Loss of circadian rhythmicity in aging mPer1−/− mCry2−/− mutant mice

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The mPer1, mPer2, mCry1, and 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 nonallelic 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−/− mCry2−/− mice at the behavioral and the molecular levels. 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.

[keywords: Circadian clock; Per; Cry; aging; transcription]

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The earth’s rotation around the Sun has strongly influenced temporal organization of the mammalian organism manifested by near-24-h 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 [Rusak and Zucker 1979, Ralph et al. 1990]. Because the internal period length generated by this pacemaker is not exactly 24 h, the clock has to be reset every day by an input pathway synchronizing the organism’s biological processes with geophysical time. The daily variation in light intensity is monitored by photoreceptors in the eye that project into the SCN via the retinohypothalamic tract [RHT; Rusak and Zucker 1979] and the intergeniculate leaflet [IGL; Jacob et al. 1999]. The oscillations generated in the SCN are translated into overt rhythms in behavior and physiology through output pathways that probably involve both chemical and electrical signals.

At the molecular level, circadian rhythms are generated by the integration of autoregulatory transcriptional/translational feedback loops (TTLs; Allada et al. 2001; Albrecht 2002, 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 upon heterodimerization bind to “E-box” enhancer elements 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. 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 stabilization of a 24-h period [Kume et al. 1999, Yagita et al. 2000, 2002; Lee et al. 2001, Miyazaki et al. 2001, Vielhaber et al. 2001, 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 mPer and mCry genes and controls transcription of BMAL1 [Preitner et al. 2002]. In mammals, three Per genes—mPer1 [Sun et al. 1997; Tei et al. 1997], mPer2 [Albrecht et al. 1997, Shearman et al. 1997], and mPer3 [Zylka et al. 1998]—and two Cry genes—mCry1 and mCry2 [Miyamoto and Sancar 1998]—have been identiﬁed. Although mPer3 seems not to be necessary for the generation of circadian rhythmicity [Shearman et al. 2000], mPer1, mPer2, and both 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 [van der Horst et al. 1999; Vitaterna et al. 1999;
Zheng et al. 1999, 2001; Albrecht et al. 2001; Bae et al. 2001, Cermakian et al. 2001). In addition to the master clock in the SCN, most cells of peripheral tissues possess a circadian oscillator with a molecular organization very similar to that of SCN neurons, but lacking light-responsiveness [Balsalobre et al. 1998; Yamazaki et al. 2000, Yagita et al. 2001].

The molecular mechanism of clock autoregulation has largely been studied in vitro [Gekakis et al. 1998; Kume et al. 1999, Miyazaki et al. 2001; Vielhaber et al. 2001, Yagita et al. 2000, 2002, Yu et al. 2002]. These studies point to multiple physical interactions between all mPER and mCRY proteins. However, the time course of protein availability, modification, and localization is difficult to resolve in cell and slice cultures [Jagota et al. 2000; Hamada et al. 2001; Lee et al. 2001]. To elucidate the functional relationship between the mPer and mCry genes in vivo, we started to inactive different combinations of mPer and mCry genes in mice (Oster et al. 2002).

Here we show that mPer1−/− mCry1−/− mice maintain a functional circadian clock and that mPer1−/− mCry2−/− mice lose circadian rhythmic behavior after a few months. This loss of rhythmicity is accompanied by altered regulation of expression of core clock components and the clock output gene arginine vasopressin (AVP).

Our results indicate that the amount of mPER and mCRY proteins and hence the composition of mPER/mCRY complexes are critical for generation and maintenance of circadian rhythms.

Results

Generation of mPer1−/− mCry1−/− and mPer1−/− mCry2−/− mice

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 or mPer1/mCry2 genes. 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 were 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/mCry1 double-mutant mice, the intervals between two litters seem to increase significantly with progressing age (data not shown).

mPer1 acts as a nonallelic suppressor of mCry1

To determine the influence of inactivation of the mCry1 gene on circadian behavior of mPer1−/− mice, mutant and wild-type animals were individually housed in circadian activity-monitoring chambers [Albrecht and Oster 2001; Albrecht and Foster 2002] for analysis of wheel-running activity. Mice were kept in a 12-h light/12-h 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 and clock gene expression patterns similar to that of wild-type mice (Fig. 1B,C, Supplementary Fig. 1). Under DD conditions, mPer1−/− mCry1−/− mutant mice displayed a period length (τ) of 23.7 ± 0.2 h [mean ± S.D., n = 15], which is similar to that of wild-type animals [τ = 23.8 ± 0.1 h, n = 17]. Thus, an additional deletion of mPer1 rescues the short-period phenotype of mCry1-deficient mice [van der Horst et al. 1999], indicating that mPer1 acts as a nonallelic suppressor of mCry1.

Loss of circadian wheel running activity rhythms in aging mPer1−/− mCry2−/− double-mutant mice

Analysis of circadian behavior of mPer1−/− mCry2−/− animals under LD conditions revealed that in young mPer1−/− mCry2−/− animals [between 2 and 6 mo old], the onset of activity was delayed as compared with wild-type animals and the highest activity could be observed in the second half of the night, with masking of activity during the first hours of the day (Fig. 1D). Under DD conditions, these animals display rhythmic behavior with a long period [τ] of 25.3 ± 0.2 h [mean ± S.D., n = 14] compared with wild-type animals [τ = 23.8 ± 0.1 h, n = 17, Fig. 1D,E).

Interestingly, mPer1−/− mCry2−/− animals that were >6 mo old displayed a markedly disturbed diurnal activity pattern under LD conditions [Fig. 1F], as evident from the very faint 24-h rhythm detected by χ² periodogram analysis [Fig. 1G]. Under DD conditions, old mPer1−/− mCry2−/− mice were completely arrhythmic [Fig. 1F,I], which sharply contrasts the robust rhythmicity of young mPer1−/− mCry2−/− mice under similar conditions. The transition from a rhythmic to an arrhythmic phenotype in mPer1−/− mCry2−/− mice correlated well with age (Fig. 1H). Whereas all mPer1−/− mCry2−/− mice at an age between 2 and 6 mo display circadian activity patterns, 40% of animals between 6 and 12 mo of age have lost circadian rhythmicity. When animals reached the age of 1 yr or older, even 87% of the mPer1−/− mCry2−/− mice have become arrhythmic. We did not observe a comparable age-related loss of rhythmicity in wild-type, mPer1−/− and mCry2−/− mice [Fig. 1H; Supplementary Fig. 2A–C] and not in mPer1−/− mCry1−/−, mPer2Brdm1 mCry1−/− and mPer2Brdm1 mCry2−/− mice, respectively [Supplementary Fig. 2D].

Alterations in expression levels of clock components and the clock output gene Avp in aging mPer1−/− mCry2−/− double-mutant mice

To extend our observations to the molecular level, we examined the expression patterns of the mPer2, mCry1, and Bmal1 genes in 6–12-month-old mPer1−/− mCry2−/−/H9270
mice under LD and DD conditions. For simplicity, we will refer to “young” and “old” mPer1+/− mCry2−/− animals on the basis of rhythmic or arrhythmic behavior, respectively.

mPer2 mRNA expression in the SCN of young mPer1+/− mCry2−/− mice was comparable to that of wild-type animals under both LD and DD conditions with peak levels at Zeitgeber time (ZT) and circadian time (CT) 12, respectively (Fig. 2A,B). Interestingly, rhythmic peak levels at time and circadian time in young mPer1+/− mCry2−/− mice kept under LD and DD conditions, with maximal expression observed around ZT12 (Fig. 2D,E). In line with the data observed for the SCN, cyclic mPer2 mRNA expression in the kidney is blunted in young mPer1+/− mCry2−/− mice (Fig. 2D,E). In conclusion, circadian oscillators lacking both mPer1 and mCry2 appear sensitive to aging.

To correlate mRNA expression to protein levels, 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,C).

**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 hybridizes to a 20-kb wild-type and a 11.8-kb mutant fragment of EcoRI-digested genomic DNA. The mCry1 probe detects a 9-kb wild-type and a 4-kb Ncol-digested fragment of the targeted locus. In mCry2 mutants, the wild-type allele is detected by hybridization of the probe to a 7-kb EcoRI fragment, whereas the mutant allele yields a 3.5-kb 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 12-h light/12-h 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 5 d in DD, wheel rotations per day were 20,000 ± 2500 (n = 17) for wild-type animals, 21,500 ± 7300 (n = 15) for mPer1+/− mCry2−/− mutants, 25,100 ± 6200 (n = 14) for young mPer1+/− mCry2−/− mutants, and 17,200 ± 7900 (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 d 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 gray bar, mPer1−/− (white bar), mCry2−/− (black bar), and mPer1+/− mCry2−/− (light gray bar)] mice. The animals tested were divided into three groups according to their age (2–6 mo, 6–12 mo, and >12 mo old). Rhythmicity in DD was determined by periodogram analysis. The values on top of each bar indicate the total numbers of animals tested per group and genotype.

Expression in old [6–12 mo] mPer1−/− and mCry2−/− single-mutant mice did not show a detectable reduction in amplitude under LD and DD conditions [Supplementary Fig. 3A,B], we conclude that the age-related loss of mPer2 mRNA oscillation in mPer1−/− mCry2−/− animals is characteristic for the double-knockout status. Analysis of the peripheral circadian oscillator in the kidney revealed a normal mPer2 mRNA expression profile in young mPer1−/− mCry2−/− mice kept under LD conditions, with maximal expression observed around ZT12 [Fig. 2D,E]. In line with the data observed for the SCN, cyclic mPer2 mRNA expression in the kidney is blunted in old mPer1−/− mCry2−/− mice (Fig. 2D,E). As mPer2

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mPER1/2 and mCRY1/2 proteins inhibit CLOCK/BMAL1-mediated transcriptional activation (Kume et al. 1999; Lee et al. 2001). Therefore, we investigated the expression pattern of mCry1 in the SCN in mPer1−/− mCry2−/− mice. mCry1 mRNA expression profiles peak at ZT12 and CT12 under LD and DD conditions, respectively (Fig. 3A,B; Okamura et al. 1999). Similar expression patterns were observed in mPer1−/−, mCry2−/−, and young mPer1−/− mCry2−/− mice (Fig. 3A,B; Supplementary Fig. 3C,D). Interestingly, mCry1 mRNA levels displayed normal cycling in old mPer1−/− mCry2−/− mice in LD [Fig. 3A], which is in marked contrast to the blunted mPer2 mRNA expression profile in these mice [Fig. 2A,B]. We thus examined mCRY1 protein levels in the SCN by immunohistochemistry. In wild-type animals, mCRY1 protein levels are oscillating 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 trough mCRY1 protein levels (at ZT24) were higher than in wild-type animals (Fig. 3C). Interestingly, mCRY1 protein levels were observed in mPer1−/− mCry2−/− mice in LD [Fig. 3A], which is in marked contrast to the blunted mPer2 mRNA expression profile in these mice [Fig. 2A,B]. We thus examined mCRY1 protein levels in the SCN by immunohistochemistry. In wild-type animals, mCRY1 protein levels are oscillating 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 trough mCRY1 protein levels at ZT24 were higher than in wild-type animals [Fig. 3C]. Strikingly, expression of mCRY1...
protein became totally blunted in old mPer1\(^{-/-}\) mCry2\(^{-/-}\) mice, leading to almost constant high levels of mCRY1 protein throughout the 24-h LD cycle (Fig. 3C). Note that age-matched mPer1\(^{-/-}\) and mCry2\(^{-/-}\) single-
mutant mice display normal mCRY1 protein cycling [Fig. 3D]. We looked at Bmal1 mRNA expression, a clock component of the positive limb, under LD and DD conditions. In wild-type and mPer1−/− animals, a maximum was seen at ZT and CT 18 in the SCN [Supplementary Fig. 3E,F] as previously observed [Honma et al. 1998]. In mCry2−/− animals, the maximum of Bmal1 expression was slightly delayed [Supplementary Fig. 3E,F]. Young mPer1−/− mCry2−/− animals displayed a wild-type expression pattern, although the peak levels tended to be slightly decreased [Fig. 3E,F]. In old mPer1−/− mCry2−/− mice, Bmal1 mRNA levels were significantly blunted [Fig. 3E].

Because expression of core clock components is altered in old mPer1−/− mCry2−/− animals, we investigated whether this translates into a change in expression of output genes. Arginine-vasopressin [Avp] expression is significantly reduced in old mPer1−/− mCry2−/− animals [Fig. 3G], indicating physiological consequences linked to the aging process. mPer1 and mCry2 mutant mice do not exhibit this change in Avp expression [Albrecht and Oster 2001; Supplementary Fig. 3G]. Dbp expression appeared also to be affected [Fig. 3H]; however, this change is caused by the Cry2 inactivation [Supplementary Fig. 3H] and is not specific to the Per1 Cry2 double mutation.

**Loss of light inducibility of mPer2 mRNA and effect on delaying the clock phase in mPer1−/− mCry2−/− mice**

mPer expression can also be induced by phase-resetting light stimuli via the CREB signaling pathway [Motzkus et al. 2000; Travnickova-Bendova et al. 2002]. To investigate whether aging affects light inducibility of the mPer2 gene in the SCN of mPer1−/− mCry2−/− mice, we exposed young and old animals to a 15-min nocturnal light pulse at ZT14. Interestingly, induction of mPer2 mRNA was significantly impaired in young mPer1−/− mCry2−/− mice when compared with wild-type animals \( p < 0.05; \) Fig. 4A,B). This defect was even more pronounced in old mPer1−/− mCry2−/− mice \( p < 0.001; \) Fig. 4A,B), indicating that the light signal transduction pathway might be affected. Therefore we set out to investigate light-dependent phosphorylation of CREB at position 133 [CREB-Ser133]. We found that in wild-type animals, phosphorylation at CREB-Ser133 was seen at ZT and CT 18 in the SCN (Supplementary Fig. 3G); however, this change appeared also to be affected (Supplementary Fig. 3H). Young mPer1−/− mCry2−/− mice showed a reduction in CREB phosphorylation at Ser133 and reduced expression of phosphorylation in CREB-Ser133 and reduced expression of mPer2 mRNA. The slow onset of wheel-running activity in LD and the strong reduction in CREB phosphorylation at Ser 133 in the SCN of old mPer1−/− mCry2−/− mice indicates that light signaling from the eye to the SCN might be defective. We therefore performed a histochemical analysis of the retina from wild-type, mPer1−/− mCry2−/−, and mPer1−/− mCry2−/− mice, respectively [Fig. 5]. No overt morphological differences between the retinas of these mice or cell death could be detected [Fig. 5A,B]. Thus, the observed effects of aging in mPer1−/− mCry2−/− animals appear restricted to the functionality of the circadian system and are not likely to originate from aberrant development or age-related morphological changes in the retina.

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−/− and mCry2−/− single-mutant animals and in 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). Taken together, these results indicate that the profound loss of circadian wheel-running behavior of old mPer1−/− mCry1−/− mice under LD conditions (Fig. 1F) is caused by impaired light signal transduction pathway performance in combination with an age-related decline in core oscillator function.

**Discussion**

Interaction of clock components has predominantly been investigated in vitro (Gekakis et al. 1998; Kume et al. 1999; Yagita et al. 2000, 2002), revealing that mPER and mCRY proteins can form complexes that influence nuclear transport or regulate transcription of clock components. In contrast, it is not known to what 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 subsequently analyzing circadian behavior, clock gene, and protein expression.

mPer1−/− mCry1−/− mice display normal circadian rhythmicity but show impaired ability to phase advance the clock.

We have shown that mPer1−/− mCry1−/− mice, in contrast to short-period mCry1−/− mice, display a period length comparable to that of wild-type littermates (Fig.
Thus, the additional loss of mPer1 in mCry1−/− mice leads to an increase in period length to near normal values in DD (23.7 ± 0.2 h for mPer1−/− mCry1−/− mice vs. 22.51 ± 0.06 h for Cry1−/− mice). This also indicates that expression of the mPER2 and mCRY2 proteins apparently is sufficient to maintain circadian rhythmicity.

The rescue of the mCry1−/− phenotype by additional loss of the mPer1 gene is also reflected at the molecular level, where mPer2 and Bmal1 show normal mRNA rhythms under both LD and DD conditions [see Supplementary Fig. 1]. Hence mPer1 acts as a nonallelic suppressor of mCry1. Interestingly, mPer1−/− mCry1−/− animals could not phase advance their behavioral rhythms after a 15-min light pulse given at ZT22 [Fig. 4E], and in this respect resemble mPer1−/− animals [Albrecht et al. 2001]. In conclusion, only circadian core clock functionality is rescued by an inactivation of mCry1 in mPer1−/− mice, but not the resetting properties of the clock.

Figure 5. Histology and light responsiveness in the retina of wild-type, young, and old mPer1−/− mCry2−/− mice. Gomori trichrome [A] and lipofuscin [B] staining of retinal sections of wild-type [first row], young mPer1−/− mCry2−/− [second row], old mPer1−/− mCry2−/− [third row], mPer1−/− [fourth row], and mCry2−/− mice [fifth 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] Immunostained retinal sections of control animals without light exposure. [Right panels] Animals 1 h after light exposure (400 lx, 15 min) at ZT14. (D) Immunohistochemistry analysis for [unphosphorylated] CREB in the retina at ZT14. Bar, 10 µm.
Breakdown of the clock in aging mPer1−/− mCry2−/− mice

Circadian organization changes with age (Valentiniuzzi 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 (Valentiniuzzi et al. 1997). In mice, aging has been found to diminish the amplitude of Per2 but not Per1 expression (Weimert et al. 2001).

Here, we provide evidence that a clock defect can make the circadian oscillator fall apart more quickly, resembling accelerated aging. Inactivation of mPer1 and mCry2 in young mPer1−/− mCry2−/− mice [2–6 mo old] leads to a decreased precision in onset of daily activity (Fig. 1D,E). Additionally, onset of activity is markedly delayed, with a sharp offset at the dark/light transition (Mrosovsky 1999). In old mPer1−/− mCry2−/− mice, the precision in onset of daily activity is even further deteriorated (Fig. 1F). Moreover, animals start to display fragmentation of activity under LD conditions, and daily rhythms become barely detectable (Fig. 1G). In constant darkness, old mPer1−/− mCry2−/− mice no longer display circadian rhythmicity, and the amplitude of wheel-running activity is decreased compared with that of wild-type and young mPer1−/− mCry2−/− mice (Fig. 1F,I). The percentage of arrhythmic mPer1−/− mCry2−/− mice increases with age (Fig. 1H), but the time of onset of the arrhythmic circadian phenotype varies among animals, indicating that additional genes or genetic background may contribute to the aging process. All these features are observed neither in mPer1−/− and mCry2−/− single-mutant mice (van der Horst et al. 1999, Zheng et al. 2001; Supplementary Fig. 2) nor in mPer1−/− mCry1−/−, mPer2−/−mCry1−/−, mPer2−/−mCry2−/− (Supplementary Fig. 2D), or heterozygous mPer1 mCry1 and mPer1 mCry2 mice (Supplementary Fig. 4).

Gene expression is known to change upon aging. Alterations in mRNA and protein levels can result from changes in transcriptional regulation (Roy et al. 2002), mRNA stability (Brewer 2002), and proteasome-mediated protein degradation (Goto et al. 2001). We have shown that the absence of mPer1 and mCry2 specifically alters the regulation of the circadian core oscillator in an age-related manner. This is illustrated by our observation that mPer2 and Bmal1 mRNA levels are strongly reduced in the SCN and in the kidney of old mPer1−/− mCry2−/− mice (Figs. 2, 3E,F). Additionally, mCry1 protein levels are elevated (Fig. 3C), pointing to an impaired degradation of mCry1 protein. Interestingly, mCry1 mRNA cycling is not affected in contrast to mPer2 and Bmal1 transcripts, indicating that regulation of mCry1 differs from that of mPer2 and Bmal1. Old mPer1−/− mCry2−/− mice display not only altered gene expression of core clock components but also altered expression of the clock output gene arginine-vasopressin (Avp, Fig. 3G), indicating that physiological pathways influenced by Avp are affected in these mice. Interestingly, Dbp seems to be regulated differently, because its gene expression is already altered in mCry2−/− mice (Fig. 3H; Supplementary Fig. 3H).

Light sensitivity is impaired in aging mPer1−/− mCry2−/− mice

Old mPer1−/− mCry2−/− mice synchronize poorly to the light dark cycle [Fig. 1F]. 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, Travinickova-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 signaling in the SCN of these mice. At the behavioral level, we could only measure the phase shifts of young mPer1−/− mCry2−/− mice, because old animals immediately became arrhythmic in DD. 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 absence of mPer1.

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 in retinal morphology [Fig. 5A]. Cell death as a reason for malfunction of the retina could most possibly be excluded, because 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 cellular signaling. The reason for the impaired transmission of the light signal is most likely not a developmental defect, because young mPer1−/− mCry2−/− mice show phosphorylation of CREB at Ser 133. Therefore, the defect is probably of transcriptional or posttranscriptional nature. The lack of phosphorylation of CREB might lead to an altered expression of melanopsin in ganglion cells. These cells are probably responsible for resetting of the clock by light (Berson et al. 2002, Hattar et al. 2002). Hence, a reduced expression of melanopsin would affect resetting. This is in line with the recent finding, that melanopin-deficient mice display attenuated clock resetting in response to brief light pulses [Panda et al. 2002, Ruby et al. 2002], similar to what we observe in mPer1−/− mCry2−/− mice [Fig. 4F]. In old mPer1−/− mCry2−/− mice, this might even lead to the poor synchronization of these mice to the LD cycle [Fig. 1F,G]. Future studies will reveal whether melanopsin expression in ganglion cells of the retina is affected in old mPer1−/− mCry2−/− mice.
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, posttranslational modification, and nuclear localization [Lee et al. 2001; Yagita et al. 2002]. The positive limb of circadian clock gene activation is influenced by the negative limb via REV-ERBα [Preitner et al. 2002], probably through a complex consisting of mPER and mCRY proteins [Albrecht 2002; Okamura et al. 2002; Yu et al. 2002]. The mPER and mCRY proteins stabilize each other when they are in a complex and inhibit the CLOCK/BMAL1 heterodimer. Such a mPER/mCRY complex would be composed of those PER and CRY proteins that are most abundant at a given time. Figure 6A depicts the temporal abundance of cycling mPer1, mPer2, mCry1, and 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 (Per and Cry) are 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 h compared with mRNA [Field et al. 2000].

Interestingly, not all PER/CRY complexes seem to be equally important in vivo [Oster et al. 2002; this study]. mPer2Brdm1 mCry2−/− mutant but not mPer2Brdm1 mCry1−/− mutant mice display circadian rhythmic behavior, indicating that mPER1/mCRY1 but not mPER1/mCRY2 is sufficient to drive the circadian clock [Oster et al. 2002]. This study indicates that mPER2/mCRY2 but—at least in older mice—not mPER2/mCRY1 can sustain circadian rhythms. Additionally, mPer1−/−mPer2Brdm1 and mCry1−/−mCry2−/− 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 activity 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 active ones, with a difference in their maxima of ∼2 h. The activity of these complexes is higher than a critical threshold level necessary to drive clock regulation (green horizontal line in Fig. 6B). In contrast, mPER1/mCRY2 complexes formed in Per2/Cry1 mutant mice probably do not reach this critical threshold. The reason for this might be that the timing of expression of these two proteins is not synchronized and/or the affinity between mPER1 and mCRY2 is low. As a consequence, Per2/Cry1 mutant mice lose clock function [Oster et al. 2002]. The complex containing mPER2 and mCRY1 seems to just reach the critical threshold necessary for clock regulation, as illustrated by the circadian wheel-running behavior of young mPer1−/−mCry2−/− mice [Fig. 1D,E]. However, with progressing age, the activity of such a complex falls below the threshold, and hence, older mPer1−/−mCry2−/− mice lose rhythmicity [Fig. 1F,G]. mPer2Brdm1 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 sufficient to drive a 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 activity of each of these complexes might then fall below the threshold critical for normal clock function.

In sum, it seems that PER/CRY complexes have different potentials to regulate the circadian clock. In wild-type animals, the formation of PER/CRY complexes is 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 may be 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 stabilized by two oscillators that are slightly out of phase [Glass and Mackey 1988; Roenneberg and Merrow 2001]. Our findings are also in agreement with the two-oscillator model proposed by Daan and coworkers [2001].

Taken together, our in vivo studies support a model based on differential presence and activity of PER/CRY protein complexes as critical regulators of circadian rhythmicity (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 be adaptable to changing environmental conditions. The importance of PER1 and CRY2 only becomes apparent in mPer1−/− mCry2−/− mice half a year after birth, illustrating a connection between the clock and aspects of aging.

Materials and methods

Generation of mPer and mCry mutant mice

We crossed mPer1+/− mice (Zheng et al. 2001) with mCry1+/− and mCry2−/− animals [van der Horst et al. 1999]. The genotype of the offspring was determined by Southern blot analysis as described (Oster et al. 2002). Hybridization probes were for mPer1 as described in Zheng et al. [2001] and for mCry1 and mCry2 as described in van der Horst et al. [1999]. Matching wild-type control animals were produced by back-crossing heterozygous animals derived from the mPer1+/− and mCry1+/− matings to minimize epigenetic effects.

Locomotor activity monitoring and circadian phenotype analysis

Mice housing and handling were performed as described [Albrecht and Oster 2001; Albrecht and Foster 2002]. 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-min bins. For activity counting and evaluation, we used the ClockLab software package (Actimetrics). Rhythmicity and period length were assessed by χ² periodogram analysis and Fourier transformation using mice running in LD or in DD for at least 10 d.

For light-induced phase shifts, we used the Aschoff Type I for mPer1+/− mCry1−/− animals) or the Type II protocol (for mPer1−/− mCry2−/− animals) as described [Albrecht and Oster 2001; Albrecht et al. 2002]. We originally chose the Type II protocol because of the convenient setup for high numbers of animals and for comparison with mPer1−/− mCry2−/− mice [Albrecht et al. 2001; Oster et al. 2002]. However, the unstable onset of activity of mPer1−/− 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 the Type II protocol. Therefore, we repeated the experiments using a Type I setup with animals free running in DD before light administration. For the Type II protocol, animals were entrained to an LD cycle for at least 7 d before light administration (15 min of bright white light, 400 lx, 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 the Type I protocol, animals were kept in DD for at least 10 d 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 1 or 2 d following the light administration were not used for the calculation because animals were thought to be in transition between both states.

In situ hybridization

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 d before decapitation. For light induction experiments, animals were exposed to a 15-min light pulse (400 lx) at ZT14 and killed at ZT15; controls were killed at ZT15 without prior light exposure. Specimen preparation, 35S-rUTP-labeled riboprobe synthesis, and hybridization 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]. The Dbp probe was made from a cDNA corresponding to nucleotides 2–951 (GenBank accession no. NM016974). The vasopressin (Avp) probe corresponds to nucleotides 1–480 (GenBank accession no. M88354). Quantification was performed by densitometric analysis of autoradiograph films [Amersham Hyperfilm MP] as described [Oster et al. 2002]. For each time point, three animals were used and three sections per SCN were analyzed. “Relative mRNA abundance” values were calculated by defining the highest value of each experiment as 100%.

Immunohistochemistry

Animals were killed and tissues were prepared as described for in situ hybridization. Eye lenses were removed before cutting. Sections were boiled in 0.01 M sodium citrate (pH 6) for 10 min to unmask hidden antigen epitopes and were processed for immunohistochemical detection using the Vectastain Elite ABC system [Vector Laboratories] and diaminobenzidine with nickel.
amplification as the chromogenic substrate. Immunostained sections were inspected with an Axioplan microscope [Zeiss], and the area of the SCN was determined by comparison to Nissl-stained parallel sections. Semi quantitative analysis for mCRY1, mPER2, and Ser133P-CREB immunoreactivity in the SCN was performed using the NIH Image program. Images were digitized, 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 intermediate aspect of the SCN were chosen at random for further analysis. Values presented are the mean of three different experiments ± S.D. Primary antibodies against mCRY1 [Al pha Diagnostics, order number CRY11-A], against CREB [Cell Signaling Technology, order no. 9192], against CREB, phosphorylated at the residue Ser 133 [New England Biolabs, order no. 9191S], and against mPER2 [Santa Cruz Biotechnology, order no. 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 Russell 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 hybridization. Labeling of probes was done using the Rediprime II labeling kit [Pharmacia] incorporating [32P]dCTP to a specific activity of 10^8 cpm/µg. Blots were hybridized 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 phosphomager plates [Bio-Rad] for 20 h, and signals were quantified using Quantity One 3.0 software [Bio-Rad]. For comparative purposes, the same blot was stripped and reused for hybridization. The relative level of RNA in each lane was determined by hybridization with mouse Gapdh cDNA.

**Histology**

All histological staining was performed as described [Burkett et al. 1993]. For Gomori’s trichrome staining, PFA-fixed, parafilm-embedded sections were dewaxed, and postfixed with Bouin’s fluid at 56°C for 30 min; nuclei were stained with ferric hematoxyline [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 salicylate. For lipofuscin staining, slides were dewaxed and colored 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, dehydrated, 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 (***)

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**References**


Field, M.D., Maywood, E.S., O’Brien, J.A., Weaver, D.R., Reppert, S.M., and Hastings, M.H. 2000. Analysis of clock pro-


Loss of circadian rhythmicity in aging *mPer1*<sup>-/-</sup> *mCry2*<sup>-/-</sup> mutant mice

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