Circadian Rhythms in Murine Pups Develop
in Absence of a Functional Maternal Circadian Clock

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Abstract   A genetic approach was used to investigate whether the emergence of circadian rhythms in murine pups is dependent on a functional maternal clock. Arrhythmic females bearing either the $mPer1^{Brdm1}/Per2^{Brdm1}$ or $mPer2^{Brdm1}/Cry1^{-/-}$ double-mutant genotype were crossed with wild-type males under constant darkness. The heterozygous offspring have the genetic constitution for a functional circadian clock. Individual pups born to arrhythmic $mPer1^{Brdm1}/Per2^{Brdm1}$ and $mPer2^{Brdm1}/Cry1^{-/-}$ mothers in constant darkness without external zeitgeber developed normal circadian rhythms, but their clocks were less synchronized to each other compared to wild-type animals. These findings indicate that development of circadian rhythms does not depend on a functional circadian clock in maternal tissue, extending previous findings obtained from pups born to SCN-lesioned mothers.

Key words   circadian rhythm, development, synchronization, Per1, Per2, Cry1

In mammals, circadian clocks control the rhythmic expression of numerous physiological processes (reviewed in Hastings et al., 2003). A self-sustaining clock mechanism is present in every individual cell (Nagoshi et al., 2004). At the molecular level, a set of clock genes drives recurrent rhythms in mRNA and protein synthesis (reviewed in Reppert and Weaver, 2002; Albrecht and Eichele, 2003). The individual cellular rhythms may then be synchronized in a tissue via gap junctions (Long et al., 2005) and other means of cellular communication (Pennartz et al., 2002). The SCNs synchronize the different tissue clocks through neuronal and endocrine outputs (Buijs and Kalsbeek, 2001) and themselves are entrainable by the daily LD cycle through the retinohypothalamic tract, which connects the eye with the SCN.

How circadian rhythms emerge during development has been of interest for many years. One focus of research has been centered on the question of whether development of circadian rhythms in mammals is under the influence of maternal entrainment or whether it is genetically predisposed. Maternal entrainment of pups to the environmental LD cycle has been demonstrated by studying metabolism of C^{14}-labeled desoxyglucose in fetal rat SCN tissue (Reppert and Schwartz, 1983). Using the same technique, Reppert and Schwartz (1984) demonstrated that oscillating metabolic activity in the SCN is observed as early as the 19th day of rat gestation. In the mouse, *Per1* and *Per2* expression is observed in the developing SCN on day 17 of gestation (Shearman et al., 1997), but rhythmic expression of these genes is not detectable until after birth (Sládek et al., 2004; Li and Davis, 2005). Rat pups born and reared under constant darkness display a circadian rhythm of pineal N-acetyltransferase (NAT) that is in phase with the circadian time of the mother, which indicates maternal synchronization of the clock in pups

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(Reppert et al., 1984). Similarly, evidence for maternal entrainment of developing rhythms was found in hamsters (Davis and Gorski, 1985). The role of the maternal SCN in entrainment of the fetal clock was investigated by SCN-lesion experiments (Reppert and Schwartz, 1986; Davis and Gorski, 1988). These investigations showed that the fetal clock was unaffected by maternal SCN lesion, but synchrony between pups was altered. These experiments indicated a prominent role of the maternal SCN in litter synchronization. However, conclusions on the role of the maternal clock on the development of the fetal circadian system are difficult to make, for the following reasons. First, lesions in dams have been performed at gestation day 7, and hence, an impact of the SCN before surgery can not be excluded. Second, removal of the SCN leaves peripheral clocks functional while synchronization between organs is abolished (Yoo et al., 2004).

To study the fetal circadian system in the complete absence of a functional maternal circadian clock, we studied the appearance of circadian wheel-running activity in heterozygous pups derived from $mPer1^{Brdm1}/Per2^{Brdm1}$ and $mPer2^{Brdm1}/Cry1^{-/-}$ double-mutant females crossed with wild-type males. We found that all offspring developed a circadian rest-activity rhythm, and within a litter, individual phases were less synchronous compared to the wild-type control litters. These findings indicate that development of the fetal circadian system is independent of a functional maternal clock.

MATERIALS AND METHODS

Animals

The $mPer2^{Brdm1}/Cry1^{-/-}$ (Oster et al., 2002) and $mPer1^{Brdm1}/Per2^{Brdm1}$ (Zheng et al., 2001) double-mutant mice used in this study were generated by crossing $mPer2^{Brdm1}$ mutant mice (Zheng et al., 1999) with $mCry1$ knockout animals (van der Horst et al., 1999) or with $mPer1^{Brdm1}$ (Zheng et al., 2001) knockout mice, respectively. Matching wild-type control animals were produced by intercrossing heterozygous animals.

Housing, Breeding, and Rearing Conditions

The $mPer2^{Brdm1}/Cry1^{-/-}$ and $mPer2^{Brdm1}/Per1^{Brdm1}$ double-mutant females and the wild-type mice were reared under a normal 12:12-h LD cycle with food and water ad libitum. They were kept and mated in transparent plastic cages (267 mm long × 207 mm wide × 140 mm high; Techniplast Makrolon type 2 1264C001)

with a stainless-steel wire lid (Techniplast 1264C116). To exclude any influence of time cues on the offspring, matings were set up under defined environmental conditions and constant darkness in isolated cabinets of our wheel-running facility (Jud et al., 2005). All manipulations were performed using night-vision goggles (Rigel 3200), and light sources that could be detected with the night-vision goggles were covered with aluminum foil prior to the experiment. The built-in infrared illuminator of the night-vision goggles was covered with dark paper to dampen it. Furthermore, the time of day of routine care was randomized. The $mPer2^{Brdm1}/Per1^{Brdm1}$ heterozygous pups were produced in 3 matings. One wild-type male was bred with 1 double-mutant female and gave birth to 2 litters (P1 and P3, see below). Another wild-type male was bred with 2 double-mutant females (litters P4 and P5). The 3rd mating consisted of 1 wild-type male and 1 double-mutant female (litter P2). The $mPer2^{Brdm1}/Cry1$ heterozygous pups were produced by 3 matings. One wild-type male and 1 double-mutant female produced litter C1. Another wild-type male and 1 double-mutant female produced litter C2. A 3rd wild-type male was mated with 2 double-mutant females, which resulted in litters C3 and C4. The wild-type control animals were produced by 3 matings, each with 1 wild-type male and 1 wild-type female (litters W1-W3). Nestlets (5 × 5 cm; EBECO) were placed into the cages to encourage nesting and improve breeding. Once double-mutant females became pregnant (determined by visual inspection) or at the latest after 2 weeks, the wild-type males were moved to a separate cage and isolation cabinet. Females received cotton nestlets and were left undisturbed for 2 to 3 d before and after delivery. A total of 18 wild-type mice, 40 $mPer2^{Brdm1}/Per1^{Brdm1}$ heterozygous mice, and 25 $mPer2^{Brdm1}/Cry1^{-/-}$ heterozygous mice were obtained (see Table 1). Pups were weaned 3 weeks after birth and kept in small groups of maximum 5 animals up to the age of 6 weeks in constant darkness.

Locomotor Activity Monitoring

Starting from the 7th week of age, each of the heterozygous offspring was placed in an individual running-wheel cage (Jud et al., 2005). To put mice in running wheels directly after weaning is not recommended because heat loss is greater than heat generation at that age, and pups die when kept individually. The heterozygous pups, the mother,

and the father were placed into the same isolation cabinet. Activity was assessed and evaluated using the ClockLab software package (Actimetrics). Activity records were double plotted in threshold format for 6-min bins. Period length and rhythmicity were assessed by χ^2 periodogram analysis.

Determination of Within-Litter Synchrony

The activity onset on the 2nd day of wheel running was determined for each individual. A regression line was drawn through onsets using ClockLab software (Actimetrics). The activity onset closest to the point where the regression line hit time axis on day 2 of the wheel-running experiment was defined as onset of activity at day 2. We determined the length of the average vector r for each litter, which represents the scatter of phases within a litter (Rayleigh test and tables for significance; see Zar, 1999). Midnight corresponded to 0°.

RESULTS

To study the emergence of circadian rhythms in absence of a maternal clock, we crossed $mPer1^{Brdm1}/Per2^{Brdm1}$ and $mPer2^{Brdm1}/Cry1^{-/-}$ double-mutant females with wild-type males in constant darkness. Because these double-mutant females display altered clock gene expression and no circadian rest-activity behavior (Zheng et al., 2001; Oster et al., 2002), it was reasonable to assume that the pups develop in the absence of a maternal clock. The pups were conceived, developed, and grew up in constant darkness and were tested at the age of 6 weeks for wheel-running activity.

We found that the wild-type control as well as the heterozygous offspring displayed a circadian rhythm (Fig. 1A, 1B, 1C) with a $\tau \pm$ SEM of 23.93 ± 0.05 h for wild-type offspring ($N = 18$) (Fig. 1M), 23.19 ± 0.05 h for $mPer1^{Brdm1}/Per2^{Brdm1}$ heterozygous offspring ($N = 40$) (Fig. 1N), and 22.98 ± 0.1 h for $mPer2^{Brdm1}/Cry1$ heterozygous offspring ($N = 25$) (Fig. 1O). The free-running period length of the heterozygous offspring was significantly different from that of their wild-type fathers (Fig. 1N, 1O) whereas this was not the case between the wild-type offspring and their parents (Fig. 1M). The difference in period of heterozygous animals to their fathers is probably caused by difference in genotype. The $mPer1^{Brdm1}/Per2^{Brdm1}$ double-mutant female shown in Figure 1H and the inset in Figure 1K shows an initial ultradian rhythm

Table 1. Within-Litter Synchrony

Litter	n	r	p
W1	8	0.95	***
W2	4	0.85	n.a.
W3	6	0.78	*
P1	9	0.87	***
P2	6	0.9	**
P3	9	0.40	n.s.
P4	9	0.54	n.s.
P5	7	0.56	n.s.
C1	8	0.55	n.s.
C2	9	0.56	n.s.
C3	2	0.73	n.a.
C4	6	0.69	n.s.

NOTE: W = wild-type; P = $mPer1^{Brdm}/Per2^{Brdm}$ heterozygous; C = $mPer2^{Brdm1}/Cry1$ heterozygous mice; n.a. = not applicable (n too small); n.s. = not significant.

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (Rayleigh test).

of 15.2 h, which was subsequently lost. In the beginning, the $mPer2^{Brdm1}/Cry1^{-/-}$ double-mutant mother shows an ultradian rhythm of 11.2 h (Fig. 1I, upper inset in 1L), which was lost later on. It then developed another ultradian rhythm of 14.8 h before it became arrhythmic (Fig. 1I, lower inset in 1L). In general, we observed that all double-mutant females periodically displayed ultradian rhythms.

Because pups are synchronized by mothers, we determined within-litter synchrony. Results are shown in Table 1 for wild-type (W1, 2, 3), $mPer1^{Brdm1}/Per2^{Brdm1}$ heterozygous (P1, 2, 3, 4, 5), and $mPer2^{Brdm1}/Cry1$ heterozygous (C1, 2, 3, 4) offspring.

DISCUSSION

In mammals, mothers are in intimate contact with their offspring to ensure their survival starting from the day of conception. Maternally provided factors such as carbohydrates, lipids, amino acids, and hormones influence the development of the embryo. Because many metabolic pathways in the maternal organism are structured in a circadian fashion, the question arises of whether the maternal circadian clock is imposed on the embryos, leading to maternally mediated circadian rhythms in the offspring. In the 1980s, experiments investigating metabolism of C^{14}-labeled desoxyglucose and pineal N-acetyltransferase in mothers and their pups indicated an influence of the maternal circadian rhythm on their offspring (Reppert and Schwartz, 1983; Reppert et al., 1984; Davis and Gorski, 1985). However, the fetal clock developed normally when the maternal SCN was
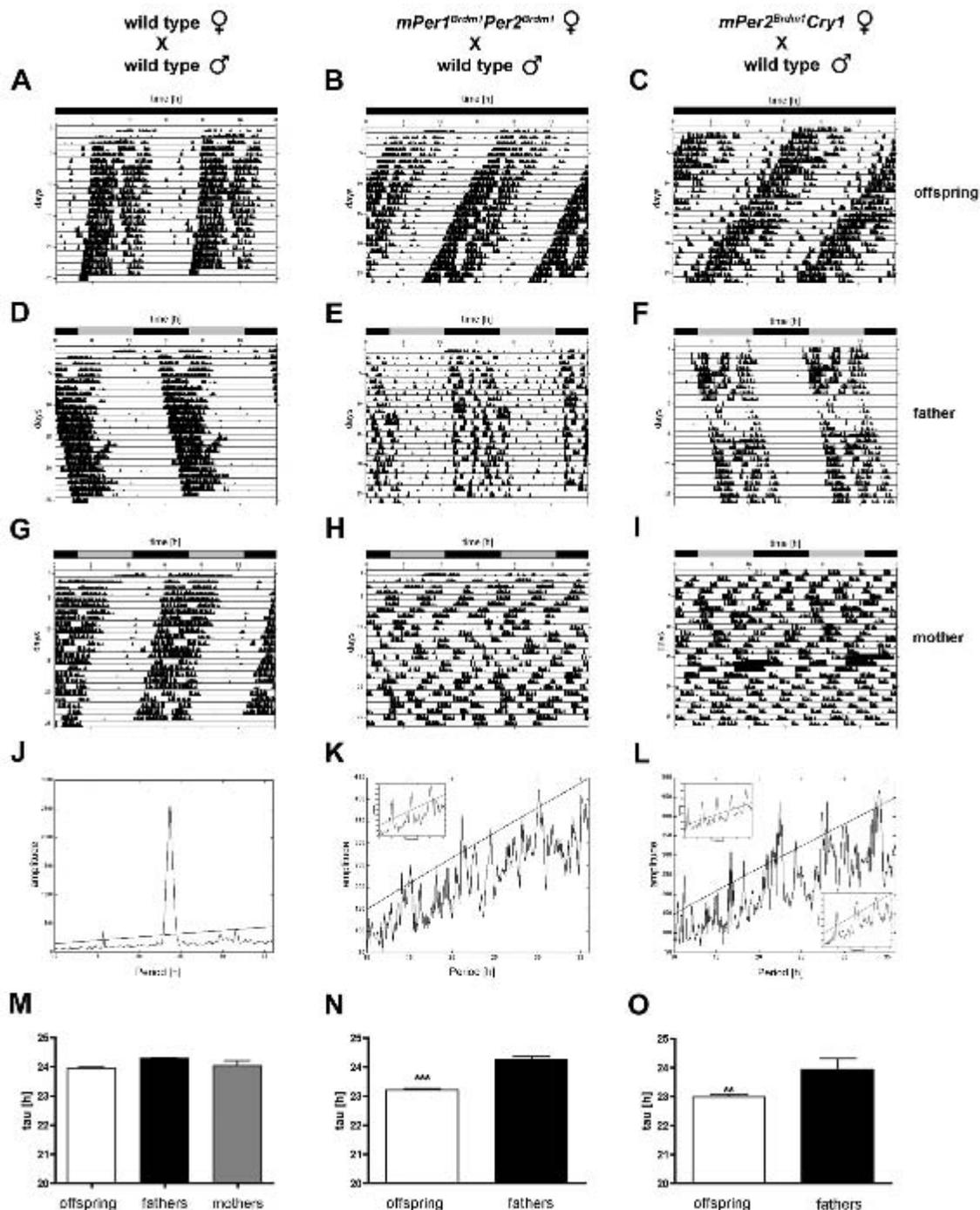
Figure 1. Representative locomotor activity records, χ^2 periodograms, and free-running period length. Locomotor activity records of wild-type (A), $mPer1^{Brdm1}/Per2^{Brdm1}$ heterozygous (B), and $mPer2^{Brdm1}/Cry1$ heterozygous (C) pups born and reared under DD conditions (black bar on the top of the graph), indicating that these animals never experienced an LD cycle. (D-F) Actograms of the corresponding wild-type fathers. Black bar, gray bar = subjective night and day, respectively. (G-I) Actograms of the corresponding mothers. (J-L) The χ^2 periodograms of the mothers; the straight line in the χ^2 periodogram represents a statistical significance of $p < 0.001$. Periodogram (J) corresponds to the activity plot of the wild-type mother shown in (G) ($\tau = 23.7$ h), (K) corresponds to the activity plot shown in (H), and (L) corresponds to the activity plot shown in (I). In (K), the big χ^2 periodogram = entire period of 26 days (arrhythmic), inset upper-left corner = days 1 to 8 ($\tau_1 = 15.2$ h, $\tau_2 = 22.8$ h, $\tau_3 = 30.3$ h). In (L), the big χ^2 periodogram = entire period of 26 days, inset upper-left corner = days 1 to 6 ($\tau_1 = 11.2$ h, $\tau_2 = 16.9$ h, $\tau_3 = 22.5$ h, $\tau_4 = 28.1$ h, $\tau_5 = 33.7$ h), inset lower-right corner = days 10 to 15 ($\tau_1 = 14.8$ h, $\tau_2 = 22.2$ h, $\tau_3 = 29.8$ h). (M-O) Average free-running period length of the offspring compared to their parents. White bar = pups; black bar = fathers; gray bar = mothers. Data are represented as mean ± SEM. Unpaired t test was performed to compare the free-running period length of the pups to their wild-type parents. **$p = 0.0056$; ***$p > 0.0001$.

surgically removed at gestation day 7 (Reppert and Schwartz, 1986; Davis and Gorski, 1988), indicating that a functional maternal SCN is not necessary for development of circadian rhythms in pups. Our observations using a genetic approach confirm these results. Female mice with mutations in *mPer1/Per2* and *mPer2/Cry1* genes do not display circadian wheel-running behavior (Zheng et al., 2001; Oster et al., 2002), and cultured fibroblasts of these double-mutant animals display no circadian rhythmicity in *Bmal1*-promoter–driven luciferase activity (Brown et al., 2005). Because in our experimental set-up, double-mutant females are arrhythmic before and after mating with a wild-type male, there is most likely no influence of the maternal clock on the fertilized egg and the developing embryos. Therefore, the observed circadian rhythms in the heterozygous offspring (Fig. 1) probably developed through an internal program starting the circadian cycling of the clock. However, we cannot exclude the possibility that the ultradian rhythms observed in double-mutant females might contribute to initiate circadian rhythmicity in their offspring.

Table 1 shows that significant within-litter synchrony is observed in wild-type pups, whereas this is not the case for most of the mutant heterozygous litters. If circadian rhythms in the heterozygous offspring were triggered by external factors, the animals within a heterozygous litter would show a good synchronization of clock phase. However, this is not observed (Table 1) except for litters P1 and P2, which were assessed during a period of construction at our university. It also appears that handling, occurring for all animals within a litter at the same time, is probably not the signal for onset of circadian clock activity. In this context, it is important to note that variations in τ are similar between wild-type (23.93 ± 0.05 h; Fig. 1M, white bar) and heterozygous (23.19 ± 0.05 h and 22.98 ± 0.1 h; Fig. 1N and 1O, white bars) animals, indicating that the differences of synchrony within a litter (Table 1) appear not to be caused by τ variation. However, we cannot exclude this possibility. A reciprocal cross with a mutant father and a wild-type mother might shed more light on litter synchronization.

Taken together, we show evidence for autonomous development of circadian rhythms in pups from mothers with a genetic defect of the circadian clock. Our findings are in agreement with previous observations made in SCN-lesioned animals and extend them, allowing to propose autonomous clock development in absence of central and peripheral maternal clocks.

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