this produced a range for the *P* value of 0.00002 to 0.0002. Although the simulation results suggest that the actual *P* value is much closer to the smaller end of this interval, *P* values were conservatively reported as less than the calculated upper bound.
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Resetting Central and Peripheral Circadian Oscillators in Transgenic Rats

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In multicellular organisms, circadian oscillators are organized into multitissue systems which function as biological clocks that regulate the activities of the organism in relation to environmental cycles and provide an internal temporal framework. To investigate the organization of a mammalian circadian system, we constructed a transgenic rat line in which luciferase is rhythmically expressed under the control of the mouse *Per1* promoter. Light emission from cultured suprachiasmatic nuclei (SCN) of these rats was invariably and robustly rhythmic and persisted for up to 32 days in vitro. Liver, lung, and skeletal muscle also expressed circadian rhythms, which damped after two to seven cycles in vitro. In response to advances and delays of the environmental light cycle, the circadian rhythm of light emission from the SCN shifted more rapidly than did the rhythm of locomotor behavior or the rhythms in peripheral tissues. We hypothesize that a self-sustained circadian pacemaker in the SCN entrains circadian oscillators in the periphery to maintain adaptive phase control, which is temporarily lost following large, abrupt shifts in the environmental light cycle.

Most (perhaps all) multicellular organisms contain multiple circadian oscillators interconnected to form a hierarchical circadian system which regulates many discrete rhythmic outputs. In mammals, the SCN contains self-sustained circadian oscillators that act as a pacemaker at the top of the hierarchy (*1, 2*). In addition, a surprising number of mammalian peripheral tissues appear to contain the molecular machinery necessary for circadian oscillation (*3, 4*) and, in a few cases, peripheral tissues exhibit damped circadian oscillations in the absence of the SCN (*5, 6*). Our working hypothesis is that the mammalian circadian system consists of self-sustained circadian oscillators in the SCN that entrain damped oscillators in the periphery. Predictions from this hypothesis include, but are not limited to: (i) the existence of damped circadian oscillators in peripheral tissues, and (ii) temporary disorganization within the circadian system in response to large, abrupt changes in the entraining light cycle. Such disruption would be a consequence of the specific and different response of each of the peripheral oscillators to common central signals.

To test the first prediction, we constructed a transgenic rat model in which the mouse *Per1* gene promoter is linked to a luciferase reporter (Fig. 1A) (*7*). We raised the resulting *Per1-luc* rats in light:dark (LD) cycles of 12 hours light and 12 hours darkness (LD 12:12), killed them 30 to 60 min before lights-off (onset of darkness), and cultured explants of SCN, skeletal muscle, liver, and lung under static conditions (i.e., without changing the medium) in constant darkness and constant temperature (36° ± 0.2°C) (*8*). We measured the light output continuously from individual cultures with a Hamamatsu photomultiplier tube detector assembly (*9, 10*).

Light emission from the SCN was invariably and robustly rhythmic (n = 62), indicating that the engineered mouse *Per1-luc* transgene was being rhythmically transcribed under the control of normal circadian mechanisms. The SCN rhythm persisted for up to 32 days in static culture (Fig. 1B). Preliminary data (*11*) from animals killed 3 or 9 hours before lights-off indicates that these times of sacrifice do not affect the phase of the SCN rhythm. Liver, lung, and skeletal muscle all showed circadian rhythms of light output that phase-lagged the SCN rhythm by 7 to 11 hours. This phase difference between the SCN and peripheral tissues is similar to the phase-lag observed in vivo (*12*). The rhythms that we recorded from peripheral tissues were not as robust as those recorded from the SCN, and always damped after two to seven cycles in culture (Figs. 1C and 2). Damping was not the consequence of tissue death or deterioration because we were able to reinitiate rhythmicity in damped cultures by simply changing the medium (Fig. 2). This reinitiation could be due to removal of toxic substances in the old medium, replacement of depleted critical components, or simply to the shocks associated with the medium change (e.g., temperature, pH, and mechanical agitation). Any or all of these stimuli could act to restart oscillators that had stopped or to resynchronize multiple oscillators within the tissue.

To test the second prediction made by our hypothesis, we investigated whether abrupt shifts of the light cycle caused disorganization of the circadian system of the *Per1-luc* rats. We advanced or delayed the LD 12:12 cycle on which the rats had been living by 6 hours (*13*) and measured the phase and, in some cases, the amplitude of circadian rhythms of locomotor behavior (*14*), and the phase and amplitude of *mPer1* expression in the SCN, liver, lung, and skeletal muscle in vitro (*15*). Locomotor rhythmicity was monitored before and after

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the shift in one group of rats (Fig. 3). For the in vitro measurements, rats were killed either before the shift (control) or after either one or six full cycles following the light cycle shift (Fig. 3), and tissues from skeletal muscle, liver, lung, and SCN were assayed for circadian rhythmicity. The SCN shifted almost completely after the first cycle, but locomotor rhythmicity shifted more slowly [compare (*16*)]; rhythmicity in each of the three peripheral tissues shifted differently, and in all cases more slowly than the SCN. In some cases, the light cycle shift partially or completely suppressed rhythmicity of one or more of the peripheral oscillators for several cycles.

Primarily on the basis of behavioral assays of rodents (wheel running) and the subjective impressions of people traveling rapidly across time zones, one would expect phase delays to be accomplished more rapidly and with less disturbance than phase advances. This has been shown previously for activity rhythms of rats (*17, 18*) and is confirmed by our locomotor data (Fig. 3). It is true also for the in vitro rhythms in muscle and lung, but, surprisingly, not for liver.

Circadian oscillation in muscle and liver peaked about 10 to 11 hours after SCN, whereas lung peaked about 7 hours after SCN (Fig. 4, A through D, and Table 1). The peripheral tissues each responded differently to the light cycle shifts. Following the delay shift, skeletal muscle and lung were phase-shifted almost 4 hours on the first complete cycle and were completely shifted (6 hours) by the sixth complete cycle. Liver, on the other hand, was not delayed at all on the first complete cycle. Four of eight liver cultures had no circadian rhythm (Fig. 4J); the other four livers were rhythmic and were either unshifted or slightly advanced. By the sixth complete cycle, the liver cultures had only delayed by about 3.5 hours. By the sixteenth complete cycle, liver cultures were still only delayed about 4 hours [n = 4 (*11*)]. Thus, in response to delays of the light cycle, muscle and lung behaved more or less as predicted by a good deal of indirect evidence, whereas the phase-shifting behavior of liver was completely unexpected.

A similar pattern was seen in the response of these three tissues to phase advances of the light cycle. As expected, advances were more difficult than delays for muscle and lung. On the first complete cycle following the phase advance, six of eight of the muscle cultures were either arrhythmic or had such severely reduced amplitude that it was difficult to accurately determine phase (Fig. 4, G and H). By the sixth cycle following the 6-hour advance, all the muscle cultures were normally rhythmic and fully shifted. The rhythm from one of the four lung cultures was disrupted on the first complete cycle following the phase advance (Fig. 4L), and the other three were slightly delayed. By the sixth cycle, all lung cultures were rhythmic and fully shifted. On the other hand, none of the six liver cultures became arrhythmic following the advance, although they only shifted by about 2 hours. By the sixth cycle, all the liver cultures were fully advanced. In a separate experiment, we exposed animals to 9-hour advances and delays and cultured only SCN and skeletal muscle. SCN was almost completely phase-shifted by the first cycle in both directions. However, none of the four skeletal muscle cultures was rhythmic on the first cycle after the advance, and the average shift on the first cycle after the delay was only 3 hours (Fig. 5).

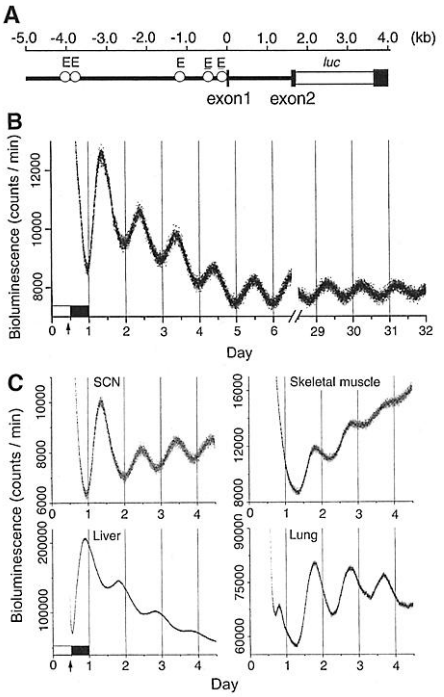


Fig. 1. (**A**) Diagram of the mouse *Per1* transgene. Heavy line, *mPer1* fragment; open bar, luciferase fragment; shaded box, polyadenylation fragment; circles represent E boxes. (**B**) Representative circadian rhythm of bioluminescence from a cultured SCN explanted from a *Per1-luc* transgenic rat. Black and white bars show the animal's previous LD conditions. The explant was made just before lights-off (arrow), and luminescence was monitored immediately. The near 24-hour rhythm peaked in the middle of the subjective day and persisted for 32 days in vitro, at which time the culture was removed from the assay. Rhythmicity persisted for more than 2 weeks in the seven SCN cultures that were maintained for this length of time; other SCN cultures were terminated after shorter times while still rhythmic. Similar rhythmicity of lower intensity was found in a second line of *Per1-luc* transgenic rats [W(perl)5], in which the SCN rhythm also peaked in the middle of the subjective day and persisted for up to 28 days in vitro. (**C**) Circadian rhythms expressed in vitro from several different tissues from the same animal. The tissues were explanted just before lights-off (arrow).

Our data are consistent with the hypothesis that the mammalian circadian system is hierarchically organized, with self-sustained oscillators in the SCN entraining damped oscillators in the periphery. We would not expect the peripheral oscillators to damp in vivo, because they receive a continuous stream of rhythmic signals from the SCN; such signals could be either neural, humoral, or both (*19, 20*) and would entrain each of the peripheral oscillators to a specific adaptive phase relationship to the SCN and to the others. The SCN, in turn, is adaptively entrained by external signals, primarily light signals, and thus determines the phase relationship of the organism to its environment. Such an arrangement would be capable of generating the complex phase relationships among multiple overt rhythms that are known to exist (*21, 22*) and are almost certainly critical to normal function.

There are other possible ways to orga-

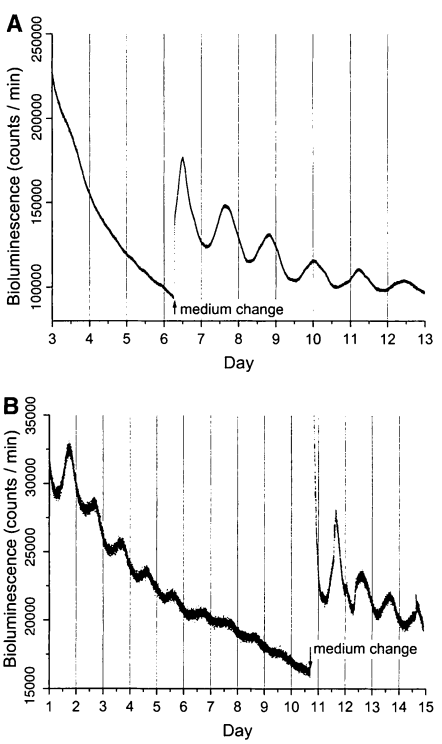


Fig. 2. Reinitiated circadian oscillation in cultured liver and lung. (**A**) The circadian rhythm in liver culture damped after 6 days in vitro and was reinitiated by changing the medium (arrow). The rhythm damped again by day 17, and another medium change on day 17 reinitiated the rhythm (*11*). Although the period of the initial oscillation was about 24 hours, that of the rhythm induced by the medium change was about 30 hours. (**B**) Cultures of lung tissue behaved similarly; however, rhythmicity could not be reinitiated in muscle by medium change alone but could be reinitiated if serum was added to the new medium (*11*).

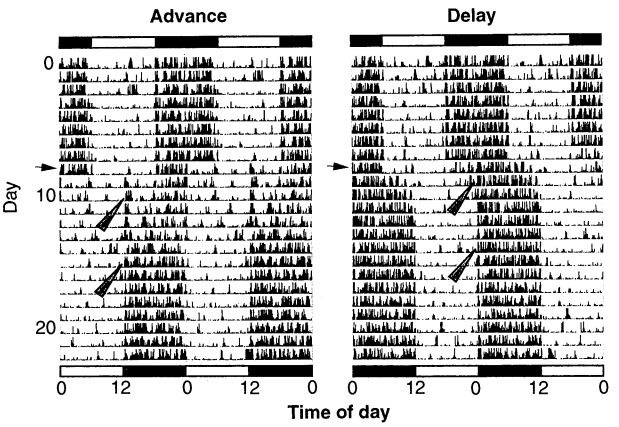


Fig. 3. Representative locomotor activity records from *Per1-luc* transgenic rats in phase-advanced and phase-delayed light cycles. General activity was monitored with an implanted transmitter and has been double plotted. The original LD cycle (black and white bars at top of figure) was shifted either 6 hours earlier (left panel) or 6 hours later (right panel) on the day indicated by the arrow (new light cycle indicated by black and white bars at bottom of figure). The burst of activity at lights-off ("masking") on the first five cycles following the advance is a direct effect of the light-to-dark transition on locomotion. Masking is commonly observed in nocturnal rodents and does not indicate the phase of clock-controlled behavior. Complete reentrainment as measured by visual inspection took about 6.2 ± 0.5 cycles (SEM, n = 10) following the phase advance or 1.8 ± 0.3 cycles (n = 10) following the phase delay. Shaded triangles indicate the times of sacrifice (on the first and sixth cycles following the phase shift) of the animals for which the time of SCN and peripheral tissue peaks is plotted in Fig. 4. Similar phase-shifting trajectories of locomotor rhythmicity have been previously observed in rats (*17, 18*).

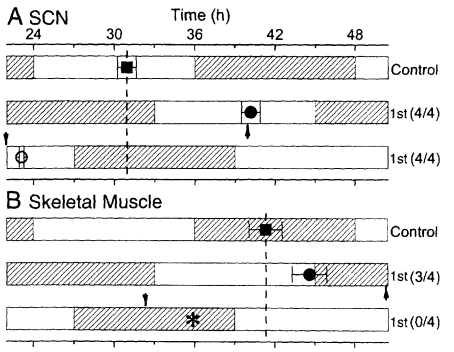
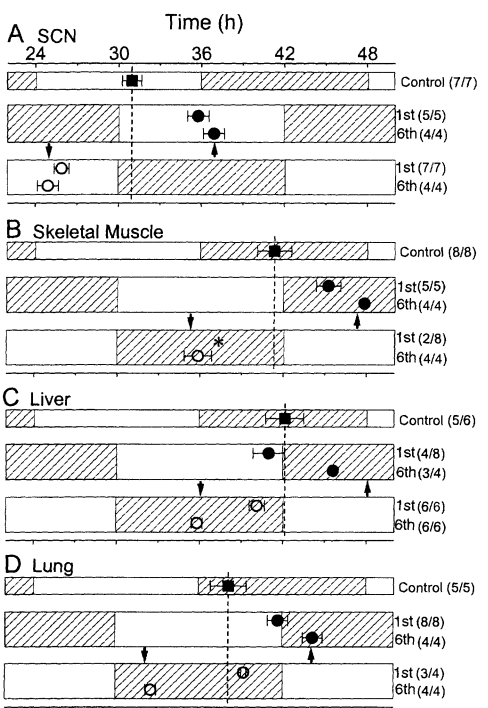


Fig. 5. Reentrainment following 9-hour delays and advances of the light cycle. The average times of peak of cultured SCN (**A**) and skeletal muscle (**B**) from unshifted control animals (filled squares), 9-hour phase-delayed animals (filled circles), and 9-hour phase-advanced animals (open circles) are plotted as in Fig. 4. Asterisk: all muscle cultures from advanced animals were arrhythmic. All cultures (except controls) were made after one full day following the light cycle shift.

nize circadian systems; indeed, somewhat different patterns of organization have been found in *Drosophila melanogaster*, in which the peripheral oscillators are light-sensitive (*23*), and in nonmammalian vertebrates, which have multiple circadian photoreceptors and central pacemakers composed of several interconnected self-sustained oscillators (*24*). Nonetheless, the existence of distributed oscillators, whatever their properties and connections, is a common attribute of all circadian systems so far described in multicellular organisms. This may be the simplest way to construct a system, which requires that many constituent rhythms have different phases, because the phase of each rhythm can be adjusted by modifying the response properties of the tissue's own peripheral oscillator to an invariant signal from the central oscillator or from the environment. Such organization provides great flexibility, both phylogenetically and ontogenetically, and as more is learned, we can expect to find



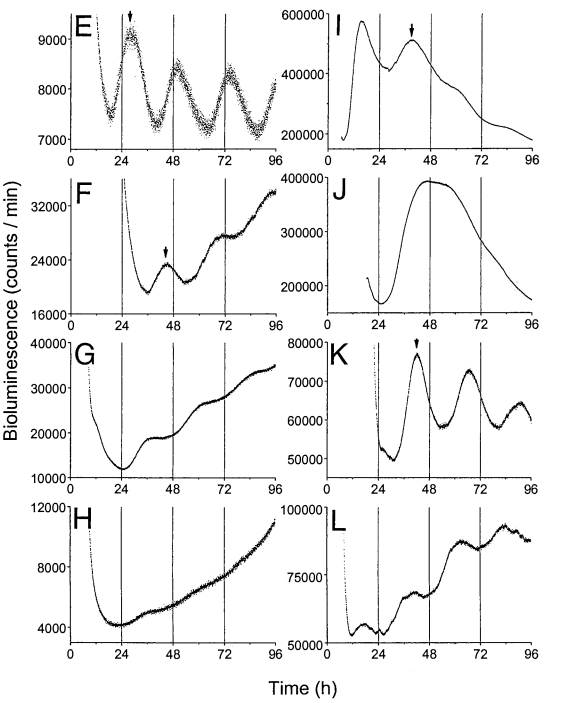


Fig. 4. (**A** through **D**) Rate of reentrainment in central and peripheral oscillators. The peak of the circadian oscillation in culture was determined by measuring the highest point in the first complete cycle in vitro. The average times (±SEM) of peaks from the unshifted control animals (filled squares) and those that were 6-hour phase-delayed (filled circles) or 6-hour phase-advanced (open circles) are plotted against original LD cycles (upper bar in each panel) and the phase-shifted LD cycles (lower bars in each panel). Arrows, the point of complete phase shift (i.e., 6 hours from the control phase for each tissue). In liver (occasionally in lung), the first peak appeared before 24 hours (e.g., in Fig. 1C, 4I). Because this peak was partially obscured by the nonspecific light emission produced by the tissue preparation process, we did not use it for analysis. The sample size is in parentheses [(number of rhythmic tissues)/(number of tissues tested)]. Asterisk, most of these muscle cultures were arrhythmic or had very low-amplitude rhythms (see Fig. 4, G and H, and Table 1). (**E** through **L**) Examples of rhythms from tissues cultured from animals on the first complete cycle after 6-hour phase delay or advance. (E) SCN from advanced animal; (F) skeletal muscle from delayed animal; (G and H) skeletal muscle from advanced animal; (I) liver from advanced animal; (J) liver from delayed animal; (K) lung from delayed animal; (L) lung from advanced animal (this was the only lung culture that showed a disrupted rhythm, and it was not included in the phase analysis). Rhythms of skeletal muscle were categorized as rhythmic (F), low-amplitude rhythm (G), or arrhythmic (H); only the data categorized as "rhythmic" were used in the analysis of peak phase in Figs. 4 and 5. Arrows in (E), (F), (I), and (K) indicate the peak phases chosen from those data sets.

Table 1. Peak phases of bioluminescence (mean ± SEM, in hours after last lights-on) in the SCN, skeletal muscle, liver, and lung. The effect of advancing or delaying the light schedule by 6 or 9 hours was measured on the first or sixth cycles following the shift in the light cycle. Average phase shifts (in parentheses) were measured relative to the average peak phase of tissues taken from control animals. The magnitude of the phase shifts of the different tissues were compared to that observed in the SCN.

Treatment	SCN	Skeletal muscle	Liver	Lung
Control	30.9 ± 0.7	41.3 ± 1.2	42.1 ± 1.4	38.1 ± 1.3
6 hour delay 1st	35.8 ± 0.8* (−4.9)	45.2 ± 0.9* (−3.9)	41.0 ± 1.1 (+1.1)†	41.7 ± 0.7* (−3.6)
6 hour delay 6th	36.9 ± 0.8* (−6.0)	47.9 ± 0.2* (−6.6)	45.6 ± 0.0 (−3.5)†	44.2 ± 0.7* (−6.1)
6 hour advance 1st	25.9 ± 0.5* (+5.0)	AR‡	40.1 ± 0.6 (+2.0)†	39.2 ± 0.2 (−1.1)†
6 hour advance 6th	24.9 ± 0.8* (+6.0)	35.9 ± 1.0* (+5.4)	35.8 ± 0.4* (+6.3)	32.6 ± 0.4* (+5.5)
9 hour delay 1st	40.2 ± 0.7* (−9.3)	44.6 ± 1.3 (−3.3)	Not tested	Not tested
9 hour advance 1st	23.1 ± 0.2* (+7.8)	AR§	Not tested	Not tested

*Significant phase shift; ANOVA followed by Dunnetts test, $P < 0.05$. †Significantly smaller shift than in the SCN, ANOVA followed by Dunnetts test, $P < 0.05$. ‡Two muscle cultures were rhythmic with peak at 34.8 (+6.5), four muscle cultures had low-amplitude rhythms with peak around 37.2 (+4.1), two muscle cultures were arrhythmic. §All muscle cultures were arrhythmic.

that major adjustments of relative phase relationships have been brought about by natural selection (e.g., in diurnal versus nocturnal organisms) and also occur in the course of development.

A system organized in this way can adjust without difficulty to small, gradual changes in the phase of the input signal (e.g., seasonal changes in day length), but may become temporarily and severely disorganized if the change in phase of the input signal is large and abrupt. Rapid travel across several time zones and abrupt changes in work schedules both produce sudden large changes in input signals to the circadian system of humans by exposing the SCN to shifted light (and other environmental) cycles. The phase-shifting paradigm that we employed in these experiments is closely analogous to trans-Atlantic flights from west to east (advance) and from east to west (delay). Of the tissues whose rhythmicity we measured, SCN, muscle, and lung (but not liver) resumed their normal phase relationships to each other and to the light cycle within 6 days of the shift in either direction. During those 6 days, however, these phase relationships have been, for some as yet undetermined number of cycles, grossly abnormal, and some individual rhythms have been severely disrupted. If the phase relationships among the many rhythms that characterize circadian organization are adaptive (and it would be surprising if they were not), then disruption of those phase relationships will have deleterious consequences. That is likely to be the general explanation of the malaise that results from rapid transmeridian travel, and more importantly, from the shift work schedules to which more than 20% of the U.S. work force is exposed (*25*).

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7. A mouse *Per1* genomic fragment of 6.7 kb was ligated directly to the second codon of the firefly luciferase cDNA flanked by the SV40 late polyadenylation signal. The *mPer1* fragment includes five functional E box regions, a transcription initiation site, the first and second exons, which are split by the first intron, and a translational start codon in the second exon (Fig. 1A). As expected, the reporter gene was induced by the concerted action of *Clock* and *Bmal1*, and repressed by either *Cry1* or *Cry2* in a transient cotransfection assay (A. Hida *et al.*, *Genomics*, in press). The linearized reporter fragment was microinjected into 302 fertilized eggs of Wistar rats (Charles River Japan Inc.); [S. Hochi, T. Ninomiya, M. Homma, A. Yuki, *Anim. Biotechnol.* **1**, 175 (1990)]. Transgenic rats were identified by polymerase chain reaction, and the copy number of the transgene was determined by Southern analysis. Six transgenic rats were obtained by the screening of 60 weaned pups. All of the six founder rats developed normally, although one was sterile and one was mosaic. Luciferase activity in brain extracts of the four transgenic lines was roughly proportional to the copy number of the reporter gene. A transgenic line W(perl)1], which showed circadian oscillation of luciferase activity in the SCN, was selected for further study. There are approximately 12 copies per genome of the transgene integrated in W(perl)1, and the luciferase activity in the brain is 1431 relative luminescence units per mg protein. We measured the period of the wheel-running activity rhythm in constant darkness for both male *Per1-luc* and male wild-type controls. The period of the transgenic rats (at 8 to 10 weeks of age) was 24.43 ± 0.02 hours (SEM, $n = 20$), which is very close to the period of the wild-type animals (24.33 ± 0.01 hours, $n = 26$) at 6 to 8 weeks of age, indicating that the transgene does not disrupt molecular circadian time keeping.
8. Paired SCN were explanted from 400-μm coronal sections of brains from transgenic rats made with a Vibroslicer and placed on a culture membrane (Millicell-CM, PICM030-50; Millipore) in a covered and sealed petri dish. Sections of muscle, liver, and lung (1 mm thickness; 1 to 2 mm square) were cultured as above, without the Millicell membrane.
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10. SCN were cultured in a 35-mm petri dish with 1.2 ml culture medium [serum-free, low sodium bicarbonate, no phenol red, Dulbecco's modified Eagle's medium (Gibco)] supplemented with 10 mM HEPES (pH 7.2), B27 (2%, Gibco), 0.1 mM luciferin (Promega), and antibiotics (25 U/ml penicillin, 25 μg/ml streptomycin). Bioluminescence was measured with photomultiplier tube (PMT) detector assemblies (Hamamatsu). The modules and cultures were maintained in a light-tight, water-jacketed incubator at 36°C and interfaced to IBM PC-type computers for continuous data acquisition. The PMT was positioned about 2 cm above the culture, and photon counts were integrated over 1-min intervals. Dark counts (nonspecific counts) from the PMTs were about 20 to 40 counts per second at 36°C.
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13. The 6-hour phase advance was accomplished by advancing the time of lights-on. The 6-hour phase delay was accomplished by delaying the time of lights-off.
14. Male heterozygous transgenic rats were weaned and transferred to individual running-wheel cages at 4 weeks of age. They were maintained in the same LD conditions to which they had been previously exposed. Two days after transfer, animals were anesthetized with sodium pentobarbital, and a radiotransmitter (Mini Mitter) was implanted into the peritoneal cavity. General activity was monitored using the Data Quest system (Data Science International). Light was produced by a 40-W fluorescent lamp (Philips) (30 to 60 μW/cm^2; about 100 to 200 lux at cage level.) After 2 weeks of entrainment, LD cycles were either advanced or delayed.
15. Heterozygous transgenic rats were raised under LD 12:12 cycles. Mother and pups were group housed until they were killed. The light conditions were identical to those used in the behavior experiments. At the time of sacrifice, animals were 15 to 41 days old. Both males and females were used, and no sex differences were observed.
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