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RESEARCH COMMUNICATION

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 nuclei* (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 arrhythmicity in DD, indicating different effects of *mCry1* and *mCry2* in the clock mechanism

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 *Bmal1* expression (Shearman et al. 2000). In vitro studies point to multiple physical interactions between all mPER and mCRY proteins and posttranslational modifications such as phosphory-

lation and ubiquitylation, thereby offering a variety of putative regulation points for timed accumulation and nuclear appearance of clock proteins (Griffin et al. 1999; Kume et al. 1999; Field et al. 2000; Shearman et al. 2000; Yagita et al. 2000; Lee et al. 2001; Miyazaki et al. 2001; Vielhaber et al. 2001; Zheng et al. 2001; Yu et al. 2002). In particular there is evidence that mPER2 (GFP-tagged) shuttles between cytoplasm and nucleus and is ubiquitylated and degraded by the proteasome unless it is retained in the nucleus by mCRY proteins (Yagita et al. 2002). 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 (Jagota et al. 2000; Hamada et al. 2001; Lee et al. 2001). In bacteria, yeast, *Caenorhabditis elegans*, and *Drosophila*, interactions of proteins have frequently been found through nonallelic supressor screens, that is, the restoration of a phenotype by introducing a mutation in another gene (Maine and Kimble 1989; Amin et al. 1999; Nakano et al. 2000; Grandin and Charbonneau 2001; LaJeunesse et al. 2001; Roy et al. 2002). We decided to further elucidate the functional relationship between *mPer* and *mCry* genes by studying their genetic interactions in the living animal. To this end, we inactivated the *mCry1* or *mCry2* gene in an *mPer2* mutant mouse strain.

Results and Discussion

*Per2^Brdm1^* 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^Brdm1^/mCry2^−/−^* and *Per2^Brdm1^/mCry1^−/−^* mice (representative genotyping shown in Fig. 1a) were obtained at the expected Mendelian ratios, appeared fertile, and were morphologically indistinguishable from wild-type animals.

To determine the influence of inactivation of either *mCry1* or *mCry2* on circadian behavior of *Per2^Brdm1^* 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 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 conditions, homozygous *Per2^Brdm1^*, *Per2^Brdm1^/mCry2^−/−^* and *Per2^Brdm1^/mCry1^−/−^* animals displayed activity patterns similar to that of wild-type mice (Fig. 1b–e). In constant darkness, *Per2^Brdm1^* mutant animals lost circadian rhythmicity after a few days (Fig. 1c), as described previously (Zheng et al. 1999, 2001; Bae et al. 2001). In contrast, *Per2^Brdm1^/mCry1^−/−^* mice lost circadian rhythmicity immediately upon release into DD (Fig. 1d), as also observed for *mPer1^−/−^/Per2^Brdm1^* and *mCry1^−/−^/mCry2^−/−^* double mutant mice (van der Horst et al. 1999; Vitaterna et al. 1999; Bae et al. 2001; Zheng et al. 2001). Surprisingly, *Per2^Brdm1^/mCry2^−/−^* animals maintained a circadian rhythm in DD (Fig. 1e). Determination of the period length (τ) by χ^2 periodogram analysis
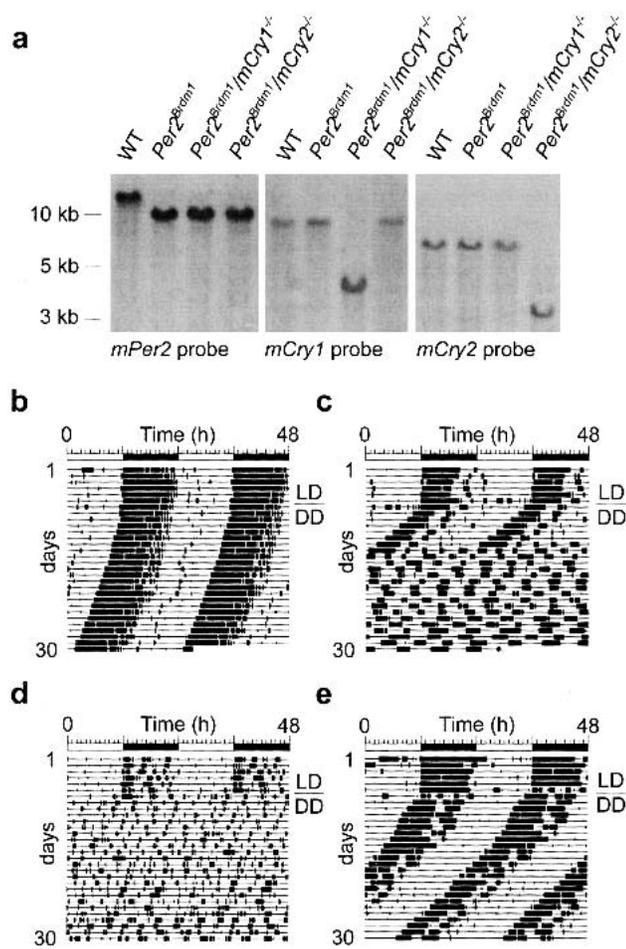
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Figure 1. Generation of *mPer/mCry* double mutant mice and representative locomotor activity records. (*a*) Southern blot analysis of wild-type (WT), *Per2^{Brdm1}*, *Per2^{Brdm1}/mCry1^{−/−}*, and *Per2^{Brdm1}/mCry2^{−/−}* tail DNA. The *Per2* probe hybridizes to a 12-kb wild-type and a 10-kb mutant fragment of *Bam*HI-digested genomic DNA. The *mCry1* probe detects a 9-kb wild-type and a 4-kb *Nco*I-digested fragment of the targeted locus. In *mCry2* mutants, the wild-type allele is detected by hybridization of the probe to a 7-kb *Eco*RI fragment, whereas the mutant allele yields a 3.5-kb fragment. The *left panel* indicates size of DNA fragments. (*b*) Representative locomotor activity records of wild-type (WT), *Per2^{Brdm1}*, *Per2^{Brdm1}/mCry1^{−/−}*, and *Per2^{Brdm1}/mCry2^{−/−}* 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 plot heights, >1, >10, and >20 wheel revolutions per 5-min period) and is double-plotted. The top bar indicates light and dark phases in LD. For the first 5 d in DD, wheel rotations per day were 20,021 ± 2,524 (n = 22) for wild-type animals, 17,656 ± 3,301 (n = 17) for *Per2^{Brdm1}* mutants, 16,025 ± 3,201 (n = 12) for *Per2^{Brdm1}/mCry1^{−/−}* mutants, and 17,859 ± 2,703 (n = 15) for *Per2^{Brdm1}/mCry2^{−/−}* mutants.

(using activity record intervals in which the circadian periodicity appeared stable on the activity record) revealed an average circadian period length of 23.8 ± 0.1 h (mean ± S.D., n = 22) for wild-type mice, 22.1 ± 0.3 h (n = 17) for *Per2^{Brdm1}* mice and 23.4 ± 0.2 h (n = 15) for *Per2^{Brdm1}/mCry2^{−/−}* animals. Interestingly, the period length of *Per2^{Brdm1}/mCry2^{−/−}* animals is the average of that of *mCry2^{−/−}* mice (24.6 ± 0.1 h, van der Horst et al. 1999) and *Per2^{Brdm1}* mice. However, this might be coincidental. Wild-type and mutant mice all showed compa-

rable levels of total wheel-running activity (see Fig. 1b legend), indicating that circadian period measurements were not influenced by aberrant running behavior.

To unravel whether the rescue of circadian rhythmicity in *Per2^{Brdm1}* mutant animals by additional inactivation of the *mCry2* gene was reflected at the molecular level, we examined the expression patterns of the *mPer1*, *mPer2*, *mCry1*, and *Bmal1* clock genes in the SCNs of wild-type and mutant animals under DD (Fig. 2 a–d) and LD (Fig. 2 e–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^{Brdm1}* mutant animals, this rhythmicity is severely blunted (Fig. 2a). As might be expected from the behavioral data, *mPer1* gene ex-
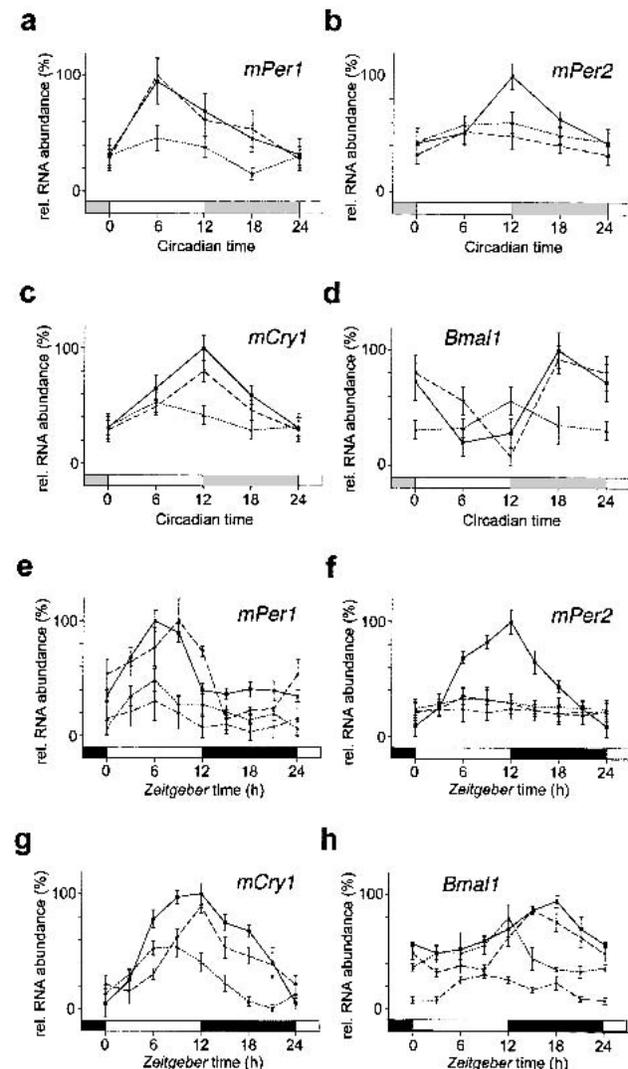
Figure 2. In situ hybridization profiles of cycling clock genes in the SCN of wild-type (solid line), *Per2^{Brdm1}* (pointed line), *Per2^{Brdm1}/mCry2^{−/−}* (dashed line), and *Per2^{Brdm1}/mCry1^{−/−}* (point/dash line) mice kept in DD (*a–d*) or in a 12-h:12-h LD cycle (*e–h*). Each value is the mean ± S.D. (n = 3). Data at circadian time (CT) and *Zeitgeber* time (ZT) 0/24 are double-plotted. Bars on *X*-axis indicate light and dark phase. (*a*) *mPer1* expression in DD; (*b*) *mPer2* expression in DD; (*c*) *mCry1* expression in DD; (*d*) *Bmal1* expression in DD; (*e*) *mPer1* expression in LD; (*f*) *mPer2* expression in LD; (*g*) *mCry1* expression in LD; (*h*) *Bmal1* expression in LD.

pression was back to a normal amplitude, reaching a maximum at CT6 in $Per2^{Brdm1}/mCry2^{-/-}$ mutants (Fig. 2a). Thus, circadian expression of $mPer1$ in $Per2^{Brdm1}$ mutants is rescued by inactivation of $mCry2$. A similar rescue was observed for $mCry1$ expression: both amplitude and timing of $mCry1$ oscillation in $Per2^{Brdm1}/mCry2^{-/-}$ mutant animals was not significantly different from that observed in wild-type animals (Fig. 2c). $Per2^{Brdm1}$ mutant mice displayed abnormal $Bmal1$ mRNA rhythms, as evident from the reduced amplitude and shift of maximal expression levels to earlier CT times (Fig. 2d; Shearman et al. 2000). In the $Per2^{Brdm1}/mCry2^{-/-}$ mutants, however, both the phase and amplitude of $Bmal1$ expression were comparable to those of the wild-type animals (Fig. 2d). Oscillation of mutant $mPer2$ expression was not rescued in $Per2^{Brdm1}/mCry2^{-/-}$ mutants (Fig. 2b). Taken together, these data strongly indicate that normal circadian behavior and core oscillator performance is possible in the absence of functional $mPer2$ and $mCry2$ genes (Fig. 1e).

We also found a rescue of the amplitude of $mPer1$, $mCry1$, and $Bmal1$ expression profiles in $Per2^{Brdm1}/mCry2^{-/-}$ animals kept under LD conditions (Fig. 2 e,g,h), whereas $mPer2$ mRNA levels remained low as in the $Per2^{Brdm1}$ mice (Fig. 2f). These results are comparable to the expression patterns observed under DD conditions (Fig. 2 a–d), except that in the $Per2^{Brdm1}$ mutant mice, the amplitude of $Bmal1$ expression was almost as high as in wild-type animals (there is no statistically significant difference between both maxima; Fig. 2h; Shearman et al. 2000). The phase advance of $Bmal1$ expression in $Per2^{Brdm1}$ mice compared to wild-type mice, which might explain the frequently observed early onset of wheel running activity before the beginning of the dark phase (Zheng et al. 1999), is also lost in $Per2^{Brdm1}/mCry2^{-/-}$ animals (Fig. 2d). These data further underline the correction of the circadian phenotype of $Per2^{Brdm1}$ mice by inactivation of $mCry2$.

As shown above, $Per2^{Brdm1}/mCry1^{-/-}$ mice are arrhythmic in DD. To investigate whether these animals lack a circadian clockwork, we studied the expression patterns of the $mPer1$, $mPer2$, and $Bmal1$ clock genes under LD conditions. We found that in $Per2^{Brdm1}/mCry1^{-/-}$ mutant animals, none of these genes is rhythmically expressed and mRNA levels are very low (Fig. 2e,f,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 cyclic $Bmal1$ expression in the $Per2^{Brdm1}$ mutant mice following inactivation of $mCry1$ suggests that $mCry1$ might play a role in transcriptional regulation of $Bmal1$.

The experiments described above indicate that an inactivation of $mCry2$ in $Per2^{Brdm1}$ 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 tissue. The expression profiles of $mPer1$, $mPer2$, $mCry1$, and $Bmal1$ (Fig. 3a–d) under DD conditions are comparable to those observed in the SCN (Fig. 2 a–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 circa-
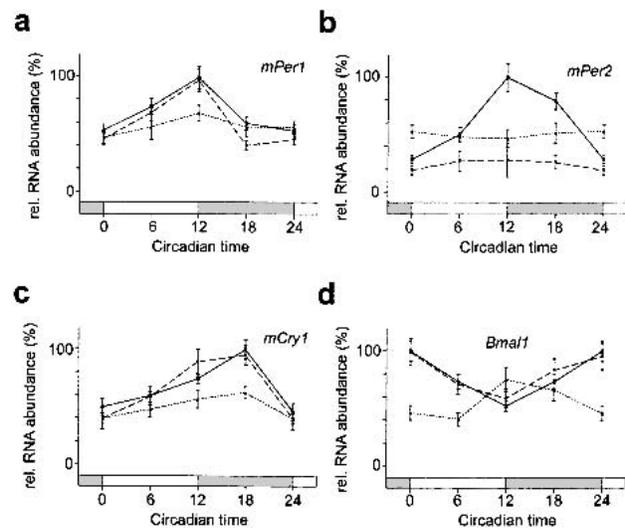
Figure 3. Quantificaion of Northern blots identifying clock genes in the kidney of wild-type (solid line), $Per2^{Brdm1}$ (pointed line), and $Per2^{Brdm1}/Cry2^{-/-}$ (dashed line) mice kept in DD. Each value is the mean ± S.D. of two (mutants) or four (wild-type) different experiments. Data at circadian time (CT) 0/24 is double-plotted. Bars on X-axis indicate subjective night (gray) and day (white). (*a*) $mPer1$ expression in DD; (*b*) $mPer2$ expression in DD; (*c*) $mCry1$ expression in DD; (*d*) $Bmal1$ expression in DD.

dian rhythmicity (Bunger et al. 2000). Since in $Per2^{Brdm1}$ mutant animals, $Bmal1$ mRNA cycling seems to be dampened under DD conditions (Figs. 2d, 3d), we investigated expression levels of the latter transcript at CT18 (wild-type and $Per2^{Brdm1}/mCry2^{-/-}$ animals) and CT12 ($Per2^{Brdm1}$ mice) at 0, 3, 5, and 10 d after transfer of animals to constant darkness conditions. We found that in wild-type and $Per2^{Brdm1}/mCry2^{-/-}$ mice, $Bmal1$ expression is maintained, whereas in $Per2^{Brdm1}$ 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 $mPer2$ mutant mice in DD (Fig. 1c; Zheng et al. 1999, 2001; Bae et al. 2001) and further supports the idea that the mPER2 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 (Yu et al. 2002). Because only $mCry1^{-/-}/mCry2^{-/-}$ and $mPer1^{-/-}/Per2^{Brdm1}$ 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 nor 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 $mPer$ and $mCry$ gene products. Analogous to $mCry1^{-/-}/mCry2^{-/-}$ (van der Horst et al. 1999; Vitaterna et al. 1999) and $mPer1^{-/-}/Per2^{Brdm1}$ double mutant mice (Bae et al. 2001; Zheng et al. 2001), inactivation of both $mPer2$ and $mCry1$ leads to immediate loss of circadian rhythmicity (Fig. 1d). This implies that $Per2^{Brdm1}/mCry1^{-/-}$ 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 $Per1$, $Cry2$, and $Bmal1$ (Figs. 2a,b,d; 3a,b,d). However, mice with inactivated $mPer2$ and $mCry2$ genes display stable circadian rhythmicity and

a

b
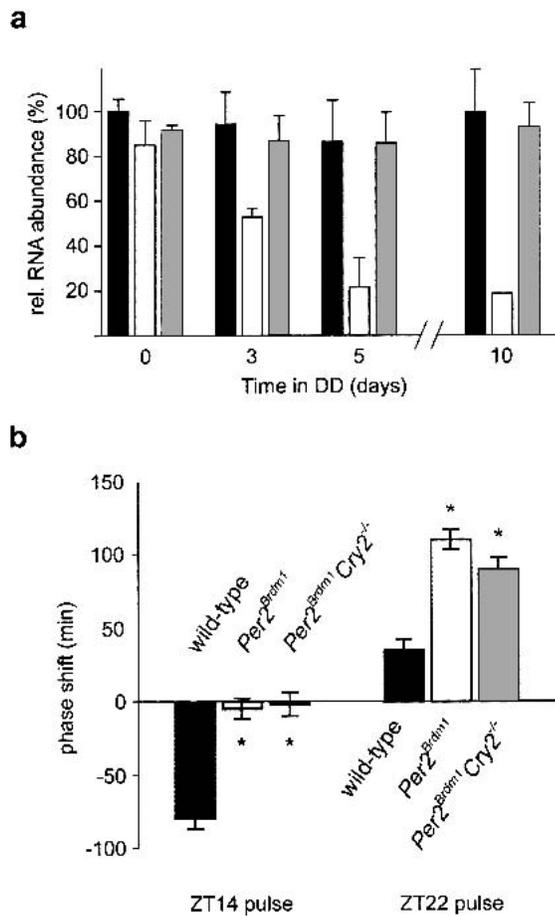
Figure 4. (*a*) *Bmal1* maximal expression in the SCN of wild-type (black column), *Per2*[Brdm1] (white column), and *Per2*[Brdm1]/*mCry2*^{−/−} (gray column) mice released into DD. Mice were adapted to a 12-h: 12-h LD cycle and subsequently put into DD for 3, 5, or 10 subjective days. Only rhythmic animals were sacrificed at time points of maximal *Bmal1* expression (CT18 for wild-type and *Per2*[Brdm1]/ *mCry2*^{−/−}; CT12 for *Per2*[Brdm1] mice). Each value is the mean ± S.D. of three animals (except for day 10 in *Per2*[Brdm1] mice with only one rhythmic animal). (*b*) Light-induced activity phase shifts in wild-type (black column), *Per2*[Brdm1] (white column), and *Per2*[Brdm1]/ *mCry2*^{−/−} (gray column) mice. After entrainment to a 12-h:12-h LD cycle, animals were exposed to a 15-min light pulse (400Lux) at the beginning (ZT14) or at the end (ZT22) of the night and subsequently released into DD. Negative values represent phase delays, and positive values phase advances. Each value is the mean ± S.D. of 10–14 animals. Only animals which remained rhythmic for at least 7 consecutive days in DD were used for quantification. Significance as indicated by asterisks was determined by one-way ANOVA with subsequent Bonferroni posttest ($p < 0.05$).

normal *Bmal1 mRNA* cycling, indicating that mPER1 and mCRY1 proteins can maintain circadian rhythmicity and rhythmic *Bmal1* expression in the absence of functional *mPer2* and *mCry2* genes. These data suggest that in *Per2*[Brdm1] single mutant mice, the functional *mCry2* gene product interferes 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 sustain the circadian clock in vivo. However, the resetting defect observed in *Per2*[Brdm1] single mutant mice (Albrecht et al. 2001) was not rescued in *Per2*[Brdm1]/ mCry2^{−/−} mice (Fig. 4b). This indicates that in *Per2*[Brdm1]/

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 four members of the negative limb (*mPer1*, *mPer2*, *mCry1*, *mCry2*) of the clock mechanism. It seems that CRY1 is a stronger repressor than CRY2, and PER2 is a more potent repressor than PER1. 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 or more precisely on activation of *Bmal1*. Assuming that all four CRY and PER proteins can be assembled into a protein complex with two CRY and two PER proteins, the activity potential of this complex is dependent on its composition. For example, a complex of two PER1 proteins and two CRY2 proteins would have a lower repressor potential than a complex composed of two PER2 and two CRY1 proteins. In turn, a complex that only forms in a wild-type animal composed of PER1, PER2, CRY1, and CRY2 would have an intermediate repressor potential. Assuming that circadian clocks are based on limit cycles of feedback-transcription to generate circadian rhythms (Glass and Mackey 1988; 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 *mCry1*^{−/−} mice further disturbs rhythmicity (van der Horst et al. 1999). In *mPer1*^{−/−} animals, both PER subunits would be PER2 proteins with a high repressor potential, which accelerates the feedback loop, as has been observed (Zheng et al. 2001). However, the overall repressor potential is still compatible with the parameters of the limit cycle, and therefore the *mPer1*^{−/−} mice are still rhythmic. In *Per2*[Brdm1] 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*[Brdm1] mice become arrhythmic. In *Per2*[Brdm1]/*mCry2*^{−/−} mice, however, the repressor complex is composed of two CRY1 proteins (strong repressors) and two PER1 proteins (weak repressors). The overall repressor potential of this complex would approach that of the wild-type complex and is thus compatible with the parameters of the limit cycle. *Per2*[Brdm1]/ *mCry2*^{−/−} mice would therefore be rhythmic, which is compatible with our observations.

Taken together, our findings suggest that *mCry2* can act as a nonallelic suppressor of *mPer2* and that there might exist PER/CRY complexes with different transcriptional activity potentials, some of which are compatible with the parameters of a limit cycle whereas others are not.

Materials and methods

Generation of mPer and mCry mutant mice

We crossed *mPer2^Brdm1* mice (Zheng et al. 1999) with *mCry1* and *mCry2* knockout animals (van der Horst et al. 1999). The genotype of the offspring was determined by Southern blot analysis as described (Ramirez-Solis et al. 1993). Hybridization probes were for *mPer2* as described by Zheng et al. (1999) and for *mCry1* and *mCry2* as described by van der Horst et al. (1999). Matching wild-type control animals were produced by back-crossing heterozygous animals.

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 day cycle's activity is shown both to the right and below that of the previous day cycle. Activity is plotted in threshold format with three different thresholds set to >1, >10, and >20 wheel revolutions per 5-min period (Fig. 1). For activity counting, we used the ACS program of Simon Fraser University. For actograms and period determination, we used the Circadia program of Simon Fraser University. Period length was assessed by χ^2 periodogram analysis using mice running in constant darkness for at least 10 d (5 d for *Per2^Brdm1*).

For light-induced phase shifts, we used the Aschoff Type II protocol as described (Albrecht et al. 2001). We chose this protocol because *mPer2^Brdm1* mice become arrhythmic in constant darkness, precluding the determination of circadian times. Animals were entrained to an LD cycle for at least 7 d before the light administration [15 min bright white light (400Lux) at *Zeitgeber* time (ZT)14 or ZT22] and subsequently released into DD. The phase shift was determined by eye-fitting a line through at least 7 consecutive d of onset of activity in LD before the light pulse and in DD after the light pulse. The difference between the two lines on the day of the light pulse determined the value of the phase shift.

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 circadian time (CT) 0, 6, 12, and 18. For DD conditions, animals were kept in the dark for 3 d before decapitation. Specimen preparation, ^{35}S-UTP labeled riboprobe synthesis, and hybridization steps were performed as described (Albrecht et al. 1998). The probes for *mPer1* and *mPer2* were as described (Albrecht et al. 1997). The *mCry1* probe was made from a cDNA corresponding to nucleotides 190–771 (accession no. AB000777) and the *Bmal1* probe corresponding to nucleotides 654–1290 (accession no. AF015953). The *mPer2* probe is located outside the region deleted in the mutant. Quantification was performed by densitometric analysis of autoradiograph films (Amersham Hyperfilm MP) using the NIH Image program after conversion into the relative optical densities by the ^{14}C-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%.

Northern blot analysis

Rhythmic animals were sacrificed at the specified circadian time points on the third day in DD. 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, 20µg of total RNA was used. cDNA probes were the same as described for in situ hybridization. Labeling of probes was done using the random prime labeling kit (Pharmacia) incorporating [P^{32}]dCTP to a specific activity of 10^8 cpm/µg. Blots were hybridized using QuickHyb solution (Stratagene) containing 100 µg/mL salmon sperm DNA. The membrane was washed at 60°C in 0.1× SSC and 0.1% SDS. Subsequently, blots were exposed to phosphoimager plates (Bio-Rad) for 20 h, and signals were quantified using Quantity One 3.0 software (Bio-Rad). Signal intensities were normalized by comparison of the 18S ribosomal RNA bands' methylene blue staining on the blotted membrane. The highest clock gene signal was determined as 100% for each experiment.

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