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Mop3 Is an Essential Component of the Master Circadian Pacemaker in Mammals

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Summary

Circadian oscillations in mammalian physiology and behavior are regulated by an endogenous biological clock. Here we show that loss of the PAS protein MOP3 (also known as BMAL1) in mice results in immediate and complete loss of circadian rhythmicity in constant darkness. Additionally, locomotor activity in light–dark (LD) cycles is impaired and activity levels are reduced in *Mop3*$^{-/-}$ mice. Analysis of *Period* gene expression in the suprachiasmatic nucleus (SCN) indicates that these behavioral phenotypes arise from loss of circadian function at the molecular level. These results provide genetic evidence that MOP3 is the bona fide heterodimeric partner of mCLOCK. Furthermore, these data demonstrate that MOP3 is a nonredundant and essential component of the circadian pacemaker in mammals.

Introduction

In nearly all organisms, behavioral and physiological processes display 24 hr rhythms that are controlled by circadian pacemakers (Rosbash, 1995; Dunlap, 1999; King and Takahashi, 2000; Wager-Smith and Kay, 2000). Circadian rhythms are regulated by three components: the circadian pacemaker or "clock," an input mechanism that allows the clock to be reset by environmental stimuli, and an output mechanism that regulates physiological and behavioral processes. In mammals, the master circadian pacemaker is located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Moore, 1997). The SCN controls neural and humoral signals that either drive output rhythms directly or synchronize peripheral oscillators with the day–night cycle (Ralph et al., 1990; Silver et al., 1996; Yamazaki et al., 2000).

Circadian oscillators in *Drosophila, Neurospora*, and mice appear to have been highly conserved throughout evolution and to involve transcription–translation negative feedback loops (Wilsbacher and Takahashi, 1998; Dunlap, 1999; King and Takahashi, 2000; Wager-Smith and Kay, 2000). The observation that PAS proteins play important roles in maintaining circadian rhythms in most organisms provides support for this idea (Kay, 1997; Reppert, 1998; Gu et al., 2000). Genetic and biochemical evidence demonstrates that the bHLH-PAS protein CLOCK is required for the proper maintenance of circadian rhythms in mammals. In mice, an ENU-induced deletion in the transactivation domain of mCLOCK results in a lengthened free-running period followed by a gradual loss of circadian rhythmicity (Vitaterna et al., 1994; Antoch et al., 1997; King et al., 1997). Much evidence suggests that the heterodimeric partner of CLOCK is another bHLH-PAS protein. Two candidate partners, known as MOP3 and MOP9 (also known as BMAL2), can form heterodimers with CLOCK that can drive transcription from E box elements found in the promoters of circadian-responsive genes and are coexpressed in neurons of the SCN (Gekakis et al., 1998; Hogenesch et al., 1998; Honma et al., 1998; Takahata et al., 1998; Jin et al., 1999; Yu et al., 1999; Hogenesch et al., 2000; Ikeda et al., 2000; Ripperger et al., 2000).

The comparative genetic approach has provided great insight into the workings of the circadian clock and its transcriptional feedback loop. Similar to mice, the circadian oscillator of *Drosophila* employs a heterodimer of two bHLH-PAS transcription factors known as dCLOCK and dCYCLE (Allada et al., 1998; Darlington et al., 1998; Rutila et al., 1998). The *Drosophila* CLOCK:CYCLE heterodimer also drives transcription from cognate E box enhancers of circadian-regulated genes such as *period* (*per*) and *timeless* (*tim*) (Hao et al., 1997; Darlington et al., 1998). The protein products of the *per* and *tim* loci act to inhibit the transcriptional activity of the CLOCK:CYCLE complex, thereby closing the feedback loop (Bae et al., 1998; Darlington et al., 1998; Lee et al., 1999). In flies, the product of the *cry* locus participates in the light-dependent sequestration and degradation of TIM, which allows the resetting of the clock by light (Emery et al., 1998; Stanewsky et al., 1998; Ceriani et al., 1999). Single mutations at each of the *dClock*, *dcycle*, *tim*, *per*, and *doubletime* (*dbt*) loci can cause arrythmicity of locomotor behavior in flies indicating the essential nature of each of these proteins in the function of the clock (Sehgal et al., 1994; Allada et al., 1998; Price et al., 1998; Rutila et al., 1998).

Applying the comparative approach from *Drosophila* to mammals is informative yet difficult due to the relative complexity of the mammalian genome. Analysis of the mammalian genome indicates that there are often several potential mammalian orthologs for each component of the *Drosophila* clock. As examples, the mouse and human genomes encode three homologs of *per* (*Per1*, *Per2*, and *Per3*) and two homologs of *cry* (*Cry1* and *Cry2*) (reviewed in King and Takahashi, 2000). These and other cases may be an indication of functional redundancy, additional regulatory complexities, or of paralo-

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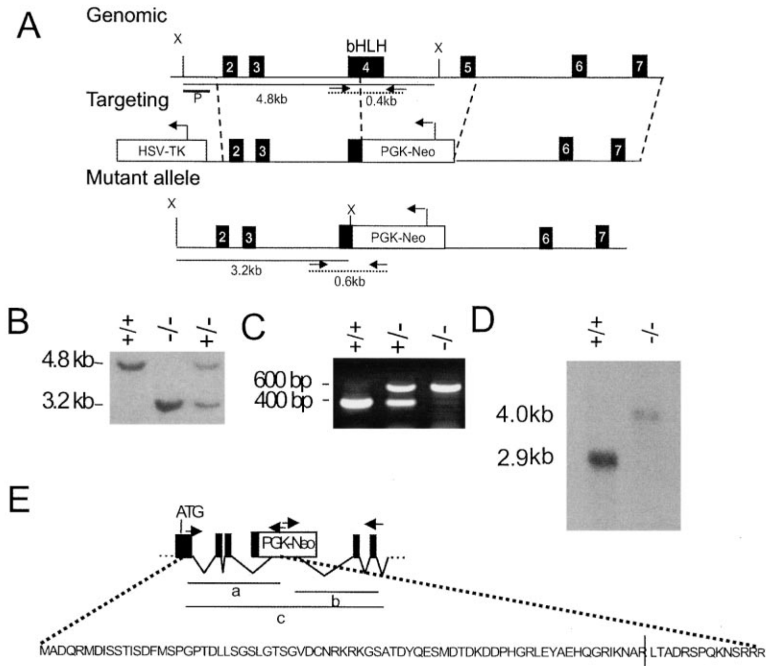

Figure 1. Generation of *mMop3*$^{-/-}$ Mice

(A) Schematic diagram of the region surrounding the bHLH domain of *mMop3*, the targeting construct, and the resulting mutant allele. Exon numbers reflect known coding exons with exon one being the furthest 5′ known start site for MOP3 protein. Dashed lines indicate the regions of homology used for homologous recombination. Solid lines indicate fragment sizes detected by probe (P) following XbaI (X) digest of genomic DNA. Dotted lines represent the fragment sizes generated by PCR genotyping of wild-type and mutant alleles. (B) Southern blot of mouse tail biopsies showing bands of 4.8 kb and 3.2 kb indicating the presence of the wild-type and mutant alleles, respectively. (C) PCR genotyping of tail biopsies showing bands of 400 bp and 600 bp indicating the presence of the wild-type and mutant alleles. (D) Northern blot of mRNA extracted from whole brain tissue. (E) Schematic of the predicted amino acid sequence of isolated transcript from the *Mop3* mutant allele. Black arrows show position of RT-PCR primers and a, b, and c correspond to the three isolated fragments. All three fragments corresponded to a single splice variant that would result in the truncation of the protein 15 amino acids after the splice junction between exon-3 and NeoR sequence (vertical bar).

gous genes that have evolved functions outside of biological rhythms in the mammalian system. In particular, the mammalian CRY proteins demonstrate significant differences in the regulation of the *Drosophila* and mammalian clocks, as mammalian CRY1 and CRY2 appear to possess greater repressor activity of CLOCK than any PER protein. Mice with a double mutation of both *mCry1* and *mCry2* are arrhythmic upon release into constant darkness and are deficient in entrainment of behavior to light cycles. However, these mice still show an increase in *mPer1* and *mPer2* gene expression in response to light indicating that in contrast to *Drosophila* CRY, mammalian CRY1 and CRY2 are not functioning as photoreceptors (Griffin et al., 1999; Kume et al., 1999; Okamura et al., 1999; van der Horst et al., 1999; Vitaterna et al., 1999). Mice with targeted mutations at the *mPer2* and *mPer3* loci have also been generated (Zheng et al., 1999; Shearman et al., 2000). The *Per2*^{Brdm} mice exhibit a shorter rhythm in constant darkness that eventually becomes arrhythmic; whereas, the *Per3* null mice maintain a circadian rhythm in constant darkness, but that rhythm is slightly shorter than wild type. The only known mammalian homolog of *Drosophila tim* appears to be required in mammalian development and may not play a significant role in circadian biology (Gotter et al., 2000). Finally, the *tau* mutation in hamsters is encoded by casein kinase 1 epsilon, a homolog of the *Drosophila* circadian gene, *dbt* (Kloss et al., 1998; Lowrey et al., 2000). Unlike mutants of the core *Drosophila* clock components, no single-gene mutation in mice results in complete abolition of circadian rhythms.

Multiple mammalian homologs of CLOCK and CYCLE also exist. The mammalian genome encodes the originally identified CLOCK protein and a close homolog known as NPAS2 (also known as MOP4), as well as two close homologs of dCYCLE, MOP3, and MOP9 (Hogenesch et al., 1997, 2000; Ikeda and Nomura, 1997; Zhou et al., 1997; Ikeda et al., 2000). The functional and orthologous nature of the murine and *Drosophila Clock* genes are clearly supported by the genetic data showing their respective roles in circadian rhythms (Vitaterna et al., 1994; Antoch et al., 1997; King et al., 1997; Allada et al., 1998). The recent observation that an *Npas2* null allele does not influence this biology is an indication that *mClock* and *mNpas2* are not functionally redundant (Garcia et al., 2000). The idea that hMOP3 is the functional ortholog of dCYCLE is primarily circumstantial and has yet to be demonstrated convincingly by genetic means. This question has taken on increased significance since the recent identification of MOP9 (Hogenesch et al., 2000; Ikeda et al., 2000). The low-level expression of mMOP9 in the SCN leads to the possibility that MOP3 and MOP9 are functionally redundant. Alternatively, it is possible that these two proteins exert differential regulation on the circadian feedback mechanism, or that either one or both of these two proteins plays no role in mammalian circadian biology.

Results

Loss of MOP3 Results in Arrhythmic Behavior under Free-Running Conditions

In an effort to understand the role of these two putative CYCLE homologs in murine circadian rhythmicity, we generated and characterized a null allele at the *mMop3* locus. A targeted disruption of *mMop3* was generated in GS-1 embryonic stem cells by replacing the helix-loop-helix domain within exon 4 and all of exon 5 with a neomycin-resistance (Neo) gene cassette (Figure 1A). No lethality was associated with the targeted *mMop3*

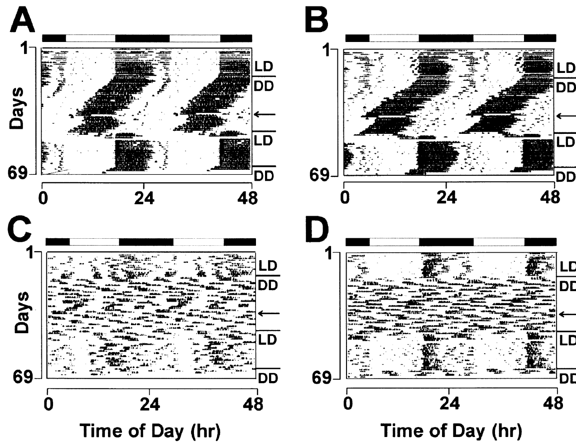

Figure 2. Wheel-Running Activity in Wild-Type and $Mop3^{-/-}$ Mice

(A–D) Representative activity records of individual wild-type (A and B) and $Mop3^{-/-}$ (C and D) littermates are presented in double-plotted format. The bar above the activity record shows the light–dark cycle. As indicated to the right of each record, animals were individually housed in a light–dark (LD) cycle for 14 days and then transferred to constant darkness (DD). On day 22 in DD, animals received a 6 hr light pulse (300 lux) given at CT16 (indicated by the arrow). 10 days after the light pulse, animals were returned to LD for 18 days and then released into constant darkness for 3 days.

allele, as null mice were born with the expected wild-type:heterozygote:null ratio (43:83:45). A larger mRNA transcript from the mutant allele is present and is expressed at low levels compared to the wild-type *mMop3* mRNA (Figure 1D). To ensure no significant protein could be expressed from the targeted *Mop3* allele, we used three sets of RT-PCR primers to amplify the predominant transcript from total brain RNA. We observed that the major transcript from the targeted allele uses cryptic splice acceptor and donor sites within the Neo cassette. The predicted protein sequence shown in Figure 1E truncates 15 amino acids following the splice site between exon 3 and the NeoR cassette. This mutant protein product contains no known functional domains as it truncates prior to the inclusion of the basic region in exon 4, and thus is unlikely to form a functional protein. This analysis and the lack of any phenotype in the $Mop3^{+/-}$ mice (see below) argue strongly that our targeting strategy generated a null allele at *mMop3*.

We hypothesized that if *Mop3* and *Mop9* were not functionally redundant in the circadian system, then a null mutation at the *Mop3* locus would significantly affect the circadian system in mice. Therefore, we examined the wheel-running activity of wild-type, $Mop3^{+/-}$, and $Mop3^{-/-}$ mice by entraining the animals to a schedule of 12 hr light–12 hr dark (LD 12:12) for 14 days and then transferring them to constant darkness (DD). In constant darkness, wild-type and $Mop3^{+/-}$ mice expressed robust circadian rhythms of activity (Figures 2A and 2B, and data not shown; Table 1). Average free-running periods in the wild-type and $Mop3^{+/-}$ mice were 23.63 hr and 23.68 hr, respectively. Spectral analysis using Fast Fourier transform (FFT) detected high-amplitude periodicity in the circadian range (18–30 hr) for both genotypes (Table 1). A 6 hr light pulse administered at CT16 elicited an average phase delay of 3.47 ± 1.17 hr in wild-type mice (Figures 2A and 2B). In contrast, $Mop3^{-/-}$ mice do not express circadian rhythms of locomotor activity in constant darkness (Figures 2C and 2D). Spectral analysis did not detect periodicity in the circadian range for the $Mop3^{-/-}$ mice (Table 1). Moreover, a 6 hr light pulse administered on the 22nd day in constant darkness had no significant effect on locomotor behavior in $Mop3^{-/-}$ mice and did not lead to phase shifts or obvious suppression of activity, as was observed in wild-type mice (Figures 2C and 2D).

The FFT analyses revealed residual, low-amplitude

Table 1. Phenotypic Characteristics of $Mop3^{-/-}$ Mice

Characteristic§	Wild-Type	$Mop3^{+/-}$	$Mop3^{-/-}$	ANOVA p Value
Free running period (hr)	23.64 ± 0.10	23.64 ± 0.12	No rhythm	0.81
Phase shift (hr)	−3.72 ± 0.39	ND	NA	NA
FFT circadian amplitude (% total variance)	16.50 ± 1.83	14.56 ± 1.94	0.26 ± 0.04*	1.00×10^{-6}
FFT ultradian amplitude (% total variance)	1.93 ± 0.20	2.32 ± 0.40	3.29 ± 0.60	0.097
Activity level (wheel counts)				
LD: Total activity OPer 24 hr, days 4–13	37,767 ± 2,980	30,077 ± 3,860	10,079 ± 2,072*	1.00×10^{-6}
% Activity in light phase, days 4–13	4.17 ± 1.00	4.67 ± 2.48	28.62 ± 5.52*	0.00018
DD: Total activity per 24 hr, days 1–20	35,773 ± 5,236	28,425 ± 4,640	18,424 ± 1,678*	0.021
Total activity per 24 hr, days 22–31 (post-light pulse)	35,070 ± 4,611	ND	10,260 ± 1,872*	0.00032
Phase of activity onset in LD, days 4–13 (hr)				
Phase angle relative to light-off (hr)	0.21 ± 0.14	−0.12 ± 0.06	−2.35 ± 0.72*	0.0029
Variance of phase angle relative to light-off (hr)	0.34	0.20	7.68*	5.29×10^{-8}¶

Values are presented as mean ± SEM except variance. Effects of genotype were analyzed by a Generalized Model (GLM) ANOVA by using NCSS (Kayesville, UT) in all cases except variance, in which an F ratio statistical analysis was used. Free running period was determined using χ^2 periodogram for days 1–20 in constant darkness. Phase shift is in response to a 6 hr (~300 lux) phase shift on day 22 in constant darkness. FFT, Fast Fourier transform. FFT circadian amplitude values represent the peak relative amplitude in the circadian range (18–30 hr) normalized a total variance of 100%. LD; 12 hr light–12 hours dark cycle. DD; constant darkness. Phase angle relative to lights-off represents the average time difference between activity onset and lights-off as determined by 24 hr average activity profile. Total activity was measured as total wheel counts per 24 hr period; light phase refers to the light-on portion of the LD cycle.

§ Wild-type, n = 7; $Mop3^{+/-}$ n = 5; and $Mop3^{-/-}$, n = 7 for free running period, FFT peak period, and total activity in DD. For total activity in LD: Wild-type, n = 18; $Mop3^{+/-}$ n = 5; and $Mop3^{-/-}$, n = 17. For phase of activity onset in LD: Wild-type, n = 18; $Mop3^{+/-}$ n = 5; and $Mop3^{-/-}$, n = 15 (due to two animals that failed to entrain). ND: not determined; NA: not applicable.

* Significantly different values as indicated by GLM ANOVA or ¶ F test.

periodicities in the 5–12 hr ultradian range in $Mop3^{-/-}$ mice (Figures 2C and 2D; Table 1) under DD conditions. Ultradian rhythms have been documented in animals with disrupted circadian rhythms, such as *Clock/Clock* mice, SCN-lesioned mice, and SCN-lesioned voles (Gerkema et al., 1990; Schwartz and Zimmerman, 1991; Vitaterna et al., 1994), but such rhythms appear not to be driven by the circadian oscillator (Lehmann, 1977; Gerkema et al., 1993). An oscillatory mechanism will produce a negative value using serial correlation coefficient analyses of activity bout length (Pittendrigh and Daan, 1976). The average serial correlation coefficient values for activity bouts were 0.00223 ± 0.096 in wild-type mice, -0.186 ± 0.152 in $Mop3^{+/-}$ mice, and 0.185 ± 0.072 in $Mop3^{-/-}$ mice. These values indicate that the ultradian bouts are not generated by an oscillatory mechanism. Furthermore, FFT analysis detected low-amplitude ultradian periodocities in the wild-type and $Mop3^{+/-}$ animals that were not significantly different from those in $Mop3^{-/-}$ mice (Table 1). The *Mop3* mutant allele is recessive, as the phenotype of $Mop3^{+/-}$ mice is not different from that of wild-type mice (Table 1).

$Mop3^{-/-}$ Mice Have Altered Activity on Light–Dark Cycles and Reduced Total Activity Levels

In addition to its extreme effect on circadian activity in constant darkness, the *Mop3* mutation also has an unexpected effect on activity in the presence of a light cycle (Figure 2C). Upon examination of wheel running activity in light–dark cycles, all wild-type and $Mop3^{+/-}$ mice began wheel-running within 0.5 hr of lights-off. In contrast, in $Mop3^{-/-}$ mice, the phase of activity onset was highly variable with respect to lights-off (Figures 2C and 2D; Table 1). A 24 hr average activity profile confirmed that of 17 $Mop3^{-/-}$ mice, only 5 began activity within 0.5 hr of lights-off and 2 failed to entrain altogether. The anticipatory phase of the activity rhythm of $MOP3^{-/-}$ mice strongly suggests that "masking" alone cannot account for the rhythmic behavior in light–dark cycles. Furthermore, distribution of activity during the light–dark cycle is significantly altered in $Mop3^{-/-}$ mice: 28% of wheel-running activity in $Mop3^{-/-}$ mice occurs during the light phase, in contrast to 4.2% and 4.1% of activity in the light phase of wild-type and $Mop3^{+/-}$ mice, respectively (Table 1). The $Mop3^{-/-}$ behavior in LD 12:12 cycles could be attributed to poor entrainment of the circadian pacemaker, because the null mice have rhythmic activity that anticipates lights-out. However, we cannot rule out the possibility that "masking" contributes to the activity pattern in response to light. Such acute effects by light and other environmental cues on behavior have been documented in SCN lesioned Syrian hamsters and rats (Wachulec et al., 1997; Redin and Mrosovsky, 1999a, 1999b).

In addition to the effects of the $Mop3^{-/-}$ mutation on circadian rhythmicity, the *Mop3* mutant mice also displayed lower average levels of wheel-running activity as compared to wild-type mice both in a light cycle and in constant darkness (Table 1). Interestingly, such decreases in total activity are not found in *Clock/Clock*, $mPer2^{Brdm1}$, or $Cry1^{-/-}$, $Cry2^{-/-}$ mutant mice (M. H. Vitaterna, personal communication; Vitaterna et al., 1994; Zheng et al., 1999). Taken together, these results indicate that mMOP3 is indeed a core clock component that is essential for the generation and maintenance of circadian rhythms in constant conditions, normal entrainment of behavior to LD cycles, as well as normal activity levels in both LD and DD conditions. Moreover, the $Mop3^{-/-}$ mutation represents an example of a single gene mutation in mammals that causes immediate loss of circadian rhythmicity in constant environmental conditions.

Molecular Analysis of Circadian Regulated Genes in the SCN and Liver of $Mop3^{-/-}$ Mice

The effects of the $Mop3^{-/-}$ mutation on circadian behavior could be the result of disruptions in either the central pacemaker or peripheral output pathways. Therefore, we examined the expression of two known CLOCK-regulated genes in the SCN, *mPer1* and *mPer2* using in situ hybridization. In *Clock/Clock* mutant mice, *mPer1* and *mPer2* expression in the SCN is blunted and nonrhythmic (Jin et al., 1999); thus, we hypothesized that if MOP3 is the true transcriptional partner of CLOCK, expression of these genes would be altered in $Mop3^{-/-}$ mice as well. Wild-type and $Mop3^{-/-}$ mice were sacrificed every 4 hr from 58 hr to 82 hr in constant darkness (3rd and 4th cycles in constant darkness) (Jin et al., 1999). Wild-type animals expressed a robust rhythm of *mPer1* and *mPer2* expression (Figures 3E and 3J). Peak expression of *mPer1* occurred at 66 hr in constant darkness, which corresponds to ~CT6 (Figures 3A and 3B). Peak expression of *mPer2* in wild-type animals occurred at 70 hr in constant darkness, or ~CT10 (Figures 3F and 3G). In contrast, expression levels of both *mPer1* and *mPer2* were not rhythmic and were near baseline levels in $Mop3^{-/-}$ mice (Figures 3C–3E and 3H–3J). The extremely low expression of *mPer1* and *mPer2* in the SCN of $Mop3^{-/-}$ mice provides direct genetic evidence that MOP3 is required for the positive regulation of gene expression in the mammalian circadian pacemaker.

To determine if the *Mop3*-dependent regulation extends to peripheral oscillators, we examined the expression of albumin D-element binding protein (*mDbp*) in liver. We chose *mDbp* because it is under circadian regulation but is not a part of the central feedback mechanism (Lopez-Molina et al., 1997). The regulation of *mDbp* in peripheral tissues appears to be due to direct transcriptional control by CLOCK through E box enhancer elements (Ripperger et al., 2000). Furthermore, *mDbp* null mutant mice show abnormalities in circadian behavior without disruption of the core oscillator in the SCN (Lopez-Molina et al., 1997). In the liver of *Mop3* wild-type mice, *mDbp* mRNA displays a robust rhythm that peaks at 70 hr in DD, or ~CT10, whereas in $Mop3^{-/-}$ mice *mDbp* levels remain low and noncycling at all times (Figure 4A). The disruption of circadian-regulated gene expression in both the SCN and the periphery of $Mop3^{-/-}$ mice demonstrates that MOP3 is essential for the generation of circadian rhythms at the molecular level in the central pacemaker as well as in peripheral oscillators.

The combined analysis of behavior and gene expression in $Mop3^{-/-}$ mice shows that the role of *Mop3* in circadian rhythm generation is not redundant; indeed, the close homolog MOP9 is insufficient to rescue this function of MOP3. To better understand *Mop9* and *Mop3*

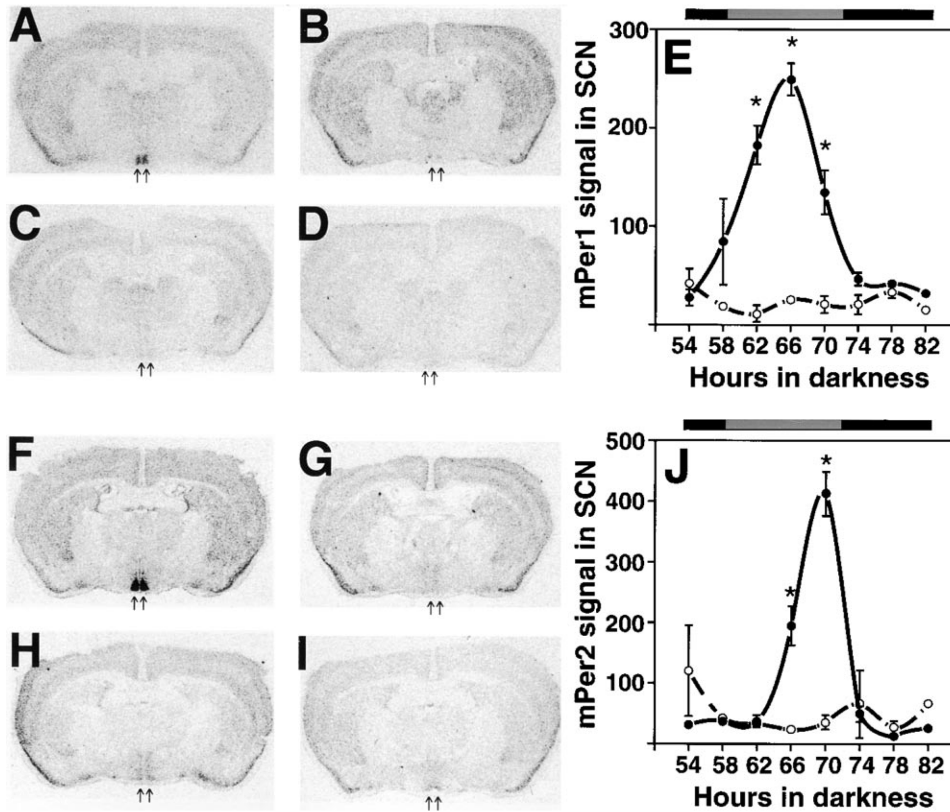

Figure 3. *mPer1* and *mPer2* Expression in the SCN of Wild-Type and *Mop3*$^{-/-}$ Mice

Coronal brain sections from mice sacrificed every 4 hr from 54 hr to 82 hr in constant darkness were hybridized with *mPer1* and *mPer2* riboprobes. (A–E) *mPer1* in situ hybridization. (A) Wild-type *mPer1* hybridization at 66 hr, which corresponds to ~CT6; (B) wild-type *mPer1* hybridization at 78 hr, or ~CT18; (C) *Mop3*$^{-/-}$ *mPer1* hybridization at 66 hr; (D) *Mop3*$^{-/-}$ *mPer1* hybridization at 78 hr; (E) time course of *mPer1* expression in the SCN of wild-type (filled circles) and *Mop3*$^{-/-}$ (open circles) mice. (F–J) *mPer2* in situ hybridization. (F) Wild-type *mPer2* hybridization at 70 hr, which corresponds to ~CT10; (G) wild-type *mPer2* hybridization at 82 hr, or ~CT22; (H) *Mop3*$^{-/-}$ *mPer2* hybridization at 70 hr; (I) *Mop3*$^{-/-}$ *mPer2* hybridization at 82 hr; (J) time course of *mPer2* expression in the SCN of wild-type (filled circles) and *Mop3*$^{-/-}$ (open circles) mice. The bar at the top indicates subjective night in black and subjective day in gray. Asterisks indicate significant differences between wild-type and *Mop3*$^{-/-}$ mice at the times shown (*mPer1* GLM ANOVA, $F(7,35) = 16.41$, $p < 1.0 \times 10^{-6}$; *mPer2* GLM ANOVA, $F(7,35) = 18.61$, $p < 1.0 \times 10^{-6}$, Tukey-Kramer posthoc comparison $p \leq 0.05$).

circadian regulation, we examined their expression levels in the liver from wild-type and *Mop3*$^{-/-}$ animals. In wild-type animals, both the *Mop9* transcript and the *Mop3* transcript cycle in the liver with peak expression at ~CT2. In the *Mop3*$^{-/-}$ mutants, however, the message levels of *Mop9* and the mutant *Mop3* were low and not cyclic (Figures 4B and 4C). In the simplest model, these data suggest that the MOP3-CLOCK protein complex positively activates both genes. Whether or not MOP9 plays a role in the circadian system remains unclear.

Discussion

The results presented here suggest that the *Mop3* locus is of critical importance at all levels of the mammalian circadian system. The results presented here also indicate that the MOP9 homolog is not functionally redundant with MOP3 but is dependent on MOP3 for circadian regulation. At the level of the circadian pacemaker, we provide genetic evidence that the MOP3 protein exerts its effects on the molecular feedback loop by acting as a positive regulator of gene expression. Given the effects of the dominant-negative *Clock* mutation, the effects of the *Mop3* null allele on *Per* gene expression are consistent with an activator role of the CLOCK-MOP3 transcription factor complex (King et al., 1997; Gekakis et al., 1998). The observation that *Mop3*$^{-/-}$ mice do not entrain to a light cycle provides genetic evidence that MOP3 plays a functional role in the input pathway. In support of this idea, a recent study shows that MOP3 (BMAL1) protein levels display a circadian rhythm in the SCN that peaks at night and are reduced upon light stimulus (Tamaru et al., 2000). Finally, the critical role of MOP3 in the circadian output pathway is clearly supported by the arrhythmicity of transcriptional output gene *mDBP* in the liver of *Mop3* mutant mice. Furthermore, *Mop3*$^{-/-}$ mice show decreased activity levels both in a light cycle and in constant darkness indicating that MOP3 may play a role in behavioral outputs beyond its role in generating behavioral rhythms.

The effect of a null mutation of *mMop3* on locomotor activity in constant dark conditions is similar to the phenotype seen in *Cryptochrome 1* and *2* mutant mice. However, on light–dark cycles, *Cry1*$^{-/-}$*Cry2*$^{-/-}$ double

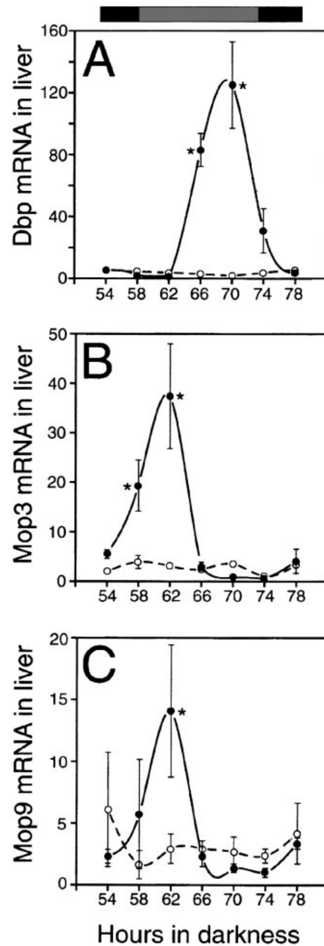

Figure 4. Analysis of *mDbp, mMop3,* and *mMop9* mRNA in Peripheral Tissues of *Mop3*$^{-/-}$ Mice

Analysis of mRNA levels analyzed using TaqMan technology™ from livers taken over a time course of 54–82 hr in constant darkness from wild-type animals (filled circles) and null animals (open circles). The bar at the top indicates subjective night in black and subjective day in gray. (A) Expression of *mDbp*. Asterisks indicate significant differences between wild-type and *Mop3*$^{-/-}$ mice at the times shown (GLM ANOVA, $F(7,34) = 9.36$, $p = 5.0 \times 10^{-5}$, Tukey-Kramer posthoc comparison $p \leq 0.05$). (B) Expression of *mMop3.* Asterisks indicate significant differences between wild-type and *Mop3*$^{-/-}$ mice at the times shown (GLM ANOVA, $F(7,34) = 3.52$, $p = 0.012$, Tukey-Kramer posthoc comparison $p \leq 0.05$). (C) Expression of *mMop9*. Asterisks indicate significant differences between wild-type and *Mop3*$^{-/-}$ mice at the times shown (GLM ANOVA, $F(7,34) = 2.75$, $p = 0.036$, Tukey-Kramer posthoc comparison $p \leq 0.05$).

mutant mice do not show anticipatory activity as was seen in MOP3$^{-/-}$ mice (Vitaterna et al., 1999; van der Horst et al., 1999). Furthermore, the *Cry1*$^{-/-}$*Cry2*$^{-/-}$ double mutant mice also respond behaviorally to light pulses by immediately stopping activity at light onset and immediately starting activity at light offset. Such strong "masking" effects of light were not observed in MOP3$^{-/-}$ mice.

The effect of a *Mop3* mutation on the level of activity is a novel phenotype that has not been reported in *Clock*, *mPer2*^{Brdm}, and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ double mutant mice (Vitaterna et al., 1994; Zheng et al., 1999; M. H. Vitaterna, personal communication). Reduced activity has also been reported in mice with a targeted mutation at the *dbp* locus, a circadian regulated transcription factor that we show here is controlled by MOP3 (Lopez-Molina et al., 1997). It is not clear from our results, however, whether the reduced activity phenotype in the *Mop3*$^{-/-}$ mice is caused by the loss of circadian rhythmicity or whether it may represent a novel role of MOP3 in regulation of metabolic or behavioral outputs. In conclusion, these data represent an example of a single-gene mutation that causes complete abolition of both behavioral and molecular circadian rhythms as well as disruptions in both input (entrainment) and output (activity level) pathways in mammals. Such widespread phenotypic consequences on the circadian system of mice argue that MOP3 rests near the top of the circadian gene hierarchy in mammals.

Experimental Procedures

Generation of *Mop3*$^{-/-}$ Mice

A 7.5 kb region of homology to MOP3 genomic DNA was isolated from BAC DNA (Genome Systems) by long-range PCR. A Neomycin resistance cassette (Neo) was inserted in reverse orientation to the *Mop3* allele into the exon containing the bHLH region deleting DNA from the middle of that exon until just 3′ of the next coding exon. Homologous recombinants were isolated following electroporation of 10 μg targeting construct into GS1 ES cells (Genome Systems). 200 clones surviving G418/Gancycolivir double selection were screened by Southern blot for homologous recombination using a 300 bp probe (PL1225) to a region just outside the 5′ end of the targeting construct on XbaI digest. Heterozygous mice were obtained from these cells by standard methods. Animal care and use procedures were approved by University of Wisconsin, Madison institutional guidelines. Southern blot analysis was performed on genomic DNA from tail biopsies to detect transmission of the targeted allele. Subsequent to the first generation, genotyping was performed using multiplex PCR with OL2646, CCACCAAGCCCAG CAACTCA; OL2647, ATTCGGCCCCCTATCTTCTGC; and OL278, TCGCCTTCTATCGCCTTCTTGACG. OL2626 and OL2647 amplify a 400 bp band corresponding to the wild-type allele. PCR was performed on genomic DNA from tail biopsies for 40 cycles of 95°C, 15 s: 60°C, 15 s: 72°C, 1 min in 1× PCR buffer (Promega) containing 3.5 mM $MgCl_2$. The region in which OL2647 was designed is deleted in the targeted allele therefore in the presence of the targeted allele OL2646 amplifies a 600 bp band with OL278 (Neomycin specific) as a reverse primer. For expression analysis, polyA mRNA was isolated from brains of wild-type, *Mop3*$^{+/-}$, and *Mop3*$^{-/-}$ mice killed at ZT-23 (ZT: Zeitgeber time) using Trizol (Gibco). Northern blot hybridization on 5 μg mRNA was performed using a 210 bp DNA probe specific for a region in the 3′ end of MOP3 coding sequence. Primers used for RT-PCR were: Fragment a OL949, 5′-TGGCAGACCAGA GAATCG and OL278, 5′-TCGCCTTCTATCGCCTTCTTGACG; fragment b, OL662, 5′-GCGCGAGCCCCTGATGCTC and OL489 5′-TTA GGATGCAGGTAGTCAAACA; fragment c, OL949 and OL489.

Behavioral Analysis

Mice were singly housed in cages equipped with running wheels on an LD 12:12 cycle for 2 weeks before being released into constant darkness. Animal care and use procedures were approved by Northwestern University institutional guidelines. A subset of wild-type and Mop3$^{-/-}$ mice ($n = 7$ each genotype) received a 6 hr light pulse (~300 lux) on day 22 in DD. Activity data were collected as previously described (Vitaterna et al., 1994; Antoch et al., 1997; King et al., 1997). χ^2 periodogram, Fast Fourier transform (FFT), least squares fit of activity onset, and total activity analyses were performed using ClockLab (Actimetrics, Evanston, IL). χ^2 periodogram was performed for days 1–20 in constant darkness at 1 min resolution over the 5–36 hr period range. FFT was performed for days 1–20 in constant

darkness as an amplitude spectrum normalized over the frequency range of 0 to 1 cycles/hour. Circadian time or CT indicates the phase of the animal's endogenous circadian rhythm while in free-running conditions, where CT0 marks the beginning of the subjective day and CT12 marks the beginning of the subjective night. Serial correlation coefficients of activity bout length were performed within ClockLab (Actimetrics, Evanston, IL) for the first 20 days in DD with maximum gap length 90 min and threshold of 20 counts/minute. As a control, we also calculated the average serial correlation coefficient for circadian activity onsets in the wild-type and $Mop3^{+/-}$ mice for the first 20 days in constant darkness. The average value of -0.519 ± 0.067 in wild-type (Student's t test; $p = 0.00025$) indicates that the daily onset of activity in constant conditions is generated by a (circadian) oscillatory mechanism. Because the $Mop3^{-/-}$ mice do not have a discernable circadian onset of activity, such an analysis cannot be performed on mutant mice. For analysis of entrainment to Light:Dark cycling of days 4–13 in LD, the 9 day average activities (wheel revolutions) were plotted against the 24 hr period using ClockLab (Actimetrics, Evanston, IL). Onset of activity was designated as the phase at which 20% of the peak activity was reached.

Gene Expression Analysis

Animals were sacrificed every 4 hr under infrared light via cervical dislocation beginning at 54 hr in constant darkness (third cycle in constant darkness). Brains were dissected under dim red light, frozen on dry ice, and stored at −80°C before sectioning. Peripheral tissues were then collected under white light, frozen on dry ice, and stored at −80°C before RNA extraction. In situ hybridization procedures were performed as described (Vitaterna et al., 1999). Riboprobes for *mPer1* and *mPer2* were generated from nucleotides 474–856 (GenBank AF099229) and nucleotides 243–701 (GenBank AF035830), respectively. Exposure time was 14 days, and hybridization signal was quantified as described (Vitaterna et al., 1999). For liver expression analysis, total liver RNA was isolated using Trizol; followed with DNase treatment using Amp-grade DNase (Gibco). Three micrograms of total RNA was reverse transcribed using Superscript II (Gibco) and 25 ng total RNA equivalent was analyzed by TaqMan technology (PE-Applied Biosystems) (Heid et al., 1996) using an ABI7700 (Perkin Elmer). Primers and probes used for *mMop3*, *mDbp*, and *mMop9* were as follows:

- *mMop3* forward, 5′-CCAAGAAAGTATGGACACAGACAAA-3′
- *mMop3* reverse, 5′-GCATTCTTGATCCTTCCTTGGT-3′
- *mMop3* probe, 5′-TGACCCTCATGGAAGGTTAGAATATGCA GAAC-3′
- *mDbp* forward, 5′-CGTGGAGGTGCTTAATGACCTTT-3′
- *mDbp* reverse, 5′-CATGGCCTGGAATGCTTGA-3′
- *mDbp* probe, 5′-AACCTGATCCCGCTGATCTCGCC-3′
- *mMop9* forward, 5′-TGGTGCCTTCGTGACTCTGA-3′
- *mMop9* reverse, 5′-GTTGACAGACACAATGTACTCCAGC-3′
- *mMop9* probe, 5′-TTCAGCTTCACAAACCCTTGGACCAAAG-3′

The obtained CT (cycle number at which amplification threshold of detection is reached) values were normalized to Rodent GADPH (PE Applied Biosystems) expression by the $\Delta\Delta$Ct method using trough wild-type levels as the calibrator value. The mean $\Delta\Delta$CT was converted to relative expression values by the equation $2^{-\Delta\Delta CT}$ and a range was calculated by $2^{-(\Delta\Delta CT + Stdev\Delta\Delta CT)}$. Values shown are $2^{-\Delta\Delta Ct}$ ($\pm$ range of $2^{-\Delta\Delta CT}$).

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