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Restoration of Circadian Rhythmicity in Circadian Clock–Deficient Mice in Constant Light

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Abstract   In mammals, circadian rhythms in behavior and physiology are controlled by a central pacemaker, the SCN, and subordinated clocks throughout the body. On the molecular level, these clocks are based on transcriptional/ translational feedback loops involving a set of clock genes that regulate their own transcription. Among the components driving the mammalian circadian clock are the *Period 1* and *2* (*Per1* and *Per2*) and *Cryptochrome 1* and *2* (*Cry1* and *Cry2*) genes. In the present study, the authors characterize the behavioral and molecular rhythms of *Per2/Cry1* double mutant mice under 3 different lighting conditions. In an LD cycle, the activity of these animals is masked by light, while in DD, the mutants lose circadian rhythmicity but exhibit strong ultradian rhythms. In LL of higher intensity, circadian rhythms are restored on the behavioral level with a drastically shortened endogenous period. Furthermore, both in the SCN and in the periphery, clock gene rhythms are restored. Based on these observations and also on the fact that light-mediated induction of *Per* gene expression is preserved in these mutants, the authors propose a mechanism by which endogenous ultradian rhythms may relay timed light exposure to the SCN, leading to a reinitiation of self-sustained circadian rhythms in LL.

Key words   circadian, constant light, corticosterone, *cry*, kidney, *per*, SCN, ultradian

In mammals, a central pacemaker residing in the SCN and subordinated peripheral clocks control most physiological and behavioral events (Lowrey and Takahashi, 2004). At the molecular level, these clocks are based on interlocking transcriptional/ translational feedback loops (TTLs) built by a set of clock genes, including 2 *Period* (*Per1* and *Per2*) and 2 *Cryptochrome* genes (*Cry1* and *Cry2*) (Reppert and Weaver, 2002). With the noted exception of *Bmal1* (Bunger et al., 2000), single gene deletions in mice are normally not sufficient to completely disrupt the circadian clockwork (van der Horst et al., 1999; Zheng et al., 1999). In contrast, double mutations such as *Per1/2*, *Per2/Cry1*, and *Cry1/2* cause immediate behavioral arrhythmicity in DD (Bae et al., 2001; Oster et al., 2002; van der Horst et al., 1999; Zheng et al., 2001). Various studies have shown that LL in comparison to DD exerts a differential effect on the regulation

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of the circadian system (Aschoff, 1960; Beaule et al., 2003; Daan and Pittendrigh, 1976; Munoz et al., 2005; Spoelstra et al., 2002; Steinlechner et al., 2002) and may further alter the coupling properties within the TTLs (Daan and Pittendrigh, 1976; Roenneberg and Merrow, 2003).

In the present study, we characterize *Per2/Cry1* mutant mice that have a disrupted clock in DD and LD conditions. In LL, these mutants regain rhythmicity at the behavioral level with a drastically shortened endogenous period. Furthermore, both the SCN and the peripheral circadian oscillations are restored. We show that light-mediated induction of *Per* genes is preserved in these animals and suggest a mechanism by which endogenous ultradian rhythms may relay timed light exposure to the SCN, thereby reinitiating circadian rhythmicity.

MATERIALS AND METHODS

Animals and Behavioral Analyses

Mice housing, handling, and locomotor activity monitoring were performed as described earlier (Albrecht and Oster, 2001; Jud et al., 2005). For all behavioral experiments, male wild-type and $Per2^{Brdm1}/Cry1^{-/-}$ (below: *Per2/Cry1*) mutant mice (Oster et al., 2002) of 3 to 6 months of age were used. All animals were on a mixed 129SV/C57Bl/6 background. Rhythmicity, circadian, and ultradian period lengths were assessed by χ^2-periodogram analysis using the ClockLab data acquisition and analysis software package (Actimetrics, Evanston, IL) on mice running in the appropriate lighting condition for at least 10 days. All procedures involving animals were approved by the district government of Hannover and in accordance with the European Union (EU) guidelines for the use of animals.

In Situ Hybridization

For DD data, mice already entrained for 2 weeks to LD 12:12 were released into constant darkness for 24 h and were sacrificed the next day by cervical dislocation under a 15-W safety red light at 6-h intervals. For LL, activity records were analyzed over a period of at least 1 week, and animals were sacrificed at the indicated time points along the individual activity cycle. For light induction, animals were kept in $LL_{\sim 20\,lux}$ for 1 week and were then subjected to

a 6-h dark pulse and subsequently released again into constant light ($LL_{\sim 10\,lux}$ or $LL_{\sim 400\,lux}$) for 1 h prior to sacrifice. Specimen preparation, ^{35}S-UTP-labeled riboprobe synthesis, and hybridization steps were performed as specified by Albrecht et al. (1998). *Per1* and *Bmal1* probes were used as described (Oster et al., 2003a; Oster et al., 2003b). Quantification was performed by densitometric analysis of autoradiograph films using the ImageJ program (National Institutes of Health [NIH], Bethesda, MD). Data of the SCN were normalized with respect to the signal intensities in an equal area of the lateral hypothalamus. Three sections per SCN were analyzed.

Quantitative RT-PCR

Quantitative PCR analysis was performed using SYBR Green detection with an iCycler PCR System (Bio-Rad, Berlin, Germany). Briefly, animals were sacrificed at the specified time points. Kidneys were dissected out, and total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA). First strand cDNA synthesis was performed using Superscript II reverse transcriptase (Invitrogen) and Oligo-dT primer. The primer set used was 5′-TGGCTCAAGTGGCAAT-GAGTC-3′and 5′-GGCTCGAGCTGACTGTTCACT-3′ for *Per1* (GenBank accession number NM_011065) and 5′-CCTAATTCTCAGGGCAGCAGAT-3′ and 5′-TCC-AGTCTTGGCATCAATGAGT-3′ for *Bmal1* (GenBank accession number NM_007489). *Elongation factor 1α* (*EF1α*; GenBank accession number BC095965; primers: 5′-AATTCACCAACACCAGCAGCAA-3′ and 5′-TGC-CCCAGGACACAGAGACTTCA-3′) was used as internal standard. Relative quantification was performed as described (Pfaffl, 2001). Expression values were normalized to the average wild-type expression for the given set up. All assays were repeated thrice with biological replicates.

Fecal and Blood Corticoid Extraction

The fecal collection and assay protocol was adapted to the mouse model from the method described by Cavigelli et al. (2005). Briefly, all animals were housed singly under the indicated lighting conditions and transferred to cages with meshed bottom without bedding 3 days *prior* to the start of the collection. Food and water were provided ad libitum. Samples were collected across 2 days at 4-h intervals and stored at –80 °C until extracted. Corticosterone metabolite extraction from the total sample was performed as described

(Cavigelli et al., 2005). For blood corticosterone determination, animals were sacrificed by cervical dislocation, and plasma extraction was performed using Microvette CB300 LH tubes (Sarstedt, Nürnbrecht, Germany). Quantification of fecal and blood corticoids was done using the ImmuChemTM Double Antibody-Corticosterone-^{125}I Radioimmunoassay Kit (MP Biomedicals, Orangeburg, NY).

Statistical Analysis

Statistical analyses of significance for all experiments were performed by unpaired Student t test using GraphPad Prism software (GraphPad, San Diego, CA), except for the emergence rhythm (Fig. 4), for which we used Rayleigh's test for random distribution and Oriana software (Kovach Computing, Anglesey, UK).

RESULTS

Disrupted Circadian Clockwork in *Per2/Cry1* Mutant Mice under LD and DD Conditions

To elucidate the influence of inactivation of both *Per2* and *Cry1* on the circadian clock, *Per2/Cry1* mutant mice were analyzed for their circadian wheel-running behavior in a 12-h light/12-h dark cycle (LD 12:12) and in DD. In LD 12:12, mutant animals displayed activity patterns similar to those of wild-type mice (i.e., the active phase was confined to the dark phase; Fig. 1A). In contrast to wild-type mice, which show robust free-running rhythms, *Per2/Cry1* mutant mice lost circadian rhythmicity immediately upon release into DD (Oster et al., 2002; Fig. 1A). To address whether the apparent rhythm in running-wheel activity in the dark phase of the LD cycle is endogenously determined (i.e., entrained) or merely an effect of masking by light, animals were subjected to a frequency demultiplication test using an LD 6:6 paradigm (Fig. 1B). As expected, wild-type mice showed only 1 block of activity per 24 h that was only partially suppressed during the 6 h of light (masking), thereby maintaining a 24-h activity rhythm. In contrast, *Per2/Cry1* mutant mice became active in each of the dark phases. Furthermore, wild-type mice showed transient activity onsets for a few days after changing to LD 6:6, indicating that the circadian oscillator was reentraining to the new LD cycle. These transients were completely absent in the mutant mice; instead, they immediately adapted to
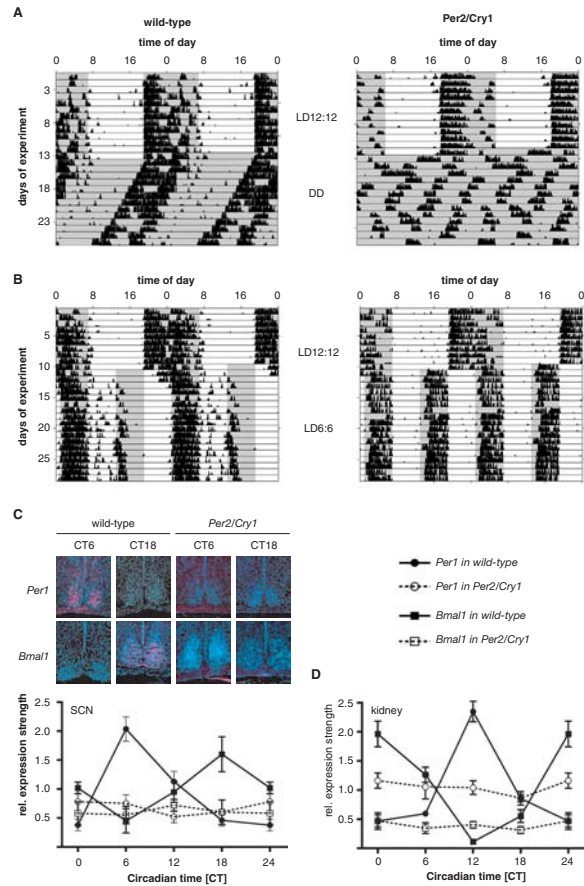

Figure 1. Locomotor activity rhythms and clock gene expression in the SCN and kidney of *Per2/Cry1* mutant mice. (A) Representative activity recordings of wild-type (left panel) and *Per2/Cry1* mutant mice (right panel) kept in a 12-h light/12-h dark (LD 12:12) cycle and subsequently released into DD. (B) Representative wild-type (left panel) and *Per2/Cry1* mutant mice (right panel) activity recordings in LD 6:6. Data are double plotted, with the dark phases in both the LD cycles indicated by gray shading. (C, D) *Per1* and *Bmal1* expression in the SCN (C) and the kidney (D) of wild-type and *Per2/Cry1* mutant mice in DD as determined by radioactive in situ hybridization (SCN) and quantitative RT-PCR (kidney). Upper panels: Representative hybridization signal dark field micrographs of SCN from wild-type and *Per2/Cry1* mutant mice in DD at CT6 and CT18 (red = hybridization signal; blue = nuclear stain). Lower panel: Circadian expression profiles for *Per1* and *Bmal1* in wild-type and *Per2/Cry1* mutant mice in DD. All values are means ± SEM (3 kidneys per time point).

the new conditions (Fig. 1B). Thus, we conclude that the *Per2/Cry1* mutants did not entrain to LD. Their activity was merely masked by light.

In DD, *Per2/Cry1* mutant mice immediately lose circadian rhythmicity. To see if this arrhythmicity is reflected at the molecular level, we examined clock gene expression (*Per1* and *Bmal1*) in the SCN of wild-type and mutant animals in DD conditions. In situ hybridization experiments revealed that both *Per1*

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and *Bmal1* expression levels were rhythmic and peaked at CT6 and CT18, respectively, in wild-type mice but were flat in the *Per2/Cry1* mutant animals (Fig. 1C). Likewise, analysis of the peripheral circadian oscillator in the kidney by quantitative PCR revealed a blunted expression rhythm for both genes in the mutants, while the wild-type controls were clearly rhythmic for these parameters (Fig. 1D). From these experiments, we conclude that the circadian clockwork of *Per2/Cry1* mutant mice was disrupted and endogenous circadian rhythms were absent in both LD and DD conditions.

Ultradian Rhythms Become More Prominent in *Per2/Cry1* Mutant Mice

Although *Per2/Cry1* mutant mice do not exhibit a circadian wheel-running rhythm in DD, ultradian rhythms were much more prominent in these animals than in wild-type controls (Fig. 2A). In DD, χ^2-periodogram analysis of the activity recordings revealed no independent circadian rhythm in the mutants but a strong ultradian component with a period length of 5 to 10 h, whereas wild-type animals exhibited a dominating circadian peak around 24 h and only weak ultradian rhythms (Fig. 2B). Remarkably, *Per2/Cry1* mutant mice were highly variable in the period of the ultradian peaks (Fig. 2C), and even within individuals, period length varied over time (Fig. 2D). Thus, the *Per2/Cry1* mutant mice provide another example where ultradian can be dissociated from circadian rhythmicity. However, the stability of the period of these ultradian oscillations seems to be dependent on a functional circadian clockwork.

Restoration of Circadian Behavioral and Molecular Rhythms in SCN and Peripheral Oscillators by Constant Light of High Intensity

Recently, it has been reported that in *Per2^Brdm1^* as well as in *Clock* mutant mice, the circadian pacemaker can be stabilized in constant illumination. Although both strains eventually become arrhythmic upon release into DD (Vitaterna et al., 1994; Zheng et al., 1999), they display robust activity rhythms in LL (Spoelstra et al., 2002; Steinlechner et al., 2002). Both these mutants are, however, different from the *Per2/Cry1* mutant mice in that the latter ones become arrhythmic immediately upon release into constant darkness, indicating the complete absence of a functional TTL. Previous studies have shown
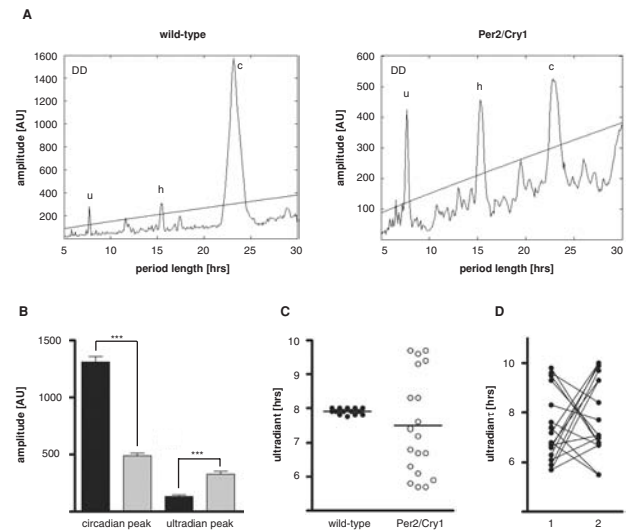

Figure 2. Ultradian rhythms in *Per2/Cry1* mutant mice in DD. (A) χ^2-periodogram analysis of wild-type (left panel) and *Per2/Cry1* mutant mice (right panel) shown in Figure 1A in DD (lower panel). Note the strong ultradian rhythm peak ($\tau \sim 7.5$ h) observed in the mutants in DD. For further analysis, peaks with a period length of 20 to 30 h were grouped as "circadian" (c), while peaks ranging between 5 and 10 h were classified as "ultradian" (u). Intermediate harmonic peaks (h) were not further evaluated. Analysis was performed on 5 consecutive days in DD. The ascending straight line in the periodograms represents a statistical significance of $p < 0.001$, as determined by the ClockLab program. (B) Amplitudes of circadian and ultradian peaks in both wild-type (black columns) and *Per2/Cry1* mutant mice (gray columns) in DD. Significant differences (***$p < 0.001$) were determined with unpaired Student's *t* test ($n = 14$-19). (C) Range of ultradian period lengths in DD. (D) Variability of the ultradian period in individual *Per2/Cry1* mutant mice for 2 different experiments (1 and 2) in DD. Values from both experiments for 1 animal are linked.

that the coupling strength between the feedback loops at the basis of the circadian system may change under different environmental conditions such as constant light versus constant darkness (Roenneberg and Merrow, 2003). We therefore determined the effect of different LL intensities on the rhythmicity of the *Per2/Cry1* mutant mice. Arrhythmicity in most of these animals was preserved in LL of low intensity. Surprisingly, *Per2/Cry1* mutant mice exhibited a robust free-running rhythm at brighter illuminations with an extremely short free-running period (τ) of approximately 20 h (Fig. 3A). In addition, the period length (τ) of these mice did not follow Aschoff's rule but became even shorter with augmenting illuminance (Fig. 3B) comparable to *Per2* mutant mice (Steinlechner et al., 2002).

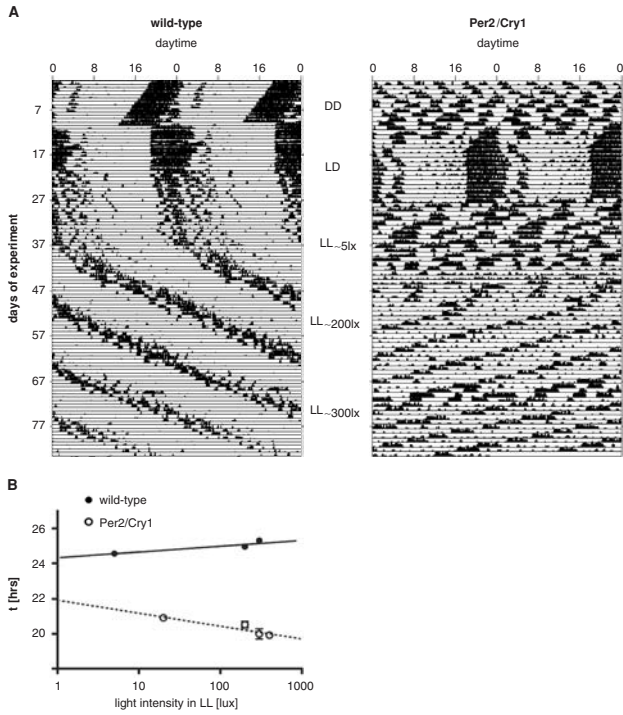To unravel whether this rescue of behavioral circadian rhythmicity was also reflected at the molecular

Figure 3. Bright light restores robust free-running rhythms in *Per2/Cry1* mutant mice. **(A)** Activity records of representative wild-type (left panel) and *Per2/Cry1* mutant mice (right panel) kept in DD before transferring into LD 12:12 and subsequently releasing into LL of increasing illuminance levels (~5 lux, ~200 lux, ~300 lux). **(B)** Free-running period lengths (τ) for LL of different intensities for wild-type (closed circles) and *Per2/Cry1* (open circles) mutant mice, as determined by χ^2-periodogram analysis on 7 consecutive days. Mutant animals are arrhythmic at lower light intensities. Data are averages ± SEM ($n = 6$ to 12).

Figure 4. Restoration of molecular and endocrine rhythms in *Per2/Cry1* mutant mice in LL. **(A-D)** Circadian expression profiles of *Per1* (A, C) and *Bmal1* (B, D) mRNAs in the SCN (A, B) and the kidney (B, D) of wild-type (solid line) and rhythmic *Per2/Cry1* mutant (dashed line) animals in constant light (LL$_{~400 lux}$), as determined by radioactive in situ hybridization (SCN) and quantitative RT-PCR (kidney). Data are double-plotted for better visualization. **(E)** Plasma rhythm of corticosterone and fecal excretion of corticoid metabolites of wild-type mice in LL$_{~100 lux}$. **(F)** Fecal corticosterone secretion of rhythmic and arrhythmic *Per2/Cry1* mutants in LL$_{~100 lux}$. Animals were released into LL and fecal samples collected, starting at CT0 for rhythmic animals and at 1300 h for arrhythmic animals (sampling time 0 h). All values presented are means ± SEM ($n = 3$ per time point for A-D and plasma samples in E; 3 animals per group were averaged for E and F).

level, we analyzed *Per1* and *Bmal1* expression in the SCN of *Per2/Cry1* mutant mice (Fig. 4A,B). In situ hybridization results indicated a clear rhythmic expression of both genes in the mutants similar to that of wild-type animals, suggesting a reestablishment of the TTL.

The mammalian circadian system is organized such that self-sustained oscillators in the SCN entrain peripheral oscillators by releasing rhythmic signals (Yamazaki et al., 2000). Indeed, in LL, the expression of *Per1* and *Bmal1* in the kidney was rhythmic in the mutant compared to the wild-type mice (Fig. 4C,D). The specific molecular substrates by which the SCN resets peripheral clocks are unknown, but glucocorticoids have been implicated as possible entraining signals (Balsalobre et al., 2000; Le Minh et al., 2001). Thus, we measured the circadian excretion of fecal corticosterone metabolites (CM) in rhythmic and arrhythmic mutants under LL conditions. Because this in vivo method has previously only been
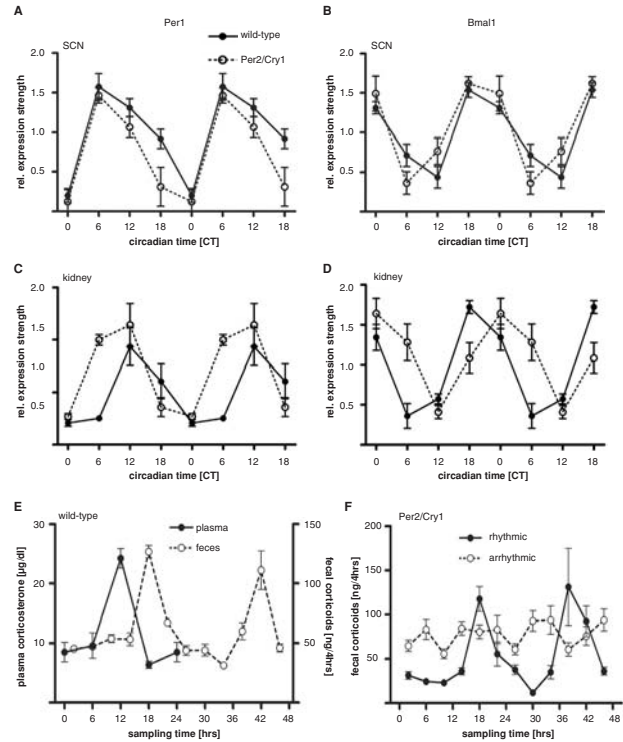
reported in rats (Cavigelli et al., 2005), we first validated our assay by comparing excretion data with those from blood samples in wild-type mice. The amplitude of the circadian fecal corticoid rhythm was indeed very similar to that for circulating corticosterone. However, the daily peak of fecal CM in feces trailed the peak in plasma by approximately 6 h (Fig. 4E). Next, we compared fecal CM abundance in behaviorally rhythmic (LL$_{~400 lux}$) with that in arrhythmic (LL$_{~20 lux}$) mutant mice. In the rhythmic animals, the levels of fecal CM peaked every 20 h, as was expected from the corresponding activity profile (Fig. 4F) and the transcription rhythms determined from the SCN and kidney. Taken together, these data

indicate that the reestablishment of circadian rhythms in LL was preserved on molecular, physiological, and behavioral levels.

Preservation of Light-Mediated Induction of *Per1* Expression and Reinitiation of Behavioral Rhythms by a Dark/Light Pulse Combination in *Per2/Cry1* Mutant Mice in LL

How can constant light exposure—holding no temporal information per se—trigger timed rhythmicity in a clockless animal? A first hint to answering this question was provided by analyzing the influence of an LD cycle on the phase of *Per2/Cry1* mutant mice released directly into LL of higher intensity (Fig. 5A). Surprisingly, the onset of activity of *Per2/Cry1* mutant mice did not exhibit a random phase angle toward the last "lights-off" in the preceding LD, although compared to wild-type animals, the phase angle in the mutants showed a much higher variance (Fig. 5B). This indicated that despite the failure of the LD cycle to entrain the circadian system in these animals, there seems to be a residual influence of light on the timing system affecting the activity phase in these mutants.

Light is believed to exert its influence on the circadian clockwork via gated activation of *Period* gene expression in the SCN. We hence speculated that light-mediated activation of *Per* expression in LL, triggered after a period of reduced light exposure (e.g., caused by closed eyelids during sleep), could reinitiate the TTL and, consequently, circadian rhythmicity. Therefore, we subjected arrhythmic mutant animals (LL$_{~20 lux}$) to a 6-h dark pulse and thereafter released them back into either LL$_{~10 lux}$ or LL$_{~400 lux}$ and measured *Per1* induction in the SCN 1 h after lights came back on (Fig. 5C). *Per1* transcription was significantly induced by light of ~10 and ~400 lux. At ~400 lux, the amount of *Per1* (and *Per2*—data not shown) transcripts in the mutant SCN was comparable to that observed in wild-type animals after a 15-min light pulse of the same intensity applied at CT14 in DD.

The above results indicate that the light input signaling pathway was preserved in *Per2/Cry1* mutant mice. To test if this transient modulation of *Period* gene transcription can actually initiate endogenous rhythmicity, which would be reflected at the behavioral level, we kept *Per2/Cry1* mutant mice in constant dim light—LL$_{~20 lux}$—and monitored wheel-running activity before and after the dark pulse. Of the animals tested, some but not all became rhythmic after the dark pulse (Fig. 5D). Specifically, after the treatment,
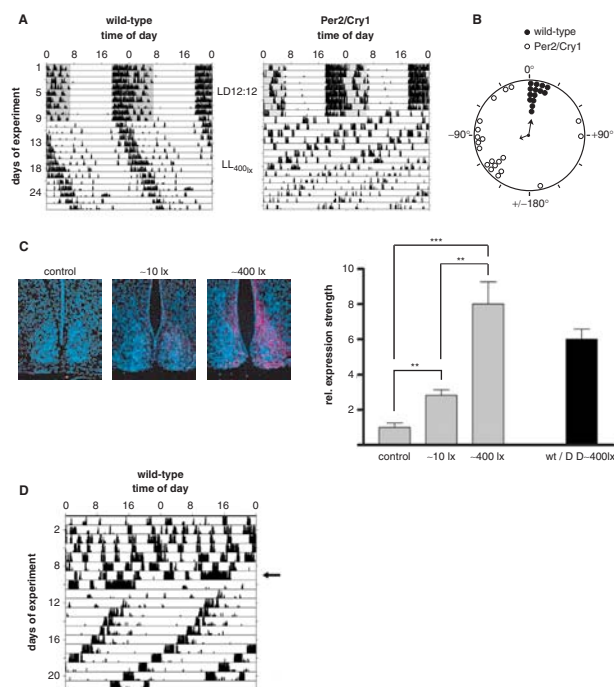

Figure 5. Light induction of *Per1* and rhythm initiation by a 6-h dark pulse in *Per2/Cry1* mutant mice. Wild-type and *Per2/Cry1* mutant mice were kept in LD 12:12 and directly released into LL$_{~400 lux}$ to assess the onset phase angle differences between LD and the first day in constant conditions. (A) Representative activity records for both genotypes. (B) Distribution of phase angle differences between wild-type (black circles) and *Per2/Cry1* mutant mice (open circles). Onset for the first day in LL was determined by extrapolation from the first 10 days showing a defined onset after the change of the light regimen. Arrows depict normalization vectors for both genotypes determined by Rayleigh's test for uniformity (90° equals 6 h; $p = 7.7*10^{-7}$ [wild-type] and $1.0*10^{-3}$ [mutant]). (C, D) *Per2/Cry1* mutant mice kept in LL$_{~20 lux}$ were subjected to a 6-h dark pulse and subsequently released again into constant light (LL$_{~10 lux}$ or LL$_{~400 lux}$). (C) Induction of *Per1* transcription in the SCN, 1 h after lights were turned back on. Left panels: representative in situ dark field micrographs (red = hybridization signal; blue = nuclear stain). Right panels: quantification of *Per1* transcriptional activation for different light intensities. Control animals were kept in darkness for another hour before killing. For comparison, wild-type data for 15-min light pulses (~400 lux) applied at CT14 in DD are shown on the right side (black bars). Significant differences (**$p < 0.01$; ***$p < 0.001$) were determined using unpaired Student t test ($n = 3$ per condition). (D) Representative actogram of a *Per2/Cry1* mutant mouse before and after a dark pulse (application time indicated by the arrow) in LL.

the percentage of rhythmic animals rose from 20% to 50%. However, the opposite effect was also seen in 1 of the mutant rhythmic animals, which became arrhythmic after the dark pulse (data not shown). Taken together, a dark pulse followed by an activation of *Per* gene expression can probably reinitiate circadian rhythmicity under LL conditions.

DISCUSSION

One of the most conspicuous qualities of circadian systems is their self-sustained rhythmicity in constant environmental conditions. In the present study, we show that in *Per2/Cry1* mutant mice, the clockwork was disrupted in LD, and these animals have completely lost their circadian rhythmicity on the behavioral and molecular levels in DD (both in the SCN and the periphery). Strikingly, these mutants display very prominent ultradian rhythms. Such short rhythms have been observed in other arrhythmic mutants such as *Clock* (Vitaterna et al., 1994), *Per2* (Zheng et al., 1999), *Bmal1* (Bunger et al., 2000), and *Per1/2* (Zheng et al., 2001). Interestingly, the period length of these ultradian rhythms seems to be regulated by the circadian clockwork and thus shows a high variation in the *Per2/Cry1* mutant mice.

Constant illumination can reinitiate the self-sustained circadian rhythmicity in these mutants both at the central SCN and peripheral levels but with an extremely shortened period. A similar phenomenon is also reported in the *Per2^{Brdm1}* mutants (Steinlechner et al., 2002). In the latter, this can be explained by the morning/evening oscillator model wherein the pacemaker is described as a system of 2 coupled oscillators locking to dawn and dusk, respectively (Daan and Pittendrigh, 1976). Specifically, *Per1* and *Cry1* would be part of the morning (M) oscillator being accelerated by light and thereby tracking dawn, and *Per2* and *Cry2* would be the part of the evening (E) oscillator being decelerated by light and thereby tracking dusk (Daan et al., 2001). Thus, the lack of *Per2* should lead to the damage of the E oscillator and therefore result in a shortened free run in LL, as is the case in the *Per2* mutants (Steinlechner et al., 2002). In the *Per2/Cry1* mutant mice, parts of both the E and M oscillators are knocked out, and still the mutant mice are rhythmic and display a shortened period length in LL. Thus, the 2-oscillator model cannot account for the observations seen in this mutant.

Instead, our data are in agreement with a network oscillator model (Roenneberg and Merrow, 2003), with the circadian machinery composed of multiple coupled molecular feedback loops that together form a "circadian network" with stable rhythmicity. The elimination of the *Per2* and *Cry1* components from this network weakens the coupling between the remaining oscillators, resulting in circadian arrhythmicity in DD. Some oscillations, however, remain intact and—at least in part—continue cycling with their own intrinsic (ultradian) period. In constant light, the interactions between these oscillators are differently organized. In this context, it has been shown that the expression of PER2 and probably CRY2 is altered in the murine SCN in LL as compared to DD (Beaule et al., 2003; Munoz et al., 2005). Thus, this differential coupling in LL may allow a relatively stable network even in the absence of both *Per2* and *Cry1*. Our experiments also indicate that this is even manifested in peripheral tissues that do not receive a direct light input in mammals.

We hypothesize that a crucial parameter in the transition between arrhythmic and rhythmic stages is the activation of light-responsive genes such as *Per1* in the SCN (Shigeyoshi et al., 1997). We show that light activation of *Per* genes in the SCN is preserved in arrhythmic *Per2/Cry1* mutant mice in LL. It is not unlikely that an initial activation of the *Per1* oscillator may "kick-start" the circadian system, which then becomes self-sustained because of the changed coupling parameters of the oscillator network in LL. The temporal gating of the light stimulus that we experimentally mimicked by application of a dark pulse could be mediated by an ultradian oscillator (e.g., sleep rhythms) still operative in these animals.

In summary, we demonstrate for the first time that constant illumination can restore self-sustained circadian rhythmicity in both the SCN and the periphery of an arrhythmic mutant. Persistent ultradian oscillators may serve to temporally gate clock gene induction in response to tonic light exposure, thereby reinitiating clock function in LL.

REFERENCES

Albrecht U, Lu HC, Revelli JP, Xu XC, Reuben L, and Eichele G (1998) Studying gene expression on tissue sections using in situ hybridization. In *Human Genome Methods*, Adolph KW, ed, pp 93-120, CRC Press, Boca Raton, FL.

Albrecht U and Oster H (2001) The circadian clock and behavior. Behav Brain Res 125:89-91.

Aschoff J (1960) Exogenous and endogenous components in circadian rhythms. Cold Spring Harb Symp Quant Biol 25:11-28.

Bae K, Jin X, Maywood ES, Hastings MH, Reppert SM, and Weaver DR (2001) Differential functions of *mPer1*, *mPer2*, and *mPer3* in the SCN circadian clock. Neuron 30:525-536.

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

Beaule C, Houle LM, and Amir S (2003) Expression profiles of PER2 immunoreactivity within the shell and core regions of the rat suprachiasmatic nucleus: lack of effect of photic entrainment and disruption by constant light. J Mol Neurosci 21:133-147.

Bunger MK, Wilsbacher LD, Moran SM, Clendenin C, Radcliffe LA, Hogenesch JB, Simon MC, Takahashi JS, and Bradfield CA (2000) *Mop3* is an essential component of the master circadian pacemaker in mammals. Cell 103:1009-1017.

Cavigelli SA, Monfort SL, Whitney TK, Mechref YS, Novotny M, and McClintock MK (2005) Frequent serial fecal corticoid measures from rats reflect circadian and ovarian corticosterone rhythms. J Endocrinol 184: 153-163.

Daan S, Albrecht U, van der Horst GT, Illnerova H, Roenneberg T, Wehr TA, and Schwartz WJ (2001) Assembling a clock for all seasons: are there M and E oscillators in the genes? J Biol Rhythms 16:105-116.

Daan S and Pittendrigh C (1976) A functional analysis of circadian pacemakers in nocturnal rodents: III. Heavy water and constant light: homeostasis of frequency? J Comp Physiol A 106:267-290.

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

Le Minh N, Damiola F, Tronche F, Schutz G, and Schibler U (2001) Glucocorticoid hormones inhibit food-induced phase-shifting of peripheral circadian oscillators. Embo J 20:7128-7136.

Lowrey PL and Takahashi JS (2004) Mammalian circadian biology: elucidating genome-wide levels of temporal organization. Annu Rev Genomics Hum Genet 5: 407-441.

Munoz M, Peirson SN, Hankins MW, and Foster RG (2005) Long-term constant light induces constitutive elevated expression of mPER2 protein in the murine SCN: a molecular basis for Aschoff's rule? J Biol Rhythms 20:3-14.

Oster H, Baeriswyl S, van der Horst GT, and Albrecht U (2003a) Loss of circadian rhythmicity in aging *mPer1⁻/⁻mCry2⁻/⁻* mutant mice. Genes Dev 17:1366-1379.

Oster H, Werner C, Magnone MC, Mayser H, Feil R, Seeliger MW, Hofmann F, and Albrecht U (2003b)

cGMP-dependent protein kinase II modulates *mPer1* and *mPer2* gene induction and influences phase shifts of the circadian clock. Curr Biol 13:725-733.

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

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45.

Reppert SM and Weaver DR (2002) Coordination of circadian timing in mammals. Nature 418:935-941.

Roenneberg T and Merrow M (2003) The network of time: understanding the molecular circadian system. Curr Biol 13:R198-R207.

Shigeyoshi Y, Taguchi K, Yamamoto S, Takekida S, Yan L, Tei H, Moriya T, Shibata S, Loros JJ, Dunlap JC, et al. (1997) Light-induced resetting of a mammalian circadian clock is associated with rapid induction of the *mPer1* transcript. Cell 91:1043-1053.

Spoelstra K, Oklejewicz M, and Daan S (2002) Restoration of self-sustained circadian rhythmicity by the mutant *Clock* allele in mice in constant illumination. J Biol Rhythms 17:520-525.

Steinlechner S, Jacobmeier B, Scherbarth F, Dernbach H, Kruse F, and Albrecht U (2002) Robust circadian rhythmicity of *Per1* and *Per2* mutant mice in constant light, and dynamics of *Per1* and *Per2* gene expression under long and short photoperiods. J Biol Rhythms 17:202-209.

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

Vitaterna MH, King DP, Chang AM, Kornhauser JM, Lowrey PL, McDonald JD, Dove WF, Pinto LH, Turek FW, and Takahashi JS (1994) Mutagenesis and mapping of a mouse gene, *Clock*, essential for circadian behavior. Science 264:719-725.

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

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

Zheng B, Larkin DW, Albrecht U, Sun ZS, Sage M, Eichele G, Lee CC, and Bradley A (1999) The *mPer2* gene encodes a functional component of the mammalian circadian clock. Nature 400:169-173.