Functional central rhythmicity and light entrainment, but not liver and muscle rhythmicity, are Clock independent

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Submitted 29 March 2006; accepted in final form 15 May 2006

Kennaway, David J., Julie A. Owens, Athena Voultsios, and Tamara J. Varcoe. Functional central rhythmicity and light entrainment, but not liver and muscle rhythmicity, are Clock independent. Am J Physiol Regul Integr Comp Physiol 291: R1172–R1180, 2006.—The circadian rhythmicity of hormone secretion, body temperature, and sleep/wakefulness results from an endogenous rhythm of neural activity generated by clock genes in the suprachiasmatic nucleus (SCN). One of these genes, Clock, has been considered essential for the generation of cellular rhythmicity centrally and in the periphery; however, melatonin-proficient Clock mice retain melatonin rhythmicity, suggesting that their central rhythmicity is intact. Here we show that melatonin production in these mutants was rhythmic in constant darkness and could be entrained by brief single daily light pulses. Under normal light-dark conditions, per2 and prokineticin2 (PK2) mRNA expression was rhythmic in the SCN of Clock mutant mice. Expression of Bmal1 and npas2 was not altered, whereas per1 expression was arrhythmic. In contrast to the SCN, per1 and per2 expression, as well as Bmal1 expression in liver and skeletal muscle, together with plasma corticosterone, was arrhythmic in Clock mutant mice in normal light-dark conditions. npas2 mRNA was also arrhythmic in liver but rhythmic in muscle. The Clock mutation does not abolish central rhythmicity and light entrainment, suggesting that a functional Clock homolog, possibly npas2, exists in the SCN. Nevertheless, the SCN of Clock mutant mice cannot maintain liver and muscle rhythmicity through rhythm outputs, including melatonin secretion, in the absence of functional Clock expression in the tissues. Therefore, liver and muscle, but not SCN, have an absolute requirement for CLOCK, with as yet unknown Clock-independent factors able to generate the latter.

cyclic; melatonin; clock genes

THE CONSTANCY OF THE DAILY and seasonal changes in the time of sunrise and sunset and the duration of the day have facilitated the development of complex timing systems in a wide range of organisms. Animals use the timing system to predict when food or a predator is likely to appear during the day, to coordinate physiological capacity with demand, or to prepare the reproductive organs for mating. Endogenous daily (circadian) rhythmicity is generated at the molecular level centrally and peripherally and entrained to the environment by retinal perception of light. A suite of transcription factors called clock genes, which include Clock (46), the period genes (per1, per2, and per3) (39, 41, 51), the cryptochromes (cry1 and cry2) (45), and Bmal1 (6), interact to generate cellular rhythms centrally in the hypothalamic suprachiasmatic nucleus (SCN) and in peripherally tissues. The CLOCK and BMAL1 proteins interact to initiate transcription of the period (per) and cryptochrome (cry) genes, and PER and CRY feed back to regulate their own transcription through an interaction with the CLOCK/BMAL1 heterodimer in generating cellular rhythms (32).

The SCN fulfills the criteria for the master clock, with endogenous metabolic (35) and neuronal rhythmicity in vivo (15) and in vitro (12). It influences a wide range of organs, including brain, pineal gland, adrenal, liver, heart, and pancreas, through neural connections and hormonal secretion into the cerebrospinal fluid (3). It is clear that the same genes that are responsible for SCN rhythmicity are rhythmically expressed throughout the body (31). Rhythmic clock gene expression has been demonstrated in many organs, including liver, heart, kidney, muscle, pancreas, lung, and ovary. Because the phasing of rhythmicity of each organ appears to be different and to lag several hours behind SCN rhythm, the following question is raised: Is peripheral rhythmicity completely endogenous, or does it remain dependent on the SCN? It is well established that lesions of the SCN result in behavioral arrhythmicity (40) and can lead to continuous pineal melatonin secretion (17). SCN lesions also resulted in the loss of rat per2 rhythmic expression in liver, heart, kidney, and several other tissues (34). In SCN-lesioned mice, a range of genes in liver that are normally rhythmically expressed were arrhythmic in continuous darkness (except for per2) (1). When direct SCN influences were removed by culture of tissues from transgenic rats expressing a per1-luciferase reporter in vitro, rhythmic gene expression persisted for only two to seven cycles (48). These findings suggest that the SCN has a primary role as the pacemaker driving peripheral oscillations. By contrast, tissues from a per2-luciferase reporter mouse retained per2-luciferase rhythmicity for many days in culture, including tissues that had been removed from behaviorally arrhythmic, SCN-lesioned animals (50). This outcome implies that the SCN has only a phase-coordinating role, rather than a role as a pacemaker driving peripheral oscillators, in contrast to the prevailing paradigm.

To help resolve this issue and to clearly define the role of the SCN and that of the clock genes in central and peripheral rhythmicity, we have developed a novel Clock Δ19 + MEL mutant mouse (21). The ClockΔ19 mutant mouse, originally developed by in vivo N-ethyl-N-nitrosourea mutagenesis (46), produces a truncated protein that is capable of binding to BMAL1 but is not capable of driving transcription (10) of per, cry, or dec1 in vitro. The wheel-running rhythm of the

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ClockΔ19 + MEL mutant mouse is poorly entrained to a light-dark (LD) cycle, but, on release into constant darkness, an abnormally long (27- to 28-h) free-running period is established that persists for 7–14 days, whereupon the mice become arrhythmic (46). This is consistent with loss of central and peripheral rhythmicity in the absence of a functional CLOCK protein. However, the original ClockΔ19 mutant was developed in a strain (C57Bl/6) that carries spontaneously generated mutations in the genes for the key enzymes involved in the synthesis of melatonin (aryalkylamine-N-acetyltransferase and hydroxyindole-O-methyltransferase) (9). This mutant, therefore, lacks a major pathway by which central rhythmicity is evident and acts to regulate rhythms throughout the body. We have crossed the ClockΔ19 (Balb/c) mutant mouse with the melatonin-producing CBA strain to produce the ClockΔ19 + MEL mutant mouse (21). Despite the mutated CLOCK protein, this strain produces melatonin rhythmically in an LD cycle and for at least two cycles in constant darkness. Moreover, wheel-running rhythmicity of approximately half of the mutants could be entrained to a single brief light pulse presented every 24 h in constant darkness. This suggested that functional SCN rhythmicity persists in the ClockΔ19 + MEL mutant mouse and that central rhythmicity may not be dependent on a functional CLOCK protein.

To test this hypothesis, we characterized Bmal1, per1, per2, prokinecin2 (PK2), and npas2 expression in the ClockΔ19 + MEL mutant mouse SCN to determine whether central cellular rhythmicity was intact. We also assessed more fully the functional rhythmicity of ClockΔ19 + MEL mutant mice by determining whether the melatonin rhythm was sustained in continuous darkness and whether it could be entrained to a brief light pulse presented every 24 h. We further hypothesized that if functional SCN rhythmicity persisted in the ClockΔ19 + MEL mutant, then liver and muscle rhythmicity may also be present, despite the lack of a functional CLOCK protein, due to similar redundant mechanisms to SCN, or the presence of the melanatonin rhythm. Rhythmicity in terms of key clock gene expression was therefore measured in liver and skeletal muscle of the ClockΔ19 + MEL mutant mouse.

MATERIALS AND METHODS

Animals and behavioral experiments. Adult male and female ClockΔ19 + MEL mutant mice (17) and their wild-type controls (2 mo old) were obtained from our breeding colony and maintained under a 12:12-h LD (12L:12D) schedule. The experiments outlined were approved by the University of Adelaide Animal Ethics Committee. The ClockΔ19 + MEL mutant strain was established by selective breeding of ClockΔ19 (Balb/c) mutant mice (46) kindly provided by Drs. J. Taka-hashi and M. Vitaterna) with melanatonin-proficient (11) CBA/CaH mice. The resulting mutant mice have functional pineal arylalkylamine-N-acetyltransferase and hydroxyindole-O-methyltransferase andsynthesize melatonin. Behavioral rhythmicity was monitored in mice housed individually in cages equipped with 11.5-cm-diameter running wheels. A data acquisition system (LabPro, Data Sciences, St. Paul, MN) was used to record the number of wheel rotations in 10-min bins. The χ² method was used for period analysis, and actograms were prepared using Actiview software (MiniMitter, Bend, OR). After acclimation to the running wheels for ~8 days in a 12L:12D photoperiod, 32 wild-type and 55 ClockΔ19 + MEL mutant mice (male and female) were released into constant darkness for 14 days. On the basis of the actogram and periodogram information, the mice were killed at predetermined times after the onset of the major wheel-running episode. On the basis of our previous studies of the times of peak melatonin production in both genotypes in the LD photoperiod, the majority of animals were killed 6–14 h after the onset of running.

In a separate experiment, 79 ClockΔ19 + MEL mutant mice (8 wk, male and female) were acclimated to the wheels in the LD photoperiod for ~8 days and then released into a “skeleton” photoperiod. Thus, on the 9th morning, the lights remained off for 6 h and then were turned on for 15 min. For the next 14 days, the mice were exposed to this single light pulse on a background of continuous darkness. On the last day of the experiment, the pulses were not given, and the mice were killed between 6 h before and 1 h after the expected pulse. On the basis of actograms and periodogram analysis, the mice were divided into those that entrained to the light pulse (i.e., a period of ~24 h), those that had a period close to that observed in constant darkness, and those that were behaviorally arrhythmic (no significant periodicity, P > 0.05).

Hormone assays. Trunk blood plasma melatonin was assayed by RIA with reagents obtained from Buhlmann Laboratories (Allschwil, Switzerland), as previously described (22). Briefly, plasma (100 μl) was added to prewashed Cl2O reverse-phase columns and sequentially washed with 10% methanol and hexane. Melatonin was eluted with pure methanol, the solvent was evaporated in a stream of air, the residue was reconstituted in 1 ml of buffer, and two 400-μl aliquots were subjected to RIA. Sensitivity of the assay was 10 pM, and intra- and interassay coefficients of variation were <10% and <15%, respectively, across the range of the standard curve. Pituitary glands were homogenized in 1 ml of RIA buffer, and 100-μl aliquots were assayed by direct RIA with a sensitivity of 21 fmol/gland. Plasma corticosterone (10 μl) was assayed by ELISA according to the manufacturer’s instructions (IDS, Boldon, UK).

Real-time RT-PCR. For investigation of the circadian expression of clock genes in an LD cycle, wild-type and ClockΔ19 + MEL mutant mice (8 wk old, 3 males and 3 females) were killed at 4-h intervals (0800, 1200, 1600, 2000, 2400, and 0400). Brain, liver, and gastrocnemius muscle were rapidly dissected and immediately placed in RNAlater (Ambion, Austin, TX) at ~20°C until they were processed (20). Total RNA from ~100 mg of liver and muscle was extracted with 1 ml of TriReagent (Sigma Chemical, St. Louis, MO) according to the manufacturer’s protocol. The samples were stored at −20°C until further use. Primers (Table 1) were designed with the ABI Prism Primer Express program (Applied Biosystems, Foster City, CA).

Brains were removed from RNAlater, trimmed, and glued to a stage, and a 400-μm coronal section encompassing the SCN was prepared using a Vibraslice (Campden Instruments, London, UK). The SCN was carefully dissected under a dissecting microscope and transferred to 100 μl of lysis solution (Ambion) and frozen at ~20°C until it was processed. Total RNA was extracted from the SCN and treated with DNase with use of RNaseous-micro (Ambion) according to the manufacturer’s instructions. For the SCN, all the RNA (20 μl) was reverse transcribed in a final volume of 100 μl (4 μl of RT buffer, 2 μl of DTT, 2 μl of dNTPs, 1 μl of transcriptase, and 69 μl of water).

Amplification of cDNA was performed on a GeneAmp 7000 sequence detection system (Applied Biosystems). cDNA (5 μl), 2 μl of 0.625 μM forward and reverse primers, 1 μl of water, and 10 μl of SYBR Green (Applied Biosystems) were added to each well. The samples were amplified in duplicate for 1 cycle of 50°C for 2 min and 95°C for 10 s, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The quantification cycle (Cq) values were used as a measure of gene expression using the comparative Cq method (20). The endogenous control gene used was hypoxanthine-guanine phosphoribosyltransferase (HPRT). The relative gene expression levels were calculated using the 2⁻ΔΔCq method (20).
95°C for 10 min and then 40 cycles of 95°C for 15 s and 60°C for 1 min. An arbitrary threshold of fluorescence was set within the exponential phase of amplification, and the cycle at which the signal exceeded this threshold was designated the cycle threshold (Ct).

The expression of each gene within each sample was normalized against β-actin and expressed relative to a calibrator sample with the use of the formula 2^(-ΔΔCt), as described by K. Livak (Sequence Detector User Bulletin 2, PE-ABI). Expression of β-actin did not vary significantly across the six time points studied (P > 0.05 by ANOVA). The calibrator sample was designated the most highly expressed time point for each gene of interest and, therefore, has a relative expression of 1.

All results for Clock^A19 + MEL mutant mice were compared with the wild-type calibrator sample set at 1. Because all samples from three mice of each genotype were assayed on a single 96-well plate for each gene, we were able to consider not only differences in the time of peak and amplitude but also relative differences in total expression of the genes between genotypes.

**Results**

**Pineal and plasma melatonin.** Wild-type mice maintained in continuous darkness for 14 days showed a peak in pineal and plasma melatonin levels ~10–13 h after commencement of the activity bouts (Fig. 1, A–C). Wheel-running activity free ran in all the wild-type mice with a period of 23.5 ± 0.05 h (n = 32). In Clock^A19 + MEL mutant mice that maintained free-running behavioral rhythmicity in continuous darkness for 14 days (51% of the animals, period = 26.85 ± 0.13 h), melatonin levels peaked ~12–13 h after the onset of running activity.

**Statistical analysis.** The times of peak pineal and plasma melatonin levels were estimated graphically by means of three-point moving averages. Gene expression data were analyzed by univariate ANOVA using SPSS version 13.0, with genotype and time of day as the fixed factors. The effects of independent factors were further analyzed by one-way ANOVA after logarithmic transformation when appropriate. P < 0.05 was considered significant.

### Table 1. Primer sequences used for the real-time RT-PCR analyses

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<th>Reverse</th>
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**Fig. 1.** Pineal and plasma melatonin concentrations and wheel revolutions in wild-type (A–C) and Clock^A19 + MEL mutant (D–F) mice maintained in constant darkness and pineal and plasma melatonin concentrations and wheel revolutions in Clock^A19 + MEL mutant mice entrained (G–I) and free running (J–L) in a skeleton photoperiod of 15 min of light every 24 h. Pineal and plasma melatonin data are from individual animals killed at specific times relative to onset of wheel-running activity. Median number of wheel revolutions (Rev) per 10 min is plotted for the night before the mice were killed. By convention, CT12 is designated the onset of running in constant conditions. In G–I, vertical arrow indicates time of the expected light pulse. For the skeleton photoperiod, by convention ZT0 is designated the time of the entraining signal, in this case, the single light pulse. For J–L, data are from mice that failed to entrain to the skeleton photoperiod and free ran instead. Data are plotted relative to the time of onset of running, with the convention of CT12 designated the time of onset of activity.

Horizontal arrow in C, F, I, and L indicates 12 h from the onset of running or back from the time of the expected pulse.
(Fig. 1, D and E). In Clock$^{+/−}$ + MEL mutant mice that entrained behaviorally to the daily 15-min light pulses (40% of the animals, period = 24.02 ± 0.06 h), melatonin levels peaked at around the time of the light pulse, which was, again, 10–12 h after initiation of running activity (Fig. 1, G and H). In the mutant mice that free ran, despite the daily light pulses (44% of the animals, period = 27.1 ± 0.1 h), melatonin levels were highest toward the end of the period of running activity (Fig. 1, J and K).

Plasma corticosterone. Plasma corticosterone increased four- to fivefold during the late light period in wild-type mice and then decreased rapidly over the next 4 h to basal levels (Fig. 2). By contrast, Clock$^{+/−}$ + MEL mutant mice failed to show any late afternoon surge in corticosterone.

Clock gene mRNA expression. Wild-type mice showed rhythmic expression of per2 mRNA in the SCN (P < 0.05), with maximal expression late in the light period (~4 h before lights off) and a fivefold rhythm amplitude (Fig. 3, Table 2). Clock$^{+/−}$ + MEL mutant mice also expressed per2 mRNA rhythmically in the SCN (P < 0.05), with a similar fivefold amplitude. In the case of per2, maximum expression in the Clock$^{+/−}$ + MEL mutant mice was approximately one-third that of the wild-type mice, and the peak occurred ~4 h later (Fig. 3).

Expression of Bmal1 mRNA in wild-type mouse SCN was highest 4 h into the dark phase, but no significant rhythmicity was detected (by ANOVA; Fig. 3). A similar level of Bmal1 mRNA expression was apparent in the Clock$^{+/−}$ + MEL mutant mice but did not change significantly across 24 h. By contrast, per1 mRNA was rhythmically expressed in wild-type SCN, with amplitude and phase similar to per2; however, no significant rhythm was detected in Clock$^{+/−}$ + MEL mice. npas2 mRNA was detected in the SCN of wild-type and Clock$^{+/−}$ + MEL mice at similar levels of expression, but there was no significant change (P > 0.05) across the day in a group of mice (Figs. 3 and 4).

PK2 mRNA in the SCN of wild-type mice was rhythmic (P < 0.05), with peak expression in the early light period and an amplitude of ~10- to 15-fold (Fig. 3). PK2 mRNA was also rhythmic (P < 0.05) in the SCN of Clock$^{+/−}$ + MEL mutant mice, with the peak at around lights off and an eightfold change in expression across 24 h. The maximum expression of PK2 was approximately one-third that of the wild-type mice, and the temporal patterns of expression of per2 and PK2 were highly correlated in both strains: r = 0.58 (P < 0.01) for wild-type and r = 0.35 (P < 0.05) for Clock$^{+/−}$ + MEL. In wild-type mice, the peak PK2 mRNA expression preceded that of per2.

Expression of Bmal1, per1, and per2 mRNA in liver of wild-type mice exhibited high-amplitude rhythms (P < 0.05; Fig. 5, Table 2). By contrast, Bmal1 mRNA was constitutively, but not rhythmically, expressed (P > 0.05) in liver of Clock$^{+/−}$ + MEL mutant mice. Expression of per1 and per2 mRNA was low and not rhythmic (P > 0.05) in liver of Clock$^{+/−}$ + MEL mutant mice. Analysis of the Clock homolog npas2 in liver revealed a high-amplitude rhythm of expression in wild-type mice, with the pattern of expression similar to that of Bmal1 (Fig. 5), but low-amplitude and arrhythmic expression in the Clock$^{+/−}$ + MEL mutant mice.

In muscle of wild-type mice, there was a similar very high-amplitude rhythm of Bmal1, per1, and per2 mRNA expression (Fig. 6, Table 2) but no significant change in their expression across 24 h in Clock$^{+/−}$ + MEL mutant mice. As observed in liver, there was a high-amplitude npas2 mRNA rhythm (>40-fold change across 24 h) in muscle of wild-type mice. Clock$^{+/−}$ + MEL mutant mice exhibited a significant (P < 0.05), but low-amplitude, change (3-fold) in npas2 expression in muscle across 24 h. Peak expression for both genotypes occurred at 0800. To determine whether this change in npas2 expression might indicate the presence of functional rhythmicity through an Npas2-Bmal1 interaction in the muscle of Clock$^{+/−}$ + MEL mice, levels of expression of the clock-controlled gene rev erba were studied. There was a significant high-amplitude (10-fold) rhythm of rev erba mRNA expression in wild-type mice and a significant (P = 0.04) low-amplitude change in expression in Clock$^{+/−}$ + MEL mice across the 24 h (Fig. 6). In contrast to the npas2 rhythm, peak expression of rev erba occurred 4 h earlier in the mutant than in the wild-type mice.

DISCUSSION

In this study, we have shown, for the first time, that endogenous central neuroendocrine (melatonin) rhythmicity was preserved in melatonin-proficient mice carrying the Clock$^{+/−}$ mutation, whereas cellular rhythmicity was largely absent in liver and muscle tissue. Clock$^{+/−}$ + MEL mutant mice are also able to entrain the neuroendocrine rhythm to a very brief (15 min) light pulse presented every 24 h. This implies that the retina, retinohypothalamic tract, and SCN output pathways (17) to the pineal gland are functional in Clock$^{+/−}$ + MEL mutants. Our previous studies of Clock$^{+/−}$ + MEL mutant mice showed that they produced melatonin rhythmically in a 12:12 photoperiod (21). The timing of the peak secretion was 2 h later than that of the wild-type melatonin-proficient mice (just before lights on), consistent with their long endogenous free-running period of behavioral rhythmicity.

In the original study describing the melatonin-deficient Clock$^{+/−}$ mutant mice, it was reported that they were able to phase shift wheel-running activity in response to a 6-h light “pulse” (46). The exposure of behaviorally arrhythmic mutants to 6 h of light, while they were maintained in otherwise continuous darkness, at least temporarily, restored long-period circadian rhythmicity (46). We previously reported that 58% of Clock$^{+/−}$ + MEL mutant mice entrained their wheel-running activity to a skeleton photoperiod (21), suggesting that the
entrainment pathway for behavior remained intact. In the present experiment, 40% of the mutants entrained their wheel-running activity to the pulses. The fact that 44% continued to free run does, however, indicate some degree of impairment of circadian rhythm control in the mutant mice, although it is also possible that they have an altered sensitivity to light and require a higher-intensity light pulse. Nevertheless, these findings, together with the present demonstration of neuroendocrine entrainment, provide compelling evidence that functional, entrainable central rhythmicity is preserved in the Clock\[^{Δ19}\]/H11001 MEL mutant mice.

Rhythmic SCN expression of a gene capable of driving the behavioral rhythm, PK2 (7), was preserved in Clock\[^{Δ19}\]/H11001 MEL mutant mice. The amplitude of the rhythm of PK2 gene expression was similar in wild-type and Clock\[^{Δ19}\] + MEL mutant mice, but the overall level of expression was reduced in the mutant mice. In a previous study, a blunted PK2 mRNA rhythm was reported in the SCN of Clock\[^{Δ19}\] (Balb/c) mutant mice (7). PK2 expression is closely associated with behavioral activity, with higher levels of the protein presumed to be secreted into the cerebrospinal fluid during the day than at night.

### Table 2. Impact of time of day on mRNA expression for clock genes in SCN, muscle, and liver of wild-type and Clock\[^{Δ19}\]+MEL mutant mice

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<th>per1</th>
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<tr>
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Values represent probability (by 1-way ANOVA). Wild-type SCN per1 Data for wild-type suprachiasmatic nucleus (SCN) were logarithmically transformed before ANOVA because of significant heterogeneity of variance. *Significant difference across 24 h (P < 0.05).
The temporal association between PK2 mRNA expression and wheel running is apparently preserved in Clock/H9004/H11001 MEL mutant mice, because the timing of peak expression (4–6 h later than in wild-type mice) is consistent with the endogenous period of 27–28 h they show in constant darkness (21).

There is also a strong temporal and quantitative relation between per2 and PK2 mRNA expression in the SCN of wild-type and Clock/H9004/H11001 MEL mutant mice. Transcription of both genes is induced through bHLH/PAS protein complex binding to E-boxes in the promoters of the genes in normal mice (7, 42); therefore, the relation we observed between the two genes in Clock/H9004/H11001 MEL mutant mice provides strong circumstantial evidence that rhythmic gene transcription in the SCN of Clock/H9004/MEL mutant mice is generated in a similar manner. Previous studies have shown that cry1, cry2 (24), Bmal1 (29, 36), per3 (16), and possibly per1 (27) are not rhythmically expressed in the SCN of Clock/H9004 mutant mice. Per2 mRNA has, however, been shown previously to be expressed rhythmically at low levels in Clock/H9004 mutant mouse SCN via in situ hybridization (16) and also to be transiently induced by an evening light pulse (37). Rhythmic expression of per2 in Clock/H9004 mutant mice has not previously been well explained. Clock/H9004 mutant mice are not null mutants and do produce a protein that is capable of heterodimerizing with its partner BMAL1 but is apparently unable to initiate transcription via an E-box (10). Although some activation by CLOCK/H9004/BMAL1 heterodimers may occur in vivo, a more likely explanation of residual rhythmicity involves the recruitment of another bHLH/PAS protein to partner with BMAL1 in SCN cells.

A likely candidate is the product of the homolog of Clock, npas2 (30). It is expressed in the forebrain, where the protein has been shown to have a functional role, along with BMAL1, in inducing per1, per2, and cry1 genes (30). The possibility that npas2 is involved in SCN rhythmicity has previously been discounted, because an in situ hybridization study of bHLH/PAS gene expression in the mouse SCN failed to detect any npas2 message in the SCN region (38). A more recent study has, however, reported low-amplitude rhythmic expression of npas2 in normal Balb/c mouse SCN (44). In the present study, we report, for the first time, the high, apparently constitutive or at least low-amplitude rhythm of npas2 expression in the SCN of Clock/H9004/H11001 MEL mutant mice. We suggest that, in the absence of a fully functional CLOCK protein, as occurs in the Clock/H9004/H11001 MEL mutants, NPAS2 heterodimerizes with BMAL1 to partially rescue rhythmicity in the SCN. The NPAS2/BMAL1 heterodimer is then sufficient to drive per2 and PK2 expression and wheel-
running rhythmicity in an LD photoperiod and continuous darkness and pineal melatonin rhythmicity in an LD photoperiod and continuous darkness.

Despite the preservation of functional central rhythmicity and light entrainment of neuroendocrine and behavioral rhythms, it is apparent from our findings that the animals with the Clock/H9004 19 mutation are unable to maintain peripheral organ rhythmicity. In this study, there was no significant rhythmicity of the expression of the core clock genes Bmal1, per1, per2, npas2, and rev erb in gastrocnemius muscle of wild-type (●) and ClockA19 + MEL mutant (○) mice. Values are means ± SE (n = 6), with time of peak normalized to 1. Data for 1200 are plotted twice. Horizontal bar represents period of darkness. Some error bars are obscured by symbols.

The maintenance of functional central rhythmicity in the ClockA19 + MEL mutant mice in the absence of peripheral rhythmicity has implications for our understanding of the organization of SCN function. ClockA19 + MEL mutant mice clearly maintain a robust melatonin rhythm in a 12L:12D photoperiod (21) that can be entrained by brief daily light pulses (present study) indicative of a functionally rhythmic SCN. Nevertheless, there was no evidence of circadian rhythmicity in corticosterone secretion in the ClockA19 + MEL mutant mice, although there is separately, unequivocal evidence of SCN involvement in adrenal rhythmicity in normal mice (4, 5, 18). The output pathways from the SCN that indirectly and directly drive the adrenal corticosterone rhythm have been mapped (4). The indirect pathway to the adrenal from the SCN involving the periventricular region of the paraventricular nucleus (PVN), the dorsal part of the PVN, and the intermediolateral column is shared with the pathway controlling melatonin rhythmicity. The intermediolateral column projects to the adrenal, as well as the superior cervical ganglion, which in turn innervates the pineal gland. AVP in the PVN drives the corticosterone rhythm but does not influence melatonin rhythmic-
ity (17). Indeed, melatonin is controlled by GABA release within the PVN. Presumably, the lack of AVP rhythmicity in the SCN of Clock\(^{Δ19}\) mutant mice (14, 16) is responsible for the absence of adrenal rhythmicity and is consistent with regional specialization of SCN cells in terms of output functions (23, 25, 49). Nevertheless, loss of rhythmicity in the adrenal could also contribute to loss of the corticosterone rhythm, even if ACTH rhythmicity were to persist in the mutant mice.

Importantly, the absence of liver and muscle rhythmicity in Clock\(^{Δ19} +\) MEL mutant mice in the present study can now be attributed to the loss of an intrinsic ability of these tissues to generate rhythmicity as well as the loss of adrenal rhythmicity that could entrain it. Of interest is the observation that dexamethasone, acting through glucocorticoid receptors, resets cellular rhythms in liver, heart, and kidney in vivo (2). The absence of melatonin rhythmicity in the original melatonin-deficient Clock\(^{Δ19}\) mutants is unlikely to have been the cause of the peripheral arrhythmicity reported previously, because the melatonin-proficient Clock\(^{Δ19} +\) MEL mutant was equally affected.

Marked differences between the SCN and other tissues in rhythmicity of expression of clock and related genes were seen in the present study. In the SCN of wild-type mice, npas2 and Bmal1 have low-amplitude rhythms of similar phase (this study), but Clock expression is known to be constitutively arrhythmic (36). The promoter for npas2 was shown to have E-box and rev erba/ROR binding elements similar to Bmal1 and Clock (44). The npas2 rhythm could continue to be driven predominantly by rev erba through the rev erba/ROR binding elements in the mutant mice, although why this does not happen in liver as well is not known. Nevertheless, the sustained SCN rhythmicity in the Clock\(^{Δ19} +\) MEL mutant mice suggests that Clock is essentially redundant in this tissue.

In liver of wild-type mice, Bmal1, Clock, and npas2 are expressed in phase with a high amplitude (44, 47). In contrast to the SCN, therefore, Clock expression is normally rhythmic in liver and, together with Bmal1, contributes to the high-amplitude npas2 rhythm through a complex series of interlinked cycles of induction and repression (44). Disabling Clock in liver removes the E-box induction of npas2, resulting in decreased expression of Bmal1 and npas2 and loss of rhythmicity. It is well established from microarray studies that the same suite of functional genes is not necessarily rhythmic in all tissues, but there is little information on how this presumed repression of E-box function is achieved.

In muscle there were high-amplitude rhythms of Bmal1, per1, per2, and npas2 mRNA expression in wild-type mice, consistent with other published reports (28). In the Clock\(^{Δ19} +\) MEL mutant mice, there was no significant rhythm of Bmal1, per1, or per2 mRNA expression. In the case of npas2, however, there was a small but significant rhythm of expression in the mutant mice. In an attempt to discover whether this npas2 rhythm represented functional cellular rhythmicity through an NPAS2-BMAL1 interaction, we analyzed the expression of rev erba, a transcription factor that is a component of the cellular clock mechanism and an output of the clock. In the wild-type mice, the timing of the rhythm of expression of rev erba and Bmal1 in muscle was consistent with that reported previously (13). However, the peak of the low-amplitude rev erba rhythm occurred earlier in the Clock\(^{Δ19} +\) MEL than in the wild-type mice, suggesting that the change across the day may not be clock controlled. Possibly, the npas2 and rev erba changes in muscle are related to muscle activity during the active dark phase.

Although disruption of circadian rhythms is strongly implicated in the initiation and outcome of metabolic and cardiovascular disease, there have been few studies of physiological consequences of CLOCK disruption. Moderate disruption of the reproductive axis leading to impaired fertility (8, 19, 26) and depression of gluconeogenesis and impaired recovery from insulin-induced hypoglycemia and metabolic syndrome have been demonstrated in the original melatonin-deficient Clock\(^{Δ19}\) mutant mouse (33, 43). The Clock\(^{Δ19} +\) MEL mutant mouse may provide a more specific model for distinguishing the wider physiological consequences of peripheral tissue arrhythmicity separately from central disturbances.

In summary, we have shown that melatonin-proficient Clock\(^{Δ19} +\) MEL mutant mice maintain centrally generated neuroendocrine and behavioral rhythmicity that can be entrained by as little as 15 min of light exposure per 24 h. By contrast, adrenal corticosterone rhythmicity, which is also normally generated centrally in the SCN, was absent in the mutant mice. Expression of the core clock genes Bmal1, per1, and per2 in liver and muscle was also not rhythmic, implying that liver and muscle rhythmicity cannot be rescued by the rhythmic SCN, the nocturnal surge of melatonin, or the Clock homolog npas2. The SCN cannot drive rhythmicity in the absence of Clock expression in these peripheral tissues, which, in contrast to central rhythmicity, has an absolute requirement for CLOCK. These findings redefine the molecular basis of rhythmicity and show that the role of the Clock gene differs between the SCN and peripheral tissues. This raises the possibility that environmental or pathophysiological factors may dissociate central and peripheral rhythmicity and contribute to development of disease. Conversely, it may be possible to intervene to selectively target central and peripheral rhythmicity to prevent or overcome this.

REFERENCES


