The mouse *Clock* mutation reduces circadian pacemaker amplitude and enhances efficacy of resetting stimuli and phase–response curve amplitude

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The mouse *Clock* gene encodes a basic helix–loop–helix-PAS transcription factor, CLOCK, that acts in concert with BMAL1 to form the positive elements of the circadian clock mechanism in mammals. The original *Clock* mutant allele is a dominant negative (antimorphic) mutation that deletes exon 19 and causes an internal deletion of 51 aa in the C-terminal activation domain of the CLOCK protein. Here we report that heterozygous *Clock*/+ mice exhibit high-amplitude phase-resetting responses to 6-h light pulses (Type 0 resetting) as compared with wild-type mice that have low amplitude (Type 1) phase resetting. The magnitude and time course of acute light induction in the suprachiasmatic nuclei of the only known light-induced core clock genes, *Per1* and *Per2*, are not affected by the *Clock*/+ mutation. However, the amplitude of the circadian rhythms of *Per* gene expression are significantly reduced in *Clock* homozygous and heterozygous mutants. Rhythms of PER2::LUCIFERASE expression in suprachiasmatic nuclei explant cultures also are reduced in amplitude in *Clock* heterozygotes. The phase–response curves to changes in culture medium are Type 0 in *Clock* heterozygotes, but Type 1 in wild types, similar to that seen for light *in vivo*. The increased efficacy of resetting stimuli and decreased PER expression amplitude can be explained in a unified manner by a model in which the *Clock* mutation reduces circadian pacemaker amplitude in the suprachiasmatic nuclei.

circadian clock | *Clock* gene | entrainment | suprachiasmatic nucleus | *Per* genes

The mouse *Clock* mutation was identified in an *N*-ethyl-*N*-nitrosourea mutagenesis screen for circadian variants (1). *Clock* is a semidominant mutation that lengthens circadian period by 1 h in heterozygotes (*Clock*/+) and by 4 h in homozygotes (*Clock*/*Clock*). With prolonged exposure, *Clock* homozygotes fail to express persistent circadian rhythms in constant darkness. The lengthened-period and loss-of-rhythm phenotypes are the hallmarks of the original mutant allele. Genetic analysis of the *Clock* mutant allele over a deletion revealed that *Clock* is an antimorph, a type of dominant negative mutation (2). Molecular cloning of *Clock*, which encodes a basic helix–loop–helix-PAS transcription factor, showed that the mutant allele is a 5′ splice donor mutation skipping exon 19 and causing an internal deletion of 51 aa in the C-terminal activation domain of the CLOCK protein (3–5).

In the last decade, significant progress has been made in our understanding of the mechanism of circadian rhythms. An autoregulatory transcriptional feedback loop forms the core mechanism of the circadian clock in animals (reviewed in refs. 6 and 7). The positive elements of the oscillator are CLOCK and BMAL1, which form a heterodimeric transcription factor to activate the mammalian *Period* and *Cryptochrome* genes (specifically, *Per1*, *Per2*, *Cry1*, and *Cry2*). The negative elements of the oscillator are the gene products of *Period* and *Cryptochrome* (PERs and CRYs) that accumulate, associate with each other, and translocate into the nucleus to inhibit the CLOCK/BMAL1 activation of their own transcription. As the negative elements turnover, CLOCK and BMAL1 then become active again to begin a new cycle of transcription of the *Period* and *Cryptochrome* genes.

To define the role of *Clock* more fully within the mammalian circadian system, we analyzed the entrainment and resetting behavior of mice to light. In principle, entrainment of the core circadian clock mechanism described above would be expected to occur as a consequence of either phasic or tonic changes in the levels of oscillating feedback elements (e.g., the PER or CRY proteins). Within the core circadian clock mechanism, the only elements that have been definitively found to be light responsive in the suprachiasmatic nucleus (SCN) are the *Per1* and *Per2* genes (8–14). Acute light exposure of mice causes phase-dependent elevations of *Per1* and *Per2* mRNA and protein levels in the SCN. We report here that the *Clock* mutation causes a drastic increase in the phase-resetting effects of light in mice. Both behavioral and molecular analyses of *Clock* mutant mice demonstrate that *Clock* alters the resetting effects of phase-shifting stimuli by reducing pacemaker amplitude rather than by changing the strength of light on the inputs to the circadian pacemaker. These results illustrate that the effects of *Clock* on circadian pacemaker amplitude have significant consequences on the entrainment behavior of mice to light and other resetting stimuli. Given that circadian pacemaker amplitude has been implicated in the entrainment of human circadian rhythms (15), these results provide a model for studying and manipulating circadian amplitude to optimize entrainment in humans and other mammals.

Results

Entrainment to Light Cycles. As reported previously, the entrainment behavior of *Clock* mutant mice to a light-dark cycle (LD) of 12 h light:12 h dark (LD12:12) appears relatively normal. Fig. 6, which is published as supporting information on the PNAS web site, shows the phase of entrainment of +/+, *Clock*/+, and *Clock*/*Clock* mice to LD12:12. All three *Clock* genotypes entrained with the same phase relationship to this light cycle, with

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Abbreviations: CT, circadian time; LD, light-dark cycle; SCN, suprachiasmatic nucleus.

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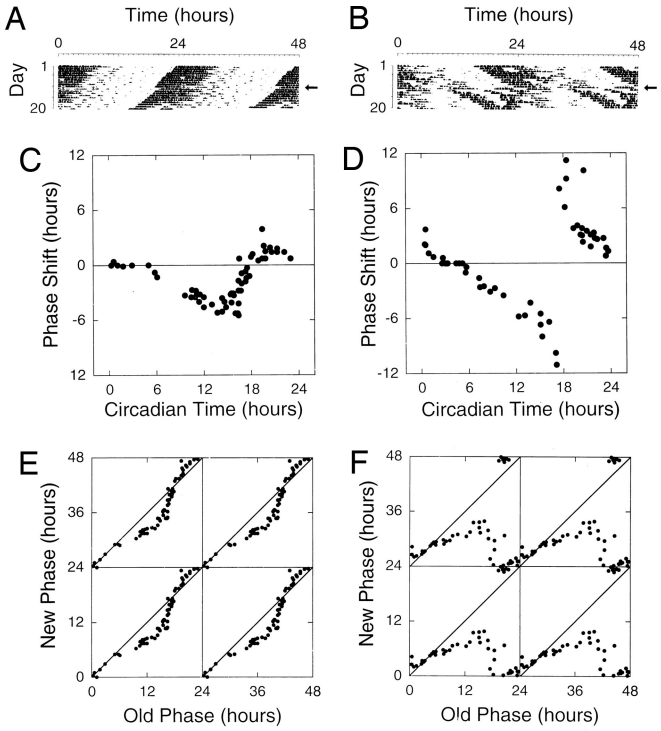

Fig. 1. Phase-shifting responses to light pulses in the circadian activity rhythms of C57BL/6J wild-type and *Clock*/+ mice. (*A* and *B*) Activity records of wild-type (*A*) and *Clock*/+ (*B*) mice given a 6-h light pulse at CT17. The arrow on the right margin indicates the day when the light pulse was given. Records are double-plotted so that 48 h are shown on each horizontal trace with a 24-h day presented beneath and to the right of the preceding day. Times of activity are indicated by dark regions. (*C* and *D*) Phase–response curve to 6-h light pulses in wild-type ($n = 61$) (*C*) and *Clock*/+ ($n = 58$) (*D*) mice. The *x* axis indicates the CT at the beginning of the light pulse. The *y* axis indicates the phase shift produced by the light pulse. (*E* and *F*) Phase–transition curve to the light pulses in wild-type (*E*) and *Clock*/+ (*F*) mice. Data from *C* and *D* are replotted so that the *x* axis indicates the phase (CT) at the beginning of the light pulse as determined from the preceding free run. The *y* axis indicates the extrapolated phase (CT) of the light pulse calculated from the reset rhythm.

activity onset closely coinciding with the time of lights off. Because the free-running periods of *Clock* mutants differ greatly from 24 h, this finding suggests that a compensatory alteration in entrainment may be present, allowing the mutants to entrain with normal phase.

Phase–Response Curve to Light. To determine whether photic entrainment may have been altered by the *Clock* mutation, we determined the resetting effects of single light pulses given to mice free running in constant darkness. Contrary to that seen in other nocturnal rodents (16, 17), we found that short light pulses (<1 h in duration) were only weakly effective in phase-shifting circadian activity rhythms in C57BL/6J mice. In addition, the variance of the phase shifts in the response to short-duration light pulses was high. By contrast, long-duration light pulses (6 h) were much more effective in resetting circadian rhythms in mice and reduced the variance in the magnitude of phase shifts. Because *Clock* homozygous mice fail to show persistent rhythms in constant darkness, we focused on wild-type and *Clock* heterozygous mice. To determine the phase–response curves to light, 6-h light pulses were given to 61 wild-type and 58 *Clock*/+ coisogenic C57BL/6J backcross progeny mice (Fig. 1 *A* and *B*; see also Fig. 7, which is published as supporting information on the PNAS web site). A higher amplitude phase–response curve was observed in the *Clock* heterozygous mice than in the wild-type mice. The largest phase shifts in both genotypes were observed near the "breakpoint," the transition from phase delays to phase advances at approximately circadian time (CT) 17, the largest phase shifts in wild-type mice were <6 h, whereas there were a number of individuals who exhibited phase shifts of >6 h among the *Clock* heterozygotes (Fig. 1 *C* and *D*).

The difference between the two genotypes is seen more clearly when the data are presented as a phase–transition curve (Fig. 1 *E* and *F*). In a phase–transition curve, the "new phase" achieved by the phase shift is plotted as function of the circadian time at which the light pulse was presented (i.e., "old phase"). In the wild-type mice, the data have an average slope of 1 (Type 1) because new phase is close to old phase. In contrast, in the *Clock* heterozygotes, the data have an average slope of 0 (Type 0), because light pulses reset to the same new phase when presented at different old phases. Similar results were obtained in (BALB/cJ × C57BL/6J)F_2 hybrid mice (Fig. 8, which is published as supporting information on the PNAS web site). Examination of the large phase shifts induced with light pulses given at CT17 revealed some interesting features. First, the large phase shifts exhibited by *Clock*/+ mice cannot clearly be categorized as advances or delays (Fig. 1*B*). In most cases, the new phase is expressed in the cycle immediately after the light pulse (Figs. 1*B* and 7*B*). This rapid change was also the case with smaller phase shifts obtained at other phases and in the wild types.

Because *Clock* homozygous mice lose circadian rhythmicity after some time in constant darkness, it is difficult to measure phase shifts to single light pulses. However, as we have shown previously (1), the majority of *Clock* homozygotes show a reinitiation of a long-period rhythm after exposure to a 6-h light pulse. Under this condition, one can infer whether entrainment might be occurring by determining whether the light pulse causes a clustering of the phases of the reinitiated rhythms. In this manner, we then could compare the responses to light pulses of *Clock* homozygotes with those of the other *Clock* genotypes. The phases of the rhythms after a 6-h light pulse are shown as Rayleigh plots (18) in Fig. 9, which is published as supporting information on the PNAS web site. The phase of the reinitiated rhythm was examined in 21 *Clock* homozygotes given light pulses according to the same procedures and at the same times as the other two *Clock* genotypes. Significant clustering of phases (Rayleigh test; $P < 0.05$) was observed in both *Clock*/+ heterozygotes and *Clock* homozygotes but not in wild types after the light pulse. Phases were not significantly clustered before the light pulse in either wild-type or *Clock* heterozygotes (Rayleigh test; $P > 0.05$). With strong, Type 0 resetting, coherence of phase is expected because phases should be clustered around a common new phase, whereas with weaker, Type 1 resetting, coherence of phases is not expected because new phases should be scattered, as were the old phases. Interestingly, there is close and significant clustering of phases in the *Clock*/*Clock* mice, consistent with either a strong phase shifting response as seen in *Clock* heterozygotes, or alternatively, with reinitiation of oscillations at a particular phase (CT6) of the cycle.

***Per* Gene Expression in SCN *in Situ*.** To determine the effects of the *Clock* mutation on circadian rhythms in the SCN, we used *Per* gene expression as a marker. Fig. 2; see also Fig. 10, which is published as supporting information on the PNAS web site, show the expression levels of *Per1* in the SCN of *Clock*/*Clock* and +/+ mice under light-dark conditions and in the first four cycles of free run in constant darkness. Although maintained in a light-dark cycle, *Per1* expression is clearly rhythmic in *Clock*/*Clock* mice but with a reduced amplitude and mean levels. During LD12:12, light induction of *Per1* by a 1-h light pulse at Zeitgeber Time (ZT) 17 was lower in *Clock*/*Clock* mice relative to wild types as seen before (19). In constant darkness, *Clock*/*Clock* mice showed reduced *Per1* expression levels at all times relative

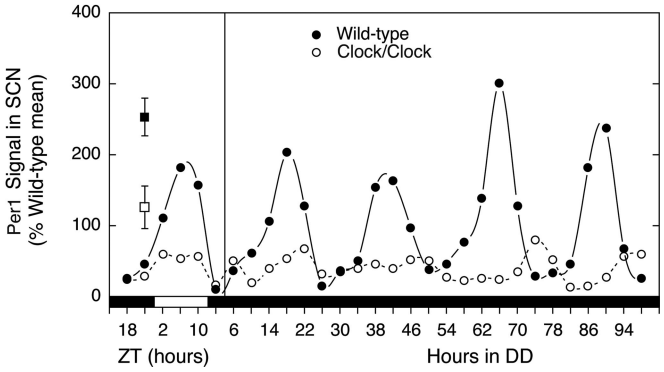

Fig. 2. Circadian rhythms of *Per1* mRNA expression in the SCN. Time course of *Per1* expression in the SCN of wild-type (●) and *Clock*/*Clock* (○) mice. Samples were collected every 4 h during the last cycle in LD and first 4 cycles of free run in constant darkness (DD). Lighting conditions are indicated by the bar at the bottom. Because data were collected in two replicate experiments, values were normalized to the wild-type mean levels. In addition, the induction by a 1-h light pulse at Zeitgeber Time (ZT) 17 are shown by the square symbols at the left. See Fig. 10 for representative images of peak and trough of *Per1 in situ* images.

to wild type. The rhythms of *Per1* expression damped out in constant darkness (although these levels are ensemble averages of four mice per genotype per time point so that some of the damping may be due to desynchrony). In addition, the amplitudes of *Per2* and *Per3* in the third cycle (58–78 h) in constant darkness also were greatly reduced in *Clock* mutants (Fig. 11, which is published as supporting information on the PNAS web site) as reported in LD12:12 conditions (20).

Expression of both *Per1* and *Per2* is induced by light (8–14). To examine the effects of the *Clock* mutation on light induction of *Per* genes in a manner more comparable to the phase response curve measurement, we determined the phase shifts and the light-induced expression of *Per1* and *Per2* in the SCN in +/+ and in *Clock*/+ C57BL/6J coisogenic mice in response to light pulses of different durations beginning at CT17 after 3 weeks in constant darkness (DD). With increasing light pulse duration up to 6 h, phase-shift magnitude continued to increase in the *Clock*/+ mice, whereas it saturated in wild-type mice after 1 h (Fig. 3*A*). In both *Clock*/+ and +/+ mice, light induced *Per1* at 1 h, but message levels returned to dark levels after 6 h of light exposure (Fig. 3*B*). In both genotypes, light also induced *Per2*, but levels remain elevated after 6 h of light exposure relative to dark controls (Fig. 3*C*). No differences in light induction of either *Per1* or *Per2* between *Clock*/+ and +/+ mice were detected. However, the amplitude of *Per1* and *Per2* rhythms in the SCN of *Clock*/+ mice was significantly lower than that of wild-type mice after 3 weeks in DD (Fig. 3 *D* and *E*). Thus, these experiments demonstrate that the *Clock*/+ mutation does not alter the photic induction of *Per1* and *Per2* but does lower the amplitude of circadian rhythms in these two genes in the SCN.

PER2::LUC Expression in SCN Explants. To examine further the effects of the *Clock* mutation on circadian pacemaker amplitude, *Clock* mutant animals were crossed to PER2::LUCIFERASE knockin mice (21). Both PER2::LUC-*Clock*$^{+/+}$ and PER2::LUC-*Clock*/+ SCN explants exhibited robust circadian oscillations of bioluminescence (Fig. 4 *A* and *B*). The luciferase expression from PER2::LUC-*Clock*/+ SCN, however, was significantly reduced in amplitude compared with that observed in wild-type littermates (30.3 counts/sec ± 3.83 SEM and 56.6 counts/sec ± 9.09 SEM, respectively; $P < 0.01$). Consistent with the behavioral phenotype observed here and in previous studies (1, 22), the period of the luciferase rhythm was lengthened by ≈1 h in PER2::LUC-*Clock*/+ SCN (24.29 h ± 0.12 SEM for wild type and 25.14 h ± 0.11 SEM for *Clock* heterozygotes; $P < 0.001$). Furthermore, PER2::LUC rhythms in *Clock*/+ SCN damped more rapidly than in wild-type SCN (mean damping rate: 12.2 days ± 1.91 SEM and 27.5 days ± 7.51 SEM, respectively, $P < 0.05$; the damping rate of an individual

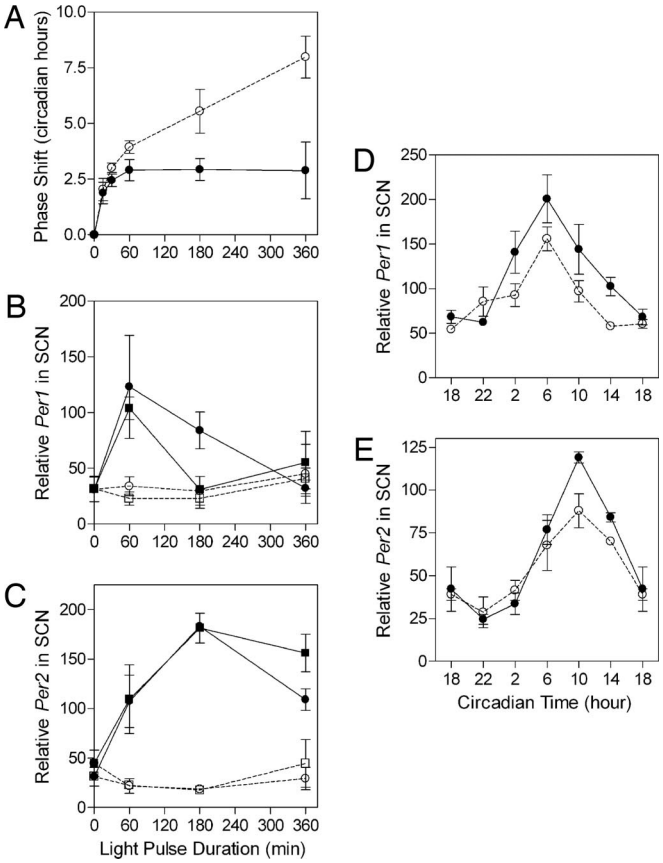

Fig. 3. Effects of light–pulse duration on the behavioral response (phase shifts) and induction of *Per1* and *Per2* in the SCN of C57BL/6J coisogenic mice. (*A*) Behavioral phase shifts (in circadian hours) as a function of light–pulse duration for wild-type (●) and *Clock*/+ (○) mice. Phase shifts were determined after 15, 30, 60, 180, and 360 min of light exposure that started at CT 17. *Clock*/+ mice exhibited significantly greater phase shifts than wild types (Tukey–Kramer; $P < 0.05$) in response to 180 and 360 min light pulses. (*B* and *C*) Relative *Per1* (*B*) and *Per2* (*C*) mRNA expression in the SCN of wild-type (● and ○) and *Clock*/+ (■ and □) mice. Samples were collected at CT 17 or after 60, 180, or 360 min of light exposure beginning at CT 17 (filled symbols). Dark-maintained controls also were sampled at 60, 180, or 360 min after CT 17 (open symbols). Overall, the induction of *Per1* and *Per2* genes to increase in light–pulse duration did not differ between the genotypes (no significant genotype effect by ANOVA. *Per1*: $F_{1,44} = 0.78$, $P > 0.05$ and *Per2* $F_{1,32} = 0.01$; $P > 0.05$). There is significant light induction of both genes (*Per1*: $F_{1,44} = 135.65$; $P < 0.001$ and *Per2*: $F_{1,32} = 89.23$; $P < 0.001$) as well as a significant effect of light pulse duration on induction (*Per1*: $F_{3,44} = 68.38$; $P < 0.001$ and *Per2*: $F_{3,32} = 3.96$; $P < 0.05$). For *Per1* expression, light-exposed levels are different from dark control levels at 60 and 180 min (Tukey–Kramer; $P < 0.05$). For *Per2* expression, light-exposed levels are different from dark control levels at 60, 180, and 360 min (Tukey–Kramer; $P < 0.05$). (*D* and *E*) Circadian rhythms of *Per1* (*D*) and *Per2* (*E*) mRNA expression in the SCN of wild-type (●) and *Clock*/+ (○) mice after 3 weeks in constant darkness. The amplitude of circadian rhythms in both *Per1* and *Per2* genes are significantly reduced in *Clock*/+ mice (*Per1*: $F_{1,71} = 0.6.71$; $P < 0.05$ and *Per2* $F_{1,71} = 5.40$; $P < 0.05$). For both *Per1* and *Per2*, there were significant differences between CTs (*Per1*: $F_{5,71} = 8.80$; $P < 0.001$ and *Per2*: $F_{5,71} = 46.85$; $P < 0.001$). For *Per1*, levels are significantly lower at CT18 than CTs 2, 6 and 10, and significantly higher at CT 6 than CTs 14, 18, and 22 (Tukey–Kramer; $P < 0.05$). For *Per2*, levels are significantly higher at CT10 than all other times, and CTs 6, 10, and 14 are significantly higher than CTs 18, 22, and 2 (Tukey–Kramer; $P < 0.05$).

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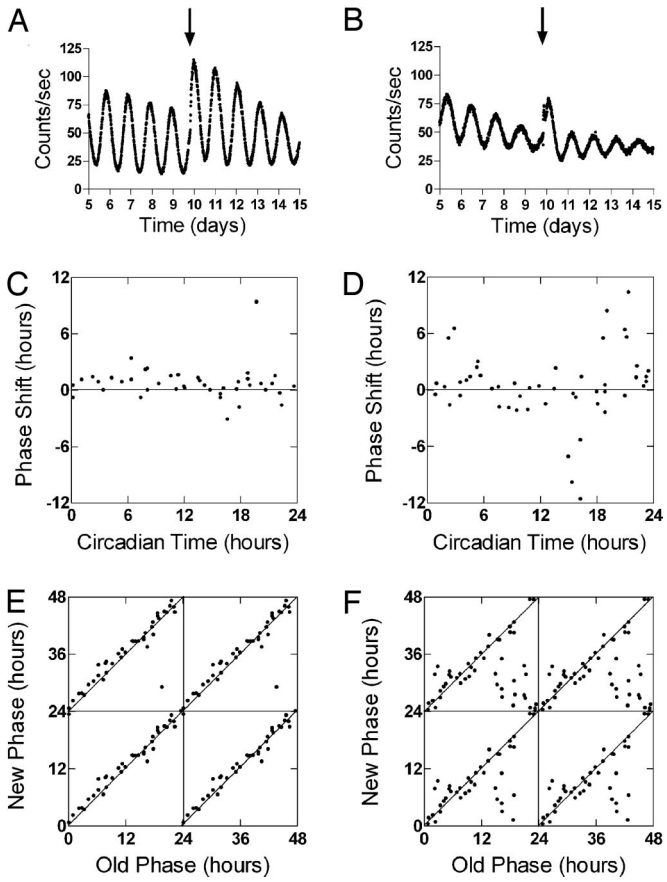

Fig. 4. Circadian rhythms of PER2::LUCIFERASE in SCN explants in response to medium change. (*A*) Representative bioluminescence rhythm in an SCN explant from PER2::LUC-wild-type mice. In this record, a medium change was administered at CT8 that resulted in no phase shift. The arrow indicates when the medium change occurred. (*B*) Representative bioluminescence rhythm in an SCN explant from PER2::LUC-*Clock*/+ mice. In this record, medium change was administered at CT10 that resulted in ≈2-h phase delay. (*C* and *D*) Phase–response curve to medium changes in SCN explants from PER2::LUC-wild-type ($n = 43$) (*C*) and PER2::LUC-*Clock*/+ ($n = 51$) (*D*) mice. The *x* axis indicates the CT when the medium changes occurred as determined from the preceding free-running period of PER2::LUC bioluminescence, with the peak of the rhythm defined as CT12. The *y* axis indicates the phase shift (in hours) produced by the medium change. (*E* and *F*) Phase–transition curve to the medium changes in SCN explants from PER2::LUC-wild-type (*E*) and PER2::LUC-*Clock*/+ (*F*) mice. Data from *C* and *D* are replotted so that the *x* axis indicates the old phase (CT) when the medium change occurred as determined from the preceding free-running period of the bioluminescence. The *y* axis indicates the new phase (CT) of the medium change calculated from the reset rhythm.

culture is the projected number of days required for the amplitude of the rhythm to decrease to $1/e$ of the starting value; ref. 23). To examine the effects of resetting agents on SCN explants, we used changes in culture medium as a perturbant. In wild-type SCN explants, medium changes induced small changes in the phase of PER2::LUC rhythms. By contrast, medium changes induced large phase shifts in *Clock*/+ SCN explants (Fig. 4 *C* and *D*). When plotted as phase–transition curves (Fig. 4 *E* and *F*), the wild-type SCN explants exhibited Type 1 resetting, whereas *Clock*/+ SCN explants exhibited responses intermediate between Type 0 and Type 1 resetting with a clustering of phases around CT0 to CT6, similar to that seen *in vivo* for the resetting effects of light pulses.

Discussion

Although phase–response curves to light have been determined in mice (24, 25), Type 0 phase resetting has not been described

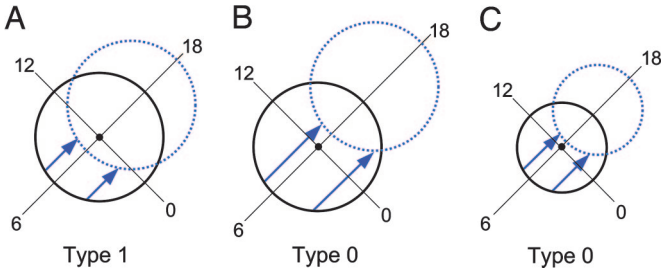

Fig. 5. Limit-cycle model of the circadian pacemaker. (*A*) A 2D limit cycle oscillator is represented diagrammatically by a circle that represents the steady-state path of the oscillatory system. In this rendition, time moves clockwise around the circle, and four phase points are indicated by the radial lines that represent ''isochrons,'' or sets of points with equivalent phase (26). The singularity, which is an unstable equilibrium point, is indicated by the black dot at the intersection of the isochrons. The blue vectors represent the perturbations caused by light pulses. During the perturbation, the system is carried off the limit cycle to a point in phase space indicated by the arrow. The dotted blue circle represents the set of points to which light would send the system at all possible phases of the cycle. In this diagram, vectors for only two examples are shown. The new phase of the system can be determined by the isochrons. The perturbed system would relax back to the limit cycle as it reaches steady state. In *A*, the strength of the light pulse is weak, and pulses cannot push the system across the singularity to the opposite phases of the cycle. Thus, new phase is similar to old phase and the resetting is Type 1. (*B*) The limit cycle is the same as that shown in *A*; however, the strength of the light input is strong and, therefore, the light can push the system across the singularity to the opposite side of the limit cycle, which results in very large phase shifts or Type 0 resetting. The stronger effect of light is represented by a larger (longer) vector. (*C*) In this limit cycle, the amplitude has been reduced by 30% and is represented by a limit cycle with a smaller diameter. Here the effect of light is the same as in wild type (the vector is the same size), but light now is strong enough to carry the system across the singularity to cause large phase shifts and Type 0 resetting.

in mice. Formally, the enhanced phase resetting effects of light in *Clock*/+ mice can be attributed to one of two different mechanisms: Either the strength of phase-shifting inputs to the circadian pacemaker is increased or, alternatively, the amplitude of the circadian pacemaker is decreased so that perturbing agents are more effective in causing phase shifts (26). Our analyses both *in vivo* and *in vitro* provide direct evidence that a reduction in circadian pacemaker amplitude is the primary mechanism by which light pulses cause Type 0 resetting in *Clock*/+ mice. The amplitude of circadian rhythms in both *Per1* and *Per2* are reduced by ≈30–50% in the SCN of *Clock*/+ mice. This change in amplitude is a relatively subtle decrease in comparison to that seen in *Clock* homozygous mice. Using *Per1* and *Per2* as indicators of the strength of photic inputs into the SCN, there was no detectable change in the magnitude or time of course of light induction in these genes in *Clock*/+ mice. Taken together, these results can be explained in a unified way by a simple limit cycle model (Fig. 5) in which the amplitude of the circadian pacemaker is reduced in *Clock* mutants, but the perturbing effects of resetting agents such as light or medium changes remain the same. Because *Per1* and *Per2* oscillate in the SCN in constant darkness and are rapidly induced by light during the subjective night, these two genes have been thought to represent the light-responsive elements of the mammalian circadian pacemaker at the molecular level (8–11, 27–30). In this model, we represent PER levels as a state variable in a simple 2D limit cycle oscillator shown diagrammatically in Fig. 5. The oscillation of PER levels is represented as a circle in phase space, and the relative amplitude of the oscillation is indicated by the diameter of the limit cycle (circle). The effects of light on PER levels are represented by a vector that increases PER levels and carries the system to a new point in phase space. Depending on

the phase and magnitude of the perturbing event (which is represented by the phase position and size of the vector), the system will be reset to a new phase. For weak inputs (when the vector is small), the system will be perturbed less, and small Type 1 phase shifts will occur. For strong inputs (when the vector is large), the system can be carried across the unstable equilibrium point known as the "singularity" and, as a consequence, very large Type 0 phase shifts will occur (26, 31). Fig. 5*A* represents a limit cycle for wild-type mice in which the amplitude is high and light inputs are weak, which results in Type 1 resetting. Fig. 5*B* represents the same limit cycle as in wild type except that light inputs are stronger, which results in Type 0 resetting. Finally, Fig. 5*C* represents the situation where amplitude is reduced by 30% (smaller diameter limit cycle) and light inputs remain the same as that seen in wild type (vector size is the same as in wild types). As seen in Fig. 5*C*, a reduction in circadian pacemaker amplitude now makes the relative effects of light stronger, which results in Type 0 resetting.

Interestingly, in the humans, Czeisler *et al.* (32) have reported Type 0 resetting of circadian rhythms in response to light pulses. Although Type 0 resetting is found in many lower organisms (33), it is less common among mammals, especially among nocturnal rodents where Type 1 resetting is normally seen (16, 24, 33). The *Clock* heterozygous mouse thus makes an interesting animal model for resetting in the human circadian system. For example, in organisms with Type 0 resetting, light pulses can reduce the amplitude of the system if exposed to "critical pulses" that drive the system near the singularity (26). Jewett *et al.* (15) have provided evidence that such critical pulses can repress circadian amplitude in human subjects. The *Clock*/+ mouse provides another mammalian circadian model to analyze the consequences of such perturbations.

In *Drosophila*, circadian amplitude is modulated by temperature, exhibits a latitudinal cline in nature, and can regulate photoperiodic responses (34). Amplitude has also been associated with circadian period mutants in *Neurospora* (35). The results presented here highlight the significance of amplitude in the function of circadian pacemakers in mice. In particular, they illustrate that the relative amplitude of the circadian pacemaker in comparison to the strength of its entraining inputs is the critical factor. Because Type 1 or low-amplitude phase–response curves are the norm in mammals (33), there appears to be adaptive value in having a robust circadian oscillator that is relatively resistant to phase perturbations (the Type 1 situation). The degradation of circadian rhythms during aging is accompanied by both a loss of amplitude and fragmentation of output rhythms (36). Perhaps such reductions in circadian amplitude can contribute to the instability of circadian rhythms and other homeostatic processes in the elderly (37).

Materials and Methods

Animals. All mice were produced in our breeding colony in the Center for Comparative Medicine at Northwestern University and were maintained and used according to Institutional Animal Care and Use Committee approved procedures. Mice used for behavioral studies and for *in situ* hybridization were coisogenic C57BL/6J or (BALB/cJ × C57BL/6J)F_2 mice. Mice used for SCN explants were 129S1 × C57BL/6J N2 and N3 backcross animals.

Activity Monitoring. Wheel-running activity rhythms were monitored as described in ref. 1. See *Supporting Text*, which is published as supporting information on the PNAS web site, for details.

Phase–Response Curve Experiments. Mice used to determine phase shifts to light pulses were housed in LD12:12 schedule for 1 week then released into constant darkness. After 3 weeks of free run, a 6-h light pulse was given, and data were recorded for an additional 10 days in constant darkness after the light pulse.

Phase of Entrainment. The phase of entrainment was determined from the initial free-running activity rhythm upon release into constant darkness. A line was eye fit through times of activity onset for the first seven cycles in constant darkness. The phase of entrainment was determined by extrapolation of the eye-fit line backwards to the last day of the light-dark cycle. This value is expressed relative to the time of lights off (38). For analysis, phases were converted to degrees from hours, and vector means were calculated (18) as described in Davis and Menaker (39). To test for significant clustering of phases, a Rayleigh test was performed (18). To assess differences between phases of different groups, a Mardia–Watson–Wheeler test was used (18).

Phase-Shift Magnitude. The phase shift in response to a 6-h light pulse was determined from the steady-state phase of onset for 7 days preceding and 7 days after the day of the light pulse. A line was eye fit through times of activity onset for the 7 days before the light pulse. The extrapolated point of intersection with the day of the light pulse was taken as the time of activity onset, or CT12. A period estimate also was taken for this same 7-day interval. The CT at the beginning of the light pulse was calculated from the period and CT12 (38). For comparison, a second line was eye fit through onsets during the 7 days after the light pulse. The time difference between the intersection points of the two lines with the day of the light pulse was the magnitude of the phase shift. This value was corrected for circadian period as estimated before the light pulse for each individual. A total of 215 animals were used in this analysis.

***In Situ* Hybridization.** Standard *in situ* hybridization procedures were used as described in ref. 40. See *Supporting Text* for details. ^{33}P-labeled antisense RNA probes were synthesized by using Ambion MaxiScript *In Vitro* Transcription Kit from templates containing nucleotides 468–821 of *Per1* (accession no. AF 022992), 15–477 of *Per2* (accession no. AF 035830), and 105–590 of *Per3* (accession no. AF 050182). Quantitation of the autoradiogram signal was performed by using NIH IMAGE software. The OD of individual SCN was normalized by subtracting the OD of an area of identical size in the lateral hypothalamus from the same side (left or right) and section. Normalized values of both SCN from three sections near the middle (anterior-posterior) were used to calculate an average for each brain.

***PER2::LUC-Clock* Bioluminescence Experiments and Data Analysis.** The SCN explants from PER2::LUC-*Clock* animals were cultured as reported in refs. 21 and 22. The entire experimental setup was placed within temperature-controlled room maintained at 36°C, and the medium changes also were conducted in the room to maintain constant temperature during the perturbation of the SCN cultures. Medium changes occurred at a predetermined time by simply lifting the Millicell culture membrane and placing it into a new culture dish prepared with fresh media. The phase of the PER2::LUC-*Clock* bioluminescence was determined after ≈10 days of initial monitoring, and the peak of bioluminescence rhythm was defined as a reference phase, CT12. The data reported here were obtained by using a LumiCycle apparatus, and all bioluminescence analyses were performed by the LumiCycle Analysis Program (Actimetrics, Wilmette, IL).

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