Daily Variation of Clock Output Gene Activation in Behaviorally Arrhythmic mPer/mCry Triple Mutant Mice

Henrik Oster,1 Gijsbertus T. J. van der Horst,2 and Urs Albrecht1,*

1Department of Medicine, Division of Biochemistry, University of Fribourg, Fribourg, Switzerland
2Department of Cell Biology and Genetics, Erasmus MC, Rotterdam, DR Rotterdam, The Netherlands

ABSTRACT

The mammalian central pacemaker, driving circadian rhythms in behavior, physiology, and metabolism, is located in the suprachiasmatic nuclei (SCN) of the hypothalamus. At the molecular level, circadian clocks are based on a system of transcriptional/translational feedback loops oscillating with a period of about 24h. In mammals the CLOCK/BMAL1 transcriptional activator complex regulates a set of central clock genes like mPer1, mPer2, mCry1, and mCry2. The corresponding gene products form protein complexes that translocate into the nucleus and inhibit CLOCK/BMAL1-driven transcription of their own genes and other E-box containing genes. To elucidate whether only one of these four genes of the negative feedback loop is sufficient to generate a 24h rhythm we generated mPer/mCry triple mutant mice. As could be expected on the basis of the arrhythmicity of mPer1/mPer2 and mCry1/mCry2 double mutant mice, we show that none of the triple mutants is able to maintain circadian rhythmicity in constant darkness. This indicates that a single mPer or mCry gene is not sufficient to drive circadian rhythms. Interestingly however, under light-dark conditions (LD) the oscillation of some output genes is persisting in these animals indicating that

*Correspondence: Urs Albrecht, Department of Medicine, Division of Biochemistry, University of Fribourg, 1700 Fribourg, Switzerland; Fax: ++41 26 300 9735; E-mail: urs.albrecht@unifr.ch.
the LD cycle is able to partially drive rhythmic signalling to the body, through an hourglass mechanism.

*Key Words:* Circadian clock; Vasopressin; Dbp; Gene expression.

**INTRODUCTION**

To anticipate daily changes in the environment [i.e., the light-dark (LD) cycle], most organisms have evolved an endogenous clock to synchronize physiology and behavior accordingly (Pittendrigh, 1993). In mammals, the central circadian pacemaker is located in the *suprachiasmatic nuclei* (SCN) of the hypothalamus (Moore and Eichler, 1972). At the molecular level, circadian clocks are built of cellular oscillators that are synchronized to each other (Welsh et al., 1995). Pacemaker genes like *Clock*, *Bmal1*, *mPer1*, 2 and 3, *mCry1* and 2 are organized in a system of transcriptional/ translational feedback loops creating precise, stabilized 24h rhythms that persist even in the absence of external time information (reviewed in Albrecht, 2002; Panda et al., 2002; Reppert and Weaver, 2001).

Under natural conditions however, the clock in the SCN is not free running but periodically synchronized on a daily basis to the external day-night cycle by light (Balsalobre, 2002; Herzog and Tosini, 2001). From the central clock, time information is conferred to other parts of the body. Subordinated clocks in peripheral organs can receive information from the SCN via the activation of clock-controlled genes (ccgs) like D-albumin binding protein (*Dbp*) and Vasopressin (*AVP*). However, restricted feeding can shift peripheral clocks without acting through the SCN pacemaker.

*Cryptochrome* (*mCry1* and 2) and *Period* (*mPer1*, 2, and 3) genes are rhythmically expressed in the neurons of the SCN with a circadian period. Their protein products interact with the CLOCK/BMAL1 transcription complex thereby negatively affecting their own transcriptional activation (Kume et al., 1999). In vitro data suggest a multimeric interaction between all mPER and mCRY proteins (Griffin et al., 1999; Yagita et al., 2000; 2002). However, studies using mutant mice indicate that no single *mPer* or *mCry* gene is essential for circadian rhythm generation (Ba et al., 2001; Cermakian et al., 2001; Oster et al., 2002; Shearman et al., 2000; van der Horst et al., 1999; Vitaterna et al., 1999; Zheng et al., 1999; 2001). Thus, partial redundancy of clock genes might serve to stabilize the circadian oscillator while the single genes additionally serve specific functions in the resetting of the pacemaker in response to external light (Albrecht et al., 2001).

We generated *mPer/mCry* triple mutant mice of various allele combinations keeping only one *mPer* (*mPer1* or *mPer2*)—*mPer*3 seems not to play a role in the central clock mechanism (Ba et al., 2001; Shearman et al., 2000)—or *mCry* gene (*mCry1* or *mCry2*) functional. We find that neither a single *mPer* nor *mCry* gene is sufficient to drive the internal clockwork. While in a LD cycle all triple mutant mice show rhythmic activity patterns, probably as a direct response to regular light exposure, their ability to maintain rhythmicity is lost in constant conditions. This is reflected at the molecular level, where cycling clock gene and clock-controlled gene expression in the SCN is strongly dampened in the mutant animals.
MATERIALS AND METHODS

Generation of \textit{mPer} and \textit{mCry} Triple Mutant Mice

We cross-bred \textit{mPer/mCry} double mutant mice (Oster et al., 2002) of comparable (mixed) genetic background. By intercrossing the double heterozygous, single homozygous fl generation we obtained triple homozygous mutants in the f2 generation. The genotype of the offspring was determined by southern blot analysis as described (Oster et al., 2002; van der Horst et al., 1999; Zheng et al., 1999; 2001). As wild-type control we used animals on a mixed 129SvEvBrd/129 Ola background derived from the crossing of \textit{Per} and \textit{Cry} mutant animals (Oster et al., 2002).

Locomotor Activity Monitoring and Circadian Phenotype Analysis

Mice housing and handling were performed as described (Albrecht and Oster, 2001). For LD-DD transitions lights remained off after the last LD cycle. Activity records of LD and DD cycles are double-plotted (day 1–2, day 2–3, day 3–4, etc.). Activity is displayed in threshold format for 5-min bins. For activity counting and evaluation we used the ClockLab software package (Actimetrics). Rhythmicity and period lengths were assessed by \(\chi^2\) periodogram and Fourier transformation using mice running in LD or in constant darkness for at least 10 days. For the shifted LD cycles, animals were kept in an LD cycle for at least 10 days before the start of the shift experiment (lights were turned on at 7:00 a.m. and off at 7:00 p.m.). Subsequently, lights were not turned on at 7:00 a.m. the next day but 8h later (3:00 p.m.). From there a shifted light cycle was presented to the animals (“lights on” = ZT0 at 3:00 p.m. and “lights off” = ZT12 at 3:00 a.m., respectively). We measured the number of days needed for an animal to adapt to the new LD cycle by looking at the onset of activity. After another 10 days the LD cycle was shifted back again (with a long night at the transition, e.g., “lights off” at 3:00 a.m. and “lights on” at 7:00 a.m. on the following day).

Animals used for the analysis in Fig. 2 were in average 6 months older than mice used in Fig. 1. This might be a reason for the higher activity in the L phase of the LD cycle observed in Fig. 2.

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. Specimen preparation, \(^{35}\text{S}-\text{UTP}\) labeled riboprobe synthesis, and hybridization steps were performed as described (Albrecht et al., 1997). The probe for \textit{Bmal1} was as described (Oster et al., 2002). The \textit{Dbp} probe was made from a cDNA corresponding to nucleotides 2–951 (GenBank NM016974). The \textit{AVP} probe corresponds to nucleotides 1–480 (GenBank M88354). Quantification was performed by densitometric analysis of autoradiograph films (Amersham Hyperfilm MP) as described (Oster et al., 2002). Three sections per SCN were analyzed. “Relative mRNA abundance” values were calculated by defining the
Figure 1. Representative locomotor activity records of wild-type (A), mPer1<sup>−/−</sup> mCry1<sup>−/−</sup> mCry2<sup>−/−</sup> (B), mPer1<sup>−/−</sup>mPer2<sup>Brdm1</sup>mCry1<sup>−/−</sup> (C), mPer1<sup>−/−</sup>mPer2<sup>Brdm1</sup>mCry2<sup>−/−</sup> (D), and mPer2<sup>Brdm1</sup>mCry1<sup>−/−</sup>mCry2<sup>−/−</sup> animals (E) kept in a 12h light 12h dark (LD) cycle and in constant darkness (DD; transition indicated by the line over the DD). Activity is represented by black bars and is double-plotted with the activity of the following day/cycle plotted to the right and below the previous day/cycle. The top bar indicates light and dark phases in LD. (F) Overall activity of wild-type and mPer/mCry triple mutant animals in DD. For the first five days in DD, wheel rotations per day were 20,000 ± 2500 (n = 10) for wild-type animals, 4300 ± 2400 (n = 10) for mPer1<sup>−/−</sup>mPer2<sup>Brdm1</sup>mCry1<sup>−/−</sup> mutants, 6800 ± 1600 (n = 6) for mPer1<sup>−/−</sup>mPer2<sup>Brdm1</sup>mCry2<sup>−/−</sup> mutants, 8800 ± 1600 (n = 10) for mPer2<sup>Brdm1</sup>mCry1<sup>−/−</sup>mCry2<sup>−/−</sup> mutants, and 8200 ± 1400 (n = 21) for mPer1<sup>−/−</sup>mCry1<sup>−/−</sup>mCry2<sup>−/−</sup> mutant mice. Data presented are mean ± s.d. highest value of each experiment as 100%. Statistical analysis was performed using GraphPad Prism software (GraphPad). Significant differences between two groups were determined by student's t-test. Values were considered significantly different with p < 0.05.
RESULTS

Animal Breeding

All combinations of mPer/mCry double mutant founder pairs produced double heterozygous/single homozygous offspring with litter numbers comparable to wild-type controls (7.5 ± 3.4; mean ± s.d.). All animals from the f1 generation were fertile, and f2 offspring was morphologically indistinguishable from wild-type controls. Genotype was assessed by Southern blot analysis. The distribution of the f2 offspring appeared with expected Mendelian ratios with the exception of mPer1−/−mCry1−/−mCry2−/− animals where only two triple homozygous females from 10 f1 breeding pairs were obtained in 1 year (total offspring number: 468; expected were 7). We tried to establish homozygous triple mutant breeding lines but did succeed only with mPer2Brdm1/mCry1−/−mCry2−/− and mPer1−/−mPer2Brdm1/mCry2−/− matings (see Table 1). Triple mutant breedings were rather unreliable with small litters, irregular breeding intervals, and high mortality rates after birth. Since the estrous cycle is known to be linked to the circadian clock (Alleva et al., 1971), it is not surprising that females with a disrupted circadian pacemaker (see below) will have difficulties with regard to their fertility.

Activity Monitoring

All triple mutant animals show daily wheel running variation in a normal 12h light, 12h dark (LD) cycle with activity predominantly during the dark phase [daytime activity was 3.4 ± 2.1% of the overall activity in LD for wild-type (n = 10), 1.8 ± 1.8% for mPer1−/−mPer2Brdm1Cry1−/− (n = 10), 2.7 ± 1.1% for mPer1−/−mPer2Brdm1Cry2−/− (n = 6), 0.9 ± 1.6% for mPer2Brdm1Cry1−/−mCry2−/− (n = 10), and 1.9 ± 1.7% for mPer1−/−Cry1−/−mCry2−/− (n = 2!) animals]. However, the distribution of activity
Figure 2. Representative locomotor activity records of wild-type (A), mPer1\(^{-/-}\) mPer2\(^{Brdm1}\) mCry1\(^{-/-}\) (B), mPer1\(^{-/-}\) mPer2\(^{Brdm1}\) mCry2\(^{-/-}\) (C), and mPer2\(^{Brdm1}\) mCry1\(^{-/-}\) mCry2\(^{-/-}\) (D) animals exposed to an 8h shifted LD cycle. The top bar indicates light and dark phases of the LD cycle before and after the experiment. Start and end of the shifted light regimen are indicated by asterisks on the right side of each diagram.

appeared more scattered in triple mutant mice [Fig. 1(A)–(E)] and overall activity was significantly lower in both LD and constant darkness (DD) conditions [Fig. 1(F)]. Nevertheless, activity onset was sufficiently sharp around the beginning of the dark phase with no differences between wild-type and triple mutant animals [Fig. 1(A)–(E)].

In DD wild-type mice displayed a stable activity rhythm with a period length of 23.8 ± 0.1h (n = 10). All triple mutant animals became arrhythmic immediately upon release into constant darkness [Fig. 1(B)–(E)] or constant light (LL) conditions (400Lux bright white light after previous entrainment to LD; data not shown) indicating that the circadian clockwork is disrupted. As mentioned above we were not able to obtain sufficient numbers of mPer1\(^{-/-}\) Cry1\(^{-/-}\) mCry2\(^{-/-}\) to perform statistically meaningful experiments.
Therefore this genotype was excluded for all further studies. However, since the actograms of both animals appeared rather similar one example is shown [Fig. 1(B)].

To test if the apparent wild-type behavior of the triple mutants in LD was clock-driven or whether daytime activity was only masked by the light we exposed the animals to a rapid shift of the LD cycle. Wild-type animals needed 3.5 ± 0.5 days \( (n = 10) \) to adjust their onset of activity to a LD cycle shifted backward for 8h [Fig. 2(A)]. In contrast, all triple mutant allele combinations tested \( \{ m_{\text{Per}}^1/-m_{\text{Per}}^2^{\text{Brdm1}}-m_{\text{Cry}}^1/- \} \) [Fig. 1(B)], \( m_{\text{Per}}^1/-m_{\text{Per}}^2^{\text{Brdm1}}-m_{\text{Cry}}^2/- \) [Fig. 1(C)], and \( m_{\text{Per}}^2^{\text{Brdm1}}-m_{\text{Cry}}^1/-m_{\text{Cry}}^2/- \) [Fig. 1(D)] adapted immediately to the shifted LD cycle. After changing the LD cycle back to the initial onset, wild-type animals needed 5.8 ± 1.2 days to adjust, indicating that phase-advancing the clockwork is less efficient (Reddy et al., 2002). In the triple mutants activity phases adapted within one day, indicating that no internal clockwork needs to be reset to resynchronize to the external LD cycle. We conclude that activity rhythms in LD are only light-adjusted and not linked to an internal pacemaker in triple mutant mice.

**Gene Expression Analysis**

The central mammalian circadian pacemaker resides in the SCN of the hypothalamus (Moore and Eichler, 1972). One of the rhythmically expressed clock genes in the SCN is \( Bmal1 \) \([MP3]; Abe et al., 1998; Honma et al., 1998; Oishi et al., 1998\). \( Bmal1 \) oscillation seems to be essential for circadian rhythm generation (Bunger et al., 2000; Oster et al., 2002). Therefore we looked for \( Bmal1 \) oscillation in the SCN of wild-type and \( m_{\text{Per}}/m_{\text{Cry}} \) triple mutant mice. We only examined gene expression in LD because the immediate arrhythmicity of the triple mutant animals in constant conditions excludes the determina-

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Average litter size</th>
<th>First interval (days)</th>
<th>Litter frequency (days)</th>
<th>Number of litters per 6 months</th>
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<tbody>
<tr>
<td>Wild-type</td>
<td>7.48 ± 3.39</td>
<td>32.0 ± 14.0</td>
<td>27.3 ± 7.9</td>
<td>5.2 ± 2.2</td>
</tr>
<tr>
<td>( m_{\text{Per}}^1/-m_{\text{Cry}}^1/- )</td>
<td>5.69 ± 2.21</td>
<td>44.0 ± 11.1</td>
<td>35.5 ± 13.5</td>
<td>3.0 ± 1.7</td>
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<tr>
<td>( m_{\text{Per}}^1/-m_{\text{Cry}}^2/- )</td>
<td>4.14 ± 2.66</td>
<td>36.7 ± 11.6</td>
<td>36.1 ± 13.8</td>
<td>1.8 ± 1.8</td>
</tr>
<tr>
<td>( m_{\text{Per}}^2^{\text{Brdm1}}-m_{\text{Cry}}^1/- )</td>
<td>6.89 ± 3.14</td>
<td>43.3 ± 36.8</td>
<td>41.6 ± 28.3</td>
<td>3.0 ± 0.8</td>
</tr>
<tr>
<td>( m_{\text{Per}}^2^{\text{Brdm1}}-m_{\text{Cry}}^2/- )</td>
<td>6.11 ± 2.16</td>
<td>32.3 ± 14.0</td>
<td>35.1 ± 11.0</td>
<td>3.6 ± 0.8</td>
</tr>
<tr>
<td>( m_{\text{Per}}^1/-m_{\text{Per}}^2^{\text{Brdm1}} )</td>
<td>8.00 ± 1.00</td>
<td>46.0 ± 5.0</td>
<td>n.a. (^a)</td>
<td>0.4 ± 0.8</td>
</tr>
<tr>
<td>( m_{\text{Per}}^2^{\text{Brdm1}}-m_{\text{Cry}}^1/-m_{\text{Cry}}^2/- )</td>
<td>5.5 ± 2.74</td>
<td>54.6 ± 15.7</td>
<td>34.6 ± 12.8</td>
<td>4.7 ± 3.8</td>
</tr>
<tr>
<td>( m_{\text{Per}}^1/-m_{\text{Per}}^2^{\text{Brdm1}}-m_{\text{Cry}}^1/-m_{\text{Cry}}^2/- )</td>
<td>n.a. (^b)</td>
<td>n.a. (^b)</td>
<td>n.a. (^b)</td>
<td>n.a. (^b)</td>
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\(^a\)matings never produced more than one litter per pair.

\(^b\)matings were not fertile.
tion of circadian time. In wild-type animals Bmal1 mRNA levels peak around the middle of the dark phase, roughly in antiphase to mPer mRNA levels [Fig. 3(A); Oishi et al., 1998]. In mPer/mCry triple mutant animals diurnal Bmal1 oscillation is dampened with no significant difference between lowest and highest levels of expression (ZT6 and ZT18, respectively; p > 0.05; student’s t-test). In the SCN the mRNA abundance of several ccgs displays a circadian pattern. These ccgs confer time information to the organism. Two of them are Dhp which is involved in regulating circadian activity and sleep consolidation (Franken et al., 2000; Lopez-Molina et al., 1997) and AVP. Vasopressin may be involved in driving rhythmic corticosterone release from the adrenal gland (Buijs et al., 1999; Kalsbeek et al., 1996) and influence signaling from the hypothalamus to the gonads via the pituitary (Palm et al., 1999). To test whether the SCN of mPer/mCry triple mutant mice can still generate rhythmic output signals to temporally organize body physiology we examined Dhp and AVP expression in the SCN of these animals. In wild-type mice Dhp mRNA shows a clear circadian rhythm with a peak around the day/night transition [Fig. 3(B)]. In mPer1−/−mPer2Brdm1/mCry1−/− and mPer1−/−mPer2Brdm1/mCry2−/− animals Dhp oscillation is severely blunted with no significant difference between minimal and maximal expression levels [ZT0 vs. ZT6 (lowest and highest expression levels) p > 0.05; student’s t-test]. Interestingly, in mPer2Brdm1/mCry1−/−mCry2−/− animals Dhp is still cycling in LD but with reduced amplitude compared to wild-type controls [ZT0 vs. ZT6 (lowest and highest expression levels) p < 0.05; student’s t-test]. It is possible that light-driven activation of mPer1 in these animals regulates a certain level of rhythmicity under LD conditions.

In contrast, rhythmic AVP expression in wild-type animals is efficiently suppressed in all mPer/mCry triple mutants examined [Fig. 3(C)]. Thus, apart from induction by CLOCK/BMAL1, AVP expression seems to be differently regulated compared to Dhp, i.e., it is not additionally influenced by the presence of mPER1.

DISCUSSION

The mPer and mCry genes are important components in the mechanism of transcriptional translational feedback loops that constitute the internal oscillator driving circadian rhythmicity in all mammals. Studies using mPer or mCry mutant mice have demonstrated that none of these genes is essential to drive the circadian pacemaker in constant light (LL; Steinlechner, 2002 and H.O. unpublished data) as well as constant darkness (Bae et al., 2001; Cermakian et al., 2001; Oster et al., 2002; van der Horst et al., 1999; Vitaterna et al., 1999; Zheng et al., 2001). Interestingly, certain combinations of double inactivation like mPer1/mPer2, mCry1/mCry2, and mPer2/mCry1 display an immediate loss of circadian rhythmicity upon release into DD (Bae et al., 2001; Cermakian et al., 2001; Oster et al., 2002; Vitaterna et al., 1999; Zheng et al., 2001). However, other combinations of double deletions allow, at least partially, stable rhythm generation as observed in mPer1/mCry1, mPer1/mCry2, and mPer2/mCry2 double mutant mice (Oster, 2002 and own unpublished data). We were interested in whether a single functional mPer or mCry gene could be sufficient to drive the circadian clockwork. To this end we generated mice missing three of the four mPer and mCry genes.

We show that mPer/mCry triple mutant mice immediately become arrhythmic upon release into constant darkness conditions (Fig. 1). Additionally, the activity profiles of
Figure 3. Expression of Bmal1, Dbp, and AVP in the SCN of triple mutant mice. (A) Diurnal expression of Bmal1 mRNA in the SCN of wild-type (---), mPer1-/-mPer2Brdm1mCry2-/- (· · · · ), mPer1-/-mPer2Brdm1mCry1-/- (---), and mPer2Brdm1mCry1-/-mCry2-/- (-----) animals in LD. In all three triple mutants Bmal1 mRNA cycling is significantly dampened (p < 0.05). (B) Diurnal expression of Dbp mRNA in the SCN of wild-type (---), mPer1-/-mPer2Brdm1mCry2-/- (· · · · ), mPer1-/-mPer2Brdm1mCry1-/- (---), and mPer2Brdm1mCry1-/-mCry2-/- (-----) animals in LD. In all three triple mutants Dbp mRNA cycling is significantly dampened (p < 0.05) with a maximum shifted toward earlier time points. (C) Diurnal expression of AVP mRNA in the SCN of wild-type (---), mPer1-/-mPer2Brdm1mCry2-/- (· · · · ), mPer1-/-mPer2Brdm1mCry1-/- (---), and mPer2Brdm1mCry1-/-mCry2-/- (-----) animals in LD. In all three triple mutants AVP mRNA cycling is significantly dampened (p < 0.05). Black and white bars on x-axis indicate dark and light phase, respectively. All data are mean±s.d. of three different experiments. X-axis shows relative signal intensities of 35S labeled in situ hybridization signal after exposure to autoradiograph film. Peak levels for wild-type were set to 100.
these animals rapidly adapt to an 8-h shifted LD cycle indicating that no functional clockwork needs to be reset in order to synchronize wheel running behavior to the LD cycle (Fig. 2). The overt activity rhythm of these animals in LD seems to be masked by the activity suppressing effect of light which is not related to internal clock function (reviewed in Mrosovsky, 1999).

These findings are supported by the blunted Bmal1 oscillation in the SCN of the triple mutant animals in LD [Fig. 3(A)]. Since Bmal1 oscillation seems to be essential for circadian rhythmicity and clock functionality (Bunger et al., 2000; Oster et al., 2002), we conclude that the internal clockwork is disrupted in mPer/mCry triple mutants animals and a single mPer or mCry gene (out of the four) is not sufficient to drive circadian rhythmicity.

Interestingly, clock output gene expression is differentially regulated in triple mutant strains. Vasopressin expression is low without significant rhythmicity as would be expected in animals lacking a functional pacemaker. In contrast, mPer2<sup>Brdm1</sup>mCry1<sup>−/−</sup>mCry2<sup>−/−</sup> mutants still show rhythmic, although dampened, Dsp mRNA levels in the SCN in LD, while mPer1<sup>−/−</sup>mPer2<sup>Brdm1</sup>mCry1<sup>−/−</sup> and mPer1<sup>−/−</sup>mPer2<sup>Brdm1</sup>mCry2<sup>−/−</sup> mutant animals display arrhythmic Dsp expression profiles. It seems that the presence of mPer1 but not mPer2 is important for Dsp activation. It is likely that the LD cycle can act as a periodic trigger that kick-starts a crippled molecular clockwork by induction of mPer1 gene expression. The absence of both mCry genes blocks the normal clock mechanism, because CLOCK/BMAL1-driven transcription is not modified by interaction with mPER/mCRY protein complexes and therefore the next circadian cycle is not initiated.

A light stimulus however will restart the clock through activation of mPer1. In DD this periodic clockwork initiation by light will not occur and a residual circadian rhythm breaks down. The described mechanism works as an “hour-glass” rather than a “clock” and supports our finding that mPer2<sup>Brdm1</sup>mCry1<sup>−/−</sup>mCry2<sup>−/−</sup> mutant mice display circadian expression of Dsp. The special role of mPer1 for the generation of certain output signals might explain why sufficient numbers of mPer1<sup>−/−</sup>mCry1<sup>−/−</sup>mCry2<sup>−/−</sup> triple mutants could not be obtained. In combination with mCry1 and mCry2 regulated genes, certain cccgS important for development seem not to be activated in mPer1<sup>−/−</sup>mCry1<sup>−/−</sup>mCry2<sup>−/−</sup> mCry2<sup>−/−</sup> mutants leading to an increased abortion rate (see above, and compare also the decreased fertility of mCry1<sup>−/−</sup>mCry2<sup>−/−</sup> double mutants (GTJ van der Horst unpublished data)).

The reduced overall locomotor activity observed in mPer/mCry triple mutant mice was somehow unexpected and is probably not directly related to the absence of an internal pacemaker since neither mPer or mCry single mutants nor the arrhythmic double mutants were reported to be less active than their wild-type littermates (Bae et al., 2001; Cermakian et al., 2001; Oster et al., 2002; van der Horst et al., 1999; Vitaterna et al., 1999; Zheng et al., 1999; 2001). However, in both Dsp and Mop3 (Bmal1) mutant mice reduced LD and DD activity levels have been observed (Bunger et al., 2000; Lopez-Molina et al., 1997). As we have shown (Fig. 3), both Dsp and Bmal1 expression are affected in the triple mutants indicating that the observed phenotype might be indirectly determined via the inhibition of cccgS (in this case: Dsp) in the triple mutant animals.

In conclusion and together with previous studies we show that certain combinations of mPer and mCry genes are necessary to drive the circadian clockwork. Although rhythmicity is totally disrupted in mPer/mCry triple mutant animals in constant conditions, the presence of mPer1 alone seems to be sufficient to induce rhythmic expression of Dsp as a response to regular light exposure under LD conditions. This “hour-glass” mechanism
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seems also to apply to light-induced increase in electric activity of SCN neurons in mCry1−/− mCry2−/− mutant mice (Albus et al., 2002).

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