Current Biology, Vol. 13, 1538–1542, September 2, 2003, ©2003 Elsevier Science Ltd. All rights reserved. DOI 10.1016/S0960-9822(03)00560-8

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.

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

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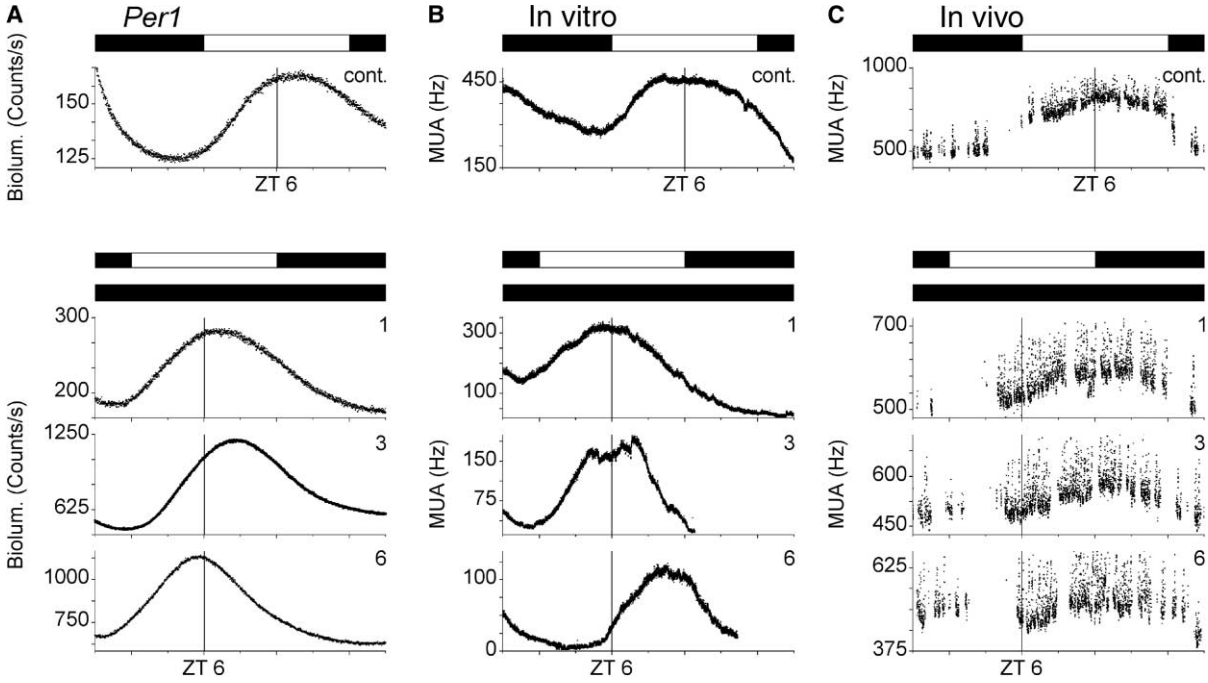

Figure 1. Examples of *Per1-luc* Bioluminescence Rhythms and SCN Electrical Activity Rhythms Recorded In Vitro and In Vivo at the Day before the Advance and Days 1, 3, and 6 after the Phase Advance of the Light-Dark Cycle

(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.

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 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

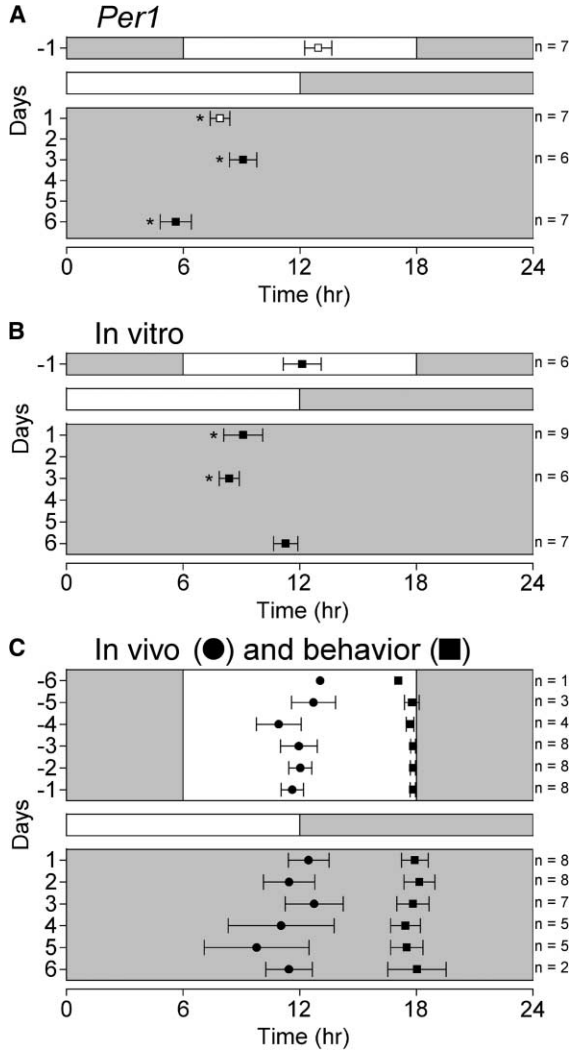

Figure 2. Average Peak Times of *Per1-luc* Bioluminescence Rhythms, Electrical Activity Measured In Vitro and In Vivo, and Average Behavioral Activity Onsets

(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.

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.

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

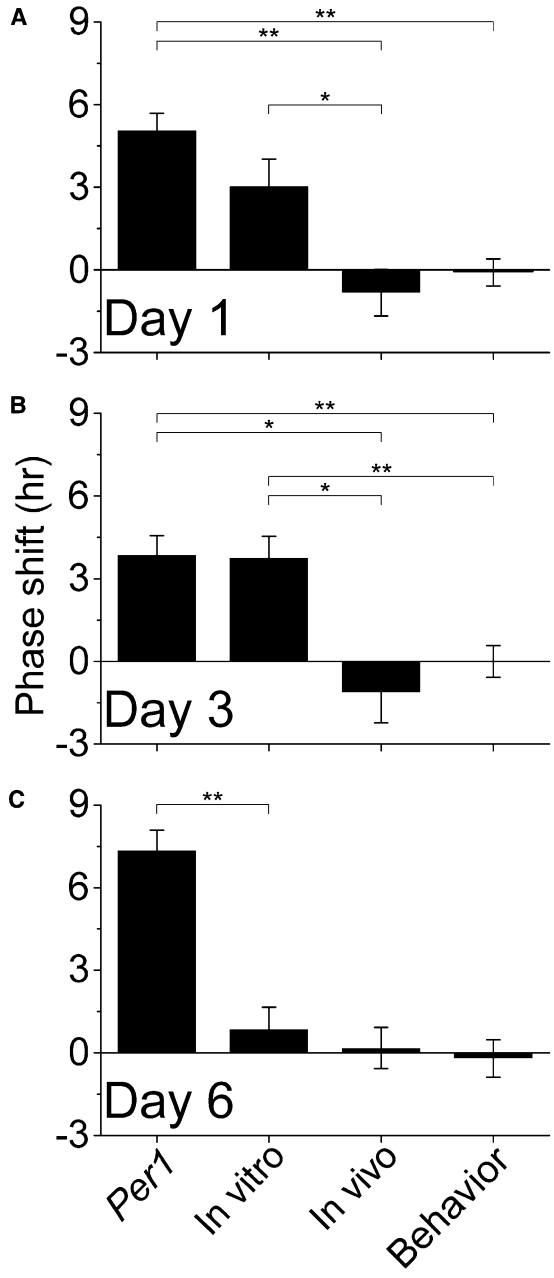

Figure 3. Magnitude of Phase Shifts in *Per1-luc* Bioluminescence, In Vitro and In Vivo Electrical Activity, and Behavioral Activity

(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.

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).

Supplemental Data

Supplemental Data including additional Experimental Procedures, methodological considerations, and statistical evaluation of differences are available at http://www.current-biology.com/cgi/content/full/13/17/1538/DC1/.

Acknowledgments

We wish to thank Hans Duindam, Jan Janse, Krista M. Greene, and Nate Schneider for excellent technical assistance. Research was supported by Leiden University Medical Center, an ASPASIA grant to J.H.M., and by National Institutes of Health grant 1R01MH62517 to G.D.B.

Received: May 9, 2003
Revised: July 8, 2003
Accepted: July 16, 2003
Published: September 2, 2003

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