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Strong resetting of the mammalian clock by constant light followed by constant darkness

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Abstract

The mammalian molecular circadian clock in the suprachiasmatic nuclei (SCN) regulates locomotor activity rhythms as well as clocks in peripheral tissues (Reppert and Weaver, 2002; Ko and Takahashi, 2006). Constant light (LL) can induce behavioral and physiological arrhythmicity, by desynchronizing clock cells in the SCN (Ohta et al., 2005). We examined how the disordered clock cells resynchronize by probing the molecular clock and measuring behavior in mice transferred from LL to constant darkness (DD). The circadian locomotor activity rhythms disrupted in LL become robustly rhythmic again from the beginning of DD, and the starting phase of the rhythm in DD is specific, not random, suggesting that the desynchronized clock cells are quickly reset in an unconventional manner by the L:D transition. By measuring mPERIOD protein rhythms, we showed that the SCN and peripheral tissue clocks quickly become rhythmic again in phase with the behavioral rhythms. We propose that this resetting mechanism may be different from conventional phase shifting, which involves light-induction of *Period* genes (Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997). Using our functional insights, we could shift the circadian phase of locomotor activity rhythms by 12 hours using a 15-hour LL treatment: essentially producing phase reversal by a single light pulse, a feat that has not been reported previously in wild-type mice and that has potential clinical utility.

Keywords

circadian rhythms; LL; entrainment; type 0 resetting; PERIOD; SCN

Introduction

In the absence of external time cues, animals continue to exhibit circadian rhythms: behavioral and physiological oscillations with a period of approximately 24 hours. Circadian rhythms are driven by endogenous circadian clocks, and include activities as diverse as sleep/wake cycles, hormone production and digestion rhythms (Allada et al., 2001; Panda et al., 2002; Reppert and Weaver, 2002; Hastings et al., 2003; Ko and Takahashi, 2006). In mammals, a circadian clock operates within individual neurons of the suprachiasmatic nuclei (SCN) in the anterior hypothalamus. These neurons form a tissue-level “master clock” that regulates behavior and synchronizes clocks in peripheral tissues through neuronal and humoral outputs (Silver et al., 1996; Ueyama et al., 1999; Terazono et al., 2003). SCN neurons entrain to the external

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environment through various cues, the most important of which is the light-dark (LD) cycle of day and night (Hattar et al., 2003; Panda et al., 2003; Panda, 2007).

While LD and constant darkness (DD) favor the expression of circadian rhythms, constant light (LL) disrupts those rhythms (Daan and Pittendrigh, 1976). Nocturnal rodents in LL exhibit reduced locomotor activity and an unusually long circadian period, and at high light intensities they become arrhythmic (Aschoff, 1960; Daan and Pittendrigh, 1976). Two main explanations have been proposed for LL-induced arrhythmicity (Winfree, 1980): 1) LL desynchronizes clock cells, disrupting only the tissue-level clock, or 2) LL stops the intracellular clock from working. The latter mechanism explains LL-induced arrhythmicity in *Drosophila* (Skopik and Pittendrigh, 1967). However, for mammals, LL desynchronizes individual SCN neurons without disrupting the intracellular clock (Ohta et al., 2005).

Since the mechanism for LL-induced arrhythmicity is now established (Ohta et al., 2005), we explored what happens when LL is lifted. We measured locomotor activity and molecular markers of circadian time (mPERIOD or mPER proteins) from mice experiencing various lighting regimes, and inferred how quickly desynchronized clocks resynchronize after transfer from LL to DD. In addition, we performed the unprecedented feat of reversing behavioral rhythms (producing a 12-hour phase shift) in wild-type mice using a single light pulse. Clock entrainment in mammals usually requires multiple days to produce large phase shifts, frequently leading to problems in modern human society. When shift workers and long-distance travelers cannot immediately adjust their internal clocks to fit their external environment and behavioral activities, they often experience drowsiness, reduced performance, and even serious health risks; for example, shift workers experience a higher rate of accidents than the general population (Vitaterna et al., 2001; Hastings et al., 2003; Knutsson, 2003; Ko and Takahashi, 2006). If rapid phase shifting by a single light pulse is possible for humans, our findings may have clinical implications in treating shift workers and sufferers of jet lag and other disorders associated with temporal clock malfunction (Vitaterna et al., 2001; Hastings et al., 2003; Knutsson, 2003).

Materials and Methods

Animals and maintenance

Eight to twelve week old male C57BL/6J mice were purchased from Charles River Laboratory. They were maintained in a climate-controlled room and used according to the FSU Animal Care and Use Committee's guidelines. All experiments involving animals were performed according to a Committee-approved protocol.

Behavioral experiments

Each mouse was housed in a cage with a running wheel. Spontaneous locomotor activity was recorded in 5 minute bins using the Stanford Software System (Santa Cruz, CA, USA). The mice were entrained in 12 hr light and 12 hr dark (LD) cycles for 2 weeks before being released into constant darkness (DD). After 9 to 12 days in DD, lights (~500 lux) were turned on for at least 9 days (LL). The light intensity in LL, while within the range of normal indoor illumination, is stronger than those most other groups have used to induce arrhythmicity in mice (Sudo et al., 2003; Munoz et al., 2005; Ohta et al., 2005). We believe that the majority of mice exhibited severely compromised behavioral rhythms in a short time due to this relatively strong light intensity, as has been shown previously (Daan and Pittendrigh, 1976). On the last day in LL, mice were divided into 4 groups and each group was moved into a DD compartment at 6 hr intervals except the last group, which was placed in DD by turning off the lights in the same room that was previously used for LL treatment. Period and amplitude were calculated by χ^2-periodogram and Fourier analysis, respectively, using the Stanford Software

System. Where possible, phase was measured in LL mice; however, the activity of most LL mice was so weakly rhythmic or arrhythmic that accurate phase determination was not possible. A couple of weakly rhythmic mice are shown in Figure 1. The first mouse in Figure 1 had 20% amplitude in LL compared to that in 1st DD. 61% of LL mice showed less than 20% of amplitude compared to that in DD. The starting phase in the 2nd DD in Figure 1 was calculated as follows. A hypothetical activity onset time on the first day in the 2nd DD was calculated by extrapolating a line through activity onset times backwards to the first day in the 2nd DD. This hypothetical time (A) was defined as circadian time (CT) 12 (CT 0 is the time of subjective dawn, while CT 12 is the time of subjective dusk; mice begin their nocturnal activities around dusk.), and (A) always preceded the transfer time (B) except in one out of 46 mice. dT was the time difference between A and B. The starting phase was CT12 + (dT/*tau*) X 24, where *tau* is the period of the 2nd DD free-running rhythm. To determine if the starting phases are different among the four groups, one-way ANOVA was applied. The phase shift in Figure 4 was determined as follows. A hypothetical activity onset time on the first day in DD was calculated as above. The amount of phase shift was the time (hr) between the previous zeitgeber time (ZT) 12 (activity onset in 12:12 LD; ZT indicates the time in LD with ZT 0 being the beginning of the light cycle) and the hypothetical onset time. For Figure 5, phase shifts were calculated as in Figure 4 except that the onset time on the first day in DD was used instead of the hypothetical one.

Tissue collection

For Figures 2 and 3, mice were entrained in LL for at least 10 days before they were transferred to DD. The mice were sacrificed at indicated times by CO_2 asphyxiation. Tissues were rapidly removed and frozen on dry ice. For SCN studies, the SCN region was punched out using a 1.25 mm diameter Harris UNI-CORE puncher (Electron Microscopy Sciences, PA, USA) from a ~1 mm coronal section as described previously (Lee et al., 2001). Five SCN punches were pooled for each time point. A single SCN punch did not provide enough protein for an immunoblot with our antibodies.

Quantitative Real Time RT-PCR

Total RNA was extracted from the liver using TRIzol Reagent according to the manufacturer's protocol (Invitrogen, CA, USA) and DNA contamination was eliminated by DNase I treatment. One mg of total RNA was reverse transcribed using Bio-Rad iScript cDNA kit. Quantitation of mRNA levels was performed by real-time PCR using an iCycler iQ PCR System (Bio-Rad, USA). Analyses were performed using the standard curve method with *β-Actin* as a normalizing endogenous control. The primer sequences were as follows:

Per1 sense 5′-TCCCTGTTTCGTCCTCCACT-3′,

antisense 5′-CTTGAGCCATTGCTGTTTGC-3′,

Per2 sense 5′-ATGAATGGATACGTGGACTTCTCCCCA-3′

antisense 5′-CAGGGTTGCCAGCGTGCTGGCCTT-3′

β-Actin sense 5′-ATG GGTCAGAAGGACTCCTATGTGGG-3′

antisense 5′-GGCCACACGCAGCTCATTGTAGAAGG-3′

Immunoblotting

Immunoblotting was performed as described previously (Lee et al., 2001). Antibodies to mPER1 (PER1-1-GP) and 2 (PER2-1-GP) were described previously (Lee et al., 2001). Anti-ACTIN antibody was purchased from Sigma.

Results

Behaviorally arrhythmic or weakly rhythmic mice in LL become immediately rhythmic when they are transferred to DD

Since locomotor activity rhythms are believed to be a direct reflection of the current status of the SCN clock, we measured these rhythms to discover how SCN rhythmicity changes in different lighting conditions. Specifically, we measured how behaviorally disrupted mice in LL become rhythmic again, when they are transferred to DD. The data may allow us to deduce two pieces of information. First, the behavioral changes would reveal how quickly the SCN can be resynchronized after its component oscillators have been desynchronized by constant light as shown by Ohta et al. (2005). We expected that mice would remain arrhythmic as long as the desynchrony is maintained, becoming weakly rhythmic and then more robustly rhythmic as the oscillators gradually become synchronized. Second, our experiment would provide clues as to what factors affect the starting phase of the behavioral rhythm in DD. For example, would the starting phase be tied to the LL-DD transition, and therefore be about circadian time (CT) 12 in most mice (the time of the light-to-dark transition in a 12:12 LD cycle)? Or would the starting phase be effectively random, because it is strongly influenced by unpredictable factors such as metabolic variations among mice or variations in the underlying desynchrony (i.e., unequal mixtures of oscillators with different phases in different mice)?

We monitored mice in LD and then DD before constant light treatment to exclude any animals that behaved erratically under those conditions. After this initial screening, mice with large amplitudes and unambiguous phases were placed in LL (~ 500 lux) for at least 9 days to induce arrhythmicity or severely attenuate the rhythmicity before they were transferred to DD (2^{nd} DD). The mice were divided into 4 groups and each group was moved at 6 hour intervals to 2^{nd} DD over 1 circadian cycle (Figure 1). Most of the mice became immediately rhythmic upon transfer with little transitional time. Interestingly, the starting phases in 2^{nd} DD relative to later stable phases were not randomly distributed over the entire circadian cycle; rather they were concentrated in a narrow time window around CT 15 (Table 1). The starting phase in 2^{nd} DD was determined by extrapolating the phase of activity onset in 2^{nd} DD back to the day of the LL-2^{nd} DD transfer; the extrapolated onset phase on the day of the transfer was considered to be CT 12 (since activity onset in mice is a marker for subjective dusk) (Schwartz and Zimmerman, 1990), and the starting phase was the time of the LL-DD transfer relative to the extrapolated CT 12. In most mice, the starting phases were around CT 15 regardless of transfer time, and phases (timing of activity onset) were stable from the second day onwards. It is clear that the starting phases were not affected by previous phases in LD and 1^{st} DD, because mice that were transferred from LL to 2^{nd} DD six hours apart exhibited similar starting phases in 2^{nd} DD, not starting phases with a six hour difference. In a minority of mice, a weak behavioral rhythm persisted in LL, but this rhythm was not consistently related to the starting phase in 2^{nd} DD (Figure 1 and Supplementary Figure 1).

mPERs in liver in LL exhibit near steady-state arrhythmicity but they also resume rhythmicity quickly in DD in phase with behavior

Our above data showed that behaviorally arrhythmic mice in LL become quickly rhythmic again with a specific starting phase (~CT 15) in subsequent DD. Like behavior, peripheral clocks are regulated by the output of the SCN master clock. Furthermore, peripheral clocks may themselves regulate behavioral rhythms. Previous work showed that when peripheral

clocks are uncoupled from the SCN by non-photic cues such as restricted feeding, behavioral rhythms become disrupted (Damiola et al., 2000; Stokkan et al., 2001). Peripheral clocks that are out of phase with the SCN may therefore affect behavioral rhythms, perhaps by interfering with output signals from the SCN. For all these reasons, we considered how peripheral clocks are affected by LL and LL->DD. Because the behavioral rhythms in the 2nd DD seemed to be as robust as in the 1st DD from the second day onward in most mice, and because the starting phase of the behavior was consistently ~CT 15, we hypothesized that peripheral clocks may also be quickly reset by the LL->DD treatment from a steady-state to a synchronized rhythm starting ~CT 15.

To test this hypothesis, we measured mPER rhythms in liver, assessed the effects of LL->DD treatment, and determined the starting phase of the rhythm in peripheral clocks. In liver, both mPER1 and 2 exhibited a near steady-state arrhythmicity in mice collected every 6 hours in LL, suggesting that peripheral tissues also lose rhythmicity in LL, probably through desynchronization of their constituent oscillators (Nagoshi et al., 2004; Ohta et al., 2005) (Figure 2A). Regardless of collection time, liver in most LL mice contained mPER across the full range of normal phosphorylation states, from hypophosphorylated mPER (like ~CT 12) to hyperphosphorylated mPER (like ~CT 0), consistent with a mixture of mPER from clocks at different phases. After mice were transferred to DD, mPER1 and 2 over the course of 28 hours were gradually phosphorylated and disappeared, similar to mPERs in control mice. mPERs 24 hours after the transfer are similar to mPERs between CT12 and CT16 in control mice, based on abundance and phosphorylation status, which implies that the extrapolated starting phase in the liver clock in DD is similar to that of behavioral rhythms (~CT 15). Thus liver oscillators seem to quickly resynchronize to the same circadian time as the behavior. Although there was a little more variation in mPER phase among mice collected at the same time during LL->DD than those collected during LD->DD (data not shown), we could see a robust rhythm even with a sample size of only two per time point. In addition, we measured mRNA rhythms of *mPer1* and *2* in liver to confirm that mRNA rhythms fit the protein rhythms. When Time 0 (LL->DD) was aligned with CT 16 in control (Figure 2B), mRNA levels of *mPer* in LL-treated mice were not as rhythmic as in control mice in the first ~12 hours after DD transfer, but became robustly rhythmic and closely matched those in control mice in the later part of the cycle,.

Taken together, our data suggest that the robust behavioral rhythms arising immediately after the LL->DD transition may result from the rapid resynchronization of desynchronized oscillators to the same circadian time in peripheral tissues as well as in SCN (see below).

mPER proteins in the SCN also resume rhythmicity rapidly after LL to DD transition

Since the behavioral rhythm phase seemed to be stabilized from the second day in 2nd DD in most mice (Figure 1) and the liver clock is also quickly rhythmic again, we hypothesized that the desynchronized oscillators in the SCN (as a key regulator of both behavioral and peripheral rhythms) must have been rapidly resynchronized by the LL to DD transition within a day or two. To test this prediction, we analyzed molecular rhythms in the SCN from mice subjected to LL->DD treatment. Specifically, we dissected SCN from these mice, and measured mPER1 and 2 by immunoblotting to see if mPER is arrhythmic in LL and becomes rhythmic again in DD. First we confirmed that mPER1 and 2 oscillate in abundance and phosphorylation in SCN on the first day in DD after LD entrainment as has been shown previously (Lee et al., 2001). The phase of mPER1 and 2 rhythms was compared between SCN and liver from the same mice to verify that the two tissues differ in phase (Lee et al., 2001). As has been demonstrated previously by immunocytochemistry (Reppert and Weaver, 2001), both mPER1 and 2 peaked several hours earlier in our SCN tissue compared to liver (Figure 3). Additionally, our

immunoblotting confirmed that both proteins oscillate robustly in abundance and phosphorylation in the SCN.

Next, we measured mPER proteins in SCN from mice in constant light and mice transferred from LL to DD (Figure 3). If constant light desynchronized the oscillators in the SCN, we expected to see an LL mPER immunoblot profile resembling a mixture of the four control samples, and the absence of an mPER tissue-level rhythm. While mPER1 in SCN of LL mice did resemble an even mixture of mPER1 from different phases, hyperphosphorylated isoforms of mPER2 were more pronounced than expected from an even mixture of the four control samples. However, as predicted, mPER1 and mPER2 protein in SCN from mice collected at two times (12 hours apart) in LL did not change significantly between the two times. Furthermore, mPER1 and mPER2 in SCN at 18 and 30 hrs after the transfer from LL to 2nd DD were similar to mPERs at CT 09 and 21, respectively, in control mice (Figure 3), which suggests that the starting phase (0 hour) of mPERs in 2nd DD is close to CT 15, the same starting phase as the behavioral rhythms. However, mPER rhythms could not be detected in SCN at the earlier time points of 6 and 12 hours (data not shown), which was surprising since rhythms in liver were detectable at those times (see Discussion). mPER levels at these times were constant, similar to those in LL.

Taken together, our data show that peripheral and SCN clocks become rapidly rhythmic again, consistent with the behavioral data, upon LL -> DD transfer. In addition, extrapolated starting phases in the 2nd DD are similar (~CT 15) in locomotor activity rhythms, liver and SCN.

The phase of behavioral rhythms can be reversed by only a 15 hr light pulse at ZT 12

Since our data showed that behavioral rhythms can be disrupted and rapidly reset to a specific phase by LL followed by DD, we set out to measure the minimum duration of constant light that can reset the behavioral rhythm to that phase. To differentiate LL-> DD induced resetting of the clock from conventional light-induced phase shifting, we examined LL durations that would generate the maximal phase shift of ~12 hours. To our knowledge, such a large phase shift by a single light pulse has not been reported in wild type mice. As LL->DD resets the circadian system from arrhythmic to ~CT 15 at the transition (Figure 1 and Table 1), a 15 hour light pulse (LL treatment) at zeitgeber time (ZT) 12 (the end of the light period in a 12:12 LD cycle), followed by release into DD, may induce the maximum phase shift of ~12 hours. The unshifted clock would be at CT 03, but the LL-treated clock would be at ~CT 15, provided that such a short LL treatment exerts the same effects as the long light treatment. In case this is not enough, we also tested 3 days (15 hours + 2 days) and 5 days (15 hours + 4 days) of constant light to see how much constant light is required to reverse the behavioral rhythm (see the diagram in Figure 4A). In every mouse (6 out of 6), 5 days of constant light induced phase reversal, consistent with our prediction and previous data (Figure 4B). Three days of constant light also elicited phase reversal in 7 out of 9 mice and induced 7–8 hours of phase shift in the other 2 mice (Figure 4B). Although not in every mouse, a 15 hour light pulse at ZT 12 also caused clear phase reversal in 8 out of 12 mice (Figure 4B). However, the same light pulse only elicited 2–3 hour phase shifts in the other 4 mice (data not shown), suggesting that the 15 hour light pulse at ZT 12 is close to a minimum LL treatment capable of resetting the rhythm. We observed that the level of light is critical to induce the phase reversal. When we used lower intensity light (~250 lux), we could not induce large phase shifts in any of the subjected mice (n = 4) with a 15-hour light pulse at ZT 12; we could induce only 3–4 hour phase shifts (data not shown). We did not test extensively if different conditions such as longer light pulses than 15 hours (e.g., 18 hours) and/or initiating the pulse at times other than ZT 12 would also produce phase reversal. However, because our data showed that LL followed by DD sets the clock to ~CT 15 regardless of the phase of prior rhythms, we expect that phase reversal depends on very specific combinations of light pulse duration and phase. Thus, an 18-hour pulse initiated

at ZT 12 or a 15-hour pulse initiated at ZT 15 would produce smaller phase shifts. Consistent with this prediction, longer light pulses do not necessarily produce larger phase shifts. Comas et al. demonstrated that a 9 hr light pulse can produce a larger phase shift than an 18 hr light pulse in mice (Comas et al., 2006).

It takes more than 5 days to reverse the behavioral phase by a reversed light-dark cycle

To evaluate the power of the LL treatment to reset the clock, we compared it to a different lighting regimen: reversing the light-dark cycle, or shifting the light-dark cycle by 12 hours. We compared the ability of this lighting condition to produce the maximal behavioral phase shift in the same environment with the same light intensity as used previously for the LL resetting. We measured behavioral and mPER protein rhythms after the LD cycle is reversed (shifted by 12 hours) (Figure 5). Mice were entrained in a 12 hour light and 12 hour dark (LD) cycle for more than 2 weeks before they were subjected to 1, 3 or 5 days of a reversed LD cycle (DL) followed by constant darkness (DD) (Figure 5A). As expected from earlier work (e.g., Daan and Pittendrigh, 1976), locomotor activity was immediately suppressed by light during DL and this “masking” effect caused the phase of the locomotor activity rhythm to appear normal (i.e., activity onset occurred around lights-off) during DL after 3 days. However, analysis of true circadian phase manifested in DD revealed that 3 days of DL only elicited 7.73 hours of phase shift on average, and only 5 days of DL induced a nearly complete phase reversal: 11.55 hours of phase shift (Figure 5A).

It is possible that the modest phase shifts produced by 1 day of the reversed DL cycle may be due to the method used for reversing the cycle, which involved repeating the “D” cycle twice (LD->DL). Thus we also tested if bigger phase shifts could be induced when mice were exposed to two consecutive “L” cycles during the reversal (DL->LD). The second “L” was followed by DD. In another words, mice were exposed to 24 hours of light before DD. However, this treatment also produced small phase shifts (3.11 +/− 0.67 hours, n=4) in behavioral rhythms similar to those produced by the 1 day after LD->DL transfer in Figure 5A (Figure 5B). None of the mice exhibited phase reversal. The DL->LD treatment is similar to the application of a 12-hour light pulse at ZT 12, which is only 3 hours shorter than the 15-hour LL treatment described above. Thus the failure of this treatment to produce phase reversal adds further support to our conclusion that the 15-hour light pulse at ZT 12 is close to the minimum duration of light pulse required (under our laboratory conditions) to induce such an unprecedented magnitude of phase resetting. The small phase shift produced by a 12-hour light pulse at ZT 12 is likely a conventional phase resetting response which is mediated by rapid induction of *Per* gene expression in the SCN (Shearman et al., 1997; Shigeyoshi et al., 1997; Albrecht et al., 2001).

Since it takes approximately 5 days to stably reverse the SCN clock using DL cycles, as judged by behavioral rhythms, we predicted that at least the same number of DL cycles is required to phase-reverse peripheral clocks. To determine how fast peripheral clocks are reset in DL, we measured mPER1 and 2 rhythms in liver on the 2^{nd}, 5^{th} and 8^{th} day in a DL cycle (Figure 5C). Consistent with behavioral rhythms, both mPER1 and 2 rhythms were not significantly changed on the 2^{nd} day in DL. However, on the 5^{th} day in DL, mPER1 and 2 rhythms were dramatically changed compared to those in control mice. The mPER profile (protein levels and phosphorylation status) on the 5^{th} day in DL was apparently completely reversed compared to that in control mice (Figure 5C). For example, mPER1 and 2 at ZT 12 in DL were similar to those at ZT 12 in LD (before the light:dark cycle was switched). In addition, there was no further change on the 8^{th} day, indicating that 5 days of DL cycle were enough to almost reverse the liver clock as was the case for behavioral rhythms. This suggests that phase resetting in liver may not lag behind that of the SCN significantly, assuming that the behavioral rhythms accurately reflect the status of the SCN clock.

Taken together, our data show that constant light is a much more efficient way to induce a large phase shift than a shifted light dark cycle such as the reversed LD cycle. More importantly, the underlying mechanism for the large phase shift response may differ from the mechanism mediating the conventional small phase shift responses.

Discussion

Although arrhythmic behavior in LL was recognized decades ago, it remained unclear how the animals would behave and how the molecular clock would be reset when the LL is removed. Here we show that the disruption of behavioral rhythms and of the molecular clock by LL is quickly corrected upon transfer to DD. Our methods (behavioral and tissue-level protein analyses) do not reveal the activities of individual cells, and therefore do not directly show desynchronization and resynchronization of those cells. However, Ohta et al. (2005) already demonstrated using single-cell reporter assays that LL-induced arrhythmicity is caused by SCN neural desynchronization. Reporter genes have proven extremely useful for revealing intrinsic properties of the circadian oscillator and the coupling of cells within the SCN (Yamazaki et al., 2000; Yoo et al., 2004; Ohta et al., 2005), since they allow measurement of rhythms in real time from individual cells as well as from cultured tissue. However, this ex vivo approach may not be appropriate to probe the in vivo clock for circadian properties such as phase and amplitude. Recently, Yoshikawa et al. explicitly demonstrated that the timing of tissue preparation for ex vivo measurement of reporter activity dramatically affects these properties (Yoshikawa et al., 2005), as can simple media changes (Yoo et al., 2004; Liu et al., 2007). Thus, although our in vivo method does not measure individual oscillators, it has the advantage of avoiding complications associated with ex vivo tissue preparation, and is appropriate and adequate for measuring the rhythmic output of synchronized cells. Since previous work has shown that LL causes desynchronization of neurons, we can conclude from our data that emergence of new rhythmicity after LL results from resynchronization.

Both locomotor behavior and mPER status in liver lost rhythmicity in LL and regained robust rhythmicity rapidly upon transfer to DD. However, we were surprised to find that oscillator resynchronization in the SCN seemed a little slower than these peripheral cell types; despite several attempts, we could not detect consistent mPER phosphorylation and abundance rhythms in SCN tissue earlier than 18–24 hours after the LL-DD transfer. We do not know if this apparent slowness is due to our SCN sampling method (see Materials and Methods), heterogeneity of SCN tissue (Lee et al., 2003), or a different mechanism of SCN synchrony by LL->DD than is found in peripheral cells. It should be noted that unlike liver cells, the oscillator cells in SCN are coupled to each other through neural connections. Thus, to restore normal rhythms in SCN, the individual cells must be not only synchronized, but also properly coupled to each other to restore network function, which may explain the apparent slowness. In any case, the relative slowness of SCN resetting begs a question regarding the hierarchy of the circadian system: are the peripheral clocks and behavioral rhythms reset independent of the SCN during the early stage of resynchronization? One might assume that the SCN drives peripheral clock resynchronization because the SCN receives light input from the retina, while the liver does not. However, certain zeitgebers can affect peripheral clocks without phase shifting the SCN clock (e.g., Damiola et al., 2000), and light/darkness can affect the body independent of the SCN (e.g., “masking” effects described by Redlin (2001)). We therefore speculate that the LL->DD transition may generate multiple internal time cues. Some of these cues (perhaps hormonal) may have reset the desynchronized liver oscillators before the SCN itself became fully resynchronized. Consistent with this idea, peripheral oscillators can be resynchronized or reset by numerous signals (Balsalobre et al., 2000b; Balsalobre et al., 2000a). If peripheral oscillators were synchronized earlier than the SCN cells, then both behavior and the SCN may have received reinforcing signals from the peripheral clocks. Our

study does not address this hierarchy of synchronization between peripheral and SCN clocks, but our results suggest that further study may be warranted.

Worthy of comment is the fact that some mice maintain weak behavioral rhythmicity in LL. We hypothesize that the weak phase was generated by a small minority of still-synchronized clock cells in the SCN. As behavioral rhythms are driven by the combined output of individual clock cells (Low-Zeddies and Takahashi, 2001), a weak amplitude oscillation from whole SCN could be generated by the small number of synchronous cells over the baseline of the majority of desynchronized cells. Upon transfer from LL->DD, the phase of the minority of cells producing the LL rhythm would become irrelevant, overwhelmed by the synchronous oscillations of the majority, whose phase is set by the transfer. Consistent with this hypothesis, the phases of weak rhythms in LL do not correlate with the new phase generated when mice are transferred to DD, and the new phase is similar to that seen in arrhythmic mice.

Our LL treatment represents an example of type 0 entrainment (Dunlap et al., 2004), in which the new phase after the stimulus (LL) is the same regardless of the old phase before the stimulus. The old phase would have been widely distributed over the circadian cycle among mice after more than 9 days in the 1st DD, but the new phase was the same after the LL treatment. Furthermore, the timing of the DD->LL transition did not affect our results, since three different times of the transition did not produce a significant difference in the starting phase of rhythms in the subsequent DD (data not shown). Type 1 entrainment, more typical of mammals, involves phase shifts of limited magnitude, so the final phase depends critically on stimulus timing (Dunlap et al., 2004). To our knowledge, type 0 phase resetting has not been reported in wild-type mice (Comas et al., 2006). There has, however, been reports of large phase shifts in *Clock*$^{\Delta 19}$ mutant mice (Vitaterna et al., 2006) and in *tau* mutant hamsters (Shimomura and Menaker, 1994). Vitaterna et al. (2006) suggested that the 6-hour light pulse can induce a type 0 response in *Clock* $^{\Delta 19}$ heterozygous mice, but not in wt mice, because the amplitude of *Per* gene oscillation is reduced in the SCN of the mutant mice; thus the same stimulus can produce a bigger effect in the mutant mice based on the limit-cycle model (Winfree, 1980; Johnson et al., 2003). Interestingly, *tau* mutant hamsters also show reduced amplitude of *Per* oscillation (Lowrey et al., 2000). It is unlikely that our type 0 entrainment is mediated through the reduction in the amplitude of PER oscillations in individual oscillators, since Ohta et al. (2005) showed that LL did not significantly reduce the amplitude of *mPer1* oscillation in individual SCN neurons. Both *Clock* $^{\Delta 19}$ and *tau* mutations damage the molecular mechanism of the clock, making the rodents bearing those mutations unusually susceptible to large phase shifts. However, our experiments show that even rodents with normal clocks can exhibit type 0 resetting when given the right stimulus.

In our LD->DL or DL->LD experiments, the lighting regimens may be viewed as a series of 12-hour phase shifts administered over 1, 3 and 5 days. Circadian phase was gradually shifted after each light pulse and reached an equilibrium after 5 days. However, a single 15-hour light pulse at ZT 12 achieved the same phase shift produced by five 12-hour light pulses. It is conceivable that a different molecular mechanism may be responsible for the two processes, as stated previously. We propose that type 0 resetting may occur in wild-type mice through rapid desynchronization and resynchronization of oscillators based on our present studies and those by Ohta et al. (Ohta et al., 2005). However, since we did not measure the circadian oscillators at the single cell level, we cannot rule out that the phase reversal by a single light pulse may be also mediated by a more exaggerated form of the conventional phase shifting mechanism; the LL treatment may have reduced the amplitudes of mPER oscillations, thus increasing the likelihood of type 0 phase resetting as discussed above. We believe that our data (as an extension of Ohta et al. (Ohta et al., 2005)) fit the desynchronization-resynchronization model better, but future studies will have to explore alternatives such as LL-mediated exaggeration of the conventional phase-shifting response.

Interestingly, type 0 phase resetting with a major shift of 10.8 hr has also been reported in humans (Czeisler et al., 1989). However, the large phase shift was induced by a 5-hour light stimulus with a very strong intensity (~10,000 lux) repeated over 3 days. If (as suggested by homology) our results in mice (by whichever mechanism) can be applied to humans, then it may be possible to shift the human clock 12 hours in just one day using an appropriate constant light stimulus. This would provide substantial benefits to shift workers, jet lag sufferers, and patients with disorders of circadian phase.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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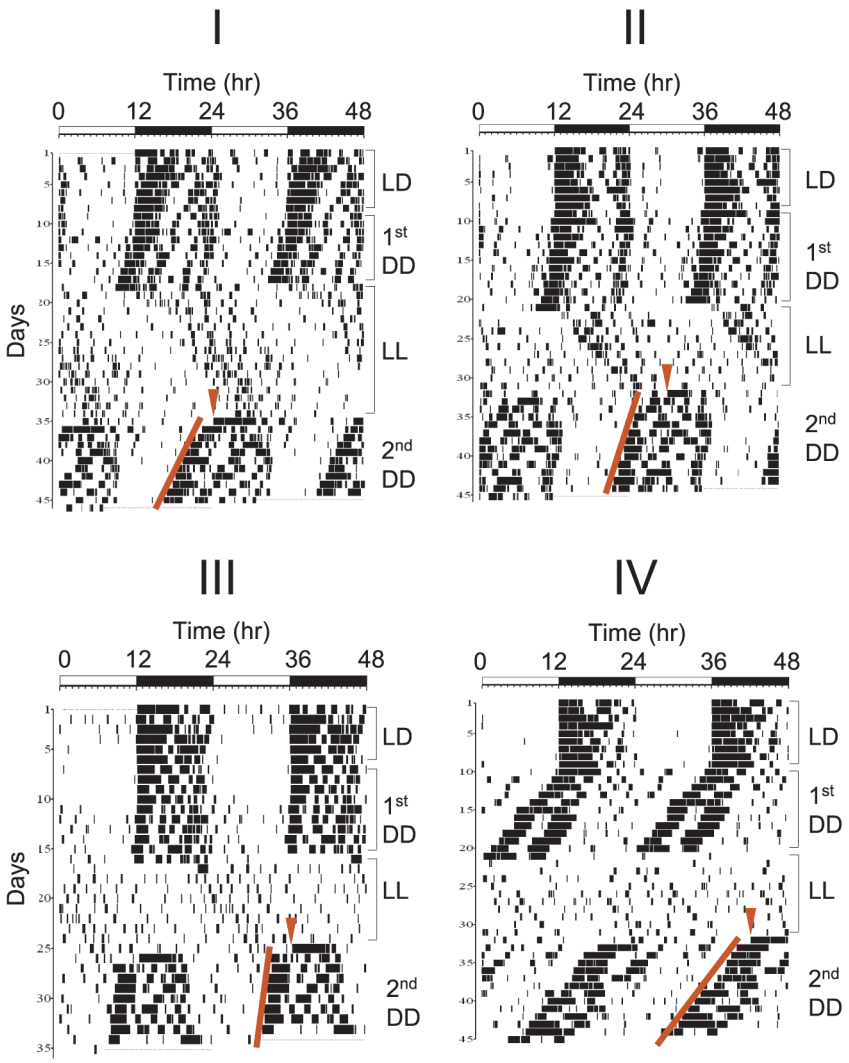

Figure 1.

Arrhythmic mice in LL become rhythmic in locomotor activity when they are transferred to DD, and their new starting phase is ~ CT 15. Representative double-plotted actograms from 4 out of a total of 46 mice (employed in 4 independent experiments) are shown. Black marks indicate wheel-running activity at each set of time coordinates. Each of the groups I, II, III and IV were moved from LL to DD in 6 hr intervals. The red arrow head indicates transfer time from LL to DD and the red line indicates the new phase of activity onset times in DD. Note that the red arrow head moves 6 hrs from group to group. The starting phase was calculated relative to the stable phase indicated by the red line after mice were moved into DD.

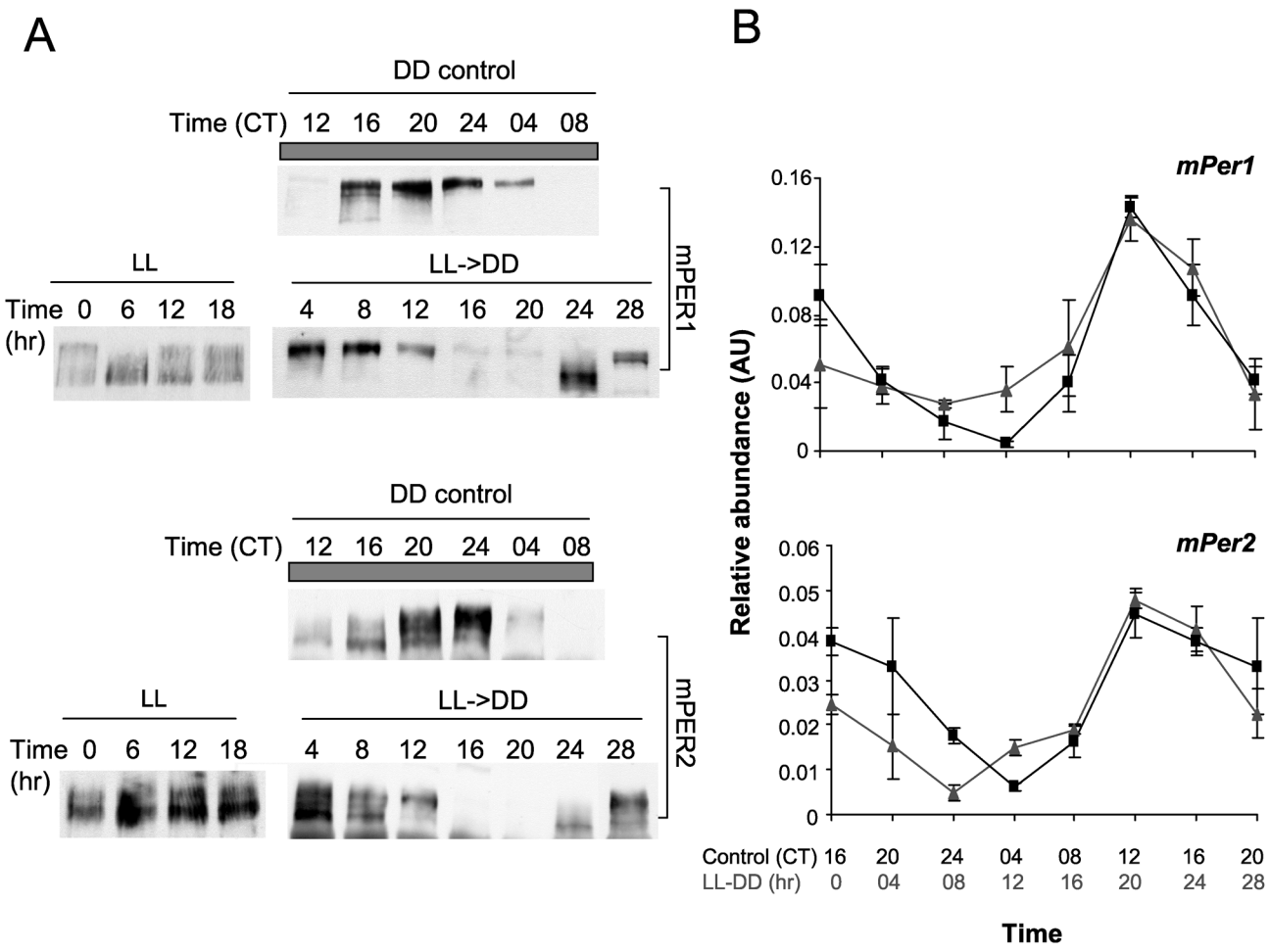

Figure 2.

Liver clock is rapidly synchronized to the same circadian time as behavior in LL->DD mice. (A) Immunoblotting for mPERs in liver. Representative results are shown from three independent experiments. DD control mice (the top panel of each pair) were sacrificed at 4-hr intervals on the first day in DD after transfer from LD. CT 0 is subjective dawn and CT 12 is subjective dusk. LL mice were sacrificed at 6-hr intervals on the last day in at least 10 days of LL before the LL->DD transition. Two other independent experiments showed similar near steady-state mPER rhythms in LL (data not shown). LL->DD mice were collected at 4-hr intervals. The first time point (4 hr) in LL->DD mice was collected 4 hr after the transfer. (B) Quantitative real-time PCR analysis of *mPer1* and *2* mRNA levels. Each point represents mean +/−SEM from three mice. For each transcript, LL->DD and control mice were aligned at Time 0 hr and CT 16, respectively to show that the starting phase (0 hr) is consistent with behavior (~CT15). The control CT 16 and 20 data were plotted twice to compare with the 24- and 28-hr samples of LL->DD mice. The gray graphs (with triangles) and black graphs (with squares) represent LL->DD and LD->DD control mice, respectively. AU=Arbitrary Unit.

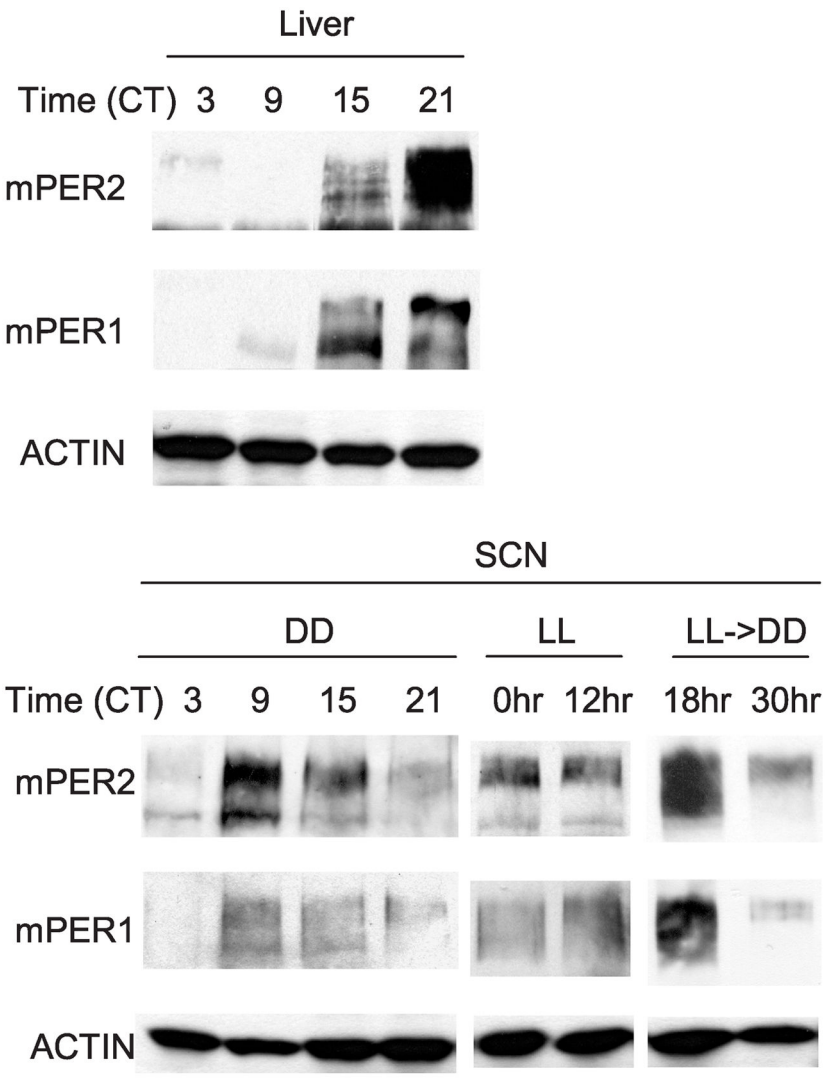

Figure 3.

Immunoblots showing mPER protein rhythms in SCN of LL and LL->DD mice. A representative result of two independent experiments. DD mice were collected on the first day in DD. LL mice were collected twice, 12 hrs apart, after at least 10 days of LL entrainment. LL->DD mice were collected at 18 hrs and 30 hrs after transfer to DD. Each of the SCN time point samples was extracted from a pool of SCN from 5 mice. Liver samples were prepared from one of the 5 DD mice. mPER1, mPER2, and (non-oscillating) ACTIN immunoblots are shown. Note that mPER protein peaks earlier in SCN than in liver.

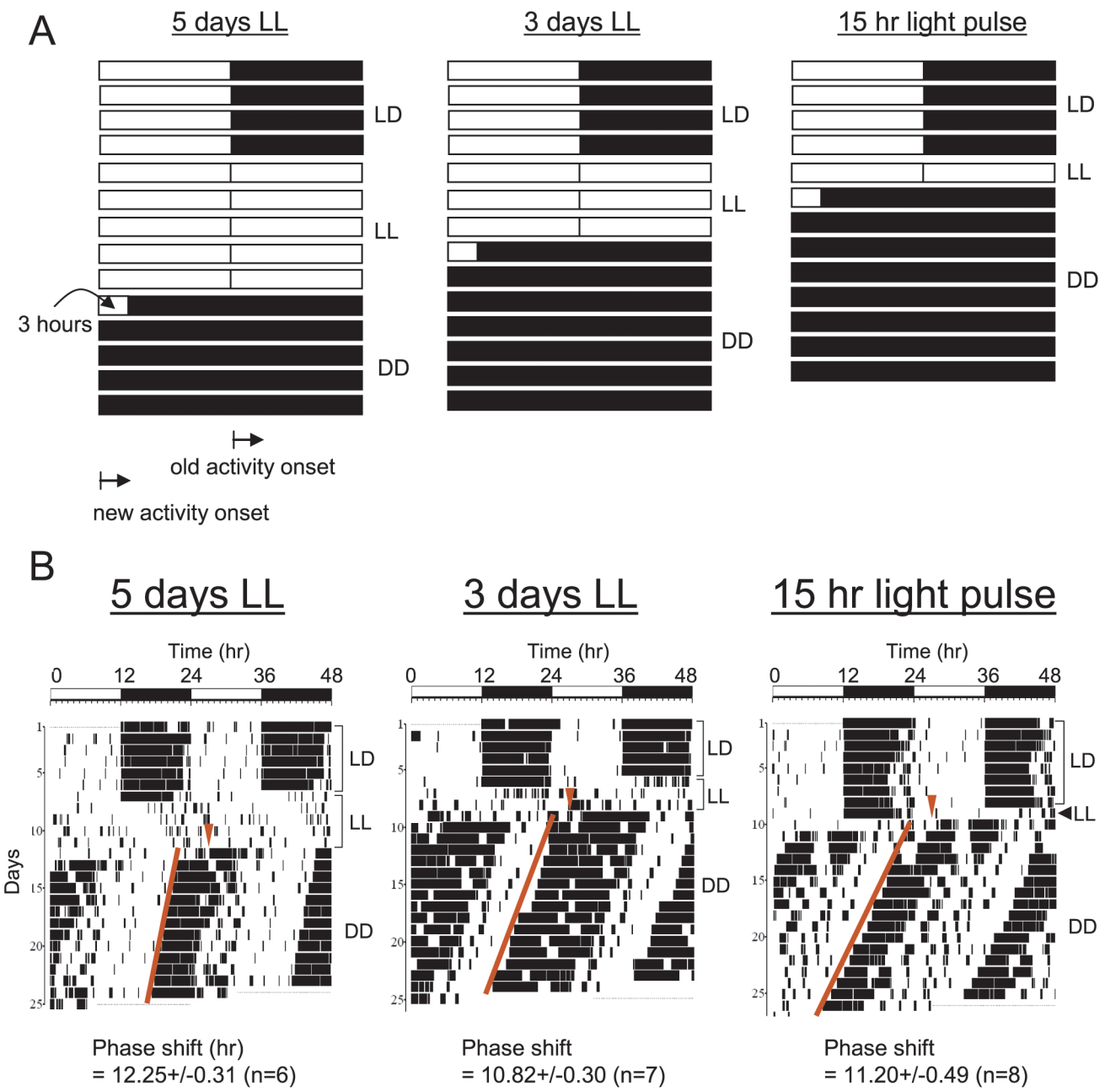

Figure 4.

The phase of behavioral rhythms can be reversed by constant light in a predictable manner. (A) Schematic diagram of 15 hours, 3 days or 5 days of constant light treatment. Each bar (full panel) represents 24 hours. The white and dark areas denote light and dark, respectively. In LD, onset of activity occurs at ZT 12 (lights off; old activity onset). However, if our hypothesis is correct, the onset of activity would be shifted ~12 hrs after the constant light treatment. (B) Representative actograms of mice before and after the constant light treatments. Mice were entrained in LD at least for 2 weeks, subjected to LL for 5 days (15+96 hrs), 3 days (15+48 hrs) or 15 hrs at ZT 12, and then placed in DD. The red line represents onset times of activity. If phase shifts were bigger than 10 hrs, they were considered phase reversals. The phase shifts on the bottom are represented as mean +/−SEM.

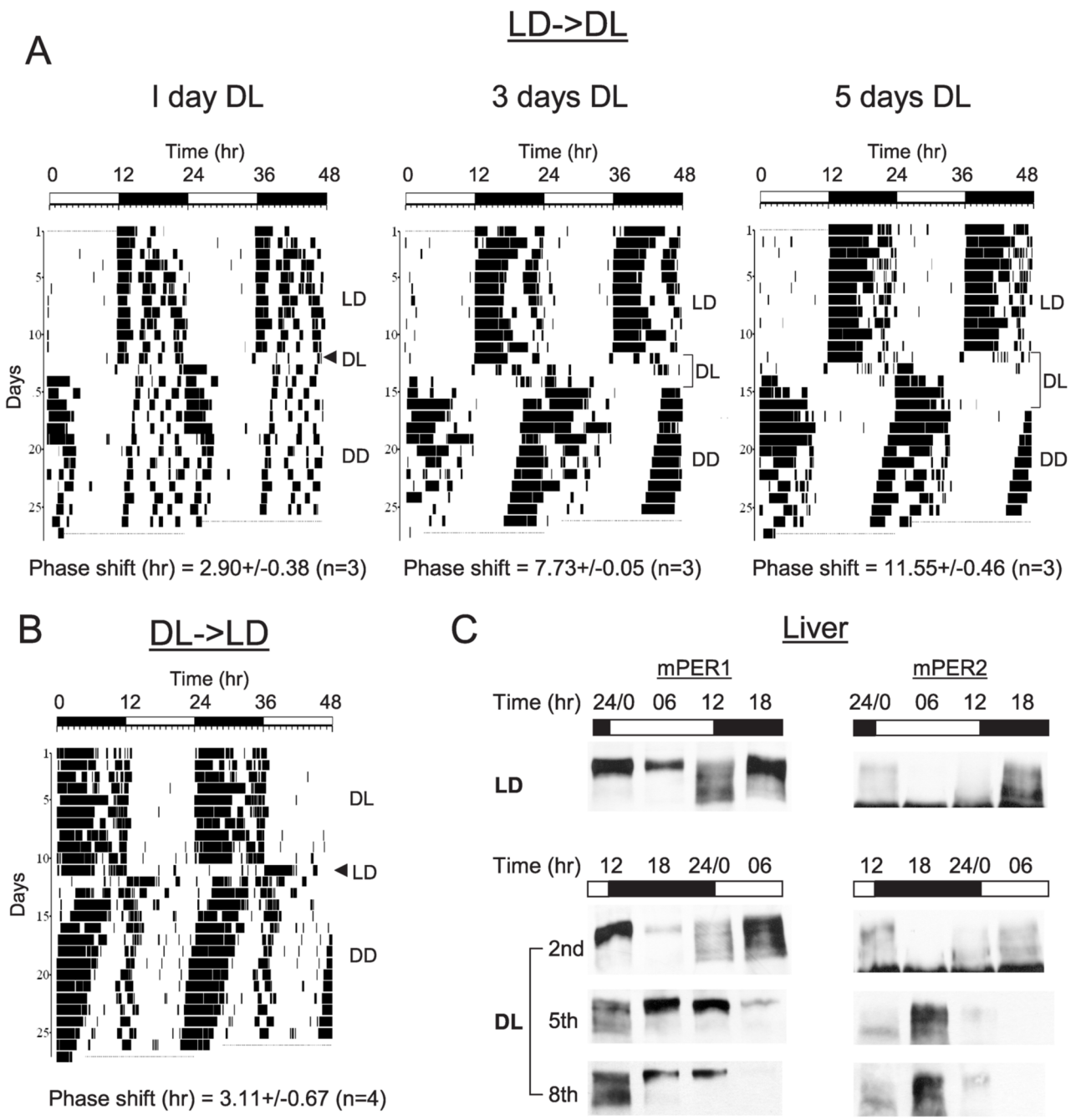

Figure 5.

A reversed light-dark cycle resets behavioral rhythms more slowly than does constant light. (A) Actograms of mice after 1, 3 or 5 days of reversed LD (DL) cycle. Mice were entrained in LD for 2 weeks. After the last dark period, lights remained off for another 12 hours followed by 12 hours of light to switch the mice to DL. Mice were in DL for 1, 3 or 5 days before they were placed in DD. Phase shifts are shown as the mean +/−SEM. (B) A representative actogram after one day of reversed LD cycle in the alternative reversal design (DL->LD). (C) Immunoblots showing mPER rhythms in liver in DL mice. The 2nd, 5th and 8th day samples were collected after 1, 4 and 7 days of DL cycle (LD->DL). These samples were compared with control samples (top panels) collected during LD.

Table 1

Summary of behavioral data obtained from the experiment presented in Figure 1. A total of 46 mice were used in four independent experiments. The extrapolated starting phase of each group in the 2nd DD is presented as Mean+/−SEM. The starting phases among the groups were not significantly different by ANOVA (F =2.783; *p*=0.53).

Group	Starting phase (Mean +/− SEM)	Number of mice
I	14.17 +/− 0.59	10
II	16.09 +/− 0.59	12
III	15.86 +/− 0.56	13
IV	14.40 +/− 0.65	11
Total	15.19 +/− 0.32	46