nucleus and the cytoplasm. In the nucleus mPER2 interacts with mCRY1 or mCRY2 thereby protecting each other from degradation by the proteasome after phosphorylation by CK1ε.

Fig. 9: Working model for mCRY-mediated nuclear accumulation of mPER2. mCRY keeps shuttling mPER2 in the nucleus and protects it from phosphorylation by CK1ε and subsequent degradation by the proteasome (taken from (Yagita et al., 2000)).

This corresponds well with the data from mCry1/ mCry2 double mutant mice where mPer transcript levels are high but mPER protein cannot be detected (Shearman et al., 2000b) and results from a human circadian disease, the familial advanced sleep phase syndrome (FASPS). FASPS patients carry a mutation of the hPer2 gene in the region of the CK1ε phosphorylation site (Toh et al., 2001). Decreased phosphorylation would lead to higher PER levels in the nucleus thereby accelerating the speed of the clock. This is exactly what is seen in FASPS patients and in the tau hamster (Jones et al., 1999; Ralph and Menaker, 1988).
1.5. Peripheral Clocks

Clock genes are not only expressed in the SCN but appear in several tissues outside the brain indicating the existence of peripheral circadian clocks subordinated to the central pacemaker of the hypothalamus (for review see Balsalobre, 2002 and Herzog and Tosini, 2001). Analysis of clock gene expression in different tissues like liver, kidney, heart or muscle revealed that most of their transcript levels indeed show a circadian profile (Balsalobre et al., 1998; Lee et al., 2001; McNamara et al., 2001; Nonaka et al., 2001; Oishi et al., 1998; Zylka et al., 1998a). Essentially all peripheral organs seem to be capable of generating circadian rhythmicity. However, circadian gene expression in peripheral cell types is delayed about 4h when compared to the SCN (Balsalobre et al., 1998). The regulative mechanisms of peripheral clocks seem identical to those present in the SCN, although a recent study in Clock mutant mice suggests a different impact of the Clock mutation on central and peripheral oscillators (Oishi et al., 2000). This probably reflects the presence of clock gene homologues in non-SCN tissues (like NPAS-2 in the forebrain (Reick et al., 2001)), which can compensate for the loss or misfunction of some of the “classical” clock genes.

Additionally peripheral oscillators dampen without continuing stimulation (Balsalobre et al., 1998). Therefore the clocks of the body need to be regularly synchronized to the master time teller of the brain. This synchronization most probably occurs via diffusible factors (Silver et al., 1996) – although neural signals seem also important for some outputs (Kalsbeek and Buijs, 2002) - probably released via the bloodstream (Oishi et al., 1998). This would explain the 4h lag of peripheral clock gene expression. SCN neurons have been shown to directly synchronize cultured fibroblasts (Allen et al., 2001). Recent work by Cheng and colleagues demonstrates that Prokineticin 2, which is synthesized in the SCN, is essential for the control of locomotor activity. PK2 receptors are found in several brain nuclei involved in output signaling from the clock. Therefore these nuclei might as well produce the humoral signals synchronizing the periphery (Cheng et al., 2002).

Several candidates exist for timing ligands in the blood. Glucocorticoids induce circadian gene expression in fibroblasts and induce phase shifts in liver, kidney and heart (Balsalobre et al., 2000a). However, the disruption of the glucocorticoid receptor does not affect circadian gene expression in the periphery indicating that glucocorticoids cannot be the only signaling compound. Fibroblast rhythmicity can be induced as well by forskolin, adenylate cyclase agonists, TPA and calcimycin (Akashi and Nishida, 2000; Balsalobre et al., 2000b; Motzkus et al., 2002; Motzkus et al., 2000; Nonaka et al., 2001; Yagita and Okamura, 2000). Retinoids
seem to be important for circadian gene expression in the vascular system (McNamara et al., 2001). But the general mechanism by which peripheral clocks are reset remains unclear. It has been shown that during light phase restricted feeding the temperature of mice was affected suggesting an impact of body temperature on the resynchronization of peripheral clocks (Damiola et al., 2000). Rutter and colleagues (Rutter et al., 2001) show that the redox state of the cell influences the efficiency of CLOCK/ BMAL1 driven transcriptional activation. Since the cell redox state is affected by metabolism, restricted feeding may thus directly reset peripheral clocks through metabolic activity (reviewed in Schibler et al., 2001).
1.6. Photoperiodism

1.6.1. Publication: “The Circadian Clock as a Molecular Calendar”

Henrik Oster, Erik Maronde, and Urs Albrecht

MINIREVIEW

THE CIRCADIAN CLOCK AS A MOLECULAR CALENDAR

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ABSTRACT

There are two dominant environmental oscillators shaping the living conditions of our world: the day–night cycle and the succession of the seasons. Organisms have adapted to these by evolving internal clocks to anticipate these variations. An orchestra of finely tuned peripheral clocks slaved to the master pacemaker of the suprachiasmatic nuclei (SCN) synchronizes the body to the daily 24h cycle. However, this circadian clockwork closely interacts with the seasonal time-teller.

Recent experiments indeed show that photoperiod—the dominant Zeitgeber of the circannual clock—might be deciphered by the organism using the tools of the circadian clock itself. From the SCN, the photoperiodic signal is transferred to the pineal where it is decoded as a varying secretion of melatonin.

Different models have been proposed to explain the mechanism by which the circadian clock measures day-length. Recent work using mutant mice suggests a set of two molecular oscillators tracking dusk and dawn, respectively, thereby translating day-length to the body. However, not every aspect of photoperiodism is covered by this theory and major adjustments will need to be made to establish a widely acceptable uniform model of circadian/circannual timekeeping. (Chronobiology International, 19(3), 507–516, 2002)

Key Words: Seasons; Per; Cry; Melatonin; Oscillator; Suprachiasmatic nuclei (SCN)

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INTRODUCTION

Life in most areas of the world is under the regime of two major periodic variations, the day—night cycle and the seasons. Organisms have learned to adapt to these oscillations by evolving mechanisms to keep track of the time thereby anticipating upcoming environmental changes. This synchronization of physiology and behavior to the environment serves to maximally benefit from the limited availability of resources in nature.

The timekeeping devices that have evolved to optimally adapt animals to the environment are the circadian clock to predict daily events and the circannual clock to enable an organism to foresee the seasons. While in the last years a vast amount of data has been accumulated about the molecular mechanism of the circadian clock, knowledge about the nature of the circannual clock remains sparse. Recent findings indicate that there might not even be a distinct circannual oscillator, but that the circadian clock itself could measure seasonal time [reviewed in Ref. (1)].

Circadian clocks are found in many organisms throughout all phyla [reviewed in Ref. (2)]. However, seasonal timekeeping has been reported only in a few species, which is not astonishing given the fact that many organisms just do not live long enough to be forced to adapt to a circannual rhythm. Nevertheless, some short-lived animals like insects [reviewed in Ref. (3)] and fungi [reviewed in Ref. (4)] show day-length adaptations.

In this review, we will focus on mechanisms of circadian and circannual timekeeping in mammals. We will give an overview of recent insights into clockwork function and model a molecular calendar that is based on the circadian clock.

CENTRAL AND PERIPHERAL CLOCKS IN MAMMALS

The mammalian organism uses more than one clock to keep track of time. In fact, almost every cell of our body holds all the components necessary to build a functional biological oscillator. Circadian rhythms were found in many tissues like the brain, liver, eyes, kidneys, skin, muscle, and even in fibroblasts. All these peripheral clocks do not run independently, but are driven by the master circadian pacemaker in the suprachiasmatic nuclei (SCN) of the hypothalamus [reviewed in Ref. (5)].

The cells of the SCN receive light information from the retina directly via the retinohypothalamic tract (RHT) and indirectly via the intergeniculate leaflet (IGL) (6). Unknown nonvisual photoreceptors in the eye transfer light/dark information via glutamatergic nerves to the SCN [reviewed in Ref. (7)]. In the pacemaker cells of the SCN, a set of light inducible genes, namely Perl and Per2, refers daytime information to the molecular clockwork (8,41). In analogy to experiments in Drosophila, it is hypothesized that the Period (PER) proteins together with the two Cryptochromes (CRYs) influence their own transcriptional activation by interfering with the CLOCK/BMAL1 transcriptional activation complex. However, there is evidence that
the regulation of the mammalian clock is different from that described in *Drosophila* (9–11). Nuclear abundance of PER and CRY proteins varies in a 24h rhythm (12). CLOCK/BMAL1 activation and deactivation cycles also control a set of clock-dependent genes whose proteins transfer the time information to the body and to the peripheral organic clocks, which themselves govern the physiological processes of the body [reviewed in Refs. (13,14)].

So far, the primary transmission pathway from the SCN to the periphery, which could either be neuronal or endocrine, remains elusive. However, most experiments favor transmission by diffusible factors (15), which would explain why the peripheral clocks normally follow the SCN with a lag of several hours. One factor that is secreted by the SCN in a diurnal fashion is TGFalpha, which has been identified as a likely inhibitor of locomotion (16). It has also been shown that glucocorticoids are able to reset vascular clocks (17), but the main resetting mechanism might be of a more indirect nature as well.

There are connections between different peripheral clocks as well as feedback to the SCN [reviewed in (18)]. Restricted feeding schedules are able to reset the clock in the liver, but not in the SCN (19). In contrast, forced activity schedules are known to reset the central pacemaker in hamsters (20).

The orchestrated interaction of interwoven cellular clocks creates the variation of the physiological status over the 24h day/night cycle, and over the seasonal cycle as well.

PHOTOPERIODICITY IN MAMMALS

Seasonality evolved to adapt the organism to expect environmental changes through the course of the year. Photoperiod is a *proximate* but not an *ultimate* factor meaning that the adaptation to the photoperiod itself does not have any evolitional advantages (like the adaptation to temperature changes and the knowledge of variations in food and water abundance) (21). However, nature chose this readout for the time of the year because of its easy accessibility and relative accuracy.

The photoperiod is transmitted to the body via the secretion of nocturnal melatonin by the pineal gland. The duration of melatonin release is reciprocal to the day-length: Short days mean longer melatonin release and vice versa (22). Melatonin-sensitive tissues convert this signal into those overt circannual rhythms we observe in most mammals like the reproductive status and activity schedules [reviewed in Refs. (23,24)].

Melatonin and the pineal seem essential for seasonality. Pineal-ectomized hamsters do not react to photoperiodic changes anymore (25). Infusions of external melatonin can mimic different photoperiods in these animals (26).

One remaining problem in reproductive photoperiodism in rodents is the occurrence of nonresponders. The adaptive value of this phenomenon is unclear, but it has been speculated that under certain environmental conditions,
nonresponsiveness to photoperiodically gated seasonality may be of adaptive benefit for the species [reviewed in Ref. (27)]. It is tempting to speculate that one reason for such variation may be mutations or polymorphisms in clock genes.

Seasonality in humans has long been debated. Nowadays, there is mutual agreement that seasonality in, e.g., birthrate can be found from the times before the inventions of artificial nocturnal illumination [reviewed in Ref. (28)].

THE ROLE OF THE SUPRACHIASMATIC NUCLEI IN PHOTOPERIODIC TIME MEASUREMENT

The SCN is a major contributor to photoperiodism because SCN lesions in rodents lead to a complete loss in photoperiodicity (29). However, this did not rule out the possibility of a secondary clockwork independent of the circadian in the same nucleus.

First evidence for a direct involvement of the circadian system in photoperiodic time measurement (PTM) came from three different experimental setups: night breaks, resonance light cycles, and T cycles. All these setups use short light periods to provoke long-day phenotypes in hamsters (30–32). They clearly show that the duration of light does not tell the body the time of the year, but the phase of light input in respect to the phase of the circadian cycle. The SCN is integrating the light signals to form photoperiodic information. From the SCN, the pineal gets its instructions to release melatonin into the body [reviewed in Ref. (33)]. In Fig. 1, we give an overview about photoperiodic signaling pathways in the brain.

There are some complications in this simplistic scheme. The SCN does not show a gradual response in firing rate corresponding to variations in melatonin occurrence under different day-lengths (34). Additionally true circannual rhythms like the body mass variation of ground squirrels are not touched by SCN lesions (35), but the SCN is involved in the photo-entrainment of this rhythm (36) and the onset of hibernation (37). Both experiments indicate a role of the SCN downstream of the pineal.

HOW THE SCN MEASURES DAY-LENGTH

Two models have been proposed to describe PTM in mammals. The external coincidence model (38) postulates that light—besides its effect in resetting the circadian system—induces long-day responses when present in certain photoinductive phases of the circadian cycle. This implies a kind of phase response curve for photoperiodism.

Secondly, the model of internal coincidence (39) postulates that two distinct oscillators in the circadian clock lock to different events of the day cycle, namely, dawn and dusk. So when the days get longer during springtime, the phase angle
between those two oscillators is growing while in fall, both get closer together again. These oscillators where termed M (morning) and E (evening) oscillator with E inducing melatonin production in the pineal and M switching off the melatonin in the morning [(39); see Fig. 2a].

In the last year, some proposals have been made in which components of the circadian clock might form these oscillators and now first data emerge to solidify these postulations. In a paper by Daan et al. (40), the authors try to flesh out the old two-oscillator model derived from splitting phenomena in hamsters with assigning certain genes as M or E components. They postulate that Per1 together with Cry1 forms the morning oscillator indicated by the peak of expression of Per1 in the early morning. Per2 and Cry2 form E, indicated by the late afternoon maximum of Per2 expression and the restricted light inducibility of Per2 to the early night proposing a role of Per2 in the delaying mechanism of the clock. Experiments from our group (41) indeed show that Per1 mutant mice are not able to phase-advance in response to a short light pulse while Per2 mutants cannot phase-delay. Additionally Per2 mutants are not following Aschoff’s rule of the tau increase with higher light intensity in constant light (LL) (42). According to the E/M model, these animals run only on the morning oscillator and are thereby not able to properly delay the clock in reaction to stronger light exposition.

In situ analysis of clock gene expression in the SCN of mice kept in long- and short-day photoperiods shows a broadening of the expression maxima of Per1 and Per2 with a maximum of Per1 in the middle of the day and of Per2 in the early evening implicating an approximation of both maxima in short days (42). In wild
Figure 2. (a) Schematic illustration of hypothetical day-length encoding in the circadian clock. Two molecular oscillators are coupled to dusk and dawn, respectively. The M (morning) oscillator inhibits melatonin secretion in the pineal while the E (evening) oscillator induces melatonin at the start of the night. The phase angle between M and E determines day-length. (b) Phase angle variations between M and E oscillator in winter (short days) and summer (long days). While short light exposure reduces the difference between M and E during the day, prolonged melatonin expression is mediated by the nightly time span between E (melatonin induction) and M (melatonin inhibition). This effect is inverted in summer where the interval between E and M is shortened by the long light exposure during the day.

type animals *Per1* and *Per2* gene expression seem to be coupled. *Per1* gene expression in *Per2* mutant mice shifted to times around dawn, whereas *Per2* gene expression in *Per1* mutant mice became linked to dusk (42). Hence, an uncoupling of *Per1* and *Per2* gene expression occurred in *Per* mutant mice. These data are consistent with the two-oscillator model in which *Per1* would be part of the M oscillator and *Per2* of the E oscillator.

This model gives an easy mechanism by which the organism can adapt to varying day-length throughout the year (Fig. 2b) by changing the phase-angle between both oscillators. However, it still shows several shortcomings. For example, the mRNA oscillations in other species like the Siberian hamster do not
show the same variations with different day-length as mice (43,44). This might be
due to the feedback on the clock by the strong melatonin rhythm in these animals.
But if the genetic clock does not work in seasonal animals, of what relevance is it
elsewhere?

Another paradigm in which gene expression is monitored while animals are
adapting to a shifted light–dark cycle shows a rapid change of Per1 and Per2
expression followed by a slow adaptation in Cry1 and Cry2 mRNA rhythms
suggesting a functional unity of both Pers and Crys, respectively (45). Still
possible is a functional compartmentalization of the SCN cells into morning and
evening oscillators. For example, multiunit recordings from horizontal SCN slices
show a bimodal firing rate maximum with peaks in the morning and in the evening
(46).

CONCLUSION

With the enormous progress made in the field of circadian timekeeping, the
mystery of circannual clocks is slowly getting unraveled as well. Work on
photoperiodic mammals like hamsters and on mutant mouse strains gave new
molecular insight on old theories about the nature of the internal calendar.

An important question remains about the impact of photoperiod on the
human organism. Despite a seasonal variation in vasopressin (VP) and melatonin
levels (47,48) as well as slightly altered fertility throughout the year (49,50), there
is not much noted about human seasonalism [reviewed in Ref. (28)].

However, the molecular aspects of the periodic function of the circadian
clock offer a simple and intriguing explanation for such common-day phenomena
like seasonal affective disorder: When during the short days of winter M and E
oscillators of the circadian clock get close together, this may result in clockwork
disturbances with psychological impact.

How the internal clock and our psychological well-being are linked and
which molecules play a role in photoperiodic signaling of the body are only
sparsely understood [reviewed in Refs. (51,52)]. But more and more gaps are
closed and the increasing understanding of the mechanisms underlying our
circadian system will soon lead towards a better comprehension of seasonal
timekeeping as well.

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Introduction


1.7. Aim of this Work

The goal of this project was to further characterize the role of the *Period* and *Cryptochrome* genes and their interaction in the mammalian circadian clockwork. Although the importance of the four genes *mPer1*, *mPer2*, *mCry1* and *mCry2* as components of the central transcriptional/ translational feedback loop has convincingly been demonstrated, the exact nature of the interactions of these genes and their specific roles in the stabilization and maintenance of the cellular oscillator remains to be discovered (Albrecht, 2002; Okamura et al., 2002).

One aspect is the putative redundancy of the *mPers* and the *mCrys* in the circadian system. Some work has been performed on the distinct role of the two *mPer* genes (Bae et al., 2001; Zheng et al., 2001). However, *mCry1* and *mCry2* are still believed to be mutually exchangeable despite the fact that the corresponding null mutant mice show clearly different circadian phenotypes (van der Horst et al., 1999; Vitaterna et al., 1999).

Another point of debate is the role of the *mPers* and *mCrys* in the resetting pathway of the clock. While the light inducibility of the *mPer* genes and resetting deficiencies in the corresponding mouse mutants make them likely candidates for the connection between the pacemaker and the environment (Albrecht et al., 1997b; Albrecht et al., 2001), it is still not clear whether the *mCrys* are important in light signaling (like their plant and *Drosophila* homologues) or not (Barinaga, 1999; Sancar, 2000).

Additionally, much of the functional data regarding the circadian system was generated using more or less artificial experimental setups. However, it is very difficult to mimic the clockwork depending on interactions organized in spatial and temporal manners *in vitro*. And indeed many of the proposed mechanisms were demonstrated to be of no significant relevance when it came to *in vivo* studies using transgenic animals (Gotter et al., 2000; Shearman et al., 2000a).

Therefore we chose to study *mPer/ mCry* interactions directly in the living animal. We crossed *mPer* and *mCry* mutant mice to produce animals lacking different combinations of the genes. This allowed us to assess *mPer* and *mCry* functional interaction under physiological conditions and deduce the different roles of these genes in the circadian clockwork.

In the second part we elucidated the molecular circadian clockwork of the blind mole rat *Spalax ehrenbergi* superspecies. Although the circadian behavior of *Spalax* has been broadly examined (Ben-Shlomo et al., 1995; Goldman et al., 1997; Rado and Terkel, 1989; Tobler et al., 1998) only sparse work has been performed on the molecular level so far (Negroni et al.,
1997; Tobler et al., 1998). The total visual blindness of the mole rats with extreme ocular degradation (Cooper et al., 1993) makes *Spalax* a highly interesting system for chronobiological studies. Additionally the ability of *Spalax* to change between a predominantly diurnal to a nocturnal activity pattern allowed us to examine differences in the clock mechanism of nocturnal and diurnal organisms in one species.
Chapter 2

Results

2.1. Publication: “Disruption of mCry2 restores circadian rhythmicity in mPer2 mutant mice”

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Disruption of mCry2 restores circadian rhythmicity in mPer2 mutant mice

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Many biochemical, physiological, and behavioral processes display daily rhythms generated by an internal timekeeping mechanism referred to as the circadian clock. The core oscillator driving this clock is located in the ventral part of the hypothalamus, the so-called suprachiasmatic nucleus (SCN). At the molecular level, this oscillator is thought to be composed of interlocking autoregulatory feedback loops involving a set of clock genes. Among the components driving the mammalian circadian clock are the Period 1 and 2 (mPer1 and mPer2) and Cryptochrome 1 and 2 (mCRY1 and mCRY2) genes. A mutation in the mPer2 gene leads to a gradual loss of circadian rhythmicity in mice kept in constant darkness (DD). Here we show that inactivation of the mCRY2 gene in mPer2 mutant mice restores circadian rhythmicity and normal clock gene expression patterns. Thus, mCry2 can act as a nonallelic suppressor of mPer2, which points to direct or indirect interactions of PER2 and CRY2 proteins. In marked contrast, inactivation of mCry1 in mPer2 mutant mice does not restore circadian rhythmicity but instead results in complete behavioral arrhythmia in DD, indicating different effects of mCRY1 and mCRY2 in the clock mechanism.

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The mammalian Period (mPer) and Cryptochrome (mCRY) genes are major components of the circadian pacemaker (King and Takahashi 2000; Albrecht 2002). mCRY proteins are part of the negative limb in the transcriptional/translational feedback loop, whereas mPER2 is thought to act positively on Znmi1 expression (Shearman et al. 2000). In vitro studies point to multiple physical interactions between all mPER and mCRY proteins and posttranslational modifications such as phosphorylation and ubiquitylation, thereby offering a variety of putative regulation points for tuned accumulation and nuclear appearance of clock proteins (Gokina et al. 1999; Rowne et al. 1999; Field et al. 2000; Shearman et al. 2000; Yagita et al. 2000; Lee et al. 2001; Miyazaki et al. 2001; Vuilleamar et al. 2001; Zheng et al. 2001; Liu et al. 2002). In particular there is evidence that mPER2 (CFP-tagged) shuttles between cytoplasm and nucleus and is ubiquitinated and degraded by the proteasome unless it is retained in the nucleus by mCRY proteins (Yagita et al. 2000). These findings implicate a regulatory effect of mCRY proteins on mPER2. However, the time course of protein availability, modification, and localization is difficult to resolve in model systems such as cell or slice cultures (Yagita et al. 2000; Harnada et al. 2001; Lee et al. 2001). In bacteria, yeast, Caenorhabditis elegans, and Drosophila, interactions of proteins have frequently been found through nonallelic suppressor screens, that is, the restoration of a phenotype by introducing a mutation in another gene (Raine and Riddle 1999; Amin et al. 1999; Nakano et al. 2000; Gandini and Chabouneau 2001; Latresanne et al. 2001; Roy et al. 2002). We decided to further elucidate the functional relationship between mPer2 and mCRY genes by studying their genetic interactions in the living animal. To this end, we inactivated the mCry1 or mCry2 genes in an mPer2 mutant mouse strain.

Results and Discussion

Per2−/− mice, carrying a mutant mPer2 gene with a deletion in the PAS domain thought to be important for protein–protein interactions (Zheng et al. 1999), were crossed with mCry1−/− or mCry2−/− mice (van der Horst et al. 1999). The double heterozygous offspring was intercrossed to produce wild-type and homozygous mutant animals. Per2−/−mCry1−/− and Per2−/−mCry2−/− mice (representative genotyping shown in Fig. 1A) were obtained at the expected Mendelian ratios, appeared normal, and were morphologically indistinguishable from wild-type animals.

To determine the influence of inactivation of either mCry1 or mCry2 on circadian behavior of Per2−/− mice, mutant and wild-type animals were individually housed in circadian activity-monitoring chambers (Albrecht and Oster 2001) for detection of wheel-running activity, an accurate measure of circadian rhythmicity. Mice were kept in a 15-h light:12-h dark cycle (LD 15:12, or LD) for several days to establish entrainment, and were subsequently kept in constant darkness (DD). Under DD conditions, homozygous Per2−/− mCry1−/− and Per2−/−mCry2−/− animals displayed activity patterns similar to that of wild-type mice (Fig. 1B). In constant darkness, Per2−/−mCry−/− mutant animals lost circadian rhythmicity after a few days (Fig. 1C), as described previously (Zheng et al. 1999–2001; Eae et al. 2001). In contrast, Per2−/−mCry1−/− mice (van der Horst et al. 1999; Vitaterna et al. 1999; Eae et al. 2001; Zheng et al. 2001). Surprisingly, Per2−/−mCry2−/− animals maintained a circadian rhythm in DD (Fig. 1E). Determination of the period length by an automated analysis.
Results

Figure 1. Generation of mPer2/mCry2 double mutant mice and representative locomotor activity records. (a) Southern blot analysis of wild-type (WT), Per2<sup>−/−</sup>, mCry2<sup>−/−</sup>, mCry1<sup>−/−</sup>, and mCry1<sup>−/−</sup>mCry2<sup>−/−</sup> tail DNA. The probe hybridizes to a 3.1-kb wild-type and a 2.9-kb mutant fragment of BamHI-digested genomic DNA. The mCry probe detects a 0.9-kb wild-type and a 4.4-kb NcoI digested fragment of the targeted locus. In mCry2 mutants, the wild-type allele is detected by hybridization of the probe to a 3.1-kb BamHI fragment, whereas the mutant allele yields a 0.9-kb band. The left panel indicates size of DNA fragments. (b) Representative locomotor activity records of wild-type (WT), Per2<sup>−/−</sup>, mPer2<sup>−/−</sup>, mCry2<sup>−/−</sup>, and mCry1<sup>−/−</sup>mCry2<sup>−/−</sup> mice. All animals were kept in a 12-h light:12-h dark cycle (LD) for at least 7 d before release into constant darkness (DD), indicated by the line over the DD. Activity is represented by black bars (three ect hight, 7th, 11th, and 23rd when resolution per 5 min period and in double-plotted). The top bar indicates light and dark phase in LD. For the first 5 d in DD, wheel rotations per day were 302 (s.e., 504) (n = 23) for wild-type animals, 17,653 ± 3,931 (n = 17) for Per2<sup>−/−</sup> mutants, 16,658 ± 3,931 (n = 13) for mPer2<sup>−/−</sup> mutants, and 17,653 ± 3,931 (n = 16) for mCry1<sup>−/−</sup>mCry2<sup>−/−</sup> mutants.

Table 1 shows that levels of total wheel-running activity (see Fig. 1b legend), indicating that circadian period measurements were not influenced by ablation running behavior. To assess whether the onset of circadian rhythmicity in Per2<sup>−/−</sup>mCry2<sup>−/−</sup> mutant animals by additional inactivation of the mCry2<sup>−/−</sup> gene was reflected at the molecular level, we examined the expression patterns of the mPer1, mPer2, mCry1, and mBmal1 clock genes in the SCN of wild-type and mutant animals under DD (Fig. 2a–d) and LD (Fig. 2e–h) conditions. In situ hybridization experiments revealed that mPer1 expression in wild-type mice peaks at CT6, which is in line with previous reports (Sun et al. 1997; Tei et al. 1997). In Per2<sup>−/−</sup>mCry2<sup>−/−</sup> mutant animals, this rhythmicity is severely blunted (Fig. 2a). As might be expected from the behavioral data, mPer1 gene ex.

Figure 2. In situ hybridization profiles of cycling clock genes in the SCN of wild-type (solid line), Per2<sup>−/−</sup> (dashed line), Per2<sup>−/−</sup>mCry2<sup>−/−</sup> (dashed line), and Per2<sup>−/−</sup>mCry1<sup>−/−</sup>mCry2<sup>−/−</sup> (dotted line) mice. Data are given as a percentage of total counts over 24 h. mPer1 expression in DD (a) mPer1 expression in DD (b) mPer1 expression in LD (c) mCry1 expression in DD (d) mCry1 expression in LD (e) mBmal1 expression in DD (f) mBmal1 expression in LD (g) mBmal1 expression in DD (h) mBmal1 expression in LD.
pression was back to a normal amplitude, reaching a maximum at C7E in Pspδ3δδ/IncrCy2−/− mutants (Fig. 2a). Thus, circadian expression of mPer1 in Pspδ3δδ mutant mice is rescued by inactivation of mCry2. A similar rescue was observed for mCry expression both amplitude and timing of mCry oscillation in Pspδ3δδ/IncrCy2−/− mutant mice, which is evident from the reduced amplitude and shift of maximal expression levels to earlier CT times (Fig. 2d). In Pspδ3δδ/IncrCy2−/− mutant mice, the expression of Bmal1 was not significantly different from that observed in wild-type animals (Fig. 2c). The results obtained in Pspδ3δδ/IncrCy2−/− mutant mice are comparable to those of the wild-type animals (Fig. 2d). Oscillation of mPer1 expression was not rescued in Pspδ3δδ/IncrCy2−/− mutant mice, whereas mCry expression was restored. Thus, the behavior of mCry expression resembles that of the wild-type animals (Fig. 2d). Taken together, these data strongly indicate that normal circadian behavior and core oscillator function are possible in the absence of functional mPer2 and mCry2 genes (Fig. 1).

We also found a rescue of the amplitude of mPer1, mCry1, and Bmal1 expression profiles in Pspδ3δδ/IncrCy2−/− animals under LD conditions (Fig. 2d), whereas mPer2 mRNA levels remained low as in the Pspδ3δδ mutant mice (Fig. 2f). These results are comparable to the expression patterns observed under DD conditions (Fig. 2a, b, c, d), except that in the Pspδ3δδ mutant mice, the amplitude of Bmal1 expression was almost as high as in wild-type animals (Fig. 2f), whereas mPer2 mRNA levels were lowest as in the Pspδ3δδ mutant mice (Fig. 2f). These results are comparable to the expression patterns observed under DD conditions (Fig. 2a, b, c, d). This suggests that mPer2 mRNA levels are high at the beginning of the dark phase (Zheng et al. 1999), as also shown in Pspδ3δδ/IncrCy2−/− animals (Fig. 2f). Thus, in this case, further underlines the correlation of the circadian phenotype of Pspδ3δδ mutant mice by inactivation of mCry2.

As shown above, Pspδ3δδ/IncrCy2−/− mice are arrhythmic in DD. To investigate whether these animals lack a circadian clockwork, we studied the expression patterns of mPer1, mCry1, and Bmal1 clock gene expression profiles under LD conditions (Fig. 2f, g, h). We found that in Pspδ3δδ/IncrCy2−/− mutant animals, none of these genes are rhythmic as expressed and mRNA levels are very low (Fig. 2f, g, h). This indicates that these animals lack a functional clock and that their diurnal behavioral activity under LD conditions (Fig. 1d) is most likely driven by the light/dark cycle. The loss of circadian expression of mCry1 in the Pspδ3δδ mutant mice following inactivation of mCry2 suggests that mCry2 might play a role in transcriptional regulation of Bmal1.

The experiments described above indicate that an inactivation of mCry2 in Pspδ3δδ mice rescues circadian rhythmicity at the behavioral level as well as at the molecular level in the SCN. To determine whether this is also valid for peripheral clocks, we performed Northern blot analysis on kidney tissues. The expression profiles of mPer1, mCry1, mCry2, and Bmal1 (Fig. 3a-d) under DD conditions are comparable to those observed in the SCN (Fig. 2a-d), except that peak expression of these genes is delayed by several hours in the kidney (Zheng et al. 2001). Thus, rescue of circadian gene expression and probably clock function is also manifest in the periphery.

Inactivation of Bmal1 causes immediate arrhythmicity, indicating that its gene product is crucial for circadian rhythmicity. Since in Pspδ3δδ mutant animals, Bmal1 mRNA cycling seems to be dampened under DD conditions (Figs. 2a, b, d), we investigated expression levels of the latter transcript at CT18 (wild-type) and Pspδ3δδ/IncrCy2−/− animals) and CT2 (Pspδ3δδ mutant mice) at 0, 5, 10, and 15 d after transfer of animals to constant darkness conditions. We found that in wild-type and Pspδ3δδ/IncrCy2−/− animals, Bmal1 expression is maintained, whereas in Pspδ3δδ/IncrCy2−/− animals, this mRNA rhythm decreases gradually until it disappears after 5–10 d (Fig. 4a). This observation parallels the gradual loss of circadian rhythmicity of mPer1 mutant mice in DD (Fig. 1e, Zhang et al. 1999, 2001; Rae et al. 2001) and further supports the idea that the mPER1 protein is involved in regulating Bmal1 expression (Shearman et al. 2000). The mCry1 and mCry2 proteins were recently reported to activate the Bmal1 promoter in vitro (Yao et al. 2002). Because only mCry1+/−/mCry2−/− and mPer1−/−/Pspδ3δδ−/− double mutant mice, but not the corresponding single mutant animals, display an immediate loss of circadian rhythmicity in constant darkness, neither mCry1 and mCry2 proteins alone or mPER1 and mPER2 proteins alone are likely to be responsible for rhythmic Bmal1 transcriptional activation. Rather, Bmal1 gene expression appears to be regulated by a combination of mPer2 and mCry2 gene products. Analogous to mCry1+/−/mCry2−/− (van der Horst et al. 1999) and mPer1−/−/Pspδ3δδ−/− double mutant mice (Rae et al. 2001; Zhang et al. 2001), inactivation of both mPer2 and mCry2 leads to immediate loss of circadian rhythmicity (Fig. 1d). This implies that Pspδ3δδ/IncrCy2−/− mutant mice are lacking a circadian clock and that mPER1 and mCry2 proteins are not sufficient for maintenance of circadian rhythmicity and expression of clock genes such as mPer1, mCry2, and Bmal1 (Rae 2001, Zhang et al. 2001). However, mice with inactivated mPer2 and mCry2 genes display stable circadian rhythmicity and
Figure 4. (a) Relative maximal expression in the SCN of wild-type (black column), Per2^−/− (white column), and Per2^−/−/mCry2^−/− (gray column) mice relative to their peak in wild-type mice. Each value is the mean ± S.E. of five animals except for day 10 in Per2^−/− mice where only one strain was used. (b) Light-induced advance phase latency in wild-type (black column), Per2^−/− (white column), and Per2^−/−/mCry2^−/− (gray column) mice. After entrainment to a 12:12-h LD cycle, animals were exposed to a 15 min light pulse (illuminator) at the beginning (2T14) or in the end (2T22) of the lights off interval relative to DD. Negative values represent phase delays and positive values phase advances. Each value is the mean ± S.E. of 10-14 animals. Only animals which retained rhythmicity for at least 7 consecutive days in DD were used for quantitation. Significance as indicated by asterisks was determined by one-way ANOVA with subsequent Bonferroni posttest (p < 0.05).

normal Retro expression suggests that mPER1 and mCRY1 proteins can maintain circadian rhythmicity and rhythmic Retro expression in the absence of functional mPER2 and mCRY2 genes. These data suggest that in Per2^−/− single mutant mice, the functional mCRY2 gene product interacts with mPER1 and/or mCRY1, which leads to a gradual loss of circadian rhythmicity. Thus, mPer1 only in combination with mCry1 and in the absence of a functional mCry2 gene seems to be able to maintain the circadian clock in vivo. However, the absence of mPer1 in mCry2^−/− mice (Albrecht et al. 2001) was not rescued in Per2^−/−/mCry2^−/− mice (Fig. 4b). This indicates that in Per2^−/−/mCry2^−/− mice, PER1 can substitute PER2 in the core clock mechanism but not in the light input signaling pathway. As a note of caution, one should keep in mind that the phenotypic effect of genetic modifications of genetically altered animals in a nonhomogeneous genetic background are prone to epigenetic effects. However, we tried to minimize this risk by using double heterozygous animals throughout the crossings from which the wild type and mutants were derived. Thus the wild-type control animals in this study have a mixed background similar to that of the mutants.

Our observations would be compatible with a hypothesis that there is a hierarchy of activity potentials for the entire murine PER2 and TIP2 protein. The activity potentials on the negative limb will have effects on the positive limb of the clock mechanism as well. Thus one could envisage a similar hierarchy of activity potentials for the positive limb of the clock mechanism as well. For example, a complex of two PER1 proteins and two CRY2 proteins would have a lower activity potential than a complex composed of two PER2 and two CRY1 proteins. In turn, complex that only forms in a CRY2 mutant composed of PER1, PER2, CRY1, and CRY2 would have an intermediate activity potential. Assuming that circadian clocks are based on limit cycles of feedback transcription to generate circadian rhythms (Chen and Mackey 1998, Leloup et al. 1999), too low or too high-repressor potentials will destabilize the limit cycle, and the system will fall into equilibrium and become arrhythmic. Such a model might be further supported by the gene dosage effects observed in mCry double mutant mice. For instance, knocking out one mCry1 allele in mCry2^−/− mice normalizes the behavioral rhythm, and knocking out one mCry2 allele in mCry2^−/− mice further disturbs rhythmicity (van der Horst et al. 1999). In mPer1^−/− animals, both PER subunits would be PER2 proteins with a high repression potential, which accelerates the feedback loop, as has been observed (Zheng et al. 2001). However, the overall repression potential is still compatible with the parameters of the limit cycle, and therefore the mPer1^−/− mice are still arrhythmic. In Per2^−/− mutant animals, both PER subunits are PER1 proteins that are weak repressors, and therefore the feedback loop is slowed down to a degree that is no longer compatible with the limit cycle, thus, Per2^−/− mice become arrhythmic. In Per2^−/−/mCry2^−/− mice, however, the repression complex is composed of two CRY1 proteins (strong repressor) and two PER1 proteins (weak repressor). The overall repression potential of this complex would approach the wild-type complex is and is thus compatible with the parameters of the limit cycle. Per2^−/−/mCry2^−/− mice would therefore be rhythmic, which is compatible with our observations.
Materials and methods

Generation of mcf/cry and mcf/cry mutant mice

We mated mcf 

Materials and methods

Generation of mcf/cry and mcf/cry mutant mice

We mated mcf 

Acknowledgments

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References


Results


2.2. Publication: “Loss of circadian rhythmicity in ageing \( mPer1^{-/-} mCry2^{-/-} \) mutant mice”

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in preparation
Abstract

The *mPer1*, *mPer2* and *mCry1, mCry2* genes play a central role in the molecular mechanism driving the central pacemaker of the mammalian circadian clock, located in the *suprachiasmatic nuclei* (SCN) of the hypothalamus. *In vitro* studies suggest a close interaction of all mPER and mCRY proteins. We investigated mPER and mCRY interactions *in vivo* by generating different combinations of *mPer/mCry* double mutant mice. We previously showed that *mCry2* acts as a non-allelic suppressor of *mPer2* in the core clock mechanism. Here we focus on the circadian phenotypes of *mPer1/mCry* double mutant animals and find a decay of the clock with age in *mPer1*<sup>-/-</sup> *mCry2*<sup>-/-</sup> mice at the behavioural and the molecular level. Our findings indicate that complexes consisting of different combinations of mPER and mCRY proteins are not redundant *in vivo* and have different potentials in transcriptional regulation in the system of autoregulatory feedback loops driving the circadian clock.
Introduction

The earth's rotation around the sun has strongly influenced temporal organisation of the mammalian organism manifested by near 24 hour rhythms of biological processes (Pittendrigh, 1993) including the sleep-wake cycle, energy metabolism, body temperature, renal activity and blood pressure. These rhythms are maintained even in the absence of external time signals (Zeitgeber). They are driven by a central clock located in the suprachiasmatic nuclei (SCN) of the ventral hypothalamus (Ralph et al., 1990; Rusak and Zucker, 1979). Since the internal period length generated by this pacemaker is not exactly 24 hours (hence the term ‘circadian’, from the Latin circa dies which translates to ‘about one day’), the clock has to be reset every day by an input pathway synchronising the organism's biological processes with geophysical time. This is accomplished by monitoring the daily variation in light intensity by photoreceptors in the eye that project directly via the retinohypothalamic tract (RHT; Rusak and Zucker, 1979) or indirectly via the intergeniculate leaflet (IGL; Jacob et al., 1999) to the SCN. The oscillations generated in the SCN are translated into overt rhythms in behaviour and physiology through output pathways that probably involve both chemical and electrical signals. These signals are essential for the maintenance of overt circadian rhythms, but most cells of peripheral tissues possess a functional circadian oscillator with a molecular organisation very similar to that of SCN neurons (Balsalobre et al., 1998; Yamazaki et al., 2000).

At the molecular level, circadian rhythms are generated by the integration of autoregulatory transcriptional/translational feedback loops (TTLs; Albrecht, 2002; Allada et al., 2001; Reppert and Weaver, 2002). In the mammalian system, the TTL can be subdivided into a positive and a negative limb. The positive limb is constituted by the PAS helix-loop-helix transcription factors CLOCK and BMAL1 that bind upon heterodimerisation to enhancer elements termed E-boxes regulating transcription of Period (mPer) and probably also Cryptochrome (mCry) genes. The mPER and mCRY proteins are components of the negative limb that attenuate the CLOCK/BMAL1-mediated activation of their own genes and hence generate a negative feedback. There is evidence that a mPER/mCRY complex interacts directly with the CLOCK/BMAL1 complex bound to chromatin (Lee et al., 2001). A number of posttranslational events such as phosphorylation, ubiquitylation, degradation and intracellular transport seem to be critical for the generation of oscillations in clock gene products and the stabilisation of a 24h period (Kume et al., 1999; Lee et al., 2001; Miyazaki et al., 2001; Vielhaber et al., 2001; Yagita et al., 2002; Yagita et al., 2000; Yu et al., 2002).
Additionally, the two limbs of the TTL are linked by the nuclear orphan receptor REV-ERBα, which is under the influence of \textit{mPer} and \textit{mCry} genes and controls transcription of \textit{Bmal1} (Preitner et al., 2002). In mammals three \textit{Per} genes, \textit{mPer1} (Sun et al., 1997; Tei et al., 1997), \textit{mPer2} (Albrecht et al., 1997; Shearman et al., 1997), and \textit{mPer3} (Zylka et al., 1998) and two \textit{Cry} genes, \textit{mCry1} and \textit{mCry2} (Miyamoto and Sancar, 1998) have been identified. While \textit{mPer3} seems not to be necessary for the generation of circadian rhythmicity (Shearman et al., 2000), \textit{mPer1}, \textit{mPer2} and both \textit{mCry} genes have been demonstrated to play essential roles in the central oscillator as well as in the light driven input pathway to the clock (Albrecht et al., 2001; Bae et al., 2001; Cermakian et al., 2001; van der Horst et al., 1999; Vitaterna et al., 1999; Zheng et al., 2001; Zheng et al., 1999).

The molecular mechanism of clock autoregulation has largely been studied \textit{in vitro} (Gekakis et al., 1998; Kume et al., 1999; Miyazaki et al., 2001; Vielhaber et al., 2001; Yagita et al., 2002; Yagita et al., 2000; Yu et al., 2002). These studies point to multiple physical interactions between all \textit{mPER} and \textit{mCRY} proteins. However, the time course of protein availability, modification and localisation is difficult to resolve in cell and slice cultures (Hamada et al., 2001; Jagota et al., 2000; Lee et al., 2001). To elucidate the functional relationship between the \textit{mPer} and \textit{mCry} genes \textit{in vivo}, we started to inactivate different combinations of \textit{mPer} and \textit{mCry} genes in mice. Disruption of \textit{mCry2} restores circadian rhythmicity in \textit{mPer2} mutant mice, suggesting that \textit{mCry2} can act as a non-allelic suppressor of \textit{mPer2} (Oster et al., 2002b). In contrast, additional inactivation of \textit{mCry1} in \textit{mPer2} mutant animals leads to an immediate loss of circadian rhythmicity (Oster et al., 2002b).

Here we show that \textit{mPer1}^{1/2} \textit{mCry1}^{1/2} mice maintain a functional circadian clock and that \textit{mPer1}^{1/2} \textit{mCry2}^{1/2} mice initially display circadian rhythmic behaviour and gene expression. After a few months however, the rhythm of \textit{mPer1}^{1/2} \textit{mCry2}^{1/2} mice breaks down. This loss of rhythmicity is accompanied by altered regulation of expression of core clock components. Additionally, the light responsiveness of the clock in \textit{mPer1}^{1/2} \textit{mCry2}^{1/2} mice is affected at the behavioural and molecular levels. Interestingly, this defect seems to have its roots in the signal transduction pathway of the ganglion cell layer in the retina. Taken together with previous observations, our results indicate that the amount of \textit{mPER} and \textit{mCRY} proteins and hence the composition of \textit{mPER/mCRY} complexes are critical for generation and maintenance of circadian rhythms. The destabilisation of these complexes in \textit{mPer1}^{1/2} \textit{mCry2}^{1/2} mice disrupts the ability of the TTL to compensate age-related changes in transcriptional and posttranscriptional efficiency resulting in a disruption of the circadian clock in older animals.
Results

mPer1 acts as a non-allelic suppressor of mCry1

To begin to understand the in vivo function of the mPer and mCry genes in the clock mechanism, we generated mice with disruptions in both the mPer1/mCry1 and mPer1/mCry2 genes respectively. Mice with a deletion of the mPer1 gene (Zheng et al., 2001) were crossed with mCry1 \(^{-/-}\) or mCry2 \(^{+/+}\) mice, respectively (van der Horst et al., 1999). The double-heterozygous offspring was intercrossed to produce wild-type and homozygous mutant animals. mPer1 \(^{-/-}\)mCry1 \(^{+/+}\) and mPer1 \(^{-/-}\)mCry2 \(^{-/-}\) mice (representative genotyping shown in Fig. 1A) were obtained at the expected Mendelian ratios and were morphologically indistinguishable from wild-type animals. The animals appeared normal in fertility, although in mPer1/mCry double mutant mice the intervals between two litters seem to increase significantly with progressing age (data not shown).

To determine the influence of inactivation of either the mCry1 or mCry2 gene on circadian behaviour of mPer1 \(^{-/-}\) mice, mutant and wild-type animals were individually housed in circadian activity-monitoring chambers (Albrecht and Oster, 2001) for analysis of wheel-running activity, an accurate measure of circadian rhythmicity. Mice were kept in a 12 hour light/12 hour dark cycle (LD 12:12, or LD) for several days to establish entrainment, and were subsequently kept in constant darkness (DD). Under LD and DD conditions mPer1 \(^{-/-}\)mCry1 \(^{+/+}\) animals displayed activity patterns and expression patterns of clock components similar to that of wild-type mice (Fig. 1B, C and supplemental Fig. 2). Under DD conditions mPer1 \(^{-/-}\)mCry1 \(^{-/-}\) mutant mice displayed a period length (\(\tau\)) of 23.7 \(\pm\) 0.2 h (mean \(\pm\) S.D., n=15) which is similar to that of wild-type animals (\(\tau\) = 23.8 \(\pm\) 0.1 h; n=17). Thus an additional deletion of mPer1 rescues the short period phenotype of mCry2 deficient mice (van der Horst et al., 1999) indicating that mPer1 acts as a non-allelic suppressor of mCry1.

Loss of circadian wheel running activity rhythms in ageing mPer1 \(^{-/-}\)mCry2 \(^{-/-}\) double mutant mice

mPer1 \(^{-/-}\)mCry2 \(^{-/-}\) animals that were between 2 and 6 months old (‘young’ mPer1 \(^{-/-}\)mCry2 \(^{-/-}\) mice) displayed a diurnal activity pattern like wild-type animals under LD conditions. However, onset of activity was delayed and highest activity could be observed in the second half of the night (Fig. 1D). Interestingly, mPer1 \(^{-/-}\)mCry2 \(^{-/-}\) animals that were more
than six months old ('old' mPer1\(^{-/-}\)mCry2\(^{-/-}\) mice) showed a disturbed diurnal activity pattern under LD conditions (Fig. 1F), but a faint 24 hour rhythm could still be detected when applying \(\chi^2\) periodogram analysis (Fig. 1G). Under DD conditions young mPer1\(^{-/-}\)mCry2\(^{-/-}\) mice display a long period (\(\tau\)) of 25.3 \(\pm\) 0.2 h (mean \(\pm\) S.D., n=14) compared to wild-type animals (\(\tau = 23.8 \pm 0.1\) h; n=17) (Fig. 1D and E). In contrast to young mPer1\(^{-/-}\)mCry2\(^{-/-}\) mice, old mPer1\(^{-/-}\)mCry2\(^{-/-}\) mice were arrhythmic under DD conditions (Fig. 1F and I). The transition from a rhythmic to an arrhythmic phenotype, however, did not occur in all animals at the same age, but age is correlated with loss of circadian wheel-running activity (Fig. 1H).

mPer1\(^{-/-}\)mCry2\(^{-/-}\) mice that are between two and six months old all display circadian activity patterns, whereas only about 60% of animals between six and twelve months of age maintain circadian rhythmicity. Interestingly, 87% of the mPer1\(^{-/-}\)mCry2\(^{-/-}\) mice older than twelve months are arrhythmic. We did not observe a comparable age-related loss of rhythmicity in wild-type, mPer1\(^{-/-}\) and mCry2\(^{-/-}\) mice (Fig. 1H and supplemental Fig. 1). However, we cannot completely rule out that arrhythmicity could be observed sometime in ageing single mutants as well but the stability of rhythmicity appears dramatically decreased in the double knock out context. Following this observation we divided all double mutant animals into two groups for use in subsequent experiments. Rhythmic animals are referred to as ‘young mPer1\(^{-/-}\)mCry2\(^{-/-}\)’ and arrhythmic animals are referred to as ‘old mPer1\(^{-/-}\)mCry2\(^{-/-}\)’. This does not necessarily correspond with the physical age of each individual because all animals used for mRNA and protein analyses were between 6 and 12 months old and the onset of arrhythmicity does not occur at the same age in every animal. However, since there is a clear correlation between age and rhythmicity the average age of ‘old’ animals is higher than of the ‘young’.

Alterations in expression levels of clock components in ageing mPer1\(^{-/-}\)mCry2\(^{-/-}\) double mutant mice

To investigate whether the loss of circadian rhythmicity in ageing mPer1\(^{-/-}\)mCry2\(^{-/-}\) mutant mice was reflected at the molecular level, we examined the expression patterns of the mPer2, mCry1 and Bmal1 genes. Under LD and DD conditions mPer2 mRNA expression in the SCN was comparable in young mPer1\(^{-/-}\)mCry2\(^{-/-}\) mice and wild-type animals with peak levels at Zeitgeber time (ZT) or circadian time (CT) 12 (Fig. 2A and B). Interestingly, mPer2 mRNA expression was markedly reduced in old mPer1\(^{-/-}\)mCry2\(^{-/-}\) mice at ZT12 with the diurnal expression pattern almost not detectable (Fig. 2A). In the kidney, a similar reduction of
mPer2 mRNA expression in old mPer1\(^{-/-}\)mCry2\(^{-/-}\) mice could be observed with a maximum of expression at ZT12 in wild-type animals (Cermakian et al., 2001; Zheng et al., 2001) and young mPer1\(^{-/-}\)mCry2\(^{-/-}\) mice (Fig. 2D and E). Expression patterns of old mPer1\(^{-/-}\)mCry2\(^{-/-}\) mice in DD were not determined because these mice loose circadian rhythm and no circadian times can be determined. To justify that the observed changes are related to the double knock out status of the animals we looked for mPer2 expression in mPer1\(^{-/-}\) and mCry2\(^{-/-}\) mice of the same age. mPer2 oscillation appeared to be normal in both LD and DD in the single mutants with no detectable reduction in the amplitude (supplemental figure 3 A and B).

To investigate whether the reduced mPer2 mRNA expression in old mPer1\(^{-/-}\)mCry2\(^{-/-}\) mice was manifested at the protein level, we examined the presence of mPER2 protein in the SCN by immunohistochemistry. In wild-type and young mPer1\(^{-/-}\)mCry2\(^{-/-}\) mice protein levels are high between ZT12 and ZT18 (Fig. 2C; Field et al., 2000) which is a few hours later than mRNA expression (Fig. 2A). In old mPer1\(^{-/-}\)mCry2\(^{-/-}\) mice however, protein levels are low comparable to mRNA expression (Fig. 2A and C).

There is evidence that mPER1/2 and mCRY1/2 can indirectly activate Bmal1 expression (Preitner et al., 2002; Yu et al., 2002) via the inhibition of Rev-Erb\(\alpha\). REV-ERB\(\alpha\) protein inhibits the transcription of Bmal1 and possibly Clock. On the other hand CLOCK/BMAL1 protein activates Rev-Erb\(\alpha\) expression (Preitner et al., 2002). mPER1/2 and mCRY1/2 proteins can interfere with CLOCK/BMAL1 mediated transcriptional activation. Therefore we investigated the expression pattern of mCry1 and Bmal1 in the SCN. In wild-type, mPer1\(^{-/-}\), mCry2\(^{-/-}\) and young mPer1\(^{-/-}\)mCry2\(^{-/-}\) mice mCry1 mRNA expression was similar in LD and DD with a maximum at ZT12 or CT12, respectively (Fig. 3A, B and supplemental figure 3 C-F). This is consistent with previous reports on wild-type animals (Okamura et al., 1999). Interestingly, mCry1 mRNA expression was normal in old mPer1\(^{-/-}\)mCry2\(^{-/-}\) mice in LD (Fig. 3A), which is in marked contrast to the reduced mPer2 mRNA expression in these mice (Fig. 2A and B). Therefore we examined mCRY1 protein levels in the SCN by immunohistochemistry. mCRY1 protein levels were cycling in wild-type animals with peak expression between ZT12 and ZT18 (Fig. 3C) as reported previously (Field et al., 2000). Similarly, young mPer1\(^{-/-}\)mCry2\(^{-/-}\) mice displayed cycling expression of mCRY1 protein, but the expression levels at ZT0 (24) were notably higher than in wild-type animals (Fig. 3C). The elevated expression of mCRY1 protein at ZT0 (24) became even more pronounced in old mPer1\(^{-/-}\)mCry2\(^{-/-}\) mice leading to almost constant high levels of mCRY1 protein throughout the 24 hour LD cycle (Fig. 3C). In age matched mPer1\(^{-/-}\) and mCry2\(^{-/-}\) mice however, mCRY1 protein cycling was observed (see Suppl Fig. 3) indicating that the abnormal regulation of the
mCRY1 protein in old mPer1\(^{-/-}\)mCry2\(^{-/-}\) mice is due to the inactivation of both mPer1 and mCry2. Next, we looked at Bmal1 mRNA expression in the SCN under LD and DD conditions. In wild-type and mPer1\(^{-/-}\) animals a maximum was seen at ZT and CT 18 (supplemental figure 3 E and F) as previously observed (Honma et al., 1998). In mCry2\(^{-/-}\) animals the maximum of Bmal1 expression was slightly delayed (supplemental figure 3 E and F). Young mPer1\(^{-/-}\)mCry2\(^{-/-}\) animals displayed a similar expression pattern, although the levels tended to be slightly decreased (Fig. 3D and E). In old mPer1\(^{-/-}\)mCry2\(^{-/-}\) mice Bmal1 mRNA levels were significantly reduced in LD (p< 0.05) (Fig. 3D) which is comparable to the low mPer2 mRNA expression observed in old mPer1\(^{-/-}\)mCry2\(^{-/-}\) animals (Fig. 2A). Taken together it seems that mPer2 mRNA levels as well as protein levels are normal in young mPer1\(^{-/-}\)mCry2\(^{-/-}\) animals, whereas in old mPer1\(^{-/-}\)mCry2\(^{-/-}\) mice the amounts are strongly reduced. The same was observed for Bmal1 mRNA but not for mCry1 mRNA. However, mCRY1 protein levels are constitutively high in old mPer1\(^{-/-}\)mCry2\(^{-/-}\) mice.

From the expression data in old mPer1\(^{-/-}\), mCry2\(^{-/-}\) and mPer1\(^{-/-}\)mCry2\(^{-/-}\) mice described above it is reasonable to assume that the circadian phenotype observed in mPer1\(^{-/-}\)mCry2\(^{-/-}\) mice is a consequence of the simultaneous inactivation of the mPer1 and mCry2 genes in these animals. Therefore we focussed in the following studies on the comparison between wild-type and double mutant animals.

Loss of light inducibility of mPer2 mRNA and effect on delaying the clock phase in mPer1\(^{-/-}\)mCry2\(^{-/-}\) mice

As described above, the amplitude of cyclic mPer2 mRNA expression declines with progressing age. In addition to CLOCK-BMAL1-driven circadian expression, phase-resetting light stimuli are known to induce mPer expression via a cAMP-responsive element in the promoter (Motzkus et al., 2000; Travnickova-Bendova et al., 2002). To investigate whether ageing affects light inducibility of the mPer2 gene in the SCN of mPer1\(^{-/-}\)mCry2\(^{-/-}\) mice, we applied a 15 minutes nocturnal light pulse at ZT14 to the animals. Wild-type mice displayed a significant increase of mPer2 mRNA (Fig. 4A and B) as described previously (Albrecht et al., 1997). Interestingly, induction of mPer2 mRNA was significantly impaired in young mPer1\(^{-/-}\)mCry2\(^{-/-}\) mice compared to wild-type animals (p < 0.05; Fig. 4A and B). This observation was even more pronounced in old mPer1\(^{-/-}\)mCry2\(^{-/-}\) mice (p<0.001; Fig. 4A and B) indicating that the light signal transduction pathway might be defective. Therefore we set out to investigate light dependent phosphorylation of CREB at position 133 (CREB-Ser\(^{133}\)). We found that in
wild-type animals phosphorylation at CREB-Ser\textsuperscript{133} was induced by light (Fig. 4C and D) as described previously (von Gall et al., 1998). Young \textit{mPer1\textsuperscript{-/-}mCry2\textsuperscript{-/-}} animals tended to show slightly reduced (but statistically not significant) phosphorylation of CREB-Ser\textsuperscript{133} (Fig. 4C and D). In contrast, old \textit{mPer1\textsuperscript{-/-}mCry2\textsuperscript{-/-}} mice hardly displayed phosphorylation at CREB-Ser\textsuperscript{133} \((p<0.001; \text{Fig. 4C and D})\) suggesting a degeneration of the light input pathway to the clock. In a next step we wanted to investigate, whether the observed reduction of \textit{mPer2} mRNA induction and CREB-Ser\textsuperscript{133} phosphorylation in \textit{mPer1\textsuperscript{-/-}mCry2\textsuperscript{-/-}} mice had behavioural consequences. We monitored wheel-running activity before and after a 15min light pulse at ZT14 and 22 as well as at CT14 and 22 and measured the phase shifts (Fig. 4E and F). In wild-type animals we observed a phase delay at ZT14 of \(82 \pm 10\) min (mean \pm S.D., \(n=14\)) and \(87.3 \pm 9.3\) min (\(n=14\)) at CT 14 and a phase advance of \(35 \pm 6.7\) min (\(n=14\)) at ZT22 and \(39.3 \pm 6.7\) min (\(n=14\)) at CT22. In \textit{mPer1\textsuperscript{-/-}mCry2\textsuperscript{-/-}} animals only the phase shifts for young animals could be determined because old animals are arrhythmic in DD, which precludes determination of phase shifts. \textit{mPer1\textsuperscript{-/-}mCry1\textsuperscript{-/-}} mice delayed their phase at ZT14 similar to wild-type animals \((86.5 \pm 12\) min; \(n=11\); Fig 4E). Remarkably, in \textit{mPer1\textsuperscript{-/-}mCry2\textsuperscript{-/-}} mice phase delays at CT14 tended to be reduced \((60 \pm 13\) min; with \(p=0.0539\) (\(n=10\)) missing the criterion of \(p < 0.05\) for significance; Fig. 4F). However, at ZT22 and CT22 phase advances in both \textit{mPer1/mCry1} \((1.3 \pm 13\) min; \(n=11\)) and \textit{mPer1/mCry2} \((7.3 \pm 10.5\) min; \(n=10\)) double mutant animals were abolished (Fig. 4E and F), which is comparable to the inability of \textit{mPer1\textsuperscript{-/-}} mice to advance clock phase after a 15min light pulse (Albrecht et al., 2001). These results suggest that the defect in advancing clock phase is due to a lack of \textit{mPer1} in both \textit{mPer1\textsuperscript{-/-}mCry1\textsuperscript{-/-}} and \textit{mPer1\textsuperscript{-/-}mCry2\textsuperscript{-/-}} mice. The impairment of delaying clock phase in \textit{mPer1\textsuperscript{-/-}mCry2\textsuperscript{-/-}} mice at CT14 is probably due to a reduction of phosphorylation in CREB-Ser\textsuperscript{133} and reduced expression of \textit{mPer2} mRNA. This is in line with previous findings that \textit{mPer2} mutant mice are defective in delaying clock phase (Albrecht et al., 2001).

\textit{CREB phosphorylation at Ser 133 is decreased in the eye of \textit{mPer1\textsuperscript{-/-}mCry2\textsuperscript{-/-}} mice}  

The sloppy onset of wheel running activity in LD and the strong reduction in CREB phosphorylation at Ser 133 in the SCN of old \textit{mPer1\textsuperscript{-/-}mCry2\textsuperscript{-/-}} mice indicated that light signalling from the eye to the SCN might be defective. We therefore performed a detailed (immuno)histochemical analysis of the retinas from wild-type, \textit{mPer1\textsuperscript{-/-}}, \textit{mCry2\textsuperscript{-/-}} and \textit{mPer1\textsuperscript{-/-}mCry2\textsuperscript{-/-}} mice, respectively (Fig. 5). Using the Gomori staining procedure we could not detect overt morphological differences between wild-type, \textit{mPer1\textsuperscript{-/-}}, \textit{mCry2\textsuperscript{-/-}}, young and old \textit{mPer1\textsuperscript{-/-}}
/-mCry2/- retina results (Fig. 5A). To reveal cell death in the different layers of the retina, we performed lipofuscin staining. We did not observe any difference between wild-type, mPer1/-, mCry2/-, young and old mPer1/-mCry2/- mice (Fig. 5B), indicating that the retinas of all mice were intact. Additionally, we performed lipofuscin as well as Congo red histological staining in different regions of the brain with a focus on areas involved in the machinery of the circadian clock. We did not detect any amyloid plaques and mPer1/-mCry2/- mice did not show any differences to wild-type animals (data not shown). Thus, the observed effects of ageing are restricted to the functionality of the circadian system and do not originate from aberrant development or age-related morphological changes in the retina of mPer1/-mCry2/- animals.

Next we investigated phosphorylation of CREB at serine residue 133 in the retina by using an anti Ser133 P-CREB antibody (Fig. 5C). In wild-type animals, in the absence of light stimuli, Ser133 P-CREB was detected in the inner nuclear layer. A light pulse given at ZT14 has been shown to result in increased numbers of immunoreactive nuclei in the inner nuclear layer and ganglion cell layer (Gau et al., 2002). In mPer1/-, mCry2/- and young mPer1/- mCry2/- mice a similar immunoreactivity was seen (Fig. 5C). Old mPer1/-mCry2/- animals however, displayed a reduced number of immunoreactive nuclei in the inner nuclear layer after a light pulse, whereas Ser133 P-CREB staining could hardly be observed in the ganglion cell layer (Fig. 5C). This indicates that phosphorylation of serine 133 in CREB is affected in the retina of old mPer1/-mCry2/- mice. Taken together, these results demonstrate that the profound loss of circadian wheel running behaviour of old mPer1/-mCry2/- mice under LD conditions (Fig. 1F) is due to impaired light signal transduction pathway performance.
Discussion

Interaction of clock components has predominantly been investigated in cultured cells, transiently (over)expressing clock components and E-box-containing reporter constructs (Gekakis et al., 1998; Kume et al., 1999; Yagita et al., 2002; Yagita et al., 2000). Such assays revealed that mPER and mCRY proteins can *in vitro* interact with themselves or each other, thereby forming stabilised complexes that influence nuclear transport of clock proteins or transcriptional regulation of clock genes. In contrast, it is not known to which extent complexes composed of various combinations of mPER and mCRY proteins contribute to circadian oscillator performance *in vivo*. We thus started to conduct genetic experiments by crossing mouse strains with inactivated *mPer* or *mCry* genes and subsequent analysis of circadian behaviour, clock gene and protein expression. We found that *mCry2* can act as a non-allelic suppressor of *mPer2* in the core clock mechanism and hence the presence of only *mPer1* and *mCry1* genes is sufficient to maintain circadian rhythmicity of the clock *in vivo* (Oster et al., 2002b). In this study we investigated the consequences of the absence of *mPer1* in combination with *mCry1* or *mCry2* on the circadian clock.

*mPer1Δ/mCry1Δ* mice display normal circadian rhythmicity but show impaired ability to phase advance the clock

Inactivation of *mPer1* and *mCry1* leads to a behavioural phenotype under LD and DD conditions similar to wild-type animals. *mPer1Δ/mCry1Δ* mice display a period length comparable to wild-type littermates (Fig 1B, C). In comparison to *mCry1Δ* mice however, it seems that the additional loss of *mPer1* in *mCry1Δ* mice leads to an increase in period length to near normal values in DD (23.7 ± 0.2 h for *mPer1Δ/mCry1Δ* mice vs. 22.51 ± 0.06h for *Cry1Δ* mice). This indicates that the loss of *mPer1* rescues the phenotype observed in *mCry1Δ* mice and that the mPER2 and mCRY2 proteins seem to be sufficient to maintain a circadian rhythm with a period that is comparable to wild-type animals. This is also reflected at the molecular level, where *mPer2* and *Bmal1* mRNA rhythms are comparable to the expression patterns in wild-type animals under both LD and DD conditions (see supplemental figure 2). Hence it seems that *mPer1* is a non-allelic suppressor of *mCry1* (or *vice versa*). Interestingly, application of a 15 minute light pulse at ZT22 does not lead to a phase advance as observed in wild-type animals (Fig. 4E). This inability of *mPer1Δ/mCry1Δ* mice to advance clock phase is
comparable to the defect observed in \textit{mPer1}\textsuperscript{−/−} animals (Albrecht et al., 2001). It seems that only circadian core clock functionality is rescued by an inactivation of \textit{mCry1} in \textit{mPer1}\textsuperscript{−/−} mice but not the resetting properties of the clock. This is similar to the observations made in \textit{mPer2\textsuperscript{Brdm1}}\textit{mCry2}\textsuperscript{−/−} mice, which appear to have a normal circadian rhythm but display a defect in delaying clock phase similar to \textit{mPer2\textsuperscript{Brdm1}} mice (Oster et al., 2002b). Taken together it seems that deletion of \textit{mCry1} in \textit{mPer1}\textsuperscript{−/−} mice and \textit{mCry2} in \textit{mPer2\textsuperscript{Brdm1}} mice rescues circadian phenotype without affecting the light driven resetting mechanism indicating that mPER1 interacts predominantly with mCRY1 and mPER2 with mCRY2 \textit{in vivo}.

\textit{Breakdown of the clock in ageing mPer1\textsuperscript{−/−} mCry2\textsuperscript{−/−} mice}

Circadian organisation changes with age (Valentinuzzi et al., 1997; Yamazaki et al., 2002). Typical changes include decrease in the amplitude of wheel-running activity, fragmentation of the activity rhythm, decreased precision in onset of daily activity and alterations in the response to the phase-shifting effects of light (Valentinuzzi et al., 1997). Within the SCN histological changes have been reported in aged rats and electrical activity rhythms in SCN slice cultures have lower amplitude and are less precise than in SCN cultures prepared from young animals (Aujard et al., 2001; Satinoff et al., 1993; Watanabe et al., 1995). At the molecular level age diminishes the amplitude of \textit{Per2} but not \textit{Per1} expression in mice (Weinert et al., 2001). However in rats similar expression patterns of molecular clock components in the SCN of young and old rats have been reported (Asai et al., 2001). The above mentioned studies have investigated the effects of ageing on the clock. Here we show evidence that a defective clock has an influence on ageing. Inactivation of \textit{mPer1} and \textit{mCry2} in mice leads in young \textit{mPer1}\textsuperscript{−/−}\textit{mCry2}\textsuperscript{−/−} animals (2-6 months old) to a decreased precision in onset of daily activity compared to wild type mice (Fig. 1D, E). Onset of activity is markedly delayed with a sharp offset at the dark/light transition probably reflecting masking (Mrosovsky, 1999). In ageing \textit{mPer1}\textsuperscript{−/−}\textit{mCry2}\textsuperscript{−/−} mice the decreased precision in onset of daily activity is even more pronounced (Fig. 1F). Additionally, a fragmentation of the activity rhythm is observed under LD conditions and a daily rhythm is barely detectable (Fig. 1G). In constant darkness old \textit{mPer1}\textsuperscript{−/−}\textit{mCry2}\textsuperscript{−/−} mice do not display a circadian rhythm and the amplitude of wheel-running activity is decreased compared to wild type and young \textit{mPer1}\textsuperscript{−/−}\textit{mCry2}\textsuperscript{−/−} mice (Fig. 1F, I). All these phenotypes are not observed in \textit{mPer1}\textsuperscript{−/−} and \textit{mCry2}\textsuperscript{−/−} single mutant mice (van der Horst et al., 1999; Zheng et al., 2001) (supplemental Fig. 1). Interestingly, \textit{mPer1}\textsuperscript{−/−}\textit{mCry2}\textsuperscript{−/−} mice display an altered response to the phase-shifting effects of
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light (see below and Fig. 4). All the above described observations indicate that the clock seems to break down in ageing \textit{mPer1}\textsuperscript{−/−}m\textit{Cry2}\textsuperscript{−/−} mice and, as a consequence, can accelerate some aspects of ageing. The breakdown of the circadian rhythm does not occur in all \textit{mPer1}\textsuperscript{−/−}m\textit{Cry2}\textsuperscript{−/−} mice at the same time indicating that additional genes or genetic background may contribute to the ageing process. However, the percentage of arrhythmic \textit{mPer1}\textsuperscript{−/−}m\textit{Cry2}\textsuperscript{−/−} animals increases with age (Fig. 1H) supporting the notion that the \textit{mPer1} and \textit{mCry2} genes in combination influence some aspects of the ageing process. Most probably transcriptional and posttranscriptional fidelity decreases with age and the absence of \textit{mPer1} and \textit{mCry2} renders the animal more prone to this infidelity. This view is supported by our observation that \textit{mPer2} and \textit{Bmal1} mRNA levels are strongly reduced in the SCN and in the kidney of old \textit{mPer1}\textsuperscript{−/−}m\textit{Cry2}\textsuperscript{−/−}mice (Figs. 2 and 3D). Additionally, mCRY1 protein levels are elevated (Fig. 2C) pointing to an impaired degradation pathway of mCRY1. Interestingly, m\textit{Cry1} mRNA cycling is not affected in contrast to \textit{mPer2} and \textit{Bmal1} transcripts indicating that regulation of \textit{mCry1} differs from \textit{mPer2} and \textit{Bmal1} transcriptional regulation.

The loss of circadian wheel running behaviour in old \textit{mPer1}\textsuperscript{−/−}m\textit{Cry2}\textsuperscript{−/−} mice is not a gradual process but occurs rapidly within a period of 3 days (see supplemental Fig. 4). This probably reflects the bimodality of transcriptional and posttranscriptional processes that are likely to act as “on/off” switches lacking intermediate states. Transcription is initiated by multimeric protein complexes (Beato, 1996; Freedman, 1999) and hence the components of transcriptional complexes have to be orchestrated in order to be present at a specific time and place in the cell. It seems that in young \textit{mPer1}\textsuperscript{−/−}m\textit{Cry2}\textsuperscript{−/−}mice the critical amplitude in the level of \textit{PER2/Cry1} heterodimers to regulate the clock is just barely reached. With progressing age synthesis and processing of these proteins are reduced. The amplitude of \textit{PER2/Cry1} heterodimer oscillation falls below a critical threshold leading to a deregulation of the clock (Fig. 6 B), probably resulting in the uncoordinated cellular and physiological events we observe in old \textit{mPer1}\textsuperscript{−/−}m\textit{Cry2}\textsuperscript{−/−}mice.

\textit{Light sensitivity is impaired in ageing \textit{mPer1}\textsuperscript{−/−}m\textit{Cry2}\textsuperscript{−/−}mice}

Old \textit{mPer1}\textsuperscript{−/−}m\textit{Cry2}\textsuperscript{−/−}mice are very poorly synchronised to the light dark cycle (Fig. 1F). Therefore we hypothesised that these animals would be defective in light driven resetting of the circadian clock. A light pulse at ZT14 revealed a reduced inducibility of \textit{mPer2} in young and even more pronounced in old \textit{mPer1}\textsuperscript{−/−}m\textit{Cry2}\textsuperscript{−/−}mice (Fig. 4 A, B). Therefore we tested whether CREB, an essential factor for numerous transcriptional processes, was activated by
phosphorylation in response to a light pulse (Motzkus et al., 2000; Travnickova-Bendova et al., 2002). CREB phosphorylation was only slightly lowered in young $mPer1^{-/-}mCry2^{-/-}$ mice but was significantly impaired in old animals (Fig. 4C, D) indicating a defect in light signalling in the SCN of these mice. At the behavioural level we could only measure the phase shifts of young $mPer1^{-/-}mCry2^{-/-}$ mice, because old animals immediately became arrhythmic in DD (thus precluding the determination of phase shifts). The young $mPer1^{-/-}mCry2^{-/-}$ mice resemble $mPer1^{-/-}$ animals in that they were not able to advance clock phase (Fig. 4F) (Albrecht et al., 2001), suggesting that this anomaly is due to the lack of $mPer1$. Interestingly, phase delays in $mPer1^{-/-}mCry2^{-/-}$ mice were also affected although the criterion of significance was barely missed ($p = 0.0539$). The reduced inducibility of $mPer2$ by light in young $mPer1^{-/-}mCry2^{-/-}$ mice and the reduction in delaying clock phase in those animals is consistent with the previous finding that $mPer2$ mutant mice are defective in delaying clock phase (Albrecht et al., 2001).

The impaired light response of $mPer1^{-/-}mCry2^{-/-}$ mice might be a consequence of a defect in transmitting light information from the eye to the SCN. To test this possibility we looked for anatomical malformations in the retina. Neither young nor old $mPer1^{-/-}mCry2^{-/-}$ mice displayed overt abnormalities (Fig. 5A) indicating that the animals were not visually blind. Cell death as a reason for malfunction of the retina could most possibly be excluded, since lipofuscin staining (Fig. 5B) and Congo red staining (data not shown) did not reveal dead cells in the retina. Comparable to the SCN however, light dependent phosphorylation of CREB at Ser 133 was affected in old $mPer1^{-/-}mCry2^{-/-}$ mice (Fig. 5C). As a consequence light perceived by the eye is probably not processed properly to induce a cellular signalling cascade coding for the light signal. The reason for the impaired transmission of the light signal is most likely not a developmental defect, since young $mPer1^{-/-}mCry2^{-/-}$ mice show phosphorylation of CREB at Ser 133. Therefore the defect is probably of transcriptional or posttranscriptional nature. For example CREB kinases might be regulated by some clock components. Candidates would be mouse homologues of the Drosophila kinase mothers against decapentaplegic (Mad). The mouse homologues, termed $Madh1$ and $Madh2$, are expressed in a circadian manner in the SCN (Panda et al., 2002) suggesting that their transcription is influenced by the clock and hence would phosphorylate CREB in a clock dependent manner.

The transcriptional potential of mPER and mCRY protein complexes and their temporal abundance determines circadian rhythmicity
The precise regulation of the circadian oscillator requires an exact choreography of clock protein synthesis, interaction and posttranslational modification. The positive limb of circadian clock gene activation is influenced by the negative limb, probably through a complex consisting of mPER and mCRY proteins (Albrecht, 2002; Okamura et al., 2002). Such a mPER/mCRY complex would be composed of those PER and CRY proteins which are most abundant at a given time. Figure 6A depicts the temporal abundance of cycling \textit{mPer1, mPer2, mCry1} and \textit{mCry2} mRNA in the SCN illustrating that the amount of mRNA of these genes differs with time (Albrecht et al., 1997; Okamura et al., 1999; Reppert and Weaver, 2002; Yan and Okamura, 2002). Because the clock components of the negative limb (\textit{Per} and \textit{Cry}) are probably regulating their own transcription, the mRNA cycling is likely to reflect the activity of the corresponding proteins. The active forms of PER and CRY proteins seem to be cycling with a delay of 4-6 hours compared to mRNA (Field et al., 2000).

Interestingly, not all PER/CRY complexes seem to be equally important \textit{in vivo} (Oster et al., 2002b) (this study). \textit{mPer2\textsuperscript{Brdm1}mCry2\textsuperscript{+/−}} mutant but not \textit{mPer2\textsuperscript{Brdm1}mCry1\textsuperscript{+/−}} mutant mice display circadian rhythmic behaviour, indicating that mPER1/mCRY1 but not mPER1/mCRY2 are sufficient to drive the circadian clock (Oster et al., 2002b). Our observations presented in this study indicate that mPER2/mCRY2 but - at least in older mice - not mPER2/mCRY1 can sustain circadian rhythms. Additionally, \textit{mPer1\textsuperscript{+/−}mPer2\textsuperscript{Brdm1}} and \textit{mCry1\textsuperscript{+/−}mCry2\textsuperscript{+/−}} double mutant mice do not show circadian rhythmicity, indicating that mPER or mCRY homodimers are not sufficient to maintain circadian rhythmicity. Based on these observations we propose abundance and timing of PER/CRY complexes as illustrated in figure 6B. According to this model the complexes composed of mPER1/mCRY1 and mPER2/mCRY2 would be the most abundant ones with a difference in their maximal presence of about 2 hours. The abundance of these complexes is higher than a critical threshold level necessary to drive clock regulation (green horizontal line in Fig. 6 B). In contrast, mPER1/mCRY2 complexes formed in \textit{Per2/Cry1} mutant mice do not reach this critical threshold. The reason for this might be that the timing of expression of these two proteins is not synchronised and/or the affinity between mPER1 and mCRY2 is low. As a consequence \textit{Per2/Cry1} mutant mice lose clock function (Oster et al., 2002b). The complex formed between mPER2 and mCRY1 seems to just reach the critical threshold necessary for clock regulation as illustrated by the circadian wheel-running behaviour of young \textit{mPer1\textsuperscript{+/−}mCry2\textsuperscript{+/−}} mice (Fig. 1D, E). However, with the progressing infidelity of transcription in ageing mice the presence of this complex falls below the critical threshold level and hence, older
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$mPer1^{-/-}mCry2^{-/-}$ mice lose circadian wheel-running behaviour (Fig. 1F, I). $mPer2^{Brdm1}$ mutant mice lose circadian rhythmicity after a few days in constant darkness. In these animals only functional mPER1/mCRY1 and mPER1/mCRY2 complexes can form, which should in principle be able to drive circadian rhythm. This seems to be the case for the first few days in constant darkness, but then competition between mCRY1 and mCRY2 for PER1 could lead to equal amounts of PER1/CRY1 and PER1/CRY2 complexes. The abundance of each of these complexes seems to fall below the threshold critical for normal clock function.

Taken together it seems that PER/CRY complexes have different potentials to regulate the circadian clock. In wild type animals theoretically all complexes can form but the formation of PER/CRY complexes is probably not random and depends on temporal abundance and strength of interaction between the complex forming partners (Fig. 6B). The sum of the regulatory potential of PER/CRY complexes over time displays a robust circadian cycling as illustrated in figure 6C. The robustness of this cycling is probably ensured by the different phasing of the oscillation of the two strong regulatory complexes PER1/CRY1 and PER2/CRY2. This notion is supported by theoretical considerations indicating that an overt oscillation is stabilised by two oscillators that are slightly out of phase (Glass and Mackey, 1988; Roenneberg and Merrow, 2001).

A model composed of two coupled molecular oscillators has been proposed by Daan and coworkers (2001). This model states that $mPer1$ and $mCry1$ are part of a morning (M) oscillator (comparable to the dark blue curve in Fig. 6B) whereas $mPer2$ and $mCry2$ are components of an evening (E) oscillator (light blue curve in Fig. 6B). Hence a deletion of the M oscillator (inactivation of $mPer1$ and $mCry1$) leaves the E oscillator untouched. The E oscillator ($mPer2$ and $mCry2$) would then drive the circadian clock alone, which would result in circadian rhythmicity. This is consistent with our observation that $mPer1^{-/-}mCry1^{-/-}$ mice display a circadian rhythm (Fig. 1C). Conversely, an inactivation of the E oscillator ($mPer2$ and $mCry2$) would leave the M oscillator ($mPer1$ and $mCry1$) alone to drive the circadian clock. In fact, inactivation of both $mPer2$ and $mCry2$ leads to normal circadian wheel running activity in mice (Oster et al., 2002b), and the rhythm seems to be stable. But what is the advantage of having two oscillators when a stable rhythm is observed with only the M or E oscillator alone? The answer lies in the adaptation of clock phase to changing environmental conditions (e.g. seasonal variation in day length) (Oster et al., 2002a; Steinlechner et al., 2002). Our results show that deletion of either the putative M or E oscillator results in normal circadian rhythmicity, but resetting in these mutant mice is not normal. Mice with inactivated mPER2 and mCRY2 proteins display a defect in delaying clock phase in response to a light
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pulse (Oster et al., 2002b) whereas mPer1\(^{-/-}\)mCry1\(^{-/-}\)mice are not able to phase advance the clock (Fig. 4E). Since these resetting phenotypes are similar to the resetting phenotypes observed in mPer2\(^{Brdm1}\) and mPer1\(^{-/-}\) single mutant mice respectively, we can conclude that both the M and E oscillators have to be intact in order to reset clock phase properly. Interestingly, hampering the M and E oscillators by inactivating Per1 and Cry2 or Per2 and Cry1 leads to loss of circadian rhythmicity. This takes several months in mPer1\(^{-/-}\)mCry2\(^{-/-}\) mice (Fig. 1H) but is immediate in mPer2\(^{Brdm1}\)mCry1\(^{-/-}\) animals (Oster et al., 2002b). The reason for this difference might be that mPer2 and mCry1 have a greater transcriptional potential than mPer1 and mCry2 (see above).

Taken together our in vivo studies support a model based on two coupled oscillators (Fig. 6). It is reasonable to conclude that not all interactions between PER and CRY proteins are equal in vivo. Although these proteins seem to be partially redundant, all of them are necessary for a functional circadian clock that can predict time and thereby being adaptable to changing environmental conditions. The importance of the PER1/CRY2 complex only becomes apparent in mPer1\(^{-/-}\)mCry2\(^{-/-}\) mice half a year after birth illustrating a connection between the clock and aspects of ageing.
Materials and Methods

Generation of mPer and mCry mutant mice.
We crossed \textit{mPer1}^{-/-} mice (Zheng et al., 2001) with \textit{mCry1}^{-/-} and \textit{mCry2}^{-/-} animals (van der Horst et al., 1999). The genotype of the offspring was determined by southern blot analysis as described (Oster et al., 2002b). Hybridisation probes were for \textit{mPer1} as described in (Zheng et al., 2001) and for \textit{mCry1} and \textit{mCry2} as described in (van der Horst et al., 1999). Matching wild-type control animals were produced by back-crossing heterozygous animals derived form the \textit{mPer1}^{-/-} and \textit{mCry}^{-/-} matings to minimise epigenetic effects. However, we can not completely rule out such effects and epistatic interactions between different gene clusters that could have clouded our observations.

Locomotor activity monitoring and circadian phenotype analysis.
Mice housing and handling were performed as described (Albrecht and Oster, 2001). For LD-DD transitions lights were turned off at the end of the light phase and not turned on again the next morning. Activity records are double plotted so that each light/dark cycle’s activity is shown both to the right and below that of the previous light/dark cycle. Activity is plotted in threshold format for 5-minute bins. For activity counting and evaluation we used the ClockLab software package (Actimetrics). Rhythmicity and period length were assessed by \(\chi^2\) periodogram analysis and Fourier transformation using mice running in LD or in constant darkness for at least 10 days.

For light induced phase shifts we used the Aschoff Type I (for \textit{mPer1}^{-/-}\textit{mCry1}^{-/-} animals) or the Type II protocol (for \textit{mPer1}^{-/-}\textit{mCry2}^{-/-} animals) as described (Albrecht and Oster, 2001; Albrecht et al., 2001). We originally chose Type II protocol because of the convenient set-up for high numbers of animals and for comparison with \textit{mPer2}^{BrNm1} mice (Albrecht et al., 2001; Oster et al., 2002b) which become arrhythmic in constant darkness precluding the determination of circadian times. However, the unstable onset of activity of \textit{mPer1}^{-/-}\textit{mCry2}^{-/-} mice in LD and the long period length of these animals in DD resulted in very high variations when determining the phase shifts with Type II protocol. Therefore we repeated the experiments using a Type I set-up with animals free running in DD before light administration. For Type II protocol animals were entrained to an LD cycle for at least 7 days before light administration (15 min bright white light (400 Lux) at ZT14 or ZT22) and subsequently released into DD. The phase shift was determined by drawing a line through at least 7 consecutive days of onset of activity in LD before the light pulse and in DD after the
light pulse as determined by the ClockLab program. The difference between the two lines on
the day of the light pulse determined the value of the phase shift. For Type I protocol animals
were kept in DD for at least ten days before the light pulse (at CT14 or CT22 respectively).
The phase shift was determined by drawing lines through at least 7 consecutive days before
and after the light pulse using the ClockLab software. The first one or two days following the
light administration were not used for the calculation since animals were thought to be in
transition between both states.

**In situ hybridisation**
Mice were sacrificed by cervical dislocation under ambient light conditions at ZT6 and ZT12
and under a 15W safety red light at ZT18 and ZT0/24 as well as at CT0/24, 6, 12 and 18. For
DD conditions animals were kept in the dark for 3 days before decapitation. For light
induction experiments animals were exposed to a 15min light pulse (400 Lux) at ZT14 and
killed at ZT15; controls were killed at ZT15 without prior light exposure. Specimen
preparation, $^{35}$S-rUTP labelled riboprobe synthesis and hybridisation steps were performed as
described (Albrecht et al., 1998). The probe for *mPer2* was as described (Albrecht et al.,
1997). The *mCry1* and the *Bmal1* probes were as described (Oster et al., 2002). Quantification
was performed by densitometric analysis of autoradiograph films (Amersham Hyperfilm MP)
as described (Oster et al., 2002b). For each time point three animals were used and three
sections per SCN were analysed. "Relative mRNA abundance" values were calculated by
defining the highest value of each experiment as 100%.

**Immunohistochemistry**
Animals were killed and tissue prepared as described for *in situ* hybridisation. Eye lenses
were removed before cutting. Sections were boiled in 0.01M sodium citrate (pH 6) for 10 min
to unmask hidden antigen epitopes and processed for immunohistochemical detection using
the Vectastain Elite ABC system (Vector Laboratories) and diaminobenzidine with nickel
amplification as chromogenic substrate. Immunostained sections were inspected with an
Axioplan microscope (Zeiss) and the area of the SCN determined by comparison to Nissl
stained parallel sections. Semiquantitative analysis for *mCRY1*, *mPER2* and *Ser133P-CREB*
immunoreactivity in the SCN was performed using NIH Image program. Images were
digitised; background staining was used to define a lower threshold. Within the whole area of
the SCN all cell nuclei exceeding the threshold value were marked. Three sections of the
Results

Intermediate aspect of the SCN were chosen at random for further analysis. Values presented are the mean of three different experiments +/- SD. Primary antibodies against mCRY1 (Alpha Diagnostics, order number CRY11-A), against CREB (Cell Signalling Technology, order number 9192), against CREB, phosphorylated at the residue Ser133 (New England Biolabs, order number 9191S), and against mPER2 (Santa Cruz Biotechnology, order number sc-7729) were used at dilutions of 1:200, 1:500, 1:1000, and 1:200, respectively.

Northern Blot Analysis

Rhythmic animals were sacrificed at the specified time points. Total RNA from kidney was extracted using RNAzol B (WAK Chemie). Northern analysis was performed using denaturing formaldehyde gels (Sambrook and Russel, 2001) with subsequent transfer to Hybond-N+ membrane (Amersham). For each sample 20 µg of total RNA was used. cDNA probes were the same as described for in situ hybridisation. Labelling of probes was done using the Rediprime II labelling kit (Pharmacia) incorporating [P\(^{32}\)]dCTP to a specific activity of 10\(^8\) cpm/µg. Blots were hybridised using UltraHyb solution (Ambion) containing 100 µg/ml salmon sperm DNA. The membrane was washed at 60°C in 0.1x SSPE and 0.1 % SDS. Subsequently, blots were exposed to phosphoimager plates (Bio-Rad) for 20 hours and signals quantified using Quantity One 3.0 software (Bio-Rad). For comparative purposes, the same blot was stripped and re-used for hybridisation. The relative level of RNA in each lane was determined by hybridisation with mouse Gapdh cDNA.

Histology

All histological staining was performed as described (Burkett et al., 1993). For Gomori’s trichrome staining PFA fixed, paraffin embedded sections were de-waxed, post-fixed with Bouin’s fluid at 56°C for 30 min, and nuclei stained with ferric haematoxyline (according to Weigert) for 10 min. After washing in water, slides were incubated for 15 min with trichrome stain (Chromotrope 2R (0.6% (w/v)) and Light Green (0.3% (w/v)) in 1% (v/v) acetic acid and 0.8% (w/v) phosphotungstic acid). After washing with 0.5% acetic acid and 1% (v/v) acetic acid/ 0.7% (w/v) phosphotungstic acid, slides were rinsed with water, dehydrated and mounted with Canada balsam/ methyl salycilate.

For lipofuscin staining slides were de-waxed and colorised with 0.75% (w/v) ferric chloride/ 0.1% (w/v) potassium ferricyanide (Aldrich) for 5 min. After washing with 1% (v/v) acetic acid and water, slides were incubated with 1% (w/v) Neutral Red for 3-4 min and
subsequently washed with water, de-hydrated and mounted with Dpx mounting media (Fluka). All reagents were from Sigma if not stated otherwise.

**Statistical analysis**

Statistical analysis of all experiments was performed using GraphPad Prism software (GraphPad). Significant differences between groups were determined with one-way ANOVA, followed by Bonferroni’s post-test. Values were considered significantly different with $p<0.05$ (*), $p<0.01$ (**) or $p<0.001$ (***).
References

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Results

Figures

Figure 1 Generation of mPer1mCry double mutant mice and representative locomotor activity records. (A) Southern blot analysis of wild-type, mPer1−/−mCry1−/−, and mPer1−/−mCry2−/− tail DNA. The mPer1 probe hybridises to a 20kb wild-type and a 11.8kb mutant fragment of EcoR I digested genomic DNA. The mCry1 probe detects a 9kb wild-type and a 4kb Nco I digested fragment of the targeted locus. In mCry2 mutants the wild-type allele is detected by hybridisation of the probe to a 7kb EcoR I fragment whereas the mutant allele yields a 3.5kb fragment. (B-D, F) Representative locomotor activity records of wild-type (B), mPer1−/−mCry1−/− (C), young mPer1−/−mCry2−/− (D), and old mPer1−/−mCry2−/− (F) animals kept in a 12h light 12h dark (LD) cycle and in constant darkness (DD; transition indicated by the horizontal line). Activity is represented by black bars and is double-plotted with the activity of the following light/dark cycle plotted to the right and below the previous light/dark cycle. The top bar indicates light and dark phases in LD. For the first five days in DD, wheel rotations per day were 20,000 ± 2,500 (n=17) for wild type animals, 21,500 ± 7,300 (n=15) for mPer1−/−mCry1−/− mutants, 25,100 ± 6,200 (n=14) for young mPer1−/−mCry2−/− mutants, and 17,200 ± 7,900 (n=9) for old mPer1−/−mCry2−/− mutants. (E, G, I) Periodogram analysis of young mPer1−/−mCry2−/− animals in DD (E corresponds to activity plot in D), and old mPer1−/−mCry2−/− animals in LD (G corresponds to activity plot in F) and DD (I corresponds to activity plot in F). Analysis was performed on 10 consecutive days in LD or DD after animals were allowed to adapt 5 days to the new light regimen. The ascending straight line in the periodograms represents a statistical significance of p < 0.001 as determined by the ClockLab program. (H) Age dependence of rhythmicity in wild-type (dark grey bar), mPer1−/− (white bar), mCry2−/− (black bar), and mPer1−/−mCry2−/− (light grey bar) mice. Animals tested were divided into three groups according to their age (2-6 months, 6-12 months and more than 12 months old). Rhythmicity in DD was determined by periodogram analysis. Values on top of each bar indicate total numbers of animals tested per group and genotype.
Figure 2  *mPer2*/mPER2 expression profiles of young and old *mPer1<sup>−/−</sup>*/mCry2<sup>−/−</sup> mice. (A) Diurnal expression of *mPer2* in the SCN of wild-type (solid line), young *mPer1<sup>−/−</sup>*/mCry2<sup>−/−</sup> (pointed line), and old *mPer1<sup>−/−</sup>*/mCry2<sup>−/−</sup> (dashed line) mice in LD. In old double mutants *mPer2* cycling is significantly dampened (p < 0.05). Black and white bars on x-axis indicate dark and light phase respectively. All data presented are mean ± S.D. for three different experiments. Right panels show representative micrographs of SCN probed with *mPer2* antisense probe at time points of minimal (ZT0) and maximal (ZT12) expression. Tissue was visualised by Hoechst dye nuclear staining (blue); silver grains are artificially coloured (red) for clarification. White bar indicates 200 µm.

(B) Circadian expression of *mPer2* in the SCN of wild-type (solid line) and young *mPer1<sup>−/−</sup>*/mCry2<sup>−/−</sup> (pointed line) mice on the fourth day in DD. Grey and black bars on x-axis indicate subjective day and night respectively. (C) Diurnal variation of mPER2 immunoreactivity in the SCN of wild-type (solid line), young *mPer1<sup>−/−</sup>*/mCry2<sup>−/−</sup> (pointed line), and old *mPer1<sup>−/−</sup>*/mCry2<sup>−/−</sup> (dashed line) mice in LD. Quantification was performed by counting immunoreactive nuclei in the area of the SCN. In old double mutants oscillation of mPER2 immunoreactivity is significantly dampened (p < 0.05) with medium numbers of immunoreactive nuclei. Right panels show representative micrographs of immunostained SCN at time points of minimal (ZT0) and maximal (ZT12) immunoreactivity. Black bar indicates 100 µm. (D) Northern analysis of diurnal expression of *mPer2* in the kidney of wild-type (solid line), young *mPer1<sup>−/−</sup>*/mCry2<sup>−/−</sup> (pointed line), and old *mPer1<sup>−/−</sup>*/mCry2<sup>−/−</sup> (dashed line) mice in LD. (E) Representative Northern blot from kidney tissue from wild-type (left), young *mPer1<sup>−/−</sup>*/mCry2<sup>−/−</sup> (middle) and old *mPer1<sup>−/−</sup>*/mCry2<sup>−/−</sup> (right) mice sequentially hybridised with *mPer2* (top row) and *Gapdh* (bottom row) antisense probe. Black and white bars bellow blots indicate dark and light phase respectively.
Figure 3 mCry1/ mCRY1 and Bmal1 expression profiles of young and old mPer1−/−mCry2−/− mice. (A) Diurnal expression of mCry1 in the SCN of wild-type (solid line), young mPer1−/−mCry2−/− (pointed line), and old mPer1−/−mCry2−/− (dashed line) mice in LD. Black and white bars on x-axis indicate dark and light phase respectively. All data presented are mean +/- S.D. for three different experiments. Right panels show representative micrographs of SCN probed with mCry1 antisense probe at time points of minimal (ZT0) and maximal (ZT12) expression. Tissue was visualised by Hoechst dye nuclear staining (blue); silver grains are artificially coloured (red) for clarification. White bar indicates 200µm. (B) Circadian expression of mCry1 in the SCN of wild-type (solid line) and young mPer1−/−mCry2−/− (pointed line) mice on the fourth day in DD. Grey and black bars on x-axis indicate subjective day and night respectively. (C) Diurnal variation of mCRY1 immunoreactivity in the SCN of wild-type (solid line), young mPer1−/−mCry2−/− (pointed line), and old mPer1−/−mCry2−/− (dashed line) mice in LD. Quantification was performed by counting immunoreactive nuclei in the area of the SCN. In old double mutants oscillation of mCRY1 immunoreactivity is significantly dampened (p < 0.05) with constantly high numbers of immunoreactive nuclei throughout the LD cycle. Right panels show representative micrographs of immunostained SCN at time points of minimal (ZT0) and maximal (ZT12) immunoreactivity. White bar indicates 100µm. (D) Diurnal expression of Bmal1 mRNA expression in the SCN of wild-type (solid line), young mPer1−/−mCry2−/− (pointed line), and old mPer1−/−mCry2−/− (dashed line) mice in LD. In old double mutants Bmal1 cycling is significantly dampened (p < 0.05). (E) Circadian expression of Bmal1 mRNA in the SCN of wild-type (solid line) and young mPer1−/−mCry2−/− (pointed line) mice on the fourth day in DD.
Figure 4 Light responsiveness in the SCN of young and old mPer1−/−mCry2−/− mice. (A) In situ hybridisation analysis of mPer2 light inducibility in the SCN of wild-type (wt, upper row), young mPer1−/−mCry2−/− (middle row), and old mPer1−/−mCry2−/− mice (lower row). Shown are representative micrographs of SCN probed with mPer2 antisense probe with (light) or without light administration (control) at ZT14 (15min light pulse, 400Lux; animals were sacrificed one hour later). Tissue was visualised by Hoechst dye nuclear staining (blue); silver grains are artificially coloured (red) for clarification. White bar indicates 200µm. (B) Quantification of mPer2 induction after a light pulse at Z14. Left panel shows control animals without light exposure. Right panel shows relative mPer2 mRNA induction after light exposure (wild-type control was set as 1). Data presented are mean +/- S.D. of three different animals each. Statistical significance is indicated by asterisks (*, p < 0.05; ***, p < 0.001). (C) Immunohistochemistry analysis of CREB Ser-133 phosphorylation by light in the SCN of wild-type (wt, upper row), young mPer1−/−mCry2−/− (middle row), and old mPer1−/−mCry2−/− mice (lower row). Shown are representative micrographs of SCN sections immunostained for Ser133-P-CREB with (light) or without light administration (control) at ZT14. As a control SCN sections for all genotypes were stained for CREB (unphosphorylated) at the same time points. (D) Quantification of CREB phosphorylation after a light pulse at ZT14. Panels show numbers of Ser133-P-CREB immunoreactive nuclei in the SCN with or without light exposure (***, p < 0.001). (E) Light induced phase shifts in mPer1−/−mCry1−/− mice using Aschoff Type II protocol to assess phase shifts. Animals were kept for at least 10 days in LD and released into DD after a light pulse at ZT14 or ZT22. Negative values indicate phase delays, positive values indicate phase advances. Data presented are mean +/- S.D. of 10 to 14 animals (***, p < 0.001). (F) Light induced phase shifts in young mPer1−/−mCry2−/− mice using Aschoff Type I protocol to assess phase shifts. Animals were kept for at least 10 days in DD before a light pulse at CT14 or CT22. Data presented are mean +/- S.D. of 10 to 13 animals.
Figure 5 Histology and light responsiveness in the retina of wild type, young and old mPer1-/-mCry2-/- mice. (A) Gomori trichrome and (B) lipofuscin staining of retinal sections of wild-type (upper row), young mPer1-/-mCry2-/- (middle row), and old mPer1-/-mCry2-/- mice (lower row). Retinal layers are indicated on the left (PRL, photoreceptor layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer). (C) Immunohistochemistry analysis of light induced CREB Ser-133 phosphorylation in the retina. Left panels show immunostained retinal sections of control animals without light exposure right panels of animals 1hr after light exposure (400Lux, 15min) at ZT14. (D) Immunohistochemistry analysis for (unphosphorylated) CREB in the retina at ZT14. Black bar indicates 10 µm.
Figure 6 Working model for PER/CRY driven inhibition of CLOCK/BMAL1. (A) mPer and mCry transcripts show diurnal/circadian cycling in the SCN. While mPer1 expression peaks between ZT/CT 4 to 8, mCry1 and mPer2 mRNA rhythms have their maxima at ZT/CT 10 to ZT/CT 12, respectively (Fig. 2 & 3; see also Oster et al. 2002; Yan et al. 2002; Kume et al. 1999). Note that mCry2 is also expressed in the SCN but without a clearly defined rhythm (Kume et al. 1999). Protein peaks are delayed by about 4-6 hours with regard to mRNA (Field et al., 2000; King and Takahashi 2000; Reppert and Weaver 2002). (B) mPER and mCRY proteins form heterodimeric complexes that form with certain preferences according to protein-protein affinity and temporal abundance. The complexes are colour coded with mPER1/mCRY1 and mPER2/mCRY2 representing the most abundant ones. The green horizontal line indicates a threshold above which a PER/CRY complex is abundant enough to influence CLOCK/BMAL1 transcription. (C) Time course of the overall inhibitory potential of the mPER/mCRY heterodimers on CLOCK/BMAL1 activity. The strong inhibition of CLOCK/BMAL1 during (subjective) night corresponds to the low transcriptional activity of (CLOCK/BMAL1 induced) mPer and mCry genes.
2.3. Additional Data

2.3.1. Breeding Statistics

To check if the deletion of certain combinations of *mPer* and/or *mCry* genes has an impact on the viability and fertility of the animals we analyzed the data obtained from our breeding colonies. In the heterozygous F1 generation we checked if the different genotypes of the F2 offspring occurred in the expected distribution according to the Mendelian laws of inheritance.

![Figure 22: Genotypic distribution in the F2 generation of the different mPer/ mCry double mutant strains. Shown are the experimental values together with the ideal (expected) numbers (black bars) of different genotypes obtained from double heterozygous parents.](image)

With minor variations all F2 genotypic ratios appeared as expected. Differences between ideal and experimental values are results of the relatively small number of F1 breedings because the colony was set up for breeding efficiency and as soon as homozygous F2 breeding pairs were available these were used to replace the F1 generation.

Once these F2 breeding pairs were established, parents were kept together as long as possible to prevent unnecessary genetic drift due to relatively small founder populations. We checked homozygous breedings for litter size and frequency.

There was no significant difference in the litter size for all strains of double and triple mutant mice with the exception of the *mPer1/2/ mCry1* and the *mPer1/ mCry1/2* mutants which never bred. *mPer1/2/ mCry2* mutants never had more than one litter per mating.
Fig. 23: Average litter size of homozygous *mPer/ mCry* double and triple mutant matings. Values on top indicate the absolute number of breeding pairs for each strain.

Fig. 24: Birthday of the first litter after mating setup of homozygous *mPer/ mCry* double and triple mutants.

While there were no significant differences in litter intervals and stability of breeding intervals between all fertile strains, wild-type breedings were generally more stable and regular but with rather high variations between different matings.
All triple mutant breedings were rather unreliable with small litters, irregular breeding intervals and high death numbers after birth. Since the estrous cycle is known to be linked to the circadian clock (Alleva et al., 1971) it seems obvious that females with a disrupted circadian clock may have difficulties with regard to their fertility.
Although the triple mutants generally appeared smaller and less aggressive and a notable number of animals showed a tendency to spastic seizures upon arousal the total numbers used in this study were insufficient to allow a scientific investigation.

2.3.2. Activity monitoring

Besides the double mutant strains presented in the publications above we generated three mPer/ mCry triple mutant strains. The fourth possible combination of triple mutation, mPer1/mCry1/2 was not fertile and probably caused some additional defects we could not examine since the F1 matings (8 pairs) did not give enough homozygous offspring. The few animals we tested however, were arrhythmic under constant conditions (DD, LL) like all the other three triple mutant strains.

Generally, triple mutants run less than their wild-type littermates. They immediately lose their rhythm in DD and LL indicating a completely disrupted circadian clockwork.

(Legend: see next page)
Results

Fig. 27: Activity profiles of mPer/mCry triple mutant mice in LD and DD. Left panels show representative actograms of mPer1/2/ mCry1−/−, mPer1/ mCry1/2−/−, mPer1/2/ mCry2−/−, and mPer2/ mCry1/2−/− mice in LD and DD (transition indicated by the line below LD). Black and white bars on top indicate light and dark phase in LD. Right panels show periodograms of the same animals in DD. The diagonal line depicts significance as given by the ClockLab program.

When released into constant light both double and triple mutants generally run less than in LD or DD demonstrating the activity suppressing (“masking”) effect of light on nocturnal animals. Interestingly, mPer1mCry2−/− mice lost their rhythmicity in LL while at least the young animals show a stable rhythm of activity in DD. Even more surprising was the fact that in mPer2mCry1 mutant mice rhythmicity could be rescued by high light intensities in LL while the animals were totally arrhythmic in DD. One explanation might be the light inducibility of mPer1: While in DD mPER1 and mCRY2 protein levels and distribution do not overlap spatially and temporally an increased mPer1 expression in LL enables mPER1 protein to interact with mCRY2. The combination of both is enough to restart the TTL, although with a very short period.
To further investigate the influence of masking on the wheel running activity, we exposed the animals to a 8h shifted LD cycle and monitored the time the animals needed to adjust to the new light regimen. While the double mutants needed a few days to adjust the triple mutants did not suffer from that “jet lag” like experience but changed their activity patterns immediately after the transition. This indicates that the rhythmicity these animals show in LD is merely driven by the masking effect of light and not by an endogenous pacemaker.
Results

Fig. 29: Activity profiles of mPer/ mCry double and triple mutant mice after a shifted LD cycle. Panels show representative actograms of wild-type (A), mCry2\(^{-/-}\) (B), mPer1/ mCry1\(^{-/-}\) (C), 'young' mPer1/ mCry2\(^{-/-}\) (D), mPer2/ mCry1\(^{-/-}\) (E), mPer2/ mCry2\(^{-/-}\) (F), mPer1/2/ mCry1\(^{-/-}\) (G), mPer1/2/ mCry2\(^{-/-}\) (H), and mPer2/ mCry1/2\(^{-/-}\) mice (I) in LD, LD 8h shifted backwards (indicated by the upper asterisk), and LD 8h shifted forwards (indicated by the lower asterisk). Black and white bars on top indicate light and dark phase of the first LD cycle.

The table below summarizes the data obtained by the general activity profile analysis of all mPer/ mCry double and triple mutant strains in this study.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Activity onset (hours after 'lights off')</th>
<th>Overall Activity (rev/day)</th>
<th>Covered Distance (km/day)</th>
<th>relative light phase activity (%)</th>
<th>duration of activity (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>0.29 +/- 0.22</td>
<td>19600 +/- 3000</td>
<td>7.14</td>
<td>3.4 +/- 2.1</td>
<td>7.7 +/- 0.7</td>
</tr>
<tr>
<td>mPer1mCry1(^{-/-})</td>
<td>0.25 +/- 0.34</td>
<td>20600 +/- 6700</td>
<td>7.51</td>
<td>3.2 +/- 3.5</td>
<td>8.2 +/- 0.6</td>
</tr>
<tr>
<td>mPer1mCry2(^{-/-})</td>
<td>5.79 +/- 0.67</td>
<td>17400 +/- 9600</td>
<td>6.34</td>
<td>10.7 +/- 11.3</td>
<td>7.4 +/- 0.3</td>
</tr>
<tr>
<td>mPer2mCry1(^{-/-})</td>
<td>-0.16 +/- 0.35</td>
<td>18000 +/- 3700</td>
<td>6.56</td>
<td>12.5 +/- 5.3</td>
<td>7.6 +/- 0.8</td>
</tr>
<tr>
<td>mPer2mCry2(^{-/-})</td>
<td>0.18 +/- 0.14</td>
<td>18000 +/- 2600</td>
<td>6.56</td>
<td>3.5 +/- 2.9</td>
<td>10.1 +/- 0.5</td>
</tr>
<tr>
<td>mPer1/2mCry1(^{-/-})</td>
<td>0.08 +/- 0.4</td>
<td>4200 +/- 2500</td>
<td>1.53</td>
<td>1.8 +/- 1.8</td>
<td>10.4 +/- 2.0</td>
</tr>
<tr>
<td>mPer1/2mCry2(^{-/-})</td>
<td>0.02 +/- 0.47</td>
<td>6300 +/- 1500</td>
<td>2.30</td>
<td>2.7 +/- 1.1</td>
<td>10.6 +/- 1.5</td>
</tr>
<tr>
<td>mPer2mCry1/2(^{-/-})</td>
<td>0.19 +/- 0.21</td>
<td>4600 +/- 2500</td>
<td>1.68</td>
<td>0.9 +/- 0.6</td>
<td>9.5 +/- 0.4</td>
</tr>
</tbody>
</table>

Table 2: Activity profiles of mPer/ mCry double and triple mutant mice in LD. All data are mean +/- SD; total numbers used for statistical evaluation are given in the right column.
In order to investigate the role of the \textit{mPer} and the \textit{mCry} genes in the light input pathway to the clock we examined phase shifting effects of nocturnal light. For most strains we applied the Aschoff Type 2 protocol (see chapter 4) for practical reasons. For \textit{mPer1mCry2} mutants however, the evaluation of these experiments turned out to be very difficult due to the long period of the free running rhythm in DD. Therefore we decided to apply the Type 1 protocol to this strain. Since we had to include wild-type animals as reference we could compare results from Type 1 and 2 protocols and show that there are no significant differences in the values obtained from both protocols.
Fig. 30: Nocturnal light pulses phase shift the rhythm of mPer1/ mCry1 and mPer2/ mCry2 mutant mice. Animals were subjected to a 15min light pulse at ZT14 (left panels) or ZT22 (right panels), indicated by the green asterisk, before release into DD. Upper row, wild-type; middle row, mPer1/ mCry1, and lower row, mPer2/ mCry2 mutants. Black and white bars on top indicate light and dark phases in LD.

The data obtained from the double mutants reflected the results obtained from mPer1 and mPer2 single mutants (Albrecht et al., 2001) with mPer1 necessary for light induced phase advances and mPer2 for light induced phase delays. This corresponds to the differential inducibility of both genes by light at different time points during the night (Albrecht et al., 1997b). The double mutant data further supports experiments indicating that the mCrys are dispensable for light entrainment but have their role in the central TTL of the circadian oscillator (Griffin et al., 1999; van der Horst et al., 1999).
Results

Fig. 31: Nocturnal light pulses phase shift the rhythm of \( mPer1/ mCry2 \) mutant mice. Animals were subjected to a 15min light pulse at CT14 (left panels) or CT22 (right panels), indicated by the green asterisk, in DD. Upper row, wild-type; lower row, \( mPer1/ mCry2 \) mutants. Grey and black bars on top indicate subjective night and day of the first day recorded.

Fig. 32: Light induced activity phase shifts in \( mPer/ mCry \) double mutant mice. Shown are results from Aschoff Type 1 (marked with an asterisk) and Type 2 protocols. Left panel shows phase delays after a 15min light pulse early in the night; right panel shows phase advances after a light pulse in the late night (***, \( p<0.001 \)).
2.3.3. Clock gene expression

To see if the disrupted activity rhythm is accompanied by an absence of clock gene oscillation in the SCN of $m$Per/ $m$Cry triple mutants we examined the diurnal expression of $m$Per2 and $Bmal1$ transcripts in these animals. Surprisingly $m$Per2 mRNA levels still cycle in all three strains but $Bmal1$ oscillation is blunted below significance. The reason might be that $m$Per2 transcription is still reactive to light. So we expect this oscillation to dampen upon release into DD. We could not define the transcript levels under constant conditions however, since the arrhythmicity of the animals excludes the determination of circadian times. Since the $m$Per2 gene was mutated in all three strains examined, the absence of $Bmal1$ induction is a further argument that the truncated mPER2 protein translated in the $m$Per2$^{Brdm1}$ mutant has no function in the circadian clockwork anymore.

Fig. 33: $m$Per2 and $Bmal1$ expression in the SCN of $m$Per/ $m$Cry triple mutant mice. (A) Diurnal mRNA profile of $m$Per2 in wild-type and $m$Per/ $m$Cry triple mutant mice. (B) Diurnal mRNA profile of $Bmal1$ in wild-type and $m$Per/ $m$Cry triple mutant mice. Black and white bars on x-axis indicate dark and light phase.
To further investigate the role of the truncated mPER2 protein in the mutant clockwork we examined mPER2 protein localization in wild-type, mPer2, mPer2/ mCry1 and mPer2/ mCry2 mutant mice by immunofluorescence. These four strains represent different configurations with regard to the role of mPER2. Wild-type animals are rhythmic and have a non-mutated mPER2 protein. mPer2^{Brdm1} animals become arrhythmic after some time in DD and have a mutated mPER2 protein. mPer2^{Brdm1}/ mCry1^{-/-} animals become immediately arrhythmic in DD and have a mutated mPER2 protein. mPer2^{Brdm1}/ mCry2^{-/-} animals are rhythmic but have a mutated mPER2 protein. We found mPER2 immunofluorescence predominantly in the nucleus in all four strains and at all time points examined.

![Image of mPER2 immunofluorescence in the SCN of mPer2/mCry mutant mice.](image)

Fig. 34: mPER2 immunofluorescence in the SCN of mPer2/mCry mutant mice. Shown are representative micrographs from ZT0 (a-d, e-h) and ZT12 (i-m, n-q) from wild-type (first row), mPer2^{Brdm1} (second row), mPer2^{Brdm1}/ mCry2^{-/-} (third row), and mPer2^{Brdm1}/ mCry1^{-/-} mice (fourth row). Second and fourth column show magnifications of the micrographs to the left.
2.3.4. Output genes

In the last set of experiments we examined clock output in double and triple mutant mice. We chose AVP and Dbp as two genes with prominent diurnal and circadian oscillation in the SCN of wild-type mice. Both genes have been shown to be directly clock controlled (Jin et al., 1999; Ripperger et al., 2000). However the exact regulation of these genes in the SCN and the periphery still needs to be elucidated.

Fig. 35: AVP expression in mPer/mCry double mutant mice. (A, B) Diurnal expression profiles of AVP transcript in the SCN. Black and white bars indicate dark and light phase. (C, D) Circadian expression of AVP transcript in the SCN. Grey and black bars indicate subjective day and night respectively. Data are mean +/- SD of three different experiments.
Results

Fig. 36: *Dbp* expression in *mPer*/*mCry* double mutant mice. (A, B) Diurnal expression profiles of *Dbp* transcript in the SCN. Black and white bars indicate dark and light phase. (C, D) Circadian expression of *Dbp* transcript in the SCN. Grey and black bars indicate subjective day and night respectively. Data are mean +/- SD of three different experiments.

![Diagram A](image1)

![Diagram B](image2)

![Diagram C](image3)

![Diagram D](image4)

Fig. 37: AVP and *Dbp* expression in *mPer*/*mCry* triple mutant mice. Diurnal expression profiles of AVP (A) and *Dbp* (B) transcript in the SCN. Black and white bars indicate dark and light phase.

AVP and *Dbp* seem to be differentially influenced by the *mPer* and *mCry* genes. While AVP expression is low and arrhythmic in *Per2* mutants in LD and DD, *Dbp* levels are still clearly cycling (see as well (Albrecht and Oster, 2001)). In the arrhythmic mutants like *mPer2mCry1*<sup>−/−</sup> and the triple mutants output gene rhythms are all blunted and generally low indicating that the LD cycle cannot substitute or re-induce clock oscillation to signalize rhythmicity to the body.
In the second project of my Ph.D. thesis I studied the molecular circadian clockwork of the blind mole rat *Spalax ehrenbergi*. This work was performed in collaboration with Dr. Aaron Avivi and Prof. Eviatar Nevo from the Institute of Evolution at Haifa University, Mount Carmel, Israel. While they performed all the animal work and sequence analysis we focused on the analysis of clock gene expression in different conditions and tissues.

**Fig. 38: Spalax ehrenbergi**

The *Spalax ehrenbergi* superspecies is a family of four actively speciating chromosomal subspecies (2n = 52, 54, 58, and 60 (Nevo, 1991)). *Spalax* is a solitary subterranean herbivore that spends more than 95% of his life in underground burrows, rarely coming to the surface. It represents an extreme example of natural eye and brain reorganization in mammals (Nevo, 1999). It is completely blind (Haim et al., 1983), yet the retina of the atrophied subcutaneous eye functions in photoentrainment of locomotor activity and thermoregulatory rhythms (Pévet et al., 1984; Rado et al., 1991). The eye is surrounded by a extremely hypertrophic Harderian gland which plays a role in the integration of photoperiodic changes (Pévet et al., 1984) and probably represents an adaptation of the mole rat's circadian system to its specialized ecotope. Despite its subterranean habitat and its degenerated visual system Spalax has a functional SCN, that can receive light/dark information from the retina via the hypothalamic tract and generate circadian rhythmicity (Ben-Shlomo et al., 1995; Nevo et al., 1982; Rado et al., 1991).

Another unique feature of *Spalax* is its polymorphic activity pattern with predominantly diurnal as well as predominantly nocturnal active individuals in the same populations (Nevo
et al., 1982). Nevertheless, activity rhythms can be entrained to changing light dark cycles in the laboratory (Tobler et al., 1998), indicating that the circadian clock of this animals retained its sensitivity to light.

When we started this project only few data was available on the molecular aspects of clocks from diurnal animals. Additionally, the ability of *Spalax* to change its activity pattern from nocturnal to diurnal and *vice versa* offered the opportunity to look for differential mechanisms of light entrainment in one species.
2.4. Publication: “Biological clock in total darkness: The Clock/MOP3 circadian system of the blind subterranean mole rat”

Aaron Avivi, Urs Albrecht, Henrik Oster, Alma Joel, Avigdor Beiles, and Eviatar Nevo

Proceedings of the National Academy of Sciences of the USA, 98 (24), 2001
Biological clock in total darkness: The Clock/MOP3 circadian system of the blind subterranean mole rat

Aaron Avivi1*, Urs Albrecht1, Henrik Oster1, Alma Joël1, Avigdor Beiles1, and Eviatar Nevo1*

1Institute of Evolution, University of Haifa, Mount Carmel, Haifa 34988, Israel and 2Max Planck Institute for Experimental Medicine, 38124 Hamberg, Germany

Contributed by Eviatar Nevo, September 20, 2001

Blind subterranean mole rats retain a degenerated, subcutaneous, visually blind but functionally circadian eye involved in phototaxic perception. Here we describe the timing, sequence, and expression of the circadian Clock and MOP3 (OMP) dimers of the Spalax ehrenbergi superspecies in Israel. Both genes are relatively conserved, although characterized by a significant number of amino acid substitutions. The glutathione-rich area of Clock, which is presumed to function in circadian rhythm, is expanded in Spalax compared with that of humans and mice, and is different in amino acid composition from that of rat. We also show that MOP3 is a bona fide partner of Spalax Clock and that the Spalax Clock/MOP3 dimer is less potent than its human counterpart in driving transcription. We suggest that this reduction in transcriptional activity may be attributed to the Spalax Clock glutathione-rich domain, which is unique in its amino acid composition compared with other studied mammalian species. Understanding Clock/MOP3 function could highlight circadian mechanisms in blind mammals and their unique pattern as a result of adapting to life underground.

Biological Clocks Underground

The behavior of all eukaryotic organisms is characterized by a 24-hour cycle of rest and activity, as a fundamental adaptation to the solar cycle of light and darkness (1–5). In mammals, the pacemaker of this circadian rhythm is localized in the central nervous system (6) and is entrained by light signals in the eye. An intriguing question is how a subterranean, visually blind mammal with a subcutaneous degenerate eye maintains its circadian system, and whether its evolution can illuminate circadian rhythm of sighted mammals above ground.

The underground adaptive ecogeographical radiation of the Spalax ehrenbergi superspecies in Israel involves four sibling species: Spalax ehrenbergi, Spalax galili, Spalax castoris, and Spalax bedoti, with diploid chromosome numbers 2n = 52, 54, 56, and 60, respectively (6), displaying progressive stages of final ecological speciation (7, 8). Their adaptive radiation in Israel, from easily fleistic to Recent times, is closely associated with increasing aridity of the climate, but, with distinct climatic diversity (9–11). Spalax bedoti habitat is in the upper northern Gobina Mountains, Spalax galili, in the cool-secluded northeastern Golani Heights; Spalax castoris, in warm-humid central Israel; and Spalax bedoti, in warm-dry southern Samaria, Judea, and the northern Negev mountains and plains.

Spalax represents an extreme example of situational and brain organization in mammals (5, 9). The animal is completely blind (10), yet the retina of the atrophied subcutaneous eye functions in photoentrainment of locomotor activity and thermoregulatory rhythms (11–13). Ocular regression of thalamic light structures conceals adaptive progression of the photosphere system (14, 15). Retinal photoreceptors (16) and corneal photoreceptors participate in photoentrainment (17, 18). Pe-rhomologous AC27GSE gene sequencing (19) shows phototactic circadian oscillation (19). This Spalax displays behavioral circadian rhythm adapted to life underground.

Activity patterns were tested in the four sibling species of the S. ehrenbergi superspecies (20). Activity patterns were found to be polyphasic and polymorphic (19, 20), with a remarkable intra- and interspecies diversity in circadian patterns (refs. 21 and 22—to one species is identified in the latter study) coupled with seasonal shifts. This complex pattern is unique in mammals analyzed to date. These patterns may display an adaptive strategy of a subterranean mammal, safeguarded from above-ground predation.

Molecular Genetic Basis of Circadian Rhythms

In recent years, tremendous progress has been achieved in revealing the molecular genetic basis of circadian activity in different organisms across phyla, including cyanobacteria, plants, fungi, insects, and mammals (1, 25). All show homologous circadian molecules, both structurally and functionally (29). The circadian genes identified in mammals are Clock/MOP3, Per1, Per2, Per3, Tim, Cry1, and Cry2 (28), all interacting in circadian rhythm. In Drosophila, the so-called period (Per) gene affects rhythmicity in circadian (~24-h), ultradian (~24-h), and infradian (~24-h) time domains (28). Work in Drosophila and mice suggested a mechanism in which expression of Per genes is driven by a CLOCK/MOP3 heterodimer, which, through their basic helix-loop-helix (BHLH) motif and the PAS domain, binds to the E-box present in the Per promoter, thereby activating Per transcription. PER/TIM and PER/CRY complexes probably block the activation of Per transcription by interfering with the activity of Clock/MOP3 (29). Indeed, it was recently proven (30) that the loss of PAS protein MOP3 in mice results in immediate and complete loss of circadian rhythmicity. Recent studies (57) more specifically clarify the detailed mechanisms of the regulation of the circadian rhythm. Studying mutant Clock, Per, and Cry of mice, Sheerman et al. (57) showed that Per1 has a dominant role in the positive regulation of MOP3, whereas the Cry are the negative regulators of the Per and Cry cycle of clock mutants appear to positively alter the regulation of MOP3 gene expression in the suprachiasmatic nucleus (SCN), but not the regulation of Clock itself. As the oscillations of the Per and Cry RNA are down-regulated in the Clock mutant mice, the effect of Clock on MOP3 levels is probably indirect and may occur through the Per and/or the Cry proteins that control levels of one or more of these genes may cause the reduced levels of MOP3 in the Clock mutant mice through loss of the positive control of MOP3 transcription.

The underlying genetic basis of circadian rhythms in the blind subterranean species may be different from that of strictly diurnal or nocturnal and sighted mammals, such as humans and mice, which presumably lack multiphasic, polymorphic, and
Results

Seasonal and circadian patterns. The coupled ecological and photogenic expression of *S. galili* makes it an intriguing model organism for assessing the molecular machinery of the biological clock.

Our objectives in this study were to compare and contrast the structure and the expression of the *Clock* gene in the blind subterranean mole rat with those of other mammals, and examine experiments for its potential effects on the unique circadian rhythm of these species. We show differences in *Clock* structure and expression between mole rats, rats, mice, and humans. We also demonstrate differences among three mole rat species whose significance may derive from the ecological differences of the species—a testable hypothesis.

Materials and Methods

**Animals.** Animals used for the cloning of the mole rat *Clock* belong to three species of the *S. chrysochloris* superspecies in Israel (64-68). All animals were healthy and free of disease. Animals were housed in individual cages, each species in a separate room. They were kept under controlled conditions of 23-25°C with seasonal light/dark hours and fed with carrots and apples. Animals in this study were adults and of similar weight (100-150 g).

**Cloning.** By reverse transcription (RT)-PCR (78), we cloned the complete ORF of *Clock* from three individuals each belonging to a different species. The cloning strategy used for the RT-PCR cloning was designed according to the published sequence of human *Clock* (GenBank accession no. AF151966) and that of the mouse (GenBank accession no. NP009908). These were 5' sense oligonucleotide 5'-ACAAAGACCAAGAACGATGA(GA)AGTA(TG)ATAGTTGTAG (3' ATO is the initiation codon) and 3' antisense oligonucleotide 5'-AGAAGAGGAAGCTAAGAACCTCAAGTTCAC (5' CTA is the termination codon). The PCR fragment was inserted into the plasmid pBluescript SK(+) (Stratagene, La Jolla, CA) and sequenced. The cloned cDNA was then purified and used as the template for sequencing.

**Results**

Cloning and NGS. A full-length *Clock* cDNA clone was isolated from *S. galili* brain tissue by using oligonucleotides designed according to the published sequence (78). The PCR products were cloned into pBluescript SK(+) and sequenced. The sequence has been submitted to GenBank (accession no. AF404665). The sequence was then compared to the published human and mouse sequences. The sequence was found to be highly conserved between species.

**Transcriptional Activity.** The cDNA ORF of *Clock* (305 nucleotides) was inserted into a plasmid expression vector (pTARGET, Promega). Plasmids expressing these constructs were cotransfected with an hEGFP or mRFP(+) expression plasmid into the human hepatoma cell line HepG2. In these experiments, the luciferase activity was measured 24 h after transfection by using a Mantle assay. The luciferase signal was normalized to β-galactosidase expression using an anti-luciferase antiserum (Clontech). The relative light units (RLU) were calculated from the luciferase activity divided by the β-galactosidase activity for each assay.

**In Situ Hybridization.** In situ hybridization was performed using a DIG-labeled PCR probe. The probe was synthesized using random hexamers and digoxigenin-labeled dUTP. The probe was hybridized to 10 µg of poly(A)-RNA from *S. galili*. The probe was then detected using an anti-digoxigenin antibody conjugated to alkaline phosphatase. The slides were then counterstained with hematoxylin.

**Results**

**Discussion**

The results presented in this study demonstrate the potential of the *S. galili* species as a model organism for the study of circadian rhythms. The high conservation of the *Clock* sequence between species suggests that the molecular mechanisms underlying circadian rhythms are conserved across species. The results also indicate that the *Clock* gene plays a key role in the regulation of circadian rhythms in these species. The findings of this study provide a valuable resource for the study of circadian rhythms and their role in physiological processes.
Results

Table 1. Summary of mutations in Clock cDNA

<table>
<thead>
<tr>
<th>SS</th>
<th>SS5</th>
<th>SS5</th>
<th>SS6</th>
<th>Mut</th>
<th>Hu</th>
</tr>
</thead>
<tbody>
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<td>—</td>
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<td>—</td>
<td>—</td>
</tr>
<tr>
<td>S56</td>
<td>3556 + 46(17)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>S56</td>
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<td>—</td>
<td>—</td>
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<td>—</td>
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<tr>
<td>Mut</td>
<td>3556 + 46(156)</td>
<td>2556 + 1556(185)</td>
<td>2456 + 54(173)</td>
<td>2456 + 54(178)</td>
<td>2556 + 1556(128)</td>
</tr>
</tbody>
</table>

The distance in Clock cDNA sequence among the three species was studied: (S52, SS5, and SS6), in mouse (Mut), and human (Hu). The distance is expressed in number of synonymous (S) and non-synonymous (mut) substitutions. The total number of substitutions appears in parentheses. Note the larger number of substitutions between Spalax and humans than that between Spalax and mouse. Also noteworthy is the unusual larger number of non-synonymous mutations compared with the synonymous substitutions among the Spalax species, indicating interspecies functional changes.

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<table>
<thead>
<tr>
<th>SS</th>
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all studied species. The most impressive difference between Spalax and human and mouse is in the Q-rich domain as can be seen in Fig. 1. The Spalax repeat is 18 as longer than that found in humans and 5 as longer than that of mice and it is different in amino acid composition from rats.

The Spalax MOP3 cDNA (accession no. AI318026) is also very similar to those of mouse and human. The putative peptide encodes 66 amino acids. Like human MOP3 (accession no. AF042288), it misses the Leu-Asp-Glu-Phe-Ala-Phe-Glu, which appears in the mouse (positions 18-24 in the mouse peptide, accession no. AB012531). There are 8 unique amino acid substitutions in the Spalax peptide (positions 9, 9, 9, 9, 9, 9, 9, 9, and 9). However, the activity domains of MHLH and PAS are completely identical in the three species.

The Clock Similarity Trees. We have used the Kimura distances (9) to generate two trees. One is based on the distances between nucleic acids and the other is based on distances between amino acids (Fig. 2). The method examines each pair of aligned sequences item by item and counts the number of exact matches, partial matches, and gap symbols. If the sequences are nucleic acids, transitions and transversions are also tallied. The Clock similarity trees show that S. judae, the youngest species, seems to evolve faster than its other sibling species. Furthermore, Spalax is somewhat closer to humans than to mice in CLOCK protein structure (see also Table 1). This result is in contrast with all other phylogenetic trees of Spalax derived from other sets of data (5-9).

Function of the Clock MOP3 Dimer. As already stated above, Spalax has a distinctly different polymorphic (polQ) repeat (Fig. 1). The Spalax MOP3 is very similar to the human MOP3 repeat, but also to the mouse protein. To functionally test whether the difference in the Q repeat affects Spalax Clock activity, we compared S. galil Clock with human Clock, which is shorter by 18 as at the polQ domain. This was done by an assay that measures transactivation by Clock:MOP3 heterodimer of E-box elements, a type of transcrip-

![Fig. 1. The Q-rich domain of Spalax Clock compared with human, rat, and mouse Clock. Spalax Clock was cloned from brain tissue of three species, Spalax galil (28 = 52, 2), cornutus (28 = 10, 2), and S. judae (28 = 60 by EST probe), with oligonucleotides from the published sequence of mouse, rat, and humans as specified in the text. Two independent clones from each strain confirmed each sequence. The figure depicts a portion of the clock Q-rich domain of the three Spalax species (S52, SS5, and SS56), human (Mut), mouse (Mut), and rat (Mut). Numbers at both sides represent the amino acids aligned. The figure displays multiple sequence alignment in the respective motifs, sharing representatives regions that agree with a calculated consensus sequence (Wisconsin package version 10.1). (D) amino acids did not align among the compared species.](image1)

![Fig. 2. Similarity tree of the Clock protein. The unrooted tree describes the similarity relationships among the Clock of the three Spalax species (S52, SS5, and SS56), mouse (Mut), rat (Mut), and humans (Mut). The calculated distances show a somewhat closer relationship of Spalax clock to human than to mouse Clock and a rather evolution of the younger S. judae (28 = 46) than its sister species S, galil (28 = 52) and S, cornutus (28 = 10).](image2)
are involved in the maintenance of the circadian system, namely brain, eye, and harderian gland. As demonstrated in Fig. 6, MOP3 also oscillates in the harderian gland, and although its oscillatory pattern is different from its pattern in the SCN, the peak of its expression is identical in both tissues.

Discussion
Circadian rhythm is localized in the central nervous system and is entrained by light signals in the eye. Light cues lead to structural reorganization of eye photoreceptors (6). Is function also lost? Maintenance of eye lid margins after million of years in darkness suggests functionality. Blind subsurface mole rats, S. eisenbergi, and S. compostus, in Israel, have undergone evolutionary thinning that has optimized molecular and structural reductions and expansions presumably through loss and gain of homologous mutations in organizing a photoperiodic system, adapting to live in total darkness underground (63). In our current work we present data on the cloning, structure, and expression of circadian genes from a visually blind, subterranean mammal.

Spalax Clock and MOP3 Structure. Despite being a rodent, Spalax, an old rodent split (7, 5), is somewhat closer to humans than to mice in Clock structure, as was already manifest for some other genes, i.e., Spalax IGF1 (33), VEGF cDNA (34), and HMG1 genes (preliminary results). Spalax presumably still retains early rodent pattern recognition and adapted to life underground. Spalax Clock demonstrates distinct interspecies polymorphism and S. judea, the youngest species of the Spalax eisenbergi superspecies in Israel (6–7), seems to evolve faster than the relatively older S. palli and S. cornelli species, suggesting distinct interspecies divergence across very short geographic distance (40 km). This may be due to its unique genetic ecology with large tracts and its polyphasic activity pattern in a stressful climate (6–8). Note that already in our first study of activity patterns of different S. eisenbergi species (20), we found that S. judea, which inhabits a significantly warmer and dryer environment than the northern species, S. palli and S. cornelli, has a significantly different activity pattern in both levels and patterns. S. judea is much more active and has more, albeit shorter, periods of activity, displaying an adaptive strategy of increasing fitness in arid environments (30).

Evolution of the Clock Gene in Spalax Species. The phylogenetic relationship of the four Spalax species was analyzed earlier by allozyme (35) and DNA-DNA hybridization (36). The trees presented here display the interspecies Clock similarly relationship of the three Spalax species is different from the phylogenetic trees derived from allozymes and DNA-DNA hybridization. Like the tree derived from cytochrome b (39), the Clock tree seems to represent not chronological relationships, but a gene tree that describes the rate of evolutionary divergence of the Clock gene in different species. This assumption is supported by the comparison between silent and nonsilent substitutions presented in Table 1. This table shows that the distances with human and mouse Clock involves 3 to 7 times more silent substitutions than nonsilent
mutation, which is the usual result when comparing sequences. However, a comparison of the three Spalax species exhibits 2 to 3 times more substitutions that cause amino acid changes than those that are synonymous. This is a very unusual observation in phylogenetic trees. The dramatic divergence of the youngest species, S. israeli, which displays much faster evolution (more amino acid changes) cannot be reconciled with its relatively recent divergence (8, 10). It appears more plausible that ecological factors were involved, such as spatiotemporal increasing stressful acidity, which may indirectly affect the circadian pattern, as was demonstrated by radiotracing in time.2

Expression and Function of the (Clock/MOP3) Dimer. Circadian oscillators appear to be highly conserved throughout evolution and involve transcription-translation feedback loop systems (3, 23). The observation that PAS proteins play an important role in maintaining circadian rhythms supports this idea (41, 42). A distinguishing characteristic of the Clock gene is the presence of several functional domains in its amino acid sequence. These are the bHLH region (amino acids 36-111), the PAS A direct repeat (amino acids 112-163), the PAS B direct repeat (amino acids 272-335), and the Q rich domain at the C terminus. Although the bHLH and PAS domains are very conserved among the different Clock DNA's, the most striking difference is in the significant variation in the Q repeat near the C terminus of Clock. The Q repeat characterizes the activation domains of many transcription factors (43), and it has been shown to influence circadian rhythmicity (44). It has been shown that Δ to Δ29 nucleotide transversion in a splice donor site, which results in exon skipping and a deletion of 21 amino acids from the Q repeat region in the mouse protein, appears to be the cause of a number of changes in the circadian phenotype (45). Most notably, mice homozygous for this deletion manifest a 3-4 h longer period (27-30 h) on initial placement in the dark and then become arrhythmic after a few weeks in darkness. A 6 h exposure to light of these arrhythmic mice restores the normal 24 h period. Hence, in addition to a bHLH region (DNA binding domain) and PAS region (protein dimerization domain) Clock also contains a transcriptional activation domain (the Q rich region). The mutant protein can therefore compete with native Clock for binding partners and or DNA binding sites, which explains the dominant negative nature of the mutant allele (43).

Evidence demonstrating that proper activity of the circadian system requires heterodimerization of two bHLH-PAS proteins: Clock and MOP3 (23, 24, 46). Our results prove that there is a reduced transcriptional activity (P < 0.001) of the Spalax Clock/MOP3 dimer compared with humans, possibly increasing metabolic activity (15). The fact that this pattern is altered after interfering with the Q rich domains of the two species supports an assumption that it is this domain that causes the difference in transcriptional activity. It was already shown that mutant Clock could form heterodimers with MOP3 that bound DNA but failed to activate transcription (46). Moreover, such a mutant, probably by down-regulating the oscillations of Per and CRY RNA, leads to reduced levels of MOP3 transcription, hence limiting the available Clock/MOP3 heterodimers at the appropriate circadian time to drive Per/Cry transcription and restart the cycle (17). This suggests that Clock, after binding to a specific transcription activation site (the E-box), upregulates the initiation rate of Per, appears to drive the positive component of Per transcriptional oscillations, which are thought to underlie circadian rhythm. Thus, the Spalax unique Clock may partially maintain transcription, which explains the ability to bind DNA and dimerize with partners. Further studies are essential to directly relate the unique polyQ repeat of Spalax Clock, with the reduced transcription of Spalax Clock or with its unique polyQ (26), polymeric (19), and seasonal (41) differences in circadian rhythms. Nevertheless, other differences in the Spalax Clock or MOP3 sequence, although evolutionarily imperceptible, cannot be related to any presently known functional sites. Hence, the reduced activity of Spalax Clock may be causally attributed to the different polyQ of its protein. Transgenic mice with Spalax Clock may elucidate its direct function.

Tissue Distribution and Oscillation of Spalax Clock and MOP3 Expression. As in other mammals, Spalax Clock does not oscillate, but Spalax MOP3 does oscillate (23). Notably, the oscillatory pattern of MOP3 in the Spalax SCN is a mirror image of that of the mouse Per3 (37) as well as the Spalax equivalent (preliminary results). That is, MOP3 expression is at its minimum at Z16, whereas Per3 expression is at its maximum. This observation may be explained by Shearman et al. (37) interpretation of the feedback mechanisms between Clock/MOP3 heterodimer level and the transcription drive of Per/Cry.

The tissue distribution of Spalax Clock is not restricted to tissues directly related to the maintenance of the bioclock, as was reported in mice (44). It may be involved, as was shown in Desmodus, in both circadian (47) and noncircadian oscillations in neoplastic tissues (48), or as hypothesized by Yamasaka et al. (48) in transgenic rats, that a self-sustained circadian pacemaker in the SCN entrains circadian oscillations in the peripheral tissues. MOP3 is expressed in all tissues studied, which is similar to what was demonstrated in mice and may reflect its involvement as an orphaned dimerizing protein in other transcription activation systems, like the hypothalamic clock factor (28).
Clock and MOPP Expression in the Harderian Gland. We are aware of the no other report of expression of circadian genes in the harderdian gland. Although the oscillatory pattern of MOPP is different from its pattern in the SCN, the peak of its expression is identical in both tissues. This may indicate a unique synchronization of these two times that has yet to be clarified, because in other tissues there is always a lag in the peak of expression of oscillatory circadian genes between the SCN and peripheral tissues. The expression of Clock and MOPP in the harderdian gland of Spalax is remarkable and should be emphasized: the harderdian gland of Spalax is tremendously hypertrophied, occupying the entire eye-sac, whereas the eye is completely atrophied. Immunohistochemistry using anti-Clock and anti-MOPP antibodies, and indirect immunofluorescence with antibodies to the harderdian gland of Spalax, has been previously described. It was previously suggested that the harderdian gland of Spalax is a possible photoreceptor and photoperiodic organ (11).

Circadian Genes in a Blind Subterranean Mammal. Expression of circadian genes in Spalax has been used to highlight the functional role of its behavioral pattern of circadian rhythmicity (19-23). We have already cloned the Spalax (CNA) homolog of Per, Rev-2, Fos, Cry, and Ctr1 (unpublished results). We plan to evaluate their function and potential conservation in other subterranean mammals. Likewise, the Spalax photoreceptor pigment rhodopsin (16) and long-wavelength coneopsin (17, 18, 20) have been cloned and sequenced, and their unique Spalax function in phototransduction was evaluated, presumably as adaptation to underground conditions by significant enrichment for wavelengths greater than 500 nm, possessing photosensitive cones, but minimizing the effects of other (18).

The mosaic evolution of the Spalax eye (17, 14, 15), harderdian gland (11), and brain (17), and its circadian genes provides a dramatic model of tinckering evolution at both the molecular and organismal levels. From an evolutionary perspective, the genetic basis of circadian rhythms must be understood; the molecular events may be different from that of strictly diurnal or nocturnal and sighted mammals. The identification of the blind Spalax Clock might help, together with its other circadian genes, characterizing the structure and evolution of the circadian organisation in mammals, including humans, at the molecular level and their ecological context. Further, it might highlight both adaptive evolution linking structure and function and behavioral-molecular interactions of regulation, progression, and convergence in subterranean mammals generally, and especially in the Fleschonine ecosystemic subterranean Spalax edenbergi species caused by increasing ecological adversity stress (6-8), which in turn affect the patterns of circadian rhythmicity. It could also elucidate, by transgenic studies in mice, the control of the Spalax unique polyphasic, polymorphic, and seasonal cycles, as well as sleep disorders, work-shifts, and jet-lag in humans.

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2.5. Publication: “Circadian genes in a blind subterranean mammal II: Conservation and uniqueness of the three Period homologs in the blind subterranean mole rat, Spalax ehrenbergi superspecies”

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Circadian genes in a blind subterranean mammal II: Conservation and uniqueness of the three Period homologs in the blind subterranean mole rat, Spalax ehrenbergi superspecies

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We demonstrated that a subterranean, visually blind mammal has a functional set of three Period genes that are important components of the circadian clockwork in mammals. The mole rat superspecies Spalax ehrenbergi is a blind subterranean animal that lives its entire life underground in darkness. It has degenerated eyes, but the retinas and highly hypertrophic habenular ganglia are involved in photoperiodic perception. All three Period genes oscillate with a periodicity of 24 h in the suprachiasmatic nuclei, eye, and habenular ganglia and are expressed in peripheral organs. This oscillation is maintained under constant conditions. The light induction of Per1 and Per2, which are similar in structure to those of other mammals, indicates the role of these genes in clock resetting. However, Per1 is unique in mammals and has two truncated isoforms, and its expression analysis leaves its function unresolved. Per1 expression analysis in the habenular ganglion suggests an important participation of this organ in the stabilization and resetting mechanism of the central pacemaker in the suprachiasmatic nuclei and in unique adaptation to life underground.

Life on Earth is adapted to cyclical phenomena imposed by the external environment (1). Most organisms have circadian systems that synchronize physiological events to the external 24-h cycle (2). The underlying molecular genetic mechanisms of these clocks exhibit an extraordinary evolutionary conservation from cyanobacteria through plants, fungi, and animals. All of these clock systems consist of autoregulatory transcriptional/translational feedback loops with positive/negative regulatory elements and similar genetic machinery (3, 4).

Two basic helix-loop-helix PAS (PER-ARNT-SIM) transcription factors, CLOCK and BMAL1, form the positive elements of the system and drive transcription of three Period (Per 1, 2, 3) and two Cryptochrome (Cry 1, 2) genes. The protein products of these genes are thought to be components of a negative feedback loop that inhibits the CLOCK/BMAL1 heterodimer, thus, stabilizing the clock loop.

The enigma of circadian rhythms in a blind subterranean mammal is intriguing (5–7). We have already shown that a CLOCK/BMAL1-driven clock exists in Spalax (8). Here we continue to decipher its circadian machinery.

The Evolutionary Model of Blind Subterranean Mammals

The blind subterranean mammals, mole rats of the Spalax ehrenbergi superspecies, consist of four species that have been studied multidisciplinarily as an evolutionary model of speciation and adaptation (5–7). Spalax lives in total darkness, yet, it perceives the daily and seasonal temporal cycles underground (9). Behaviorally, Spalax displays polyphasic and polytypic day-night activity patterns (20, 14) coupled with polymorphic (12) and seasonal variation, supported by a unique photoperiodic perception mechanism (9). Spalax had a degenerated a.c. functional eye (13, 14), which, together with the habenular ganglion, participates in photoperiodic perception (9, 15–18). The retina harbors Rhodopsin (19, 20) and Coneopsin (21), adaptively effective in photophase perception (22, 23), and expresses alpha-B-crystallin (24). The photic signals contain a function in the suprachiasmatic nucleus (SCN)/Zeisberger (25) and can possibly activate circadian genes.

Evolutionarily, Spalax’s genealogical brain structures (SCN and anterior) were expanded and light pathways were dramatically (>90%) reduced. The visual cortex was replaced by somatosensory cortex (26–28), homologous AC1/NCN/ACNN-repeat cycles in the hypothalamus (29) and melatonin precursors occur in the habenular and pineal glands and retina (30).

What is the genetic basis of circadian rhythmicity in Spalax? We cloned, sequenced, and unraveled the expression of the circadian Clock and MO16 cDNAs of three species of the S. ehrenbergi superspecies in Israel (5). Both genes are relatively conserved, yet Clock displays a unique O-rich region as compared with other mammals, assumed to function in circadian rhythmicity, and Spalax Clock/MO16enser is less potent than its human counterpart in driving transcription.

Here we describe the cloning, sequencing, and expression of the three Period cDNAs of Spalax. Its three Per cDNAs are conserved, yet they show unique features in Spalax especially in Per1, Per2, and Per3 cycles in the SCN, eye, and habenular gland. Per2 is structurally unique among studied alignment mammals and arrests functional circadian rhythms.

Materials and Methods

Animals. We analyzed adults (100–150 g), belonging to Spalax judear, (20–60) from Arza, Samaria (7). Field-trapped animals were kept at 22–24°C with seasonal photoperiod. We selected diurnal animals that were kept under a 12-h light:12-h dark cycle. For analysis of Per transcriptional activity in constant darkness, light was turned off at Zeitgeber time (ZT) 12, and animals were kept in the dark for at least 2 days before being killed under dim illumination (15-W safety red light). Light-inducibility experiments were done on animals kept in light (ZT 12) for a week with a short light pulse (15 min, >200 J) at specified ZT followed by release into constant darkness. For gene induction analysis brains were taken 1 h after illumination. Each experiment was done on three sets of animals.

Abbreviations: mRNA, poly-A mRNA; cDNA, cDNA; ZT, Zeitgeber time; POL, reverse transcription–PCR, DNA; OPL, Oligo (dT)25, RNA; OPL, Oligo (dT)25, DNA. For details on the sequence see published paper. All sequences deposited in the GenBank database (accession nos. AY195898–AY195942).

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Results

Cloning of Spinus Per (dASNs). We cloned the three Spinus Per dASNs by reverse transcription PCR (RT-PCR) (31). Oligos were synthesized according to the ORF of the known human and mouse homologs (sequences GenBank accession nos. AF022991, AF020872, and AB010686 for human Per1, Per2, and Per5, respectively, and AF020965 for mouse Per1, Per2, and Per3, respectively). Whole brain total RNA was purified by using the Trizol RNA isolation agent (Molecular Research Center, Cincinnati). First-strand cDNA was synthesized with oligo(dT) as a primer and SuperScript II reverse transcriptase enzyme (Gibco/BRL). This cDNA product was taken for PCR by using TaqDNA polymerase (Applied Biosystems, Foster City). The annealing temperature, elongation time, and MgCl2 concentration were adjusted for each specific PCR. In the case of Per2 isolation, we verified our RT-PCR results by also cloning through cDNA library screening (32). Spinus brain cDNA library in Lambda TriPlEx II was screened using a partial mouse Per2 cDNA as a probe. Sequencing was determined by thermocycling sequencing using di-deoxy nucleotide terminators (370 DNA Analyzer, Perkin-Elmer Applied Biosystems) at the sequencing unit of the Weizmann Institute of Science (Rehovot, Israel).

Evolutionary Analysis. The evolutionary analysis of the Per dASN s presented here is based on distant and divergence calculations (Wisconsin package version 10, GCG). The DISTANCES program (33) calculates pairwise distances between aligned sequences expressed as substitutions per 100 bases or amino acids. To correct the distances for multiple substitutions at a site, we used Kumm's nucleic acid (33) and protein (34) methods.

The PHYLIP program estimates the pairwise number of synonymous and nonsynonymous substitutions per site between two or more coding aligned nucleic acid sequences (35, 36).

In Situ Hybridization (ISH). Tissues used for ISH were treated and counted as described (37). The Spinus Per2 probe corresponded to nucleotides 641-1300, the probe for Spinus Per1 corresponded to nucleotides 1751-2791.

Quantification was performed by densitometric analysis of hybridization signals on autoradiographs with Scion (Frederick, MD) IImage 3.0 software. The signal was visualized by fluorescein isothiocyanate (FITC)-stained nuclei, and silver grains signals were artificially colored for clarity. Quantification of ISH results was analyzed with GraphPad (San Diego) Prism software. Data sets were compared by ANOVA with subsequent Bonferroni correction for multiple comparisons, with R < 0.05 as the criterion of significance.

Quantitative RT-PCR. For quantification of the dASN expression in the barbadeau gland and the liver of Spinus, a quantitative RT-PCR was performed. Equal amounts of total RNA from animals killed at the relevant ZT points were taken for first-strand cDNA synthesis (see above). The cDNAs were synthesized using a mixture of (dT)15/CTP to ensure equal amounts of cDNA templates in the PCRs. For the PCR we used oligos synthesized according to the sequence of the different dASNs isolated from Spinus. For each Per gene, we used one oligo sense 5' oligo and two antisense 5' oligos (1'1 and 2'1), giving rise to two distinct products (450-400 bp). One set of oligos (5'2 and 2'2) was used for the barbadeau gland and the other set of oligos (5'3 and 3'2) for the liver, in the same PCR tube. A second PCR was carried out by changing the 5' oligo between the barbadeau and liver tissues. In each PCR, a 100 bp fragment of actin, as an internal control, was also synthesized, using specific actin oligos. Each cDNA sample was tested for different PCRs amplifying cyclins with the different sets of oligos. The final experimental PCRs were performed at the logarithmic phase of the reaction for each specific cDNA of interest (30-22 cycles). Each experiment was carried out on two RNA samples taken from two different individuals, and each PCR was repeated three times. The PCR products were then run on ethidium bromide (1.3%) agarose gels. The gels were subjected to quantitation of the specific bands by the Eagle Eye II system (Stratagene). The most intense band from each lane was measured and used as the value for that lane. The results were recorded for each individual animal for each of the 10 different combinations of hybridization conditions.

Results

Cloning and Structural Analysis of the Three Spinus Per (dASNs). Analysis of the ORFs of Spinus Per (dASNs) revealed transcripts of 1,062 aa residues for dPER1 (GenBank accession no. AB34262) and 1,216 aa for dPER2 (GenBank accession no. AB34560). For dPER1 we isolated two truncated deduced proteins, one with 496 aa (isofrom a, GenBank accession no. AB34560) and the other with 583 aa (isofrom b, GenBank accession no. AB34560). Identified functional domains like the PAS domain and the basic helix-loop-helix motif are highly similar in dPER1 and dPER2, but the homology in dPER3 is low. The recently identified casein kinase 1ε (CK1ε) binding site of human PER2 and the five putative phosphorylation sites (AA 666, 671, 674, 677, and 680) (30) are conserved in PER proteins of Spinus, mouse, and humans with the exception of dPER3. Hence dPER3 is probably not a substrate for the Spinus CK1ε ortholog.

Evolutionary Analysis of the Spinus Per Genes. Protein trees of PER1, PER2, and PER3 in Spinus, mouse, and rats are shown in Fig. 1. The Dendroica Per1 (GenBank accession no. X63696) was also compared, but it was very different from the three mammalian Per proteins. The computer program used (32) estimated the distance of the dPER from its mammalian counterparts as maximal and beyond the accuracy of the method. The estimated divergence time between Spinus and mammalian rodents is much shorter than the divergence between humans and rodents. Therefore, we expected that the genetic distance between Spinus and mammals would be similar to that between humans and rodents. Any divergence from these expectations could suggest that additional factors influence the rate of evolution of the Per genes of Spinus and, therefore, deviate from the phenotypic divergence time.

Below we present the evolutionary analysis for each Per gene separately.

Per1. There is agreement between the above evolutionary expectations and the PER1 protein (Fig. 1 Top) and PER1 nucleotide sequence (tree not shown). The relative distances as in protein and nucleotides between Spinus and mice are 75% (685 vs. 912) and 80% (1013 vs. 1316) of the distance between Spinus and human, respectively. The distances between rodents (Spinus and mice) and humans are similar: 912 and 8014 in the protein and 1316 and 1326 in the nucleotide sequence.

Per2. Rat PER2 (GenBank accession no. NM013360) was also included and expected to be similar to mouse PER2. As in PER1, PER2 results were in agreement with the phenotypic expectations (Fig. 1 Middle). The relative distances between Spinus and mice were 42% (1155 vs. 2754) or 59% (1307 vs. 2296) of the distances between Spinus and humans in protein and nucleotides, respectively. The distances between Spinus and rats were 46% (1269 vs. 2754) or 46% (1616 vs. 2296) of the distances between Spinus and humans in protein and nucleotides, respectively.
Fig. 1. Slightly tree of the three Per disulphide proteins. The unrooted tree depicts the similarity relationships of the three PER proteins (amino acid) in Spatal, zebrafish, rat, human, and Drosophila.

and humans were also similar: 27.00, 27.70, and 27.54 for protein and 23.56, 23.50, and 22.09 for nucleotide, respectively.

As mentioned, we cloned two truncated clones of Spatal PER2 (accession a and b). Both clones start at the equivalent of mouse 468 bp to the mouse ATG initiation codon. We could not isolate any further 5' sequences either through RT-PCR or cDNA library screening. Both clones contain an insertion of 168 bp at position 1211 bp of mouse sequence. At the start of this deletion in Spatal PER2, the 168 bp has a cluster of termination codons at any of the three reading frames. The apparent initiation ATG is located immediately after the deletion in the 5' ORF of these termination codons in Spatal PER2. Omitting the changes in the 5' ORF clone yielded a Spatal PER2 ORF, which is similar to that of mice and humans. It should be emphasized that similar products have never been obtained in negative control amplifications with templates generated without reverse transcriptase enzyme, eliminating the possibility of genomic DNA contamination. Furthermore, the Spatal clones that were isolated from the Spatal brain cDNA library contain a shorter 3' untranslated region than those of mice and humans, and in contrast to them, contain an adaption site 340 bp 3' to the termination TAA codon. The published 3' untranslated region of mice (1,446 bp) and humans (2,421 bp) do not match the adaptation site. Southern blot analysis suggests that the Spatal PER2 is probably a pseudogene (results not shown).

The nucleotide distances between Spatal PER2 and that of mice or humans are similar. The protein distances (Fig. 1, Bottom) between the two Spatal PER3 and mice or humans were 59.23 vs. 59.50 for PER2, respectively, and 60.41 vs. 57.74 for PER1, respectively. Kumar's two-parameter nucleotide distance analysis (33) gave more than two substitution per bp. Thus, the exact calculated value is meaningless and depends heavily on the assumptions of the correction factors. Nevertheless, the calculated distance of Spatal vs. mice is even larger than the calculated distance of Spatal vs. humans. The same is true for the distances calculated separately for synonymous and non-synonymous substitutions.

Synonymous vs. Heteronymous Substitutions in the Per Family. Our calculations show that PER2 has a ratio around 0.2, indicating that it attained optimum function before the divergence of the species. PER2 and PER3 have a ratio of 0.3 to 0.75, a relatively high ratio suggesting adaptive evolution. The Drosophila PER showed a ratio >1.0, indicating positive selection for a functional change.

aPer Genes Oscillate in the Spatal SCN. IIB with antisense riboprobes in the brain revealed a rhythmic pattern of expression for aPer2 and aPer2, mainly in the SCN but aPer1 is widely spread in the brain (Fig. 2). Maximal expression for aPer2 was at ZT0 and for aPer2 and aPer3 at ZT12. The amplitude of aPer2 expression was modestly lower (P < 0.05) than that of aPer2 and aPer2. The same (center) riboprobes of the three aPer2 had a reproducible background hybridization that did not overlap with the antisense probe. No rhythmic expression with the sense riboprobe hybridization intensity was noted.

aPer Genes Exhibit a Diurnal Oscillation in Spatal Peripheral Tissues. Significant expression of the three aPer genes was noted, using RT-PCR, in brain, intestine, liver, harlequin gland, eye, brain, and skeletal muscle (data not shown).
Results

The Spalding Barren Gland. Expression maxima in the barren gland could be observed by quantitative RT-PCR (Fig. 3A) and ISH (data not shown) at the following ZTs: sPer1 at ZT16 and sPer2 and sPer3 at ZT13. Quantitative RT-PCR analysis in the liver (Fig. 3B) revealed rhythmic expression of sPer genes with maxima of sPer1 at ZT12, and of sPer2 and sPer3 at ZT13. The oscillation in the Spalding liver, as in its eye, shows a 6-9 h delay compared with the Spalding SCN. However, the circadian rhythm in the Spalding barren gland is synchronous with the expression patterns in the Spalding SCN.

The Circadian Oscillation of sPer Genes Is Maintained in Constant Darkness. sPer gene RNA levels in the SCN, eye, and barren gland were studied at four time points over a 24 h period, on the second day in constant darkness (not shown). ISH revealed that RNA levels of all three sPer genes were rhythmic and the oscillation pattern under constant darkness was similar to that under 12 l:12 d light-dark conditions. The levels were observed during the subjective day in the SCN at circadian time 6 for sPer2 and circadian time 12 for sPer1 and sPer3. The peak levels of mRNAs in the eye were 6 h later than in the SCN, but were synchronized with the SCN in the barren gland. The amplitude of sPer1 rhythmicity was markedly lower than that of sPer2 and sPer3 in all three tissues studied and only weakly significant (P > 0.05).

Differential Light Regulation of Spalding Per Genes. Previous studies have shown that after and sPer2 expression in the SCN is induced by exposure to light at night (39, 40), whereas sPer3 is unaffected (41). We examined indelibility of the sPer genes in the SCN, eye, and barren gland by nocturnal light probes at ZT14 and ZT22 (Fig. 4). These time points were chosen for study as light pulses at these times produce phase shifts and advances in locomotor activity.

Quantification of the in vivo results showed that 1 h after a light pulse at ZT14, sPer1 and sPer2 were significantly induced in all three tissues. Remarkably, the level of sPer1 induction in the barren gland was significantly (P < 0.05) higher than in the SCN or the eye, reinforcing its great importance for the Spalding clock. One hour after the light pulse at ZT22 only sPer1 was significantly induced in the three tissues examined. Like the sClock gene, sPer2 gene was not light inducible either at ZT14 or ZT22 in the three tissues tested.

Discussion

Adaptive Selection on Per Genes in Spalding to Life Underground in Total Darkness. Like other mammals, the subterranean blind Spalding has three Per genes.

The distances between sPer1 and sPer2 and those of other rodents or humans are as expected from their divergence time, which is estimated to be 40 million years ago and 80 million years ago, respectively (6). The distance between Spalding or mice and humans are similar, as expected. Generally, the distances calculated for Per2 are longer than the distances for Per1. In our analysis of synonymous vs. non-synonymous substitutions we relied on Liberof et al. (42), who suggested using this ratio to several selection for change as an enzymatic function. Data of Makowski and Boppaki (43) show that most rodents and humans sequences have a ratio of 0.2. This finding indicates that such proteins, selected over millions of years, attained an optimum function before the divergence of rodents and primates and subsequent evolution was relatively conservative. They also considered ratios between 0.6 and 1.0 as suggesting a reduction of functional constraint and selection. Our results show that sPer1 and sPer2 have a ratio of 0.6 to 0.6 and sPer3 has a ratio of about 0.2. Hence, presumably, sPer2 has not been changed to
function in a visually blind mammal living in a dark environment with negligible light cues. However, the figures obtained for xPer and xPer might suggest that molecular changes in these genes were necessary to fulfill their expected adaptive function in darkness. If we combine the calculated distances and the high ratio of non-synonymous substitutions, the result supports the hypothesis of adaptive changes caused by natural selection, possibly in response to light in darkness underwent major changes of deletions and insertions, resulting in two isoforms exhibiting truncated coding regions, somewhat similar results were reported for the xPer pseudogene (44). However, xPer2 is very different from xPer1. Its insertion could not be identified with any known sequence, in contrast to the formal MER3-2 mobile element that is within the xPer locus (44). When the changes in the xPer2 are omitted, an ORF similar to mPer2 is obtained. When the distances were calculated from the aligned short-sequence sequences the phylegetic expectations were not met. The distances between xPer1 and mPer2 are similar or even larger than the distances between xPer2 and mPer2. This finding supports adaptive selective changes in the evolution of this gene and not just the neutral accumulation of substitutions over time. The comparison of xPer expression profiles with the mPer expression profiles was described here and is discussed below, raising questions as to the role that xPer plays in the circadian clock system.

The Functional Circadian Domains of Spalax Per Genes. xPer1 and xPer2 contain all functionally relevant domains and could encode mammalian PER proteins so far. The basic helix-loop-helix motif as well as the PAS A domain consisting of PAS A, PAS B (38, 45), and PAS C3R1 (14) binding and phosphorylation sites (36) are conserved, suggesting their role at a CRY1 substrate in the central mechanism of the Spalax circadian clockwork. Remarkably, the whole putative CRY1 binding site as well as the N-terminal beta-loop-beta motif are missing in xPer2. This finding supports a speculation about its role in the biological clock (45).

Because Spalax is visually blind and lives entirely underground, hence denied outside Zeitgeber information (6), a robust and precise internal clock is necessary for the animal to keep track of time under negligible light cues. Indeed, in situ data revealed that Spalax Per genes' expression is clearly rhythmic and maintained under constant conditions and in constant darkness. xPer1 and xPer2 expression in the Spalax brain is based on the SCN. However, xPer2 is widely spread in different areas of the brain, similar to what has been reported for the mouse Per2 (43), and its oscillation levels are more prominent than those of xPer1 and xPer2. These findings raise questions as to the role of xPer2 in the circadian system. The central pacemaker of the SCN signals time to the retina and the periphery, as in the liver, where the circadian gene expression follows its rhythm with a delay of 4-6 h (44, 46). The blind Spalax Per genes' expression is similar, and our results also show a lag of 6 h in the peak expression of the Per genes in the retina and liver. Although the exact role of Spalax Per genes in the maintenance of the circadian rhythm remains unresolved, it may prove substantial for time keeping underground.

The Harderian gland: A Circadian Center in Spalax. Noteworthy, mRNA levels in the functional glandular domain coincided with the rhythm of the entraining signal (SCN) (47) and an essential component of the circadian pacemaker in mammalians (48). Furthermore, xPer2 inducibility in the harderian gland after light pulse during the dark phase was much higher than in the SCN. The harderian gland of Spalax is extraordinarily enlarged and occupies the entire eye socket, presumably as an adaptation to total darkness. Previous studies (15) suggested that the harderian gland of Spalax is a possible photoreceptor and photosensitive organ. Given its position directly below the atrophied mite eye, it seems likely that the harderian gland has a prominent role in the Spalax circadian clock mechanism. This gland may demonstrate the extreme of evolutionary progression during the adaptive morphological and molecular reorganization for light-underground (6).

Differential Regulation of Spalax Per Genes. Light inducible experiments in Spalax revealed that xPer1 is light-inducible both early (ZT14) and late (ZT22) at night, whereas xPer2 is light inducible only at the beginning of the night. This result is in accordance with the findings of Ahsan et al. (49). xPer levels are reproducible in light pulses applied throughout the dark phase of the circadian cycle. This differential regulation among the three xPer genes suggests that each has a different regulatory function in the SCN. The behavioral effects of photic stimuli at these two time points (ZT14 and ZT22) have been characterized in mice (49, 50). Light pulse at ZT14 causes phase delays in behavioral rhythms and light pulse at ZT22 causes phase advances. Our results indicate a role of xPer1 in both phases and of xPer2 in the phase-delay mechanism. Our in situ data provide a molecular confirmation of previous
behavioural studies in *Spinalus* (11) and the activity pattern of these species with 117 light and dark cycles. The poor 117 oscillation and the absence of light influence on 117 expression levels may suggest a role for 117 outside the central nervous system. This finding is consistent with data from 117-deficient mice (34, 57) but may prove an adaption to the underground and deserve further critical studies.

All three *Spinalus* Par genes are expressed in a wide variety of nonmesenchymal tissues as previously shown in *Drosophila* (53, 54) and mice (55, 56). In three-dimensional studies (Hear, eyes, and hardman gland) we found that the DNA levels for the three *Spinalus* genes oscillate. Oscillation of *Spinalus* gene in the eye, the target organ of light absorption, is rational. As we already suggested for oscillation of the *Spinalus* genes in the Spinalus hardman gland is in accordance with previous results, suggesting an important role of this tissue in circadian maintenance (15) and marks further intensive study. The oscillation in the liver and the widespread expression in other peripheral tissues that were examined suggest the existence of clocks outside the SCN.

**Molecular Genetic Testing of Circadian Genes in a Blinded Mammal**

This study substantiates the claim that the blind submammalian *Spinalus* needs a photoendocrine system to perceive daily and seasonal cycles. It has evaluated a functional retina with effective visual pigment genes signaling to the SCN (16, 17, 20, 25, 64) presumably induced by the small amount of photons that penetrate underground to an otherwise dark environment. Circadian genes in the retina, hardman gland, SCN, and other sites, including Clock (8) and the three Par genes described here, process the light signals and translate them into the intracellular rhythm. *Eyes* of *Spinalus* including photic, polyphasic, and seasonal behavioral phenomena. In this respect we should emphasize that *Spinalus* exhibits naturally occurring circadian rhythm or nocturnal or diurnal in individuals. Currently we are studying the expression pattern of the Par genes in naturally occurring nocturnal animals or on different animals after a phase shift of light. The mosaic evolutions of the *Spinalus* eye (17, 18), hardman gland (8, 64), and brain (26, 29) and its circadian gene provide a striking model of clock evolution at both the molecular and organismal levels. From an evolutionary perspective the genetic basis of circadian rhythms in the blind submammalian *Spinalus* may be different from that of directly diurnal or nocturnal and sighted mammals. Identification of the circadian genes of blind *Spinalus* might advance insights into the structure and evolution of the circadian organization in mammals, including humans, at the molecular level and their ecological causation. Remarkably, the colonization of the submammalian chaosological zone by *Spinalus* did not obliterate the conservative circadian rhythmicity and its basic genetic basis of photoreception and clock genes. All of the circadian machinery was adaptively transformed to perceive light in darkness.

We thank Dr. Z. S. Sun for his initial help with the drawing of the Spinalus Forte probe. We are also grateful to Mr. Michael Milgrom for his help with the computer graphic work. This work was supported by the National Institute of Mental Health and Society and the Deutsche Forschungsgemeinschaft Grant AL529/1-2 (to U.A.) and the Axel Tschierske Internal Foundation for Genetics and Molecular Evolution (to E.N.)
2.6. Publication: “A Switch from Diurnal to Nocturnal Activity in the Mole Rat
*Spalax Ehrenbergi* Superspecies is Accompanied by an Uncoupling of the
Light Input and the Circadian Clock”

Henrik Oster*, Aaron Avivi*, Alma Joel, Urs Albrecht, and Eviatar Nevo

* these authors contributed equally to this work

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A Switch from Diurnal to Nocturnal Activity in S. ehrenbergi Is Accompanied by an Uncoupling of Light Input and the Circadian Clock

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Results

The subterranean mole rat Spalax ehrenbergi super-species represents an extreme example of adaptive visual and neuronal reorganization [1, 2]. Despite its total visual blindness, its daily activity rhythm is entrainable to light-dark cycles [3], indicating that it can confer light information to the clock. Although most individuals are active during the light phase under laboratory conditions (diurnal animals), some individuals switch their activity period to the night (nocturnal animals) [3, 4]. Similar to other rodents [5], the Spalax circadian clock is driven by a set of clock genes, including the period (Per) genes [6, 7]. In this work, we show that diurnal mole rats express the Per genes sPer1 and sPer2 with a peak during the light period. Light can synchronize sPer gene expression to an altered light-dark cycle and thereby reset the clock. In contrast, nocturnal Spalax express sPer2 in the dark period and sPer1 in a biphasic manner, with a light-dependent maximum during the day and a second light-independent maximum during the night. Altogether, sPer1 expression remains light inducible, this is not sufficient to reset the molecular clockwork. Hence, the strict coupling of light, Per expression, and the circadian clock is lost. This indicates that Spalax can dissociate the light-driven resetting pathway from the central clock oscillator.

Discussion

The circadian clock coordinates the body's physiological, endocrinological, and behavioral status and enables the organism to maximally benefit from temporarily available natural resources [6]. At the molecular level, the clock is based on transcriptional/translational feedback loops (TTLs), a principle that is conserved throughout all phylogeny, even though the single components of the loops vary [8]. In mammals, the master circadian clock is located in the hypothalamic suprachiasmatic nucleus (SCN) [10]. From here, subordinated clocks in the peripheral organs of the body are synchronized to generate a concerted rhythm for the whole organism [11]. Among the genes driving the clock in the SCN are the two Period genes Per1 and Per2 [12–14]. Both homologs show a circadian rhythm of activation within the SCN and can serve as markers for the phase of the circadian clock [15–17].

Recent data indicate that, at both the behavioral and the molecular level, the blind mole rat Spalax ehrenbergi super-species has a functional circadian clock despite its isolated subterranean ectopos [3, 6, 7]. Twenty-five million years of selective adaptation to this environment have resulted in a radical degeneration of its visual system, leading to atrophied (900 µm wide) totally fur-covered eyes that lack any image-forming ability [19]. Interestingly, the degenerated retina contains spirals and melanopin, which might be responsible for light detection [19, 20]. The SCN, however, is well developed and receives clock-related signals from the retina via the retinohypothalamic tract [7, 11, 21]. Mole rats show a unique polyphasic activity pattern in that they can switch from day activity (diurnal animals) to night activity (nocturnal animals) depending on environmental conditions [4, 22]. Although the majority of all populations studied are more active during the day, activity patterns seem to be highly influenced by temperature and activity [23]. Compared with above-ground mammals, for a totally blind subterranean herbivore, a change in the time of activity is less crucial for its ability to find food or for its susceptibility to predators. Therefore, the polyphasic nature of Spalax rhythmicity may have been evolutionarily stabilized by balancing the need for social interaction in the mating season on one hand and metabolic economy on the other [24].

In the laboratory, activity of animals can be entrained to shifted light-dark cycles, indicating a sensitivity of the circadian clock to light [25, 26]. At the molecular level, three Period genes (sPer1, sPer2, and sPer3) as well as a Clock and a MOPs homolog have been characterized and show circadian expression rhythms and light inducibility similar to their counterparts in other rodents like mice, rats, and hamsters [6, 7]. Experiments with diurnal species like Anomoeops noctivagans and Meriones unguiculatus revealed that clock gene expression in these animals is the same as in nocturnal animals; this indicates that the center managing activity is located downstream from the core pacemaker [26, 27].

The great majority of Spalax individuals (~90% of 63 total) used in this study displayed a diurnal activity pattern (see [22] as well). To test whether the behavioral adaptation to a shifted light-dark cycle [3] is reflected at the molecular level, we looked for sPer gene expression in the SCN of diurnal animals before and after an inversion (12 hr shift of a 12 hr light/12 hr dark (LD) cycle). As has been shown before [7], in diurnal animals, sPer1/Per1 expression rises during the early day, with a maximum around noon, and has a steady decline throughout the night (Figures 1A and 2C). The sPer2 expression peaks at the day-night transition, with low levels during
Results

the dark period and the early morning (Figures 1A and 2C). Similar expression patterns have been observed in mice [15-17].

After the shift of the LD cycle ("lights on" at 19:00), we examined sPer1 and -2 expression in the SCN after the animals' activity patterns were synchronized to this new light schedule. We found an inverted rhythm in Period gene expression, with peaks at 1:00 and 7:00, indicating that the molecular clockwork is reacting to light and adapts readily to new environmental conditions.

Since the Spalax clock is light sensitive, what is the explanation for the nocturnal activity pattern of some animals when most are diurnal? To pursue this question, we scanned our colonies for night-active animals. About 28% of all individuals tested displayed a stable nocturnal activity rhythm and were chosen for further experiments (Figures 2A and 2B). We examined Period gene expression in these animals to determine whether the observed phenotype was caused by an event in, up-, or downstream of the central oscillator. In LD, sPer1 shows a bimodal expression — with two maxima, one in the middle of the day and the other at midnight — in nocturnal animals (Figures 2D and 2F). As in diurnal animals, sPer2 expression shows only one peak of expression, occurring at the end of the activity phase, in nocturnal mole rats (Figures 2E-2F). Maximal sPer2 expression for nocturnal animals is at Zeitgeber time (ZT) 6 (7:00). Hence, in relation to the light/dark cycle, the expression pattern for sPer2 is inverted in nocturnal Spalax as compared to diurnal Spalax, except for the second sPer2 maximum at ZT6 (13:00).

Figure 1. sPer Gene Expression in the SCN
(A and B) Expression in (A) diurnal animals and (B) nocturnal animals entrained to a 12 hr shifted LD cycle. Diurnal animals were adapted to a 12 hr light/12 hr dark (LD) cycle and were sacrificed at four different time points throughout the day (7:00, ZT0; 13:00, 19:00, and 1:00). The phase-shifted animals were exposed to a 12 hr shifted LD cycle (19:00, ZT0) and were sacrificed after behavioral adaptation (1 week, data not shown). Quantitative analysis of sPer1 (solid line) and sPer2 expression (dotted line) in the SCN is shown. All values are mean ± SD of three different experiments. Black and white bars indicate dark and light phases, respectively.

Figure 2. Activity and sPer Gene Expression of Nocturnal Animals
(A and B) Typical activity plots of [A] day- and (B) night-active animals measured by crossing events of an infrared beam in the animal's cage.
(C and D) Representative bright-field micrographs of [C] liquid time autoradiographs on coronal brain sections probed for sPer1 (upper row) and sPer2 (lower row). (C) Diurnal animals; (D) nocturnal animals in LD. The localization of the SCN was verified by bismuth-zincate staining (not shown).
(E and F) Quantitative analysis of (n = 3) sPer1 (solid line) and sPer2 expression (dotted line) in the SCN of (E) day- and (F) night-active animals kept in LD (7:00, ZT0). The scale bar represents 100 μm.
The two distinct SpER1 activity maxima in nocturnal animals could be the consequence of an uncoupling of different cell groups within the individual SCN or of an uncoupling of the two SCNs oscillating in antiphase. Thorough examination of the whole SCN, however, did not reveal any clusters of cells with just one expression maximum or differences between the left and the right nucleus, as has been shown in the mouse SCN [28] and in hamsters with a split activity pattern [29].

To test whether the second SpER2 peak was clock driven or induced by the LD cycle, we released nocturnal animals into constant darkness (DD) and looked for the Period1 gene expression on the first day in constant conditions (Figure 3). White SpER2 gene expression continues to oscillate in a circadian manner, the morning peak in SpER1 expression vanishes in DD. This demonstrates that, in animals with a predominantly nocturnal activity pattern, the molecular clock is shifted for 12 h, with the activity pattern clearly following gene expression in the SCN. Under normal LD conditions, SpER1 expression is regulated by light. This induction, however, is not sufficient to reset the clock (Figure 2D).

As a rapidly light-inducible gene in mice, Per1 is thought to integrate light-driven signaling pathways from the retina via the retinohypothalamic tract and the intergeniculate leaflet [30, 31]. The PER1 proteins would then phase shift the oscillation of the circadian clock, thereby synchronizing it to the environment [5, 32]. Here, we give the first example of a mammalian species in which Per1 is, under some circumstances, not sufficient to shift the circadian pacemaker. We show that, in the laboratory, the molecular circadian clock of the diurnal Spalax is light responsive and that diurnal animals can adapt to changes in the external light cycle. However, in animals with a nocturnal activity pattern, photic signaling can be overcome by factors other than light. We propose that the variability of the mole rat’s circadian clock to react to light reflects its subterranean ecology. Radiotracking field studies revealed that mole rats are predominantly diurnal during the rainy, short-day winter (mating season) and are predominantly nocturnal during the dry, long-day, hot summer seasons [33]. We tried to simulate this natural ambience by applying different photoperiods, e.g., long day/short night environments, to diurnal animals but failed to induce transition into a nocturnal activity profile by varying only one environmental parameter (light; data not shown). This indicates that other factors like temperature and humidity could have an impact on resetting the Spalax clock.

The results indicate that the Spalax clock contains a switch controlling the preference for diurnal to nocturnal activity that is located in the input pathway upstream from the core clock mechanism. This is in marked contrast to other mammalian species in which the mechanism that determines the activity phase lies downstream from the clock, because, in all diurnal and nocturnal species studied so far, Per gene expression is invariant with maxima during the light phase. We suggest that the Spalax clock can vary its sensitivity to light-induced input signals, probably as a response to changes in humidity or temperature. Our findings give a good example for the highly adaptive nature of the clock entrainment mechanism with regard to a species-specific environment.

Experimental Procedures

Animal Handling and Activity Monitoring

We analyzed 33 adult (100–150 g) belonging to Spalax judei (JJ × 40) from Arava, Samara [34]. Field-trapped animals were kept at 22°C–24°C under a 12-h light/12-h dark (LD) cycle. Selection of animals was done after monitoring their activity. The animals were kept in 20 × 20 cm cages supplied with two 50 cm transparent tubes. Each tube was equipped with two infrared, two sensors that signaled whenever the animal crossed the sensor. For analysis of spER RNA levels in total darkness (DD), light was turned off at Zeitgeber time (ZT) 12 for at least 2 days before animals were sacrificed.

In Situ Hybridization

Animals were sacrificed by cervical dislocation under ambient light conditions at 1200 and 1800 in LD and under a 15 W safety red light at all other indicated time points. Specimen preparation, 5′-UTP-labeled riboprobe synthesis, and hybridization steps were performed as described in [35]. The probes for SpER1 and spER2 were as described in [7]. Quantification was performed by densitometric analysis of autoradiographs using Amersham Hyperfilm MP by using the NIH Image 1.6 program after conversion into the relative optical densities by the 125I autoradiographic microscale (Amersham). Data from the SCN were normalized with respect to the signal intensities in an equal area of the lateral hypothalamus. Three sections per SCN were analyzed. "Relative mRNA abundance" values were calculated by defining the highest value of each experiment as 100%.
To conclude the work on the central circadian clock of *Spalax* we examined *sCry1* and 2 expression in the SCN, the Harderian gland and the eye. From a *Spalax* brain cDNA library Dr. Avivi extracted two different fragments for both *sCry* genes. We looked for daily expression profiles with different probes for all four fragments by *in situ* hybridization. The two probes for *sCry1* were named 5’ and 3’-probe since they aligned to the 5’ and 3’ region of the corresponding mouse gene. The two probes for *sCry2* were named ‘S’ (for ‘short’) and ‘L’ (for ‘long’) because of the different lengths of the two fragments extracted from the cDNA library.

While only the 5’ probe of *sCry1* showed a diurnal oscillation, the 3’ probe showed stable expression throughout the day. This might indicate a post-transcriptional modification of the mRNA with the 5’ part but not the 3’ (more stable) part used for circadian function in the central TTL. *sCry2* transcripts did not cycle prominently as expected from other rodents.
(Vitaterna et al., 1999). Interesting however, is the rather high level of the ‘S’ probe signal with a significant double peak at the light/dark and dark/light transitions in the eye. One might speculate about a role of the ‘S’ fragment in light reception like it is still proposed for the mammalian Cry (Sancar, 2000; Thresher et al., 1998) while the ‘L’ fragment serves in the central oscillator. This would reflect the situation in zebrafish, where some of the Cry genes act as photoreceptors while others drive the feedback of the TTL (Kobayashi et al., 2000).
Chapter 3

Conclusion and Perspectives

Most of the interpretations that arise from the work presented here have been elaborated in the included publications. Therefore I will focus in this part on some comprehensive conclusions drawn from the phenotypical data derived from the mPer/ mCry double mutants with special regard to the free-running period lengths in DD. I will introduce a new model emphasizing the repressive action of the mPER and mCRY proteins in the TTL as a variation of the limit cycle model suggested in the second double mutant publication (chapter 2.2.).

On the mixed genetic background we used for the double mutants we generated several

Fig. 53: Free running period lengths of clock mutant mice (from the mixed genetic background used in this study) in DD. Data presented are mean +/- SD; total numbers of animals used are given below the x-axis; significance was determined by one way ANOVA followed by Bonferroni post-test (**, p< 0.01; ***, p< 0.001).
different mutant lines of which five were rhythmic for at least some time in DD. Four of these lines showed a period length significantly different from that of the corresponding wild-type controls (see figure 53).

From the current understanding of the central circadian oscillator (see chapter 1.4.) a major role of the PER and CRY proteins is to form a multi-protein complex which interacts with the CLOCK/ BMAL1 transcriptional activator thereby inhibiting the transcription of their own genes. If one or more of the PER and/ or CRY proteins are removed from this complex by genetic disruption of the corresponding gene loci, the overall activity of the remaining complex is changed.

If we define the efficiency of the PER/ CRY protein complex by its ability to accelerate the pace of the oscillator (by its efficiency to inhibit transcription of CLOCK/ BMAL1 induced genes), all components of this complex contribute to the overall potential of the Cluster. The easiest way to separately define the contributions of the single PER and CRY proteins is to assign ‘accelerator potentials’ (APs) to each gene/ protein with the sum of all four single unit potentials being the overall accelerator potential of the multimeric complex. This overall accelerator potential (OAP) determines the pace of the central oscillator under constant conditions measured as the internal period $\tau$. A high overall accelerator potential speeds up the clock resulting in a short period length (like in $mCry1$ mutants) while a low OAP decelerates the clock which corresponds to a long $\tau$ in DD (like in $mCry2$ mutants). To have a measure of the OAPs we subtracted the experimentally defined mutant free-running period from that of the wild-type. This results in positive values for mutants with a shortened $\tau$ and negative values for mutants with a longer $\tau$ as given in the table below (termed "experimental accelerator potentials" or EAPs).

<table>
<thead>
<tr>
<th>mutant</th>
<th>$\tau$ in DD (in hours)</th>
<th>experimental accelerator potential (in min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>23.8</td>
<td>0</td>
</tr>
<tr>
<td>$mPer2$</td>
<td>22.1</td>
<td>102</td>
</tr>
<tr>
<td>$mPer1/mCry1$</td>
<td>23.7</td>
<td>6</td>
</tr>
<tr>
<td>$mPer1/mCry2$</td>
<td>25.3</td>
<td>-90</td>
</tr>
<tr>
<td>$mPer2/mCry2$</td>
<td>23.4</td>
<td>24</td>
</tr>
<tr>
<td>$mCry2$</td>
<td>24.1</td>
<td>-18</td>
</tr>
</tbody>
</table>

Tab. 6: Period lengths and EAPs of clock gene mutant mice from this study. Internal period length $\tau$ was determined as described (chapter 4.1.3.3.). Experimental accelerator potentials (ERPs) were calculated as the difference between mutant and wild-type $\tau$. Only mice of the mixed genetic background that was used throughout this study are shown.
From the assumptions above one can derive formulas to calculate theoretically expected OAPs (theoretical overall activator potentials; TAPs) from the single APs. For the mutants examined in this study the formulas to calculate the TAPs would be:

- **wild-type:** \((RP_{Per1} + RP_{Per2} + RP_{Cry1} + RP_{Cry2})\)
- **mPer2 mutant:** \((RP_{Per1} + RP_{Cry1} + RP_{Cry2})\)
- **mPer1/ mCry1 mutant:** \((RP_{Per2} + RP_{Cry2})\)
- **mPer2/ mCry2 mutant:** \((RP_{Per1} + RP_{Cry1})\)
- **mCry2 mutant:** \((RP_{Per1} + RP_{Per2} + RP_{Cry1})\)

To calculate optimized accelerator potentials for the single genes/proteins we determined a regression error (RE) with \(RE = \sqrt{\sum (TAP - EAP)^2}\) for all different strains.

We now assigned random values for the single gene/protein APs, calculated the TAPs and subsequently RE. Now we applied the Solver routine of the Excel program (Microsoft) using a Newton regression algorithm to minimize RE by changing the values for the single gene/protein accelerator potentials. After 100 iterations the values were as follows:

<table>
<thead>
<tr>
<th>gene/protein</th>
<th>optimized accelerator potential.</th>
</tr>
</thead>
<tbody>
<tr>
<td>mPer1</td>
<td>62.50</td>
</tr>
<tr>
<td>mPer2</td>
<td>-51.00</td>
</tr>
<tr>
<td>mCry1</td>
<td>-39.00</td>
</tr>
<tr>
<td>mCry2</td>
<td>47.00</td>
</tr>
</tbody>
</table>

Tab. 7: Optimized accelerator potentials (APs) for Per and Cry genes/proteins after Newton regression using the Solver routine of the Excel program (100 iterations).
If we applied these optimized APs to the different mutants using the formulas given above we get the following theoretical overall accelerator potentials (TAPs):

<table>
<thead>
<tr>
<th>Mutant</th>
<th>optimized TAPs</th>
<th>EAPs</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>19,5</td>
<td>0</td>
<td>-19,5</td>
</tr>
<tr>
<td>mPer2/-</td>
<td>70,5</td>
<td>80</td>
<td>9,5</td>
</tr>
<tr>
<td>mPer1mCry1/-</td>
<td>-4,0</td>
<td>6</td>
<td>10,0</td>
</tr>
<tr>
<td>mPer1mCry2/-</td>
<td>-90,0</td>
<td>-90</td>
<td>0,0</td>
</tr>
<tr>
<td>mPer2mCry2/-</td>
<td>23,5</td>
<td>24</td>
<td>0,5</td>
</tr>
<tr>
<td>mCry2/-</td>
<td>-27,5</td>
<td>-18</td>
<td>9,5</td>
</tr>
</tbody>
</table>

Tab. 8: Optimized theoretical overall accelerator potentials for clock mutants used in this study. The difference between optimized TAPs and the experimental results (EAPs) visualizes the quality of the iteration.

From the regression we get a clearly defined ranking of accelerator potentials (APs) with mPer1 and mCry2 as the most potent accelerator – a fact reflected in the mCry2 mutant, which shows the longest τ of all single mutants (van der Horst et al., 1999; Vitaterna et al., 1999) – followed by mCry1 with mPer2 being the weakest accelerator. This was expected from the short free running period of the mPer2Brdm1 mutant in DD (Zheng et al., 1999).

To compare our model with results from other groups we applied the obtained TAPs to different clock gene mutants previously published:

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Source</th>
<th>EAPs</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>mCry1/-</td>
<td>v.d. Horst et al., 1999</td>
<td>76</td>
<td>17,50</td>
</tr>
<tr>
<td>mCry2/-</td>
<td>&quot;</td>
<td>-52</td>
<td>-24,50</td>
</tr>
<tr>
<td>mCry1/-</td>
<td>Vitaterna et al., 1999</td>
<td>41</td>
<td>-17,50</td>
</tr>
<tr>
<td>mCry2/-</td>
<td>&quot;</td>
<td>-61</td>
<td>-33,50</td>
</tr>
<tr>
<td>mPer1/-</td>
<td>Zheng et al., 2001</td>
<td>66</td>
<td>109,00</td>
</tr>
<tr>
<td>mPer2/-</td>
<td>&quot;, 1999</td>
<td>96</td>
<td>25,50</td>
</tr>
<tr>
<td>mPer1/-</td>
<td>Bae et al., 2001</td>
<td>32</td>
<td>75,00</td>
</tr>
<tr>
<td>mPer2/-</td>
<td>&quot;</td>
<td>10</td>
<td>-60,50</td>
</tr>
<tr>
<td>mPer1/-</td>
<td>Cermakian et al., 2001</td>
<td>35</td>
<td>78,00</td>
</tr>
</tbody>
</table>

Tab. 9: Comparison between calculated (TAP) and experimental determined accelerator potentials (EAP) from the literature.

As expected, the differences between theoretical and experimental results are higher than in our own studies. However, most of the free-running periods are comparable to the predictions from the model. For example the predicted τ of the mCry1 mutant lies exactly between the two values published before (van der Horst et al., 1999; Vitaterna et al., 1999). The same is true for the mCry2 mutant, where the published mutants (van der Horst et al., 1999; Vitaterna
et al., 1999) both have longer period lengths than predicted but our own mCry2 mutant shows a shorter $\tau$. Again, in the $mPer2$ mutant the predicted value is located between two different versions of published data (Bae et al., 2001; Zheng et al., 1999).

The only mutation the model completely fails to predict is $mPer1$. This is somehow expected since $mPer1$ single mutants show a normal or slightly shortened $\tau$ (Bae et al., 2001; Cermakian et al., 2001; Zheng et al., 2001) while we could show that an additional mutation of $mPer1$ in $mCry1$ or $mCry2$ mutants leads to an elongation of the internal period. This may as well reflect the posttranslational role of mPER1 protein in the circadian pacemaker which cannot be taken into consideration by a simple model of accelerator potentials (Bae et al., 2001; Lee et al., 2001; Zheng et al., 2001).

But what happens in the $mPer2$, the $mPer2/mCry1$ and the $mPer1/mCry2$ mutants which become arrhythmic immediately or after some time in DD? Calculating the TAPs for these three strains we get very high or very low values (71, 110 and –90 min respectively) indicating corresponding short or long internal periods respectively. If the circadian clockwork functions as a stabilized oscillator the disturbance introduced by the deletion of these genes in the negative component of the TTL might overwhelm the compensation capacities of the clockwork. With the loss of stability the oscillation dampens and ultimately reaches equilibrium or arrhythmicity.

Taken together we demonstrate in this work that the finely tuned interaction of both $Per$ and $Cry$ genes creates a stabilized negative branch of the transcriptional/ translational feedback loop at the heart of the circadian pacemaker. Although there is some redundancy in these components when looking at mere rhythmicity, every gene has its specific function in the whole clock mechanism. Together they ensure the stability and adaptivity the internal pacemaker needs to serve as a reliable time-teller and synchronizer for the complex mammalian organism.

With the discovery of $Rev-Erb\alpha$ as the transcription factor controlling $Bmal1$ expression (Preitner et al., 2002) most of the genes which are supposed to be necessary to build up the mammalian cellular clockwork have been discovered. Some questions remain on the physical interactions of the clock proteins and especially their spatio-temporal organization. Novel animal models like transgenic mice carrying reporter genes tagged to clock controlled promoters will probably help to elucidate the exact time course of clock organization in the living animal (Wilsbacher et al., 2002).

With the discovery of peripheral oscillators and the identification of clock gene relatives in several tissues outside the hypothalamus much work has been spent on these body clocks and
their connections to the SCN (reviewed in Balsalobre, 2002). Although some signaling candidates have been discovered like glucocorticoids (Balsalobre et al., 2000a), retinoids (McNamara et al., 2001) or Prokineticin 2 (Cheng et al., 2002), the pathway by which the SCN synchronizes the body to the environment is still not clear.

The quest for these signaling molecules and the properties of the peripheral clocks organizing the metabolism of the body will most probably be the main focus of molecular chronobiology in the upcoming years. It will offer us the pharmacological tools to manipulate these oscillators thereby providing new strategies for the treatment of diseases caused or complicated by desynchronization or misentrainment of circadian clocks.
Chapter 4

Material and Methods

4.1. Animal Handling and Breeding

All animal work was performed according to the guidelines of the Bundestierschutzgesetz (BGBI. I S. 1105, ber. S. 1818, Abschnitt 2 (§2+3) 5 (§7-9) and 8(§11) for Hannover and the Schweizer Tierschutzgesetz (TSchG, SR455, Abschnitt 2 (Art. 5 und 7), 5 (Art. 11) and 6 (Art. 12-19) for Fribourg.

4.1.1. Mouse Strains

Colony founders for the mPer/mCry multiple mutant lines came from two different genetic backgrounds. We started with mPer1/- and homozygous mPer2^{Brdm2} mice carrying a targeted gene from a 129S5/S7 genomic library in a C57BL/6 background (Zheng et al., 2001; Zheng et al., 1999) and mCry1^+/mCry2^+/ heterozygous mice carrying a targeted gene from a Ola129 derived genomic library in a C57BL/6 background (van der Horst et al., 1999). While in the mPer1, the mCry1 and the mCry2 targeting vectors almost all of the translated coding sequence was deleted the Per2^{Brdm2} mutants lack a major part of their PAS domain responsible for protein protein interaction resulting in a truncated non-functioning protein (Oster et al., 2002; Zheng et al., 1999).
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Fig. 54: Targeted disruption of the *Per* and *Cry* genes (a) Genomic structure of the murine *mPer1* gene, the targeting vector, and the predicted structure of the targeted allele. Exons are indicated by vertical black bars with the first and last exons numbered. WT, wild-type; *R*, *EcoRI*; *Hprt*, hypoxanthine phosphoribosyltransferase gene; *TK*, *Herpes Simplex Virus thymidine kinase* gene. A 1.6 kb 3' external probe that detects a 20 kb wild-type *EcoRI* fragment and an 11.8 kb mutant *EcoRI* fragment were used to detect targeted ES cell clones and to genotype test mutant mice. (b) Genomic structure of a portion of the mouse *mPer2* gene, the targeting vector and the predicted structure of the targeted allele. *H*, *HindIII*; *B*, *BamHI*; *Neo*, neomycin resistance gene. Figure 1 Targeted disruption of the *cry* genes and generation of Cry-deficient mice. (c) Physical map of the wildtype *Cry1* locus, the targeting construct and the disrupted *Cry1* locus. Exons are indicated by black filled boxes. Note that the use of PCR-derived genomic DNA does not allow proper exon numbering. The probe used for screening homologous recombinants and genotyping mice, localized external to the construct, is represented by a gray box. Primers used for RNA analysis by RT-long-range PCR are depicted as black arrowheads. (d) Physical map of the wildtype *Cry2* locus, the targeting construct and the disrupted *Cry2* locus (from van der Horst et al., 1999; Zheng et al., 2001; Zheng et al., 1999).

### 4.1.2. Mouse Breeding

Mice were bred in BioZone ventilated caging systems (Cage Model CA20, VR Classic TM, BioZone, Margate, UK) in Hannover and in filter top open cages (Type 2 Polycarbonate Cage with top wiring, Tecniplast, Italy) in custom made racks in Fribourg in a 12h Light 12h dark cycle. Matings were normally setup as pairs or triples with rotation of the females in a one to two week turn during the expansion of the colonies. Litters were weaned three to four weeks after birth and separated for their gender. Store cages held up to 6 animals. Genotype was determined by Southern Blot Hybridization with genomic DNA extracted from tail tissue before weaning.
4.1.3. Activity Monitoring

4.1.3.1. Facilities and General Guidelines

Animals were housed individually in transparent plastic cages (Tecniplast 1155M) that were equipped with a steel running wheel of 115 mm in diameter (Trixie 6083, Trixie GmbH, Germany). The axis of the wheel was equipped with a plastic disc holding a small magnet (article number 34.6401300702, Fehrenkemper Magnetsysteme, Germany). The magnet opens and closes a magnetic switch (Reed-Relais 60, Conrad Electronic, Germany) upon rotation of the running wheel. The switch was connected to a computer that counts the revolutions of the wheel (using the Activity Counting System Program, Simon Fraser University, Burnaby, Canada in Hannover and ClockLab, Actimetrics, Austin St. Evanston, USA in Fribourg). Twelve cages of this type were placed in one isolation cabinet (custom made, length = 180cm; height = 54cm; depth = 69cm). Running data analysis was performed using the Circadia Program (Simon Fraser University) in Hannover and ClockLab plug-in for MatLab (The Mathworks, Natick, USA) in Fribourg (Albrecht and Foster, 2002).

Mice used for activity monitoring were generally 2 to 6 month old males (except stated otherwise). An equal number of wild-type controls was included in each isolation chamber. Animals were provided food and water *ad libitum* at all times. Cages were changed every three to four weeks at the beginning of the activity phase to minimize phase shifts induced by a new environment (Mrosovsky, 1996).
4.1.3.2. Publication: “The Circadian Clock and Behavior”

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The circadian clock and behavior

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1. Introduction

Activity in rodents is evaluated either by monitoring general movement (e.g., with photo sensors), or by measuring wheel-running activity. Assessment by photo sensors or infrared sensors registers general movement including movement that occurs during feeding or grooming. In contrast, wheel-running activity represents only intended running of the animal and therefore provides activity patterns lacking background activity. This is one of the reasons, why this type of activity measurement is preferred over monitoring. In the following, we will focus only on wheel-running activity and describe general protocols.

In our wheel-running experiments, animals are housed individually in transparent plastic cages (200 mm long × 105 mm wide × 125 mm high, Techniplast, ITSM) that are equipped with a steel running wheel of 115 mm in diameter. The axis of the wheel is equipped with a magnet that opens and closes a magnetic switch upon rotation of the running wheel. The switch is hooked up to a computer that counts the revolutions of the wheel. Twelve cages are placed in a black, light-tight box, which is ventilated and containing fluorescent light that can be controlled via a timer from the outside. The box is placed in a room that is controlled for temperature, humidity, light and free of noise and vibrations. If environmental noise is not well under control, a constant background sound (white noise) should be installed to drown any outside noise, e.g., traffic or trains. Living conditions that tend to disease can be readily observed. Outbursts of viral infections are reflected in daily wheel-running activity. After the viral burst, the circadian activity returns to the normal nocturnal activity until the next viral burst occurs. Hence, abnormal activity patterns can be a result of infections.

Wheel-running activity in mice is measured under a standard light-dark (LD) cycle that consists of 12 h of light (we use 500 lux, but 50 lux is sufficient). Light intensity is measured with a lux-meter. Tastass (Luzern, Switzerland) and 12 h of darkness, e.g., lights on 07:00, lights off 19:00. Activity recording is usually started just before lights off (e.g., 19:00) when mice are at the beginning of their activity phase. Hyperactivity due to the placement in a novel environment (anorma) has then a minimal effect on the animals' clock [6]. It is important that at this point the data transmission is checked to correct potential problems that occur with data acquisition. If adjustments are necessary later on in the experiment, it is advisable to do them just before lights off, e.g., 19:00. This is also the guideline for food and
water supply, which is necessary every 3–4 weeks. Important to note here is that the cage and the wheel should not be replaced by a new cage, because a new wheel is a novel stimulus that can phase-shift the clock [8]. After the animals have been held in the LD 12/12 cycle for at least 10 days, they are “entrained” and adjusted to this particular LD cycle and the measurements from that point on are reliable. These animals are still under the daily influence of light and therefore can use the light as a Zeitgeber or timegiver. Measurements under these conditions are determined by the Zeitgeber time (ZT), where ZT0 is lights on. The specificity of the LD cycle determines the ZT for lights off, e.g., ZT12 in an LD 12/12 cycle. Animals held under these conditions can also be used for molecular analysis. Good markers for the circadian clock are the mPer genes [1,7], which are expressed in a diurnal manner and remain expressed in a circadian manner under constant conditions (see next paragraph).

To investigate the circadian clock, mice have to be held under constant darkness conditions. Their clock is then ‘free-running’ and independent of light changes. After animals were in an LD 12/12 cycle for at least 10 days, the lights are not turned on the next day and remain off for the rest of the experiment. The first 5 days show an unstable period length of a particular animals clock, because the animal is in transition and has to adjust to the constant darkness or dark–dark (DD) conditions. The period (tau) of an animal is established by analyzing 10 days of stable circadian data using the Chi-squared periodogram. A wild type (WT) animal has a period length of ~23.8 h (depending on the strain), which is close to a day of 24 h. In contrast, an mPer2 mutant animal has a period length of only 22 h [9]. To study arrhythmicity, animals are left undisturbed for 28 days in DD. For example, an mPer2 mutant animal can take 14–20 days until its circadian rhythm is lost [9]. After the period length has been determined, the circadian times (CT) for an animal are defined and time measurements under DD conditions are described by CT. For example, CT24 is at the end of the period of the animals clock, hence, after 23.8 h for a WT animal or after 22 h for an mPer2 mutant animal. Times given in CT indicate constant darkness conditions and are strictly speaking animal specific. Given that the individual variation in tau for a strain is not very large, the CT is based on the average tau of that strain and can be used for all individuals. Strains that immediately lose circadian rhythm in DD, like the mPer1/mPer2 double mutants [8] cannot be temporally defined by a CT, because no tau can be determined.

Phase shifts are the consequence of disturbance of the clock by an external signal, such as light, noise or providing a new running wheel. To study the effects of light on the phase shifting of the circadian clock, light pulses are applied to the animals. We apply a light pulse that has a duration of 15 min and an intensity of 500 lux. The animals that are investigated must establish a clear stable rhythm in DD for 10–12 days before a light pulse is applied. Animals that become arrhythmic in DD have to establish a stable rhythm in LD. A light pulse has only an effect on the clock if it is applied in the activity phase of the mouse, i.e., between CT12 and CT24 (subjective night) or ZT12 and ZT24, respectively [3]. To evoke a phase delay of the clock, the light pulse must be applied in the early night (between CT12 and CT18–20). For phase advances, the light pulse is applied in the late night (between CT18–20 and CT24). These are just approximate CT values, because they

Fig. 1. (a) Wheel-running activity of WT and NOS1−/− mice before and after a 15 min light pulse. Mice were entrained for 10 days to an LD 12/12 cycle and then released into constant darkness (DD). Shown are 10 days of activity in DD before a light pulse was applied at CT14 (top arrow) or at CT22 (bottom arrow), respectively. The vertical grey lines indicate activity onset. The light pulse at CT14 causes a delay of wheel-running activity (vertical line moves to the right), whereas a light pulse at CT22 causes onset of activity at earlier times (vertical line shifted to the left). WT and NOS1−/− mice did not behave differently in response to a light pulse, but wheel-running activity was more fragmented in NOS1−/− mice. (b) Induction of mPer1 and mPer2 in NOS1−/− mice by a light pulse. NOS1−/− and WT mice were exposed to 500 lux of light for 15 min at ZT14 and ZT22, respectively. Induction of mPer1 and mPer2 after 1 h in NOS1−/− animals was comparable to WT animals indicating that lack of NOS1 has no influence on mPer gene light inducibility. Induction of mPer1 and mPer2 in WT animals at ZT14 is similar to mPer1 induction at ZT22. mPer2 is not inducible by light at ZT22 [1].

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![Diagram showing expression of vasopressin prepropeptidin mRNA in WT, mPer1, mPer2, and mPer1/m2 mutants.]

Fig. 2. Expression of vasopressin prepropeptidin mRNA in WT, mPer1, mPer2, and mPer1/m2 mutant mice that were kept in an LD 12/12 cycle. Constitutive expression is detected in the SON and PVN. In the SCN, diurnal expression is observed in WT and mPer1/m2 mice at ZT6, but this diurnal expression is absent in mPer2 and mPer1/m2 mutant mice. SON = supraoptic nucleus, PVN = paraventricular nucleus, and SCN = suprachiasmatic nucleus.

Results

Vary depending on the mouse strain [2,6]. After the light pulse, the animals are left in DD. Phase delays can already be recognized 1 day after the light pulse has occurred, but phase advances take up to 6 days to be recognizable and they are smaller than phase delays. At least seven consecutive days have to be measured to determine the amount of the phase advance or delay, respectively. Fig. 1 shows that NOS1 knock-out animals behave like WT animals in clock resetting [4], Fig. 1(a). In molecular terms, NOS1−/− mice displayed mPer1 and mPer2 light inducibility comparable to WT animals (Fig. 1(b)). Thus, NOS1 is not necessary for Per1 and Per2 gene induction, which is consistent with the normal resetting behavior in NOS−/− mice. However, it cannot be ruled out that NO is synthesized by other isoforms of NOS, like endothelial NOS or unknown NOS isoforms.

The mPer1 and mPer2 genes are important components of the mammalian circadian clock [1,7-9]. Disruption of the circadian clock can lead to a deminishing of an organism’s physiology. To illustrate this point, we examined the transcriptional regulation of the clock-controlled vasopressin prepropeptidin in the suprachiasmatic nucleus (SCN). We found that the vasopressin mRNA rhythm was severely blunted in the SCN of mPer2 mutant and in mPer1/m2 double mutant animals, but not in mPer1 mutants (Fig. 2). This indicates that vasopressin mRNA is positively regulated by mPer2, but not by mPer1. Vasopressin is well known for its peripheral effects on salt and water balance and has a number of distinct actions within the central nervous system. Therefore, a number of physiological abnormalities can be expected in mPer2 and mPer1/m2 mutants. This clearly shows that in a WT animal the physiological state of the organism is not constant throughout the day. We recommend that behavioral studies are accompanied by a ZT with the corresponding LD cycle to ensure reproducibility of behavioral measurements.

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References

4.1.3.3. Experiments

4.1.3.3.1. Light/ Dark (LD) Cycle Entrainment

Animals were kept in a 12h light (250-300 Lx bright white light)/ 12h dark cycle (“Lights on” = ZT0 at 7:00 in summer and 6:00 in winter; “Lights off” = ZT12 at 19:00 and 18:00 respectively) for three to five weeks (LD12:12). After one week of training activity profiles were taken for characterizing the entrainment of the animals. We studied the following criteria:

a. Activity onset: A minimum of 100 wheel revolutions per 5min bin after a minimum of 240min of rest. The average was taken for 7 consecutive days after one week of adaptation.

b. Overall activity: average number of wheel evolutions and approximate covered distance in 24h

c. Night time activity: like b. but only during the dark phase

d. Day time activity: like b. but only during the light phase

e. Activity phase ($\alpha$): time between onset and offset of activity

4.1.3.3.2. Shifted LD Cycles

After entrainment to an LD12:12 cycle for at least 10 days lights were not turned on at 7:00/6:00 the next day but 8h later (15:00/14:00). From there a shifted light cycle was presented to the animals (“Lights on” = ZT0 at 15:00 in summer and 14:00 in winter; “Lights off” = ZT12 at 3:00 and 2:00 respectively). We measured the number of days needed for an animal to adapt to the new LD cycle by looking at the onset of activity. After another 10 days the LD cycle was shifted back again (with a long night at the transition, e.g. “lights off” at 3:00/2:00 and “lights on” at 7:00/6:00 on the following day.)
4.1.3.3.3. Free Running in Constant Darkness

After entrainment to an LD12:12 cycle for at least 10 days lights were not turned on again at the following morning and animals were kept in constant darkness for several weeks (DD). We studied the following criteria:

a. Internal period (τ): Length of a subjective day determined by the time points of activity onset on two consecutive days. The average was taken from at least 7 consecutive days of stable rhythmicity in DD.

b. Overall activity: average number of wheel evolutions and approximate covered distance in 24h

c. Activity phase (α): time between onset and offset of activity

4.1.3.3.4. Free Running in Constant Light

Activity monitoring in constant light (LL) was performed like described for DD. In some experiments increasing light intensities were applied (as given in the diagrams). Therefore isolation boxes were equipped with dimmable halogen lamps (one for each cage). Light intensity was measured with a Luxmeter (Testo, Germany) and averaged for all cages (deviations were less than 10% in all cases).

4.1.3.3.5. Phase Shifting Experiments

Two different protocols were used for determining light induced phase shifts (Pittendrigh and Daan, 1976). For Type 1 mice were kept in DD for at least 10 days before a 15min Light pulse (300Lx) was applied to every single animal at CT14 (2 subjective hours after activity onset) or at CT22 (10 subjective hours after activity onset). For that the animals were removed from the isolation box and placed under a fluorescent light after replacing the lid of the cage with an empty wiring lid to ensure equal illumination of the whole cage area. The phase shift was determined by fitting a line through the onsets of activity before and after the light pulse with ignoring the first days after the treatment when the clock is still in transition. The difference between the two lines at the day after the pulse depicts the phase shift. Phase delays count as negative while phase advances count as positive phase shifts.

For the Type 2 protocol mice were kept in LD 12:12 for at least 10 days. A nocturnal light pulse (at ZT14 or at ZT22) was applied for 15min to all animals in one chamber and the lights
remained turned off on the following days. Two lines were drawn through the onsets of activity before and after the light pulse. The line after the light treatment was elongated to the day of the pulse. The difference between the two lines was determined. The same procedure was performed with control animals by applying the same LD/DD schedule without the light pulse. The difference between treated and control animals was determined as the phase shift.

These values are generally very similar to the ones obtained with a Type 1 experiment with slightly smaller numbers. An advantage of Type 2 are the easy and efficient treatment of many animals of different strains since no $\tau$ has to be taken into consideration for the timing of the light pulse and the animals need not to be removed from the isolation chamber for illumination. Additionally this protocol is applicable to mutants which eventually become arrhythmic in DD like the $mPer2^{Brdml}$ mutant used in this work or the Clock mutant (Vitaterna et al., 1994).

4.1.4. Cross Breeding

$mCry1^{+/+}/mCry2^{+/+}$ animals were crossed with $mPer1^{-/-}$ and homozygous $mPer2^{Brdml}$ animals respectively. From the f1 offspring $mPer/mCry$ double heterozygous animals (e.g. $mPer1^{+/+}/mCry1^{+/+}$) were selected and cross-bread to yield double mutants and corresponding wild-type animals in the f2 generation. For $mPer/mCry$ triple mutants homozygous animals from two double mutant strains sharing one mutation were chosen (e.g. for $mPer1^{-/-}/mCry1^{-/-}/mCry2^{-/-}$ mutants we started with $mPer1^{-/-}/mCry1^{+/+}$ and $mPer1^{+/+}/mCry2^{+/+}$ mice). The double heterozygous f1 offspring was intercrossed to yield the desired triple mutant strain in the f2 generation. As wild-type controls we chose the same animals as used for the double mutants (depicted “PC-WT”).
Fig. 57: Breeding schemes for double and triple mutant mice. (a) Breeding scheme for \textit{mPer1/mCry1} mutants (b) breeding scheme for \textit{mPer1/mCry1/mCry2} mutants.

4.1.5. \textit{Spalax} Strains

All animal work with \textit{Spalax} was performed in collaboration with Dr. Aaron Avivi at the Institute of Evolution, University of Haifa, Israel. For the work on \textit{sClock} and \textit{sMOP3} we used brain tissue, eyes and Harderian glands from \textit{Spalax S. judaei} (2n= 60) and \textit{Spalax S. galili} (2n= 52). For the studies on the \textit{sPers} and the \textit{sCrys} we used only \textit{Spalax S. judaei} (2n= 60). Animals were trapped in the field, kept under specific lighting conditions in the lab and sacrificed at the depicted time points. We received fixed and dehydrated tissue for \textit{in situ} Hybridization.
4.2. Molecular Biological Experiments

4.2.1. Genotyping

4.2.1.1. DNA Templates

The \textit{mPer2} probe hybridizes to a 12kb wild-type and a 10kb mutant fragment of \textit{BamH I} digested genomic DNA. The \textit{mCry1} probe detects a 9kb wild-type and a 4kb \textit{Nco I} digested fragment of the targeted locus. In \textit{mCry2} mutants the wild-type allele is detected by hybridization of the probe to a 7kb \textit{EcoR I} fragment whereas the mutant allele yields a 3.5kb fragment. For further descriptions see (van der Horst et al., 1999; Zheng et al., 2001; Zheng et al., 1999).

4.2.1.2. Probe Preparation

Templates for southern probes were as described for \textit{mPer1} \textsuperscript{-/-} (Zheng et al., 2001), \textit{mPer2}\textsuperscript{Brdm1} (Zheng et al., 1999) and for \textit{mCry1} \textsuperscript{-/-} and \textit{mCry2} \textsuperscript{-/-} (van der Horst et al., 1999). Probes were labeled with \textsuperscript{32}P-dCTP (NEN, Boston, USA) using the Rediprime II Random Prime Labeling Kit (Amersham Pharmacia, Little Chalfont, UK). Unincorporated nucleotides were removed with ProbeQuant G-50 Micro Columns (Amersham Pharmacia). Incorporation efficiency was assessed by measuring the activity of the probe with a liquid scintillation counter (Canberra Packard, Zürich, Switzerland).

4.2.1.3. DNA Extraction

Tail tips (~1cm) taken at the age of weaning (3-4 weeks) were used as tissue samples with a sharp scalpel blade or scissors. Excessive bleeding was stopped by holding a flamed blade briefly to the truncated tail. Tips were digested in a roller bottle over night at 55-60°C in 500\textmu l of 100mM Tris/HCl, 5mM EDTA, 200mM NaCl, 0.2% SDS, 100\textmu g/ml Proteinase K (pH 8.5). Genomic DNA was precipitated with Ethanol. The cloudy DNA was transferred to a new tube using a pipette tip and washed once with 70% Ethanol and 100% Ethanol before drying. The pellet was then dissolved in 50\textmu l TE buffer (10mM Tris/ HCl, 1mM EDTA, pH 7.5) and stored at 4°C.
4.2.1.4. Southern Blotting and Hybridization

- start with 10µg genomic DNA in TE buffer
- + water up to 20µl
- + 2.5µl 10x restriction enzyme buffer (New England Biolabs, Boston, USA)
- + 2.5µl (50U) restriction enzyme (EcoRI for Per1 and Cry2, BamHI for Per2, NotI for Cry1, all from NEB)
  Incubation ON at 37°C
- + 5µl 6x DNA Loading Buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 30% (v/v) glycerol)
  mix gently with the pipette tip to avoid shearing of the genomic DNA
- load samples on a 0.7% agarose gel (no ethidium bromide!) in a Gibco Sunrise electrophoresis chamber (Invitrogen, San Diego, USA)
- run at 60V (~ 6-8h) until the bromophenol blue band hits the next loading slot row
- cut gel and mark orientation
- incubate gel for 10min in 0.05‰ ethidium bromide in TE buffer
- photograph gel on a UV screen (Vilber Lourmat, Marne Le Valée, France)
- partly hydrolyze the DNA in 0.25M hydrochloric acid (exactly 2x 5min!)
- denature in 0.4M sodium hydroxide (20min)
- assemble blotting chamber (from bottom to top: 0.4M sodium hydroxide for transfer, blotting paper, gel (turn upside down for better transfer efficiency), blotting membrane (Hybond+®, NEN), blotting paper, paper towels, weight) and leave ON
- disassemble chamber, mark membrane and cross-link (Stratagene Linker, Stratagene, La Jolla, USA)
- wash 2x with water and put into hybridization bottle
- add appropriate volume of hybridization buffer (QuikHyb, Stratagene) and rotate at 68°C (minimum 20min) prior to hybridization
- denature probe and hybridize 1h at 68°C
- remove excess buffer and wash three times with 2xSSC (0.3M sodium chloride, 0.03M sodium citrate, pH 7), 0.1% SDS
- wash 2x 15min with 2xSSC, 0.1% SDS at RT
- wash 2x 20min with 0.1xSSC, 0.1%SDS at 60-63°C in a shaking water bath
• wrap membrane with Saran and expose on film (-80°C with enhancer screen (Amersham Pharmacia)) ON

4.2.2. RNA Blotting

4.2.2.1. cDNA Templates

The probes for mPer1 and mPer2 were as described (Albrecht et al., 1997b). The mPer1 probe corresponds to nucleotides 1 to 619 (GenBank accession number AF022992). The mPer2 probe was made from a cDNA corresponding to nucleotides 229–768 (AF036893). The mPer2 probe is located outside the region deleted in the mutant. The c-Fos probe was made from a mouse cDNA whose nucleotide sequence corresponds to amino acid positions 237–332. The AVP probe was made from a cDNA corresponding to nucleotides 1 to 480 (GenBank M88354). The mCry1 probe was made from a cDNA corresponding to nucleotides 190-771 (accession number AB000777) and the Bmal1 probe corresponding to nucleotides 654-1290 (accession number AF015953).

cDNA was obtained by RT-PCR from brain total RNA (for preparation see below) using Superscript II (Gibco/ Invitrogen) and Taq Polymerase (Qiagen, Hilden, Germany) and standard protocols (Sambrook and Russel, 2001). PCR product was cloned into pCR II Topo vector using TOPO TA Cloning Kit (Invitrogen). Single colonies were picked and grown in LB medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) sodium chloride, 0.1 mg/ml ampicillin) for Maxi preparation of plasmid DNA using QIAfilter Plasmid Maxi Kit (Qiagen). Inserts were cut from the vector with EcoRI and extracted from an agarose gel (1%, with ethidium) using QIAEX II Gel Extraction Kit (Qiagen).

4.2.2.2. Probe Preparation

See probe preparation for genotyping (4.2.1.2.).

4.2.2.3. RNA Purification

Animals were killed by cervical dislocation and tissue removed and transferred into RNAzol B (WAK Chemie, Bad Soden, Germany; ~1ml per 0.25g of tissue) on ice. After
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homogenization (Polytron PT 1200, Kinematica, Littau, Switzerland) 1/10 volumes chloroform was added and mixed before incubation on ice for 5 min. After centrifugation (15 min, 13k rpm, 4°C) supernatant was transferred to a fresh tube and RNA precipitated with an equal volume of 2-Propanol (15 min on ice). Total RNA pellets were stored at –80°C under 50% 2-Propanol and dissolved in water before use. For mRNA preparation we used Oligotex mRNA Kit (Qiagen) according to the protocol of the manufacturer.

To remove excess glycogen from liver total RNA pellets were washed in 4M lithium chloride before use.

4.2.2.4. Northern Blotting and Hybridization

- start with 20µg total RNA or up to 10µg mRNA in 5µl water
- + 2.2µl 5x Running Buffer (0.1M MOPS (pH 7), 25mM sodium acetate, 5mM EDTA)
  + 3.9µl 37% (w/v) formaldehyde
  + 11.1µl formamide
  + 2.2µl 6x RNA Loading Buffer (see DNA Loading Buffer but without xylene cyanol FF)
- incubation for 15 min at 65°C
- load samples on a 1% agarose gel (no ethidium!) containing formaldehyde (see (Sambrook and Russel, 2001))
- run at 10-15V (ON) until the bromophenol blue band hits the end of the gel
- cut gel and mark orientation
- incubate gel for 5 min in water
- partly hydrolyze the RNA in 50mM sodium hydroxide, 10mM sodium chloride (45 min)
- neutralize in 0.1M Tris/HCl (pH 7.5) for 45 min
- equilibrate gel in 20xSSC (see above) for 1 h
- assemble blotting chamber (from bottom to top: 10xSSC for transfer, blotting paper, gel (turn upside down for better transfer efficiency), blotting membrane (Hybond+, NEN), blotting paper, paper towels, weight) and leave ON
- disassemble chamber, mark membrane and UV crosslink
- wash 2x with water and put into hybridization bottle
- add appropriate volume of hybridization buffer (QuikHyb, Stratagene or UltraHyb, Ambion, Austin, USA) and rotate at 68°C (QuikHyb, minimum 20 min) or 42°C (UltraHyb, 1-2 h) prior to hybridization
Material and Methods

- denature probe and hybridize 1-2h at 68° (QuikHyb) or at 42°C (UltraHyb)
- remove excess buffer and wash three times with 2xSSC, 0.1% SDS
- wash 20min with 2xSSC, 0.1% SDS at RT
- wash 2x 20min with 0.1xSSC, 0.1%SDS at 60°C in a shaking water bath
- wrap membrane with Saran and expose on film (-80°C with enhancer screen (Amersham Pharmacia)) or on phosphoimager screen.

4.2.2.5. Quantification

Exposed phosphoimager plates (Bio-Rad, Hercules, USA) were scanned with a phosphoimager (Bio-Rad) and quantified using Quantity One V3.0 software (Bio-Rad).

4.2.3. In situ Hybridization

In situ Hybridization was performed according to Albrecht et al., 1997a.

4.2.3.1. cDNA Templates

For most templates see 4.2.2.1. The c-Fos probe was made from a mouse cDNA whose nucleotide sequence corresponds to amino acid positions 237–332. The AVP probe was made from a cDNA corresponding to nucleotides 1 to 480 (GenBank M88354).

4.2.3.2. Probe Preparation

\[ ^{35} \text{S-UTP (NEN) labeled RNA probes were made using RNA Transcription Kit (Stratagene) with T7, T3 or SP6 RNA polymerases (Stratagene, NEB and Promega, Madison, USA). 1\mu g linearized plasmid template was used in a 30\mu l setup sufficient for 24 slides according to the manufacturer’s protocol.} \]

Incorporation was determined by liquid scintillation and probes were diluted to 2-6M cpm per slide in HybMix (25% (v/v formamide, 0.3M NaCl, 20mM Tris/HCl (pH 8), 5mM EDTA, 10% (w/v) dextrane sulfate, 0.02% Ficoll, 0.02% BSA, 0.02% polyvinylpyrolidone, 0.5mg/ml yeast RNA, 0.1M DTT, 250\mu M \alpha S-ATP).
4.2.3.3. Tissue Preparation

Either paraffin embedded or frozen tissue was used. For paraffin embedding animals were killed by cervical dislocation, tissue was removed and immersion fixed in 4% paraformaldehyde (PFA) in PBS (pH 7.4) at 4°C for 12-18h. Tissues were then dehydrated with ethanol (30, 50, 70 and 100%, 3h each at 4°C) and transferred to xylene. Xylene was changed once and replaced by 50% xylene/50% paraffin and 3x paraffin at 60°C before pouring the samples in embedding forms. Tissues were cut on the following day or later using a microtome (R. Jung, Hamburg, Germany) at 7µm thickness and stored at RT before use.

For cryo-sections tissue was shock frozen in liquid nitrogen, cut embedded in TissueTek (Sakura Finetec, Zoeterwoude, The Netherlands) on a cryostat (Leica Microsystems, Wetzlar, Germany) at 20µm thickness and stored at –80°C before use.

4.2.3.4. Hybridization

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

- 2x Histoclear (Vogel, Giessen, Germany) 10min
- 2x 100% Ethanol 2min
- re-hydrate with ethanol 20s each (95/80/70/50/30%)
- 0.9% sodium chloride 5min
- PBS 5min
- 4% PFA (pH7.4 in PBS) 20min
- 2x PBS 5min
- Proteinase K (20µg/ml in 50mM Tris/HCl, 5mM EDTA pH8.5) 5min
- PBS 5min
- 4% PFA pH7.4 20min
- acetylation in 0.1M Triethanolamine/HCl pH8: add 600µl acetic anhydride on 250ml; stir 3min; add another 600µl acetic anhydride and stir 7min
- PBS 5min
- 0.9% sodium chloride 5min
- de-hydrate with ethanol 20sec each (30/50/70/80/95/100%)
• let slides air-dry at a RNase-free place

Frozen sections were fixed in PFA and treated like paraffin sections subsequently. Hybridization was performed in humidified chambers (5x SSC, 50% (v/v) formamide) in a hybridization oven ON at 55-58°C. Probe was put on the slides and spread over the whole area using a pipette tip before covering with a cover slip. Post-hybridization was performed in Tissue Tek II cuvettes (Sakura Finetec).

• Removal Wash: 30min in 5xSSC/ 20mM mercaptoethanol (ME) at 64°C in a shaking water bath; after 10min cover slips were removed using forceps

• 2xSSC/ 50% formamide/ 40mM ME 30min at 64°C

• 3x NTE (50mM sodium chloride, 10mM Tris/ HCl, 5mM EDTA, pH 8) 15min at 37°C

• RNase A (20µg/ml in NTE) 30min at 37°C

• NTE 15min at 37°C

• 2xSSC/ 50% formamide/ 40mM ME 30min at 64°C

• 2xSSC 15min at RT

• 0,1xSSC 15min

• de-hydration with ethanol 30sec each (30/60/80% EtOH/ 0.3M ammonium acetate, 95/100/100% EtOH)

• let slides air-dry before exposure to film or coating with liquid film (Kodak)

4.2.3.5. Quantification

Exposed films were scanned with a flatbed scanner (Hewlett Packard, Palo Alto, USA). SCN cut outs were selected using Adobe PhotoShop (Adobe, San Jose, USA) and analyzed densitometrically with NIH Image 1.62 (National Institutes of Health, USA). Three sections per brain were used and background subtracted from adjacent hypothalamic areas on the same slide. Measurements from different animals/ experiments were combined for statistical analysis performed with GraphPad Prism software (GraphPad Software, San Diego, USA).
4.3. Immunological Experiments

4.3.1. Immunohistochemistry and –fluorescence

4.3.1.1. Antibodies

<table>
<thead>
<tr>
<th>Antigen / Antibody</th>
<th>Company</th>
<th>Catalog Number</th>
<th>Working dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit-αPER2 (mouse)</td>
<td>Alpha Diagnostics</td>
<td>PER21-A</td>
<td>1:200</td>
</tr>
<tr>
<td>Rabbit-αCRY1 (mouse)</td>
<td>Alpha Diagnostics</td>
<td>CRY11-A</td>
<td>1:200</td>
</tr>
<tr>
<td>Rabbit-αCREB (mouse)</td>
<td>Cell Signalling Tech</td>
<td>9192S</td>
<td>1:500</td>
</tr>
<tr>
<td>Rabbit-αP^33_-CREB (mouse)</td>
<td>New England Biolabs</td>
<td>9191S</td>
<td>1:500</td>
</tr>
<tr>
<td>Biotinylated Goat-αIgG (rabbit)</td>
<td>Vector Laboratories</td>
<td>PK6101</td>
<td>1:200</td>
</tr>
</tbody>
</table>

Tab. 10: Used Antibodies and Dilutions.

The secondary antibodies were included in the Vectastain Elite Kit used for all immunostaining protocols (Vector Laboratories, Burlingame, USA).

4.3.1.2. Tissue Preparation

Tissue was prepared as described for in situ hybridization (4.2.2.3.).

4.3.1.3. Immunohistochemistry

- 2x 10min xylene
- 2min ethanol
- re-hydration with ethanol (100/70/50/30%) 20sec each
- 2min water
- 10min 3% (v/v) hydrogen peroxide in methanol
- 3x 2min water
- 2min 0,01M sodium citrate (pH6)
- 10min boiling in citrate (s.a.)
- 10min cool down
- 2min TNT (0.1M Tris/ HCl pH 7.5, 150mM sodium chloride, 0.05% Tween20); put slides in flow chambers (Rediflow, Tecan, Durham, USA)
- 2x 2min TNT
1h normal goat serum (Vectastain Kit, in TNT)
1
1st antibody ON at RT (in TNT; store horizontally in a humidified chamber)
3x 5min TNT
2nd antibody 1h at RT (Vectastain Kit, in TNT)
3x 5min TNT
1h AB-Complex (Vectastain Kit, in TNT) RT
3x 5min TNT
10min Ni/DAB (1g nickel ammonium sulfate; 17.5mg diaminobenzidine/HCl (both from Fluka, Germany) in 100ml 0.1M sodium acetate (pH 6)
add 100µl 30% H₂O₂; incubate for 5min
3x 5min TNT
2x 20sec water
air dry
apply cover slips with Canada Balsam

4.3.1.4. Immunofluorescence

All steps for immunofluorescence follow the immunohistochemistry protocol up to the incubation with the AB-Complex. Further protocol as follows:

3x 5min TNT
20min Tyramide-Fluoresceine in NEN amplification buffer (Tyramide Amplification Kit, NEN)
3x 5min TNT
dehydrate with ethanol 20sec each (30/50/70/100% ethanol)
2min xylene
mount coverslips with DPX (Fluka, Germany)
4.3.1.5. Quantification

Semiquantitative analysis of immunohistochemistry was performed with NIH Image software 1.62 (National Institutes of Health). In selected slice areas positively stained nuclei were counted after thresholding. Three slices per SCN of similar regions were selected and the average value determined. Values from multiple experiments were taken for statistical analysis with GraphPad Prism software (GraphPad Software).

4.4. Histological Experiments

4.4.1. Nissl Staining

To visualize cell nuclei in brain and eye slices samples were stained with Cresyl Violet according to Nissl:
Slides were de-waxed and re-hydrated and subsequently colorised 5min in Cresyl Violet (0.15g in 250ml 0.3M sodium acetate buffer, pH 5.5, 60°C). After de-hydration slides were incubated in xylene and coverslips mounted with DPX.

4.4.2. Gomori’s Trichrome Staining

Slides were de-waxed and re-hydrated and post-fixated in Bouin’s Fluid (70% (v/v) saturated picric acid, 10% (w/v) formaldehyde and 5% (v/v) acetic acid) for 30min at 56°C (. After washing with water slides were stained for 15-20min with Trichrome Stain (0.6% (w/v) Chromotrope 2R, 0.3% (w/v) Light Green, 1% (v/v) acetic acid and 0.8% (w/v) phosphotungstic acid). Subsequently samples were washed with 0.5% (v/v) acetic acid for 2min and 1% (v/v) acetic acid containing 0.7% (w/v) phosphotungstic acid if the staining was still too dark. After that slides were de-hydrated, incubated in xylene and coverslips were mounted with DPX.

4.4.3. Lipofuscin Staining

Slides were de-waxed and re-hydrated with decreasing concentrations of ethanol. After a brief rinsing in distilled water they were incubated for 5min in 0.75% (w/v) ferric chloride/ 0.1%
(w/v) potassium ferricyanide. Subsequently they were transferred to 1% (v/v) acetic acid (5min), washed in distilled water (10min) and colonized with neutral red (1% (w/v) for 3.5min. After another wash with distilled water slides were de-hydrated in increasing ethanol concentrations. After incubation with xylene coverslips were applied using DPX embedding medium.

4.4.4. Congo Red Staining

Congo red staining was performed using Accustain Amyloid Stain, Congo Red (Sigma Diagnostics, St. Louis, USA). Slides were de-waxed and re-hydrated with decreasing concentrations of ethanol. After a brief rinsing in distilled water they were incubated for 10min in Mayer's hematoxylin. Subsequently slides were incubated for 5min in distilled water, for 20min in 0.01% (w/v) sodium hydroxide in saturated sodium chloride solution, and for 20min in 0.01% (w/v) sodium hydroxide, 0.2% (w/v) Congo red in 80% saturated sodium chloride solution. After that slides were washed three times (1min each) with ethanol and two times with xylene. Coverslips were applied using DPX embedding medium.
Chapter 5

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Chapter 6

Curriculum Vitae

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08.83-07.92 High school attendance at the Treviris Gymnasium Trier and the
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06.92 Abitur at the Detlefsenschule Glückstadt
08.92-10.93 Public service at the Diakonie-Sozialstation Glückstadt
10.93-06.99 University courses in biochemistry at the University of Hannover/ Germany
05.96-01.97 Project work in the group of Prof. G. Brabant at Hannover Medical School
(MHH) on cell adhesion molecules and DNA methylation in human thyroid
carcinoma
03.97-05.97 Project work in the group of Prof. T.J. Jentsch at the Center for Molecular
Neurobiology Hamburg/ Germany (ZMNH) on the morphological and
functional characterization of the voltage regulated chloride channel hCLC-
1
03.98-05.98 Project work in the group of Prof. J. Barber at the Imperial College for
Science, Medicine and Technology London/ UK on the structural
characterization of CP43 in the plant photosystem II
11.98-06.99 Graduation project in the group of Dr. R. Bauerfeind (Institute of
Physiological Chemistry, Prof. H.H. Niemann) at Hannover Medical School
on molecular interactions of Amphiphysin 1 in the endocytosis machinery
06.99 Diploma in biochemistry at Hannover Medical School
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mammalian circadian clock
Chapter 7

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Fribourg, the 22nd of October 2002