Hierarchical organization of the circadian timing system

Steensel, M.J. van

Chapter 5

Dissociation between circadian *Per1* and neuronal and behavioral rhythms following a shifted environmental cycle

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SUMMARY

The suprachiasmatic nucleus (SCN) of the anterior hypothalamus contains a major circadian pacemaker that imposes or entrains rhythmicity on other structures by generating a circadian pattern in electrical activity [1, 2]. The identification of “clock genes” within the SCN [3–6] and the ability to dynamically measure their rhythmicity by using transgenic animals open up new opportunities to study the relationship between molecular rhythmicity and other well-documented rhythms within the SCN. We investigated SCN circadian rhythms in *Per1-luc* bioluminescence, electrical activity in vitro and in vivo, as well as the behavioral activity of rats exposed to a 6-hr advance in the light-dark cycle followed by constant darkness. The data indicate large and persisting phase advances in *Per1-luc* bioluminescence rhythmicity, transient phase advances in SCN electrical activity in vitro, and an absence of phase advances in SCN behavioral or electrical activity measured in vivo. Surprisingly, the in vitro phase-advanced electrical rhythm returns to the phase measured in vivo when the SCN remains in situ. Our study indicates that hierarchical levels of organization within the circadian timing system influence SCN output and suggests a strong and unforeseen role of extra-SCN areas in regulating pacemaker function.

RESULTS AND DISCUSSION

Per1-luc Bioluminescence

Previous studies on the response of *Per1-luc* bioluminescence to phase advances in the light-dark schedule revealed that on the first cycle following a 6-hr phase advance, the *Per1-luc* luminescence rhythm is advanced by 5.0 ± 0.5 hr, relative to an average control peak time at ZT 6.9 ± 0.7 (n = 7) [7]. In the present experiment, we sought to determine whether this initial phase shift is stable by allowing the animal to remain in DD (constant darkness) following the phase-advanced light-dark schedule (Figure 1A). Phase shifts were measured on days 3 and 6 in DD following the phase advance in the light-dark schedule (Figure 2A). The peak times of *Per1-luc* bioluminescence were advanced by 3.9 ± 0.7 hr (day 3, n = 6) and 7.3 ± 0.8 hr (day 6, n = 7). The peak times on these days differed significantly from the peak time on the day prior to the phase advance of the light-dark cycle; this finding indicates that the *Per1* phase shift persisted after several cycles in DD ($p < 0.01$, ANOVA with post hoc Dunnett's test).

In Vitro Electrophysiology

Electrical activity recordings were performed simultaneously in the dorsal and ventral SCN. No consistent differences between these areas were detected (see the Supplemental Results and Discussion in the Supplemental Data available with this article online; Figure 1B). In slices that were prepared on the day before the phase advance, peak electrical activity occurred at ZT 6.1 ± 1.0 (n = 6) (Figure 2B). At days 1 and 3 after the advance, the average peak in electrical activity shifted by 3.0 ± 1.0 hr (n = 9) and 3.8 ± 1.0 hr (n = 6), respectively. The average peak time differed significantly from the peak time before the phase advance ($p < 0.05$, ANOVA with post hoc Dunnett's test). In contrast, at day 6 in DD, the peak in electrical activity was advanced by only 0.8 ± 0.8 hr (n = 7), which did not differ significantly from the peak time before the advance ($p > 0.05$, ANOVA with post hoc Dunnett's test).

In Vivo Electrophysiology and Behavior

Electrical activity from the rat SCN was recorded in vivo in freely moving animals. Recordings were obtained for several days prior to and after the phase advance in the light schedule. In addition, behavioral activity was recorded, which allowed us to compare, within the same animal, SCN electrical activity and behavioral data. Electrical activity rhythms were recorded successfully from the SCN of five wild-type animals and three transgenic W(perl)1 animals (Figure 1C). On the last day prior to the advance in the light schedule, the average peak time occurred at ZT 5.6 ± 0.6 (n = 8, Figure 2C). After the advance, the peak times were not significantly different from the peak time before the phase advance ($p > 0.9$, ANOVA with post hoc Dunnett's test), and no differences were observed between wild-type and transgenic animals.

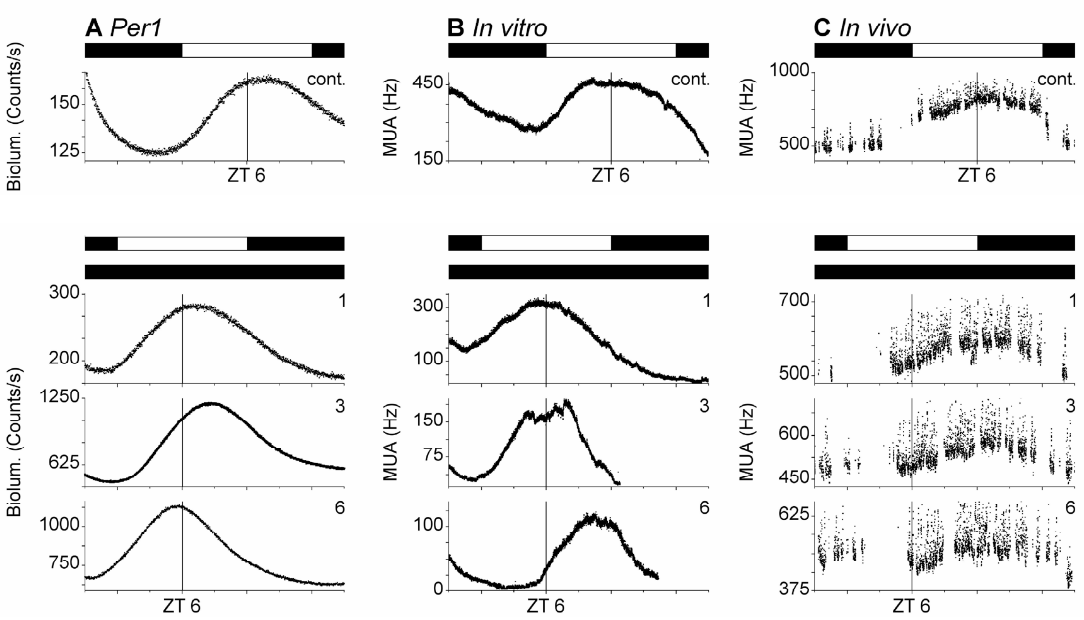

Figure 1. Examples of *Per1-luc* Bioluminescence Rhythms and SCN Electrical Activity Rhythms Recorded In Vitro and In Vivo at the Day before the Advance and Days 1, 3, and 6 after the Phase Advance of the Light-Dark Cycle

(A) Examples of *Per1-luc* bioluminescence rhythms. The graphs from top to bottom indicate the day before the advance (cont.) and days 1, 3, and 6 in DD, respectively (see the Experimental Procedures). The bioluminescence rhythm in Hz is indicated per minute. The vertical lines in the figure panels indicate ZT 6 in the unshifted state and after the phase advance. The bars above the panels indicate lights on (white) and lights off (black) before, during, and after the phase advance. The bioluminescence rhythms of the control day and day 1 were taken from the dataset used in Yamazaki et al. [7].

(B) Examples of SCN electrical activity rhythms recorded in vitro. The figure layout is as in (A). The multiunit activity in Hz is indicated every 10 s.

(C) Example of the SCN electrical activity rhythm of a rat recorded in vivo. The figure layout is as in (A). The multiunit activity in Hz is indicated every 10 s. Episodes of multiunit activity that contain movement artifacts were deleted, resulting in missing values in the dataset.

The average time of behavioral activity onset occurred shortly before the time of lights off at ZT 11.8 ± 0.1 (n = 8, Figure 2C). After the advance, none of the average activity onset times were significantly different from the mean activity onset time measured on the day prior to the shift in the light schedule ($p > 0.9$, ANOVA with post hoc Dunnett's test).

Our data reveal surprising and significant differences in the kinetics of phase readjustments of molecular and neuronal rhythms within the SCN and overt behavioral activity (Figure 3; see the Supplemental Data for analysis and Methodological Considerations 1). Considering first the behavior, given the phase shifting effects of short light pulses in rats [8, 9], the absence of a phase advance in behavioral activity is surprising. We confirmed this observation, however, in a separate behavioral experiment (see Figure S1 in the

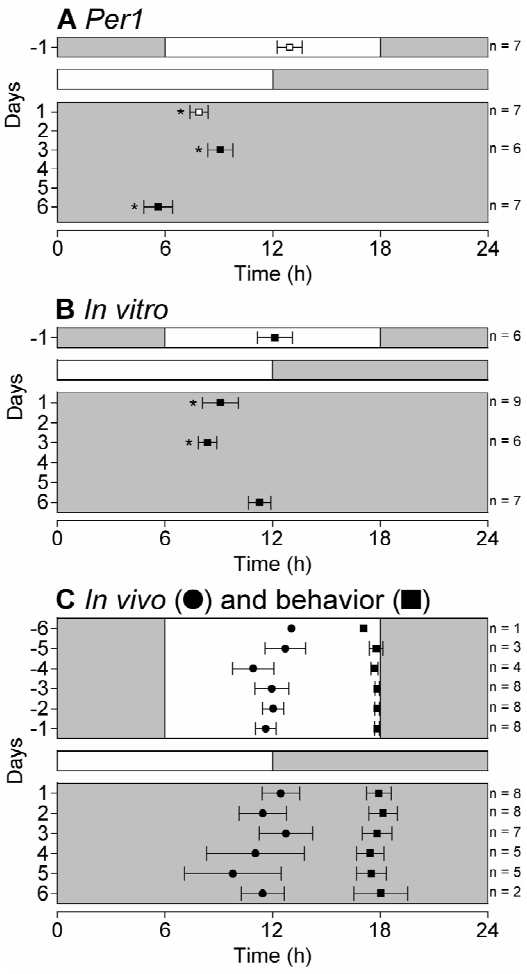

Figure 2. Average Peak Times of *Per1-luc* Bioluminescence Rhythms, Electrical Activity Measured In Vitro and In Vivo, and Average Behavioral Activity Onsets

(A) Average (± SE) peak times of *Per1-luc* bioluminescence. The horizontal axis indicates 24 hr. The days on the *y* axis are relative to the phase advance of the light-dark cycle, i.e., day -1 is the last day before the advance and day 1 is first day after the advance. Lights-on is indicated in white, and lights-off is indicated in gray. The number of animals contributing to every data point is indicated at the right. The asterisk indicates that the average peak time is significantly different from the average peak time at the day before the advance ($p < 0.05$, ANOVA with post hoc Dunnett's test). The open squares indicate averages that were taken from Yamazaki et al. [7]. The closed squares indicate new data.

(B) Average (± SE) peak times of electrical activity of the rat SCN in vitro before and after the advance of the light-dark cycle. The figure layout is similar to (A). The number of SCN slices contributing to a data point is indicated at the right.

(C) Average (± SE) peak times in electrical activity of the rat SCN in vivo and behavioral activity onsets from the same animals before and after the 6-hr phase advance. The figure layout is similar to (A). The mean peak times of electrical activity are indicated by black circles. After the advance, the average peak time shifted for -0.8 ± 0.8 (n = 8), 0.2 ± 1.0 (n = 8), -1.1 ± 1.1 (n = 7), 0.6 ± 1.7 (n = 5), 1.8 ± 1.7 (n = 5) and 0.2 ± 0.7 (n = 2) hr, at days 1 to 6 in DD, respectively. Average activity onset times are indicated by black squares. After the advance, the mean activity onset time was shifted by -0.1 ± 0.5 (n = 8), -0.3 ± 0.6 (n = 8), 0.0 ± 0.6 (n = 7), 0.4 ± 0.5 (n = 5), 0.3 ± 0.5 (n = 5), and -0.2 ± 0.7 (n = 2) hr, at days 1–6, respectively. For each day, the number of animals that contributes to the averages is indicated at the right.

Supplemental Data). Similar results have also been reported in Sprague-Dawley rats that were subjected to an 8-hr advance of the light-dark cycle before being released in DD [10]. Complete advances were obtained only after exposure to the advanced LD (light-dark) regime for three cycles. A possible explanation for the absence of phase advances in our study is that behavioral phase shifting is attenuated when animals are exposed to LD cycles and increases when animals are exposed to DD, as is typical in protocols used to generate phase response curves [11, 12]. In retrospect, the applied protocol has been unexpectedly helpful in revealing that behavioral, neuronal, and molecular processes can dissociate following a change in the light schedule.

There is increasing evidence that the SCN is a functionally heterogeneous tissue at cellular and molecular levels [13–19]. In the present study, the discrepancy observed between the electrical responses and *Per1-luc* bioluminescence raises the possibility that the *Per1-luc* bioluminescence rhythm reports a subset of neurons distinct from the subset from which electrical activity was recorded. Although we did not find different results when recording from the dorsal and the ventral SCN in vitro, we cannot exclude the possibility that cells of different subsets are intermingled within the SCN. A second possibility is that within single neurons, *Per1* luciferase and electrical activity respond differently to the phase advance in the light schedule. In either case, the electrical activity rhythm of the SCN and the animal's behavioral activity do not track the rhythmic behavior of *Per1-luc* bioluminescence.

There is evidence that *Per1* may play a different role in peripheral mammalian tissues [20]. In addition, the precise role of *Per1* in the SCN has been questioned [21–25]. The apparent dissociation between molecular rhythmicity on the one hand and the in vivo neuronal and behavioral responses on the other raises a fundamental question about the role of *Per1* in the control of behavioral circadian rhythmicity. Especially significant is the finding that the dissociation between *Per1* and the in vivo neuronal/behavioral response persists for at least 6 days of DD. Immediate and complete shifts in *mPer1* have been observed in mice that were exposed to a 6-hr advance of the environmental light-dark cycle [26]. This is consistent with our findings on day 1 after the shift in the light schedule and with data obtained in cultured mouse SCN [27]. In the phase advance protocol used by Reddy et al. [26], mice were kept on the shifted light-dark cycle throughout the experiment, and it was shown that behavioral activity shifted after several light-dark cycles to reestablish the normal phase relationship with *Per*. In contrast, in our experiments, animals were kept in DD after the shift. In this way, we could exclude the continuing effects of the light-dark cycle on any eventual phase readjustment. Our results indicate that *Per1* and electrical activity/behavior dissociate during transient cycles and show little evidence of reestablishing their normal phase relationship even after 6 days in darkness. This suggests that any coupling between *Per1* and the clock controlling SCN electrical and behavioral rhythmicity must be weak.

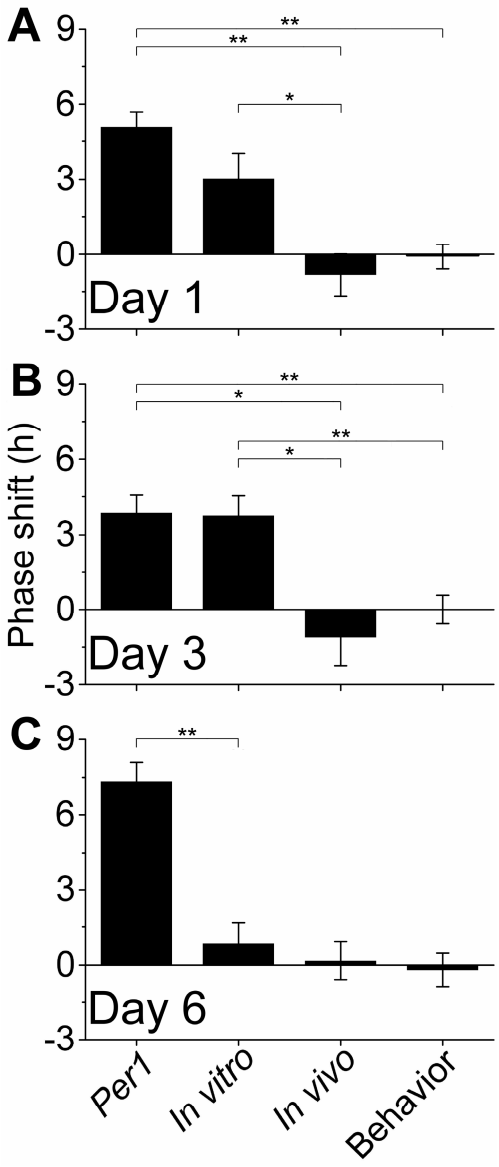

Figure 3. Magnitude of Phase Shifts in *Per1-luc* Bioluminescence, In Vitro and In Vivo Electrical Activity, and Behavioral Activity

(A–C) The magnitude of phase shifts (mean ± SE) of the different rhythms are compared at (A) days 1, (B) 3, and (C) 6 (single asterisks: $p < 0.05$, double asterisks: $p < 0.01$, two-way ANOVA). (A and B) The advances in the *Per1-luc* bioluminescence rhythm as well as in the in vitro electrical activity were significantly different from the responses of in vivo electrical activity and behavioral activity at days 1 and 3 after the phase advance of the light-dark cycle (Note: in vitro versus behavior at day 1: no significance was reached, but a trend was present, $p = 0.058$). The responses of in vivo electrical activity and behavioral activity were not significantly different. The phase shift in the *Per1-luc* luminescence rhythm at day 1 is from Yamazaki et al. [7]. (C) At day six, the *Per1-luc* bioluminescence rhythm was significantly different from in vitro electrical activity, which had returned to baseline levels.

The transient phase shifts observed in electrical activity in vitro contrast with the absence of phase advances in electrical activity measured in vivo. The slice procedure itself cannot account for these differences, given the time of slice preparation and the fact that the rhythm returns to its prior phase by day 6 (see Methodological Considerations 2 in the Supplemental Data). It seems unlikely that the neuronal populations measured in vitro are different from those measured in vivo, given the similarity in recording methodology and the fact that these phase differences gradually disappear in slices measured at days 1–6 in DD. We believe it more plausible that the differences between the phase shifting responses of electrical activity observed in vivo and in vitro are the consequence of the SCN remaining connected to the rest of the nervous system during in vivo electrical recording. We suspect that extra-SCN regions inhibit the ability of the SCN to fully shift in response to phase advances in the light schedule.

Although we cannot completely exclude the possibility that intrinsic SCN mechanisms have played a role in returning the SCN to the unshifted phase by day 6, this explanation seems remote since recordings in different areas of the SCN in vitro revealed no evidence for any rhythms remaining at the old phase. In addition, electrical recordings on day 1 expressed the unshifted phase when measurements were made in situ, whereas recordings from the isolated SCN revealed a phase advance. Taken together, it seems most likely that the unshifted oscillators reside outside the SCN.

Our finding can explain the results from previous studies demonstrating that phase shifts obtained in vitro are larger than those obtained in vivo [28–30]. Our results are a first indication that extra-SCN areas can affect the phase of the electrical activity rhythm in the SCN. The functional importance of this finding is evidenced by the fact that the behavioral output tracks the electrical behavior of the SCN in situ rather than the intrinsic phase of the electrical rhythm as measured in vitro.

It has been shown that behavioral activity results in changes in the neuronal firing activity of SCN neurons in vivo [31, 32]. It is possible that this is the pathway by which extra-SCN areas affect electrical activity rhythms in the SCN as revealed in the present experiments. The question remains whether the neuronal rhythm of the SCN in vivo reflects the phase of the underlying molecular pacemaker. Given the phase advances observed at days 1 and 3 in vitro, we think it likely that the SCN electrical activity recorded in vivo is masked by activity generated by extra-SCN areas. Isolation of the SCN at day 6 demonstrates that the endogenous rhythm eventually comes into phase with the in vivo-recorded neuronal rhythm and provides evidence that the SCN is ultimately entrained by the extra-SCN areas.

CONCLUSIONS

Taken together, our results lead us to the following hypothesis. The phase advance in the light-dark schedule leads to a nearly complete phase advance of the *Per1-luc* bioluminescence rhythm and a transient advance in the SCN pacemaker mechanism, controlling electrical activity. Extra-SCN oscillators are not phase advanced by the shifted light-dark cycle and influence SCN electrical activity. Eventually, the extra-SCN oscillators are effective in entraining the SCN pacemaker to their phase. This is a novel hypothesis in that it postulates a powerful role for non-SCN regions in phase control of the SCN and has important implications for understanding problems associated with shift work and transmeridian air travel.

EXPERIMENTAL PROCEDURES

Animals and the Light-Dark Regime

Male wild-type Wistar and transgenic W(perl)1 (see [7]) rats were kept on a 12:12 light-dark regime (100 lux during lights-on). The experimental protocol consisted of a 6-hr phase advance of the light-dark regime by advancing the time of lights-on. After one complete shifted cycle, the animals were kept in constant darkness (DD). Food and water were available ad libitum throughout the experiments. All experiments were performed under the approval of the Animal Experiments Committee of the Leiden University and the Committee on Animal Care and Use at the University of Virginia.

Per1-luc Bioluminescence

Brains of transgenic W(perl)1 rats were prepared at the following time points: at ZT 12 (i.e., the time of lights-off) of the unshifted light-dark cycle [7], immediately after the phase advance of the light-dark regime at the onset of DD [7], after 2 days of DD, and after 5 days of DD. Preparation at these time points provides data at the day before the phase advance and at days 1, 3, and 6 of DD, respectively. When animals were in DD, the eyes were first removed under Halothane anesthesia and infrared light by using an infrared viewer, after which the brain could be prepared under lights-on. *Per1-luc* luminescence from SCN explants was monitored as previously described ([7, 33] and see details in the Supplemental Experimental Procedures).

In Vitro Electrophysiology

The multiunit electrical activity rhythms of SCN neurons were recorded as described previously [34, 35]. In short, brains of wild-type Wistar rats were rapidly dissected from the skull at the same days and Zeitgeber times as in the bioluminescence experiments. When animals were in DD, the brains were removed in dim red light (for further details, see the Supplemental Data).

In Vivo Electrophysiology and Behavior

In vivo recordings of SCN electrical activity and behavioral activity of transgenic W(perl)1 rats and wild-type Wistar rats were performed as described before [32, 34] (for further details, see the Supplemental Data).

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SUPPLEMENTAL DATA

SUPPLEMENTAL RESULTS AND DISCUSSION

Results from In Vitro Recordings

Analysis of the data revealed that no consistent differences in peak times existed between the dorsal and ventral SCN, either before or after the phase advance. A strong correlation existed between separate recordings from one slice ($R = 0.74$, $p < 0.001$). Therefore, in the analysis, average values per slice were used. Similar shifts were obtained for calculations using two other phase reference points, troughs, and the half-maximum values of the rising phases of the peaks for all recording days (data not shown).

Comparison of Different Variables; Statistical Analysis

The phase advances in *Per1-luc* bioluminescence, in vitro and in vivo electrical activity, and behavioral activity were compared at days 1, 3, and 6 in DD (Figure 3).

Per1-luc Bioluminescence versus Electrical Activity and Behavior

Two-way ANOVAs revealed that the phase advances in *Per1-luc* bioluminescence were significantly different from the phase shifts in in vivo electrical activity and behavior at days 1 and 3 (*Per1* versus in vivo: $p < 0.01$, $F = 14.793$; $p < 0.05$, $F = 6.997$, respectively; *Per1* versus behavior: $p < 0.01$, $F = 21.281$; $p < 0.01$, $F = 9.476$, respectively, significant effect of day x variable). The phase advances in *Per1-luc* bioluminescence were not significantly different from the advances in electrical activity obtained in vitro at days 1 and 3 ($p > 0.2$, $F = 1.357$; $p > 0.9$, $F = 0.006$, respectively, no significant effect of day x variable). At day 6, in vitro electrical activity had shifted back and showed significant differences in phase advance with *Per1-luc* bioluminescence ($p < 0.01$, $F = 17.523$, significant effect of day x variable).

Electrical Activity In Vitro versus Electrical Activity in Vivo and Behavior

Two-way ANOVAs revealed significant differences between the phase advance of in vitro electrical activity and the response of in vivo electrical activity at days 1 and 3 (in vitro versus in vivo: $p < 0.05$, $F = 4.214$; $p < 0.05$, $F = 6.103$, respectively, significant effect of day x variable). Similar results were found for the difference between the phase advance of in vitro electrical activity and the response of the behavioral activity (in vitro versus behavior; day 1: $p = 0.058$, $F = 3.904$, trend for effect of day x variable; day 3: $p < 0.01$, $F = 8.067$, significant effect of day x variable, respectively). At day 6, however, the phase advance of the in vitro electrical activity was strongly decreased (see Figure 2B), and the response was similar to the response of in vivo electrical activity and behavior (see Figure S1).

Methodological Consideration 1: Validation of the Luciferase Per1-luc Model and Procedure of Experiments

The data indicate that the behavioral response of the animal and the in vivo electrical activity in the SCN do not follow the expression profile of *Per1*. Moreover, SCN electrical activity in vitro, although transiently shifted, also does not track the phase shifts observed in *Per1* activity. The question is whether methodological considerations can account for these differences.

First, it may be argued that the large phase advances obtained for *Per1-luc* bioluminescence are a result of the preparation procedure. This seems unlikely, however, because in the control experiments, *Per1* expression peaked in the mid-subjective day, which corresponds with the results found after in situ hybridization [S1] (see also Methodological Consideration 2).

Second, the experiments on *Per1* expression were performed on transgenic rats, while the in vitro experiments have been performed on wild-type rats. In order to be able to compare the experiments, the in vivo and behavioral studies were performed with both transgenic and wild-type rats. No differences were observed, which indicates that the circadian clock in vivo, as well as the behavioral output, responds in the same manner in transgenic and wild-type rats. In a separate series of experiments, we performed electrophysiological measurements in slices of transgenic rats. The results showed that on day 1 after the shift, the mean advance in electrical activity is 4.9 hr ± 0.5 (n = 3 rats, with dual recordings in dorsal and ventral SCN). No differences in phase were observed between dorsal and ventral SCN, and this is similar to what is seen in wild-type animals. At day 6 after the shift, the mean advance was -0.1 hr ± 0.4 (n = 4 rats), with no difference between dorsal and ventral SCN. These data show that the pattern of phase shifting resembles that of wild-type animals.

Third, it may be argued that the rhythms in *Per1-luc* bioluminescence that we measured do not reflect the endogenous *Per1* mRNA rhythms. However, the phase of *Per1-luc* bioluminescence in steady-state situations is the same as the phase of *Per1* mRNA [S1–S3]. Second, rapid readjustment of both *Per1-luc* bioluminescence rhythms as well as *Per1* mRNA rhythms has been observed after a 6-hr advance of the light-dark schedule [S2, S4]. Of note, in situ hybridization experiments cannot provide an answer to the latter issue insomuch as animals would have to be sacrificed at different times of the day. This would lead to the SCN being attached for variable times to the brain before preparation. This procedure would differ significantly from our real-time bioluminescence and electrophysiological recording protocol in which we prepare all tissue at the same phase and determine the phase of the rhythm. Our experiments indicate that this is not an unimportant difference insofar as preparation at days 1, 3, or 6 (hence, keeping brains attached for 1, 3, or 6 days) yield different results.

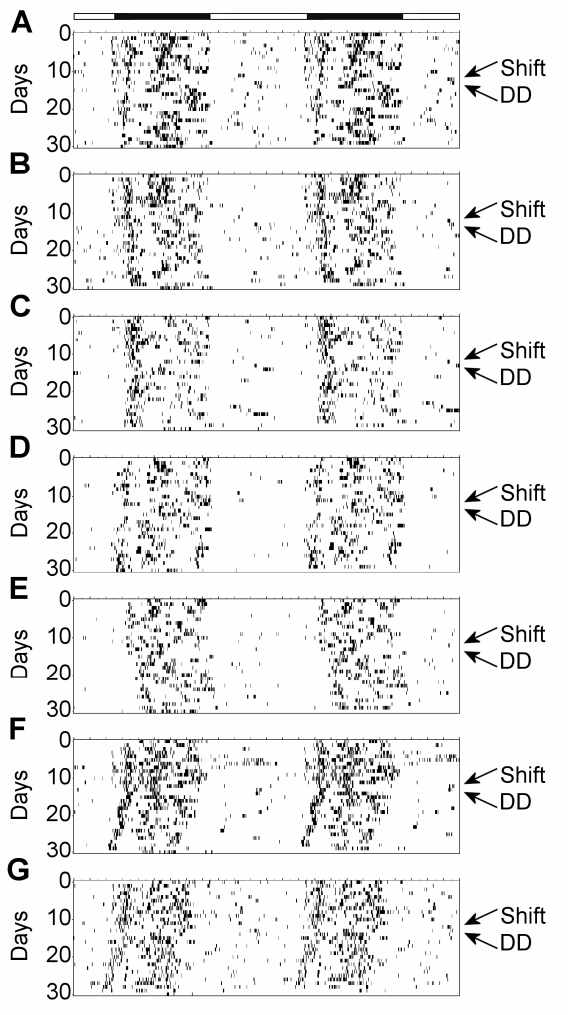

Figure S1. Behavioral Activity Recordings of Rats

(A–G) Running wheel activity was recorded in 1-min bins and is double plotted to enable visual inspection of the rhythm. The initial light-dark cycle is indicated above record (A). The time of a shift in the light-dark schedule, as well as the onset of constant darkness (DD), is indicated by an arrow.

Methodological Consideration 2: Slice Procedure and Phase Shifting

An important issue is whether phase shifts in vitro were introduced by the slice preparation procedure itself [S5]. The estimated preparation time of the brain slices ranges from ZT 6 (when the neuronal rhythm was not shifted) to ZT 12 (when the rhythm was completely shifted). This range falls within the dead zone of the phase response curve of slice preparations, which is similar to the photic PRC [S5]. If any phase shift occurred, we would have expected it to be a phase delay. Moreover, at day 6, the rhythm in in vitro electrical activity had shifted back to the prior (unshifted) phase, a result that cannot be explained by phase

shifts brought about by slice preparation. We conclude that the phase advances we measured were not induced by the slice preparation procedure.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Per1-luc Bioluminescence

Coronal slices of 400 μm thickness (0.8 x 1.0 x 1.0 mm triangle), containing the SCN, were made of the brains, and the paired SCN were explanted and placed on a culture membrane (Millicell-CM, PICM30-50m Millipore) in a sealed petridish [S2, S6]. The SCN was cultured with serum-free culture medium (low sodium bicarbonate, no phenol red, Dulbecco's modified Eagle's medium [GIBCO-BRL]) supplemented with 10 mM HEPES (pH 7.2), 2% B27 (GIBCO-BRL), 0.1 mM luciferin (Promega), 25 U/ml penicillin, and 25 μg/ml streptomycin. Bioluminescence was measured with photomultiplier tube (PMT) detector assemblies (Hamamatsu). The modules and cultures were maintained in darkness at 36ºC and were connected to a computer for continuous recording. The PMT was placed about 2 cm above the culture, and photons were counted in 1-min bins. Nonspecific dark counts were about 20–40 counts/s at 36ºC.

Statistics for Per1-luc Bioluminescence

The peaks in *Per1-luc* bioluminescence from the SCN were determined by using a moving average with a 2-hr window. The highest point in the first complete cycle in vitro is the peak [S2, S6]. Average peak times were taken of animals from the different groups. Differences between the groups were tested for statistical significance by using ANOVA with a post hoc Dunnett's test. All averages are indicated as mean ± SE.

In Vitro Electrophysiology

Coronal hypothalamic slices of 500 μm thickness (4 x 3.5 mm), containing the SCN, were sectioned and transferred to an interface chamber. Smaller slices gave similar results but a lower success rate (data not shown). Slices were constantly oxygenated with humidified 95% O2/5% CO2. Multiunit neuronal recordings from the SCN were performed at 35ºC with 90% platinum/10% iridium electrodes (R ~ 100 kΩ). The signal was passed through a high-impedance amplifier (bandpass 300 Hz–3 kHz). The action potentials were converted into pulses by a window discriminator and were counted by a computer every 10 s for off-line analysis.

Statistics for In Vitro Analysis

The time of peak electrical activity was determined by smoothing the data by using penalized least squares [S7]. The existence of differences in peak times between the dorsal and ventral parts of the SCN was investigated with Student's t tests. The correlation coefficient of the data from the dorsal and ventral SCN was calculated to determine whether the data of two electrodes could be considered as independent recordings. Averages were taken of the

peak times of electrical activity obtained from animals sacrificed at different times, and differences between groups were tested by using ANOVA with a post hoc Dunnett's test. All averages are indicated as mean ± SE.

In Vivo Electrophysiology

The W(perl)1 rats were implanted with Teflon-coated stainless steel electrodes with an interelectrode distance of 150 μm for differential recording. The wild-type Wistar rats were implanted with Formvar-insulated stainless steel electrodes, with an interelectrode distance of 0.4 mm, for recording from one electrode at a time. In both cases, a third uncoated electrode was implanted in the cortex for reference. At the onset of the experiment, the animals were connected to the recording system. A flexible cable, attached to a swivel system, minimized the effect of the connection on the animal's freedom of movement. The signal was amplified and band width filtered. The signal from the W(perl)1 rats was fed into an AD board and a recording computer. A window was set that contained multiunit activity and no noise or artifacts. Multiunit activity was recorded in 1-min bins. The signal from the wild-type rats was fed into two window discriminators. The first window discriminator converted the action potentials into pulses. The second window discriminator was set at a higher level to detect artifacts caused by movements of the animals. Action potentials and artifacts were recorded in 10-s bins. The bins that contained artifacts were excluded from analysis.

During the measurements, the wheel-running activity of the W(perl)1 rats and the drinking activity of the wild-type rats were recorded every minute. When an animal showed a clear circadian rhythm in the electrical activity for at least 3 or 4 days, the light-dark cycle was advanced. At the end of the experiments, the animals were sacrificed, and the recording site was verified histologically. In six out of eight animals, we could histologically confirm that at least one of the electrodes was implanted in the SCN. In the two other animals, histology was unclear. Before the phase advance, all eight animals showed peaks in electrical activity during the light period, which is typical for SCN electrical activity rhythms [S8–S10].

Statistics for In Vivo Analysis

In vivo electrical activity was smoothed by using penalized least squares [S7] to determine the peak times. The onset times of behavioral activity were determined by drawing straight lines through the onsets of wheel-running and drinking activity and measuring the exact times of the activity onset at the days before and after the shift. The existence of significant differences between the peak times of electrical activity or activity onset at days 1, 3, and 6 of DD and the day before the phase advance was determined by using ANOVA with a post hoc Dunnett's test. All averages are indicated as mean ± SE. The magnitudes of the phase shifts in *Per1-luc* bioluminescence, in vitro and in vivo electrical activity, and behavior were compared at days 1, 3, and 6 in DD by using two-way ANOVAs.

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