Melatonin and activity rhythm responses to light pulses in mice with the Clock mutation

David J. Kennaway, Athena Voultsios, Tamara J. Varcoe, and Robert W. Moyer

Department of Obstetrics and Gynaecology, University of Adelaide
Medical School, Adelaide, South Australia 5005, Australia

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Kennaway, David J., Athena Voultsios, Tamara J. Varcoe, and Robert W. Moyer. Melatonin and activity rhythm responses to light pulses in mice with the Clock mutation. Am J Physiol Regul Integr Comp Physiol 284: R1231–R1240, 2003. First published January 9, 2003; 10.1152/ajpregu.00697.2002.—Melatonin and wheel-running rhythmicity and the effects of acute and chronic light pulses on these rhythms were studied in Clock<sup>19</sup> mutant mice selectively bred to synthesize melatonin. Homozygous melatonin-proficient Clock<sup>19</sup> mutant mice (Clock<sup>19</sup>-MEL) produced melatonin rhythmically, with peak production 2 h later than the wild-type controls (i.e., just before lights on). By contrast, the time of onset of wheel-running activity occurred within a 20-min period around lights off, irrespective of the genotype. Melatonin production in the mutants spontaneously decreased within 1 h of the expected time of lights on. On placement of the mice in continuous darkness, the melatonin rhythm persisted, and the peak occurred 2 h later in each cycle over the first two cycles, consistent with the endogenous period of the mutant. This contrasted with the onset of wheel-running activity, which did not shift for several days in constant darkness. A light pulse around the time of expected lights on followed by constant darkness reduced the expected 2-h delay of the melatonin peak of the mutants to ~1 h and advanced the time of the melatonin peak in the wild-type mice. When the Clock<sup>19</sup>-MEL mice were maintained in a skeleton photoperiod of daily 15-min light pulses, a higher proportion entrained to the schedule (57%) than melatonin-deficient mutants (9%). These results provide compelling evidence that mice with the Clock<sup>19</sup> mutation express essentially normal rhythmicity, albeit with an underlying endogenous period of ~26–27 h, and they can be entrained by brief exposure to light. They also raise important questions about the role of Clock in rhythmicity and the usefulness of monitoring behavioral rhythms compared with hormonal rhythms.

IN THE SUPRACHIASMATIC NUCLEUS (SCN), Clock is considered an essential transcription factor for cellular rhythmic processes. In the Clock<sup>19</sup> mutant mouse (32), an A → T transversion in the splice donor site downstream from exon 19 leads to the skipping of this exon in the Clock mRNA and elimination of 51 amino acids in the COOH-terminal glutamine-rich region of the CLOCK protein (17). The BMAL1/CLOCK<sup>19</sup> heterodimer lacks the ability to promote per transcription (9); consequently, per1, (13) BMAL1 (26), cry1 and cry2 (19), and arginine vasopressin (AVP) genes (13, 29) are arrhythmic in the SCN. Nevertheless, homozygous Clock<sup>19</sup> mutants can express daily behavioral rhythms, and the rhythms may persist for ≥1 wk in constant darkness, with a period of ~27 h, before they become arrhythmic (20). Behavioral rhythms, however, can be masked by exogenous influences, and it can be quite challenging to distinguish between a masked and a genuine circadian rhythm.

The robust rhythm of pineal gland melatonin secretion is driven by the SCN in the anterior hypothalamus. The presence of melatonin receptors in the SCN (31) and their effects on SCN rhythmicity (21) suggest that the hormone may play an important role in the maintenance and entrainment of rhythms. The monitoring of melatonin rhythmicity is considered the “gold standard” for human studies, because it provides noise-free insight into SCN rhythmicity and is particularly useful in studies of rhythm disorders. In animals, it has proved useful in monitoring alterations in the melatonin rhythm in studies of the role of neurotransmitters in the control of rhythmicity by light (14).

Mice are widely used in circadian rhythm research because of the growth in our understanding of mouse genetics, particularly the ability to disrupt genes. They exhibit a robust rhythm of wheel-running activity, which allows simple, noninvasive quantitation. Unfortunately, the strains of laboratory mice that are so valuable for gene knockout studies, including the growing number of clock gene knockouts, are melatonin deficient because of mutations in arylalkylamine-N-acetyltransferase (AA-NAT) and hydroxyindole-O-methyltransferase (HIOMT) (8). The Clock<sup>19</sup> mutant mouse was developed in melatonin-deficient C57Bl mice (32). The use of this background strain has seriously restricted the usefulness of the mutants for understanding the control of rhythmicity, because the SCN is a major target site for melatonin as well as the generator of rhythmicity. The CBA strain has a robust melatonin rhythm (12), which can be manipulated by light pulses (15).

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We therefore set out to produce homozygous Clock$^{19/19}$ mutant animals with normal AA-NAT and Hiomt alleles producing functional melatonin production. On the affirmative answer to this question, we then asked whether melatonin production and secretion are rhythmically cycling in a 12:12 light-dark photoperiod, whether the rhythm is endogenously generated, and whether light can suppress melatonin production and entrain the melatonin rhythm. Finally, we examined whether the wheel-running rhythms of Clock$^{19}$ mutants that were melatonin proficient or deficient could be entrained with equal efficiency under conditions that minimized the effects of behavioral masking by light (skeleton photoperiods).

MATERIALS AND METHODS

Animals. On arrival in Adelaide, mice heterozygous for the Clock$^{19}$ mutation (Clock$^{+/19}$) on a BALB/c background were paired to produce Clock$^{+/19}$, Clock$^{+/19}$, and Clock$^{19/19}$ mice. CBA/6CaH mice, a strain derived from stock originally provided by the Animal Resources Centre of the University of Western Australia (http://members.iinet.net.au/~arcweb/), were obtained from the University of Adelaide Central Animal House. Animals were housed in our specific pathogen-free facility in a 12:12-h light-dark photoperiod and fed standard laboratory chow ad libitum.

Genotyping. Inheritance of the Clock$^{19}$ mutation was monitored in the breeding program by PCR of tail DNA (27). Briefly, mouse genomic DNA was extracted from tail biopsies and subjected to PCR analysis using primers reported previously (27). The products were digested with HincII and electrophoresed on a 1.5% agarose gel. The mutant allele produced a 460-bp product, the wild-type allele gave a 398-bp band.

Melatonin assays. Plasma was assayed by RIA using reagents obtained from Buhlmann Laboratories (Allschwil, Switzerland), as previously described (15). Briefly, plasma (100 μl) was added to the prewashed columns and sequentially washed with 10% methanol; hexane and melatonin were finally eluted with pure methanol. After evaporation of the solvent, the residue was reconstituted in 1 ml of buffer, and aliquots (100 μl) were subjected to RIA. Sensitivity of the assay was 10 pM. Intra- and interassay coefficients of variation were <10% and <15%, respectively, across the range of the standard curve.

Pineal glands were stored frozen in 1 ml of RIA buffer. Before assay, the buffer was thawed, and the pineal gland was homogenized. Aliquots (100 μl) were assayed in duplicate by direct RIA with a sensitivity of 21 fmol/gland. Intra- and interassay coefficients of variation were <10% and <15%, respectively, across the range of the standard curve.

Phenotyping for melatonin production. Because the mutations that disable AA-NAT vary between strains (23) or have not been characterized (HIOMT), we developed an in vivo phenotyping procedure for melatonin production based on the observation that early-morning administration of the β-adrenergic agonist isoproterenol increased plasma melatonin levels in CBA, but not C57Bl or BALB/c, mice (15). Briefly, the test involves administration of isoproterenol (20 mg/kg sc) 2 and 4 h after lights on. Under light halothane anesthesia, blood (~300 μl) was collected from the retroorbital sinus 30 min after the last injection, and plasma was assayed for melatonin by RIA. Preliminary evidence indicated that mice carrying one mutant set of AA-NAT and Clock$^{19}$ alleles produced half as much melatonin as those carrying two functional alleles (8). Thus we selected the highest melatonin producers at all stages of the breeding program to produce our target genotype, which we have designated Clock$^{19/19}$MEL (see RESULTS).

Melatonin rhythmicity. To monitor the endogenous production and rhythmicity of melatonin in mice with a disabled Clock gene, Clock$^{+/+}$MEL and Clock$^{19/19}$MEL mice previously maintained in a 12:12-h light-dark photoperiod were released into constant darkness, and blood and pineal glands were collected 4–6 h later from groups of 4–11 animals. Sampling times were concentrated around the time of subjective dawn during the first 2 days of continuous darkness. Tissue and blood were collected after brief exposure to dim red light.

In these and subsequent experiments, the time of day is reported as zeitgeber time (ZT) or circadian time (CT). ZT12 is the time of lights off in a 12:12-h light-dark photoperiod; CT12 is the time of expected lights off in continuous darkness (usually also coinciding with the onset of wheel-running activity).

Phase shifting of a light pulse on melatonin. Exposure of animals to an unexpected light pulse during the night causes an immediate cessation of melatonin synthesis through inhibition of AA-NAT activity (18). To test whether melatonin synthesis in Clock$^{19/19}$MEL mice could be suppressed by light, groups of five animals were killed in the dark around the expected time of peak melatonin production, specifically at ZT23.5, at ZT24, or 15 min after 15 min of exposure to a 100-lux light pulse that started at ZT23.5. As a control, Clock$^{+/+}$MEL mice were killed around the time of their peak melatonin production, specifically at ZT21.5, at ZT22, or 15 min after a 15-min light pulse that started at ZT21.5.

Phase shifting of melatonin rhythms by light. To address whether Clock$^{19/19}$MEL mice could be phase advanced or prevented from phase delaying (i.e., entrained) by exposure to light, groups of five mice were exposed to light (100 lux for 15 min) around the time of peak melatonin production, specifically at ZT23.5 and ZT23.5 for Clock$^{+/+}$MEL and Clock$^{19/19}$MEL mice, respectively, and kept in darkness until they were killed 21–28 h later. As a control, groups of mice from each line (n = 3–5 per time point) were not exposed to light. Potential phase shifting of the rhythm was analyzed by ANOVA from CT20 to CT23 and from CT2 to CT5 for Clock$^{+/+}$-MEL and Clock$^{19/19}$-MEL mice, respectively, with significance set at P < 0.05. The degree of shift was determined by changes in the time of the peak.

Wheel running. Mice were 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. After the data were downloaded, the Actiview software package (MiniMitter, Bend, OR) was used to display the actograms and to perform the χ2 method of period analysis.

To investigate the pattern of wheel running in a 12:12-h light-dark photoperiod, 77 Clock$^{+/+}$, 61 Clock$^{19/19}$, 71 Clock$^{+/+}$MEL, and 83 Clock$^{19/19}$MEL mice were studied on the 5th–10th days of access to wheels. The onset of running was evaluated in 12:12-h light-darkness by recording the time of day the wheel revolutions exceed 50 revolutions per 10 min for at least three successive 10-min periods within a 1-h period before and after lights off. Over the 5 days, the within- and between-animal mean onset times and the standard deviations were calculated. A subset of these animals was released into constant darkness for a further 10 days, and periodogram analysis was performed to estimate period length. The free-running period was calculated for 17
male mice (not fathers) to produce the first backcross (BC1) line. This cross produced a total of 71 pups from 7 pregnancies, of which 37 were heterozygous and 32 were homozygous for the Clock\(^{Δ19}\) mutation (2 died before genotyping). A second independent BC1 line produced 35 Clock\(^{+/Δ19}\) and 54 Clock\(^{Δ19/Δ19}\) mice. Figure 1 shows the frequency distributions for the plasma melatonin levels after the isoproterenol phenotyping for the BC1 mice. On the basis of the expectation that the AA-NAT and HIOMT genes are not linked (8), we expected that 25% of the BC1 animals would be heterozygous for the enzyme. Thus animals with melatonin levels in the 75th–100th percentile could be assigned this genotype (Fig. 1). Clock\(^{+/Δ19}\) and Clock\(^{Δ19/Δ19}\) genotypes had similar distributions. Indeed, the top 25% secretors had melatonin levels of 29.5 ± 4.4 pM (n = 24) and 28.4 ± 3 pM (n = 30), respectively, after isoproterenol administration.

The four highest melatonin-producing male and female mice of the first backcross carrying the Clock\(^{Δ19}\) mutation were then intercrossed to provide the BC1 intercross line. This mating produced 40 pups from 4 pregnancies (18 female and 22 male mice). The second BC1 line was also intercrossed (3 pairs) to produce 11 male and 7 female mice that survived to phenotyping. Figure 2 shows the frequency distribution of the plasma melatonin. Assuming again that all 3 genes segregated independently, we expected 1 in 16 mice to be homozygous for the Clock\(^{Δ19}\) mutation and to carry 2 functional alleles of AA-NAT and HIOMT.

The two male mice with plasma melatonin levels higher than the 94th percentile after isoproterenol administration (135 and 78 pM) were therefore tentatively assigned the genotype Clock\(^{Δ19/Δ19, AA-NAT^{+/+}}\).

### RESULTS

#### Breeding program for Clock\(^{Δ19/Δ19-MEL}\) mice. Male Clock\(^{Δ19/Δ19}\) mice were crossed with five female CBA mice to produce heterozygotes for the Clock\(^{Δ19}\) AA-NAT, and HIOMT mutations (3 litters of 7, 5, and 6 pups). The female offspring from each of the litters (n = 3, 3, and 2) were then backcrossed to Clock\(^{Δ19/Δ19}\).
HIOMT^{+/+} (hereafter designated Clock^{Δ19/Δ19-MEL}). These male mice were mated with 14 CBA female mice to produce heterozygotes for the Clock^{Δ19} mutation and homozygotes for the AA-NAT and HIOMT genes (53 male, 66 female, and 11 dead pups). The offspring from these matings were intercrossed to give Clock^{+/+}-MEL, Clock^{+/Δ19-MEL}, and Clock^{Δ19/Δ19-MEL} mice. To establish the Clock^{Δ19/Δ19-MEL} line, we used 13 male and 17 female mice. To establish the Clock^{+/+}-MEL line, we used 9 male and 13 female mice. We currently maintain the lines using at least seven breeding pairs per generation and have produced five generations with no evidence that the original putative Clock^{Δ19/Δ19-MEL} male mice were misclassified (i.e., no melatonin-deficient litters have been produced).

Melatonin rhythms in Clock^{Δ19/Δ19-MEL} mice. The animals produced after the backcross and intercrossing provided an opportunity to conduct preliminary investigations on the endogenous production of melatonin in Clock^{Δ19} mutants. Figure 3 shows the mean plasma melatonin levels after the isoproterenol test in those BC1 animals in which levels were higher than the 75th percentile; at these levels, the animals were likely to be carrying at least one functional allele of AA-NAT and HIOMT. Nighttime (ZT22) plasma melatonin concentration and pineal content are also shown in Fig. 3. The stimulated daytime levels were almost identical in the two genotypes, but 2 h before lights on (the time of peak melatonin production in the founder CBA strain), plasma and pineal melatonin levels were 50% lower in the Clock^{Δ19/Δ19-MEL} mice than in the heterozygotes. Low, but detectable, plasma and pineal melatonin levels were recorded in some of the presumptive melatonin-deficient mice at ZT22, but even in this group, Clock^{Δ19/Δ19-MEL} mice had lower levels at ZT22. We presume that the measurement of endogenous melatonin production in some animals categorized as deficient reflects a low false-negative assignment for the isoproterenol test.

For the intercross animals, we expected three melatonin phenotypes: deficient mice (50%), those carrying at least one allele each of AA-NAT and HIOMT (44%), and those carrying two functional AA-NAT and HIOMT alleles (6.25%). Because only four of the latter group were available for analysis at ZT22, no firm conclusions could be drawn about melatonin production at this time of day in mice with this genotype.

When sufficient Clock^{Δ19/Δ19-MEL} animals became available, the endogenous melatonin rhythm in 12:12-h light-darkness and during the first two cycles of continuous darkness was studied. Figure 4 shows that
It was clear that Clock\textsuperscript{19/19-MEL} mice placed in constant darkness delayed the peak of melatonin production by 2 h each cycle. It was therefore of interest to test whether light exposure at the time of peak production would advance or at least prevent the 2-h delay in the melatonin rhythm. Figure 6 shows peak melatonin production between CT0 and CT4 in Clock\textsuperscript{19/19-MEL} mice after one cycle of continuous darkness, as observed in the first experiment. After brief exposure to light, the mutant mice had a different pattern of plasma and pineal melatonin, with significantly lower levels at CT2 and CT3 (P < 0.05 by ANOVA). The Clock\textsuperscript{+/+}-MEL mice also responded to the light pulse with significantly lower plasma melatonin levels and pineal melatonin contents at CT20, CT21, and CT22 (P < 0.05). From the previous experiment where we observed a 2-h delay in the time of the peak melatonin production in the Clock\textsuperscript{19/19-MEL} mice, we expected that light presentation at the time of peak melatonin production would prevent this delay. In the case of the Clock\textsuperscript{+/+}-MEL mice, we would expect the rhythm to be advanced. The results shown in Fig. 6 are consistent with these hypotheses.

Wheel-running rhythms. Wheel running in all four mouse lines in a 12:12-h light-dark photoperiod was characterized by intense running during the dark phase, with the greatest concentration of running early in the night and decreasing steadily toward lights on (Fig. 7). In the Clock\textsuperscript{+/+} and Clock\textsuperscript{+/+}-MEL mouse lines, the onset of running occurred 6 ± 29 (SD) min after darkness and 11 ± 25 min before lights off, respectively. In the Clock\textsuperscript{19/19} and Clock\textsuperscript{19/19-MEL} lines, the mean onset times were 6 ± 42 and 8 ± 42 min before lights off. The intra- and interanimal variance was higher in the mutants than in the wild-type animals. When the average number of wheel revolutions during the last 6 h of light was compared between genotypes, Clock\textsuperscript{+/+} and Clock\textsuperscript{+/+}-MEL mice accumulated 4% and 3.7%, respectively, of their total wheel revolutions per day during this time. By contrast, 13.7% of the total daily running activity of Clock\textsuperscript{19/19} mice and 14% of the activity of Clock\textsuperscript{19/19-MEL} mice occurred during this period. The melatonin-deficient Clock\textsuperscript{+/+} and Clock\textsuperscript{19/19-MEL} mice also accumulated 12.3% and 11.6%, respectively, of their total running during the first 6 h of light, in contrast to their melatonin-proficient genotypes (2.3% and 6.3%). In Fig. 7, the differences in the time of offset of running on light onset in the melatonin-deficient and -proficient Clock\textsuperscript{+/+} lines are clearly evident.

On release into continuous darkness, rhythmicity was maintained in the melatonin-deficient and -proficient Clock\textsuperscript{+/+} lines for ≥14 days, with periods of 23.8 ± 0.04 h (n = 16) and 23.3 ± 0.07 h (n = 10), respectively. By contrast, rhythmicity was sustained in 76% (n = 25) and 44% (n = 9) of the Clock\textsuperscript{19/19} and Clock\textsuperscript{19/19-MEL} mice, respectively. In those animals that showed rhythmicity, the free-running periods were 27.0 ± 0.17 and 26.9 ± 0.24 h, respectively. When the revolutions per 10 min were averaged in constant darkness, it was clear that the onset of running activity
in the Clock^+/^+ mice changed little over the first four cycles (Fig. 7), consistent with their free-running period of 23.8 h. Similarly, the Clock^+/^+MEL mice changed their onset times over the first four cycles, consistent with their shorter periodicity, most evident from the second onset in constant darkness. Despite having free-running periods of ~27 h, it was not until the second and fourth cycles in constant darkness that the Clock^19/19 MEL mice showed clear changes (delays) in onset.

Entrainment of running activity by light pulses. When melatonin-deficient and -proficient Clock^+/^+ mice were released into the skeleton photoperiod with the pulse occurring at the time of the previous lights on (ZTO), both lines free ran with a period of 23.5 ± 0.04 and 23.4 ± 0.25 h, respectively, over the duration of the experiment (Fig. 8). Over the duration of the experiment, the pulses never coincided with wheel-running activity in these mice. Therefore a double-pulse skeleton photoperiod (0.25:23.75:0.25:11.5-h light-darkness-light-darkness) was utilized (with the pulses occurring at the previous lights on and lights off). Under these conditions, it was hypothesized that the Clock^+/^+ mice would be entrained to the “evening” pulse. As expected, the Clock^+/^+ lines had periods of 24.0 ± 0.01 and 24.0 ± 0.03 h, showing that the pulses were sufficient to entrain the mice.

Under single- and double-pulse regimens, it was expected that only the morning pulse would influence entrainment of the mutants because of the long free-running period. Data from both regimens are shown in Table 1. Because the results obtained were similar for single and double pulses, they were combined. In the case of the Clock^19/19 MEL mice, 2 of 22 entrained to the pulses (period 23.5–24.5 h), whereas 13 of 22 free ran with a period of 27.1 ± 0.32 h and 7 of 22 became arrhythmic immediately (Fig. 9). By contrast, 12 of 26 Clock^19/19 MEL mice were entrained and 3 of 26 were entrained after free running for ~8 days (late entrainment), indicating that 58% of the mice entrained. Another 6 of 26 mice free ran with a period of 26.3 ± 0.35 h, and 5 of 26 mice became arrhythmic immediately on release into the pulse conditions.

To further test the ability of Clock^19/19 MEL mice to entrain to single light pulses, we maintained mice in a 12:12-h light-dark photoperiod and then released them into the skeleton (0.25:23.75-h light-dark) photoperiod with light pulses timed for 3 or 6 h after expected lights on for 13 days. Figure 10 shows that two of five Clock^19/19 MEL mice were entrained to the single pulses presented 3 h after expected lights on, one free ran, and two became arrhythmic. Four of five mice entrained to the single pulses presented 6 h after expected lights on, and one free ran. The Clock^+/^+ MEL mice free ran until the onset of wheel running coincided with the pulse, after which they all entrained (data not shown).
A principal aim of the experiments was to investigate hormonal (melatonin) rhythmicity for the first time in mice carrying the Clock\(^{\Delta 19}\) mutation. Unfortunately, the original Clock\(^{\Delta 19}\) mutants were produced in C57Bl/6J mice and maintained on a BALB/c background and, therefore, are melatonin deficiency. The CBA mouse strain produces melatonin rhythmically (12, 33), and the rhythm shifts in response to light pulses (15); therefore, we chose to introduce functional AA-NAT and HIOMT genes into Clock\(^{\Delta 19}\) mice by selective breeding. It was possible that the mutants would not synthesize melatonin, because BMAL1/CLOCK heterodimer binding in the promoter region of AA-NAT is required for induction of the enzyme in the chicken pineal gland (7) and rat retina (5). Although there appears to be no such requirement in the rat pineal gland in vitro (5), this had not been tested in vivo. Our program showed that Clock is not required for AA-NAT induction in vivo and that Clock\(^{\Delta 19}\) MEL mice produced and secreted melatonin in amounts comparable to wild-type mice.

The selective breeding program was based on the assumption that the three genes segregated independently. AA-NAT has been mapped to mouse chromosome 11 (11) and Clock to mouse chromosome 5 (16), and AA-NAT and HIOMT genes are known to segregate independently. AA-NAT has been mapped to mouse chromosome 11 (11) and Clock to mouse chromosome 5 (16), and AA-NAT and HIOMT genes are known to segregate independently.
gate independently (8). To our knowledge, mouse HIOMT has not been mapped; therefore, we had to assume that it was not close to Clock on chromosome 5. Therefore, a conservative selection procedure was used, with only top-ranking mice being used for breeding, especially after the intercross stage. The two putative Clock^{19/19} MEL male mice were crossed again with CBA female mice to rapidly expand the colony size and reduce inbreeding and the chances of transmitting a mutant enzyme allele. After a mutant enzyme allele. After

percentage of total number of mice of each phenotype. Note high proportion of Clock^{19/19} MEL mice that were entrained compared with melatonin-deficient mutants.

Fig. 9. Proportions of Clock^{19/19} and Clock^{19/19} MEL mice that entrained, free ran, or became arrhythmic in 0.25:23.75-h light-dark or 0.25:12:0.25:11.5-h light-dark-light-dark photoperiod. Values are percentages of total number of mice of each phenotype. Note high proportion of Clock^{19/19} MEL mice that were entrained compared with melatonin-deficient mutants.

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<th>Clock^{19/19} MEL</th>
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<tr>
<td>Single pulse</td>
<td>1/11 (9%)</td>
<td>0/11 (0%)</td>
<td>6/11 (54%)</td>
<td>4/11 (36%)</td>
<td>Clock^{+/+} 8/8 free ran</td>
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<tr>
<td>Double pulse</td>
<td>1/12 (8%)</td>
<td>0/12 (0%)</td>
<td>7/12 (58%)</td>
<td>4/12 (33%)</td>
<td>Clock^{+/+} 8/8 entrained</td>
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Animals that were entrained, arrhythmic, or free ran in single- and double-pulse regimens

Table 1. Animals that were entrained, arrhythmic, or free ran in single- and double-pulse regimens

Single pulse, 0.25:23.75-h light-dark photoperiod; double pulse, 0.25:23.75:0.25:11.5-h light-dark photoperiod.

MEL mice were exposed to a light pulse at the time of the peak of melatonin production and followed over the next cycle in constant darkness, the melatonin production ceased significantly earlier than nonpulsed mice, as shown previously in CBA mice (15). Exposure of Clock^{19/19} MEL mice to light at the time of the melatonin peak resulted in significantly decreased pineal melatonin content and plasma melatonin levels at CT3 and CT4 compared with the nonpulsed animals. The fact that the peak at CT1 was still 1 h later than the time of the peak in a light-dark environment and 1 h earlier than in the control mutant animals suggests that the response to the light pulse was weak or that only a portion of the population responded. Nevertheless, we conclude that a light pulse can shorten the period of the melatonin rhythm and potentially entrain it. These responses to light are particularly interesting in view of the reported poor responsiveness of Clock^{19/19} mutants to light pulses, at least with respect to induction of the immediate-early gene c-fos and per1 and per2 (27).

Using the more traditional method of wheel running to monitor rhythmicity, we confirmed the entrainment of Clock^{19/19} mice (32) to a 12:12-h light-dark photoperiod and report similar results for Clock^{19/19} MEL mice. The precision of the entrainment in both mutant lines was poor compared with the wild-type animals, and running during the light period was common. Because Clock^{19} mutants have a period of 26–27 h, the onset of running activity should be 2–3 h after lights off (1), but in the Clock^{19/19} and Clock^{19/19} MEL mice it occurred within ±10 min of lights off, similar to the wild-type mice. By contrast, the melatonin rhythm peak occurred 2 h later than in wild-type animals, which is expected from Aschoff’s predictions (1). A similar lack of correlation between phase angle difference and period of the wheel running rhythm is evident in other Clock gene knockouts: per1 (2, 4), per2 (2, 34), per3 (25), per1per2, per2per3, and per1per3 (2), cry1, cry2, and cry1cry2 (30), and BMAL1 (3). Furthermore, when Clock^{19} mutants were released into constant darkness, there was a lag of one to two cycles before the onset shifted (so-called transients), a phenomenon not observed with the melatonin rhythm. By contrast, Clock^{19/19} maintained on a Jc:ICR genetic background have activity and body temperature rhythm acrophases in 12:12-h light-darkness consistent with the long endogenous period (24). This raises the question whether wheel running is a universally suitable output measure of the circadian clock in mice.
Given that the melatonin and wheel-running rhythms of $\text{Clock}^{\Delta 19/\Delta 19}$-$\text{MEL}$ mice were strongly entrained to a 12:12-h light-dark photoperiod, we were interested in the extent to which rhythmicity could be entrained by short light pulses in a skeleton photoperiod. Vitaterna et al. (32) reported that a single 6-h light “pulse” could restore a long free-running period in $\text{Clock}^{\Delta 19/\Delta 19}$ mice that had become arrhythmic in constant darkness. We found that daily 15-min light pulses presented at the time of the previous lights on or 3 or 6 h later prevented animals from free running or eventually entrained them with a period close to 24 h. We observed that the $\text{Clock}^{\Delta 19/\Delta 19}$-$\text{MEL}$ mice were more likely to be entrained than the melatonin-deficient mutants, although $\geq 40\%$ still failed to entrain or became arrhythmic. Because we have introduced genes other than AA-NAT and HIOMT from the CBA strain, we cannot attribute the increased proportion of entrained animals solely to melatonin.

How can these results of robust hormone rhythmicity and entrainment in $\text{Clock}^{\Delta 19/\Delta 19}$-$\text{MEL}$ mutant mice be reconciled with what is known about the molecular biology of the “essential” $\text{Clock}$ gene? The $\text{Clock}^{\Delta 19}$ mutation is an antimorph that inhibits wild-type function (16). Indeed, not only is the BMAL1/CLOCK heterodimer incapable of driving transcription of an mper1-luciferase reporter, it is less active than BMAL1 alone (9). As a result, $\text{Clock}^{\Delta 19}$ mutant mice should have decreased transcription of mper1 and other clock genes. This has been shown to be the case, with major disturbances in the rhythm of per1, per2, and per3 (13), cry1 and cry2 (19), BMAL1 (26), AVP (13, 29), and, more recently, prokineticin2 (6). One clock gene, mper2, appears to retain significant rhythmicity (13), and per1 and per2 have been shown to be induced, albeit at a reduced level, by a 15-min light pulse at ZT16 (27). The BMAL1/CLOCK heterodimer is the key driver of cellular rhythmicity; yet the $\text{Clock}^{\Delta 19/\Delta 19}$-$\text{MEL}$ mutant mice show hormonal and behavioral circadian rhythmicity and entrainment to very brief light pulses. How can these hormonal and behavioral rhythms be sustained, especially at the unchanged amplitudes maintained by the mutants? One possibility is that the BMAL1/CLOCK heterodimer does have some intrinsic transcriptional activity at the E-boxes of some of the clock genes, but, as indicated earlier, at least for per1, there is no evidence for this (9). We are aware of no similar studies on per2. Another possibility is that another partner exists for BMAL1 that may drive transcription with decreased efficiency in the SCN or in centers that project to the SCN, resulting in the relatively poor precision of entrainment and long period length. NPAS2 would appear to be such a candidate protein, because it has been shown to be a functional analog of CLOCK and can partner with BMAL1 and drive transcription of appropriate reporter genes (22). However, npas2 is not expressed in the normal mouse SCN (28); instead, it is widely expressed in the forebrain. Although it is possible that npas2 is expressed in the SCN of $\text{Clock}^{\Delta 19}$ mice, and not normal mice, it is more likely that other parts of the brain that maintain rhythmicity via BMAL1/NPAS2 drive, project to the SCN, and maintain essential sleep-wakefulness and hormonal rhythms.

In conclusion, we have produced $\text{Clock}^{\Delta 19}$ mutant mice that can synthesize melatonin. We have shown that, despite producing a protein that fails to initiate transcription of $\text{Clock}$ and clock-controlled genes, the animals have a normal endogenous melatonin rhythm than can be altered by light pulses. The timing of the melatonin rhythm is consistent with the period expressed in constant darkness, unlike the case with locomotor activity. Finally, we have shown that the $\text{Clock}^{\Delta 19/\Delta 19}$-$\text{MEL}$ mice can be entrained to skeleton photoperiods, where the duration of the light period is 15 min.

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