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Reorganization of Suprachiasmatic Nucleus Networks under 24-h LDLD Conditions

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Abstract The suprachiasmatic nucleus (SCN), locus of the master circadian clock in the brain, is comprised of multioscillator neural networks that are highly plastic in responding to environmental lighting conditions. Under a 24-h light:dark:light:dark (LDLD) cycle, hamsters bifurcate their circadian locomotor activity such that wheel running occurs in each of the 2 daily dark periods with complete inactivity in between. In the present study, we explored the neural underpinning of this behavioral bifurcation. Using calbindin (CalB)–containing cells of the SCN as a regional marker, we characterized PER1 and c-FOS expression in the core and shell SCN subregions. In LD-housed animals, it is known that PER1 and c-FOS in the core and shell region are in phase with each other. In contrast, in behaviorally bifurcated animals housed in LDLD, the core and shell SCN exhibit antiphase rhythms of PER1. Furthermore, cells in the core show high FOS expression in each photophase of the LDLD cycle. The activation of FOS in the core is light driven and disappears rapidly when the photophase is replaced by darkness. The results suggest that bifurcated activity bouts in daytime and nighttime are associated with oscillating groups of cells in the core and shell subregions, respectively, and support the notion that reorganization of SCN networks underlies changes in behavioral responses under different environmental lighting conditions.

Key words circadian rhythms, suprachiasmatic nucleus, LDLD, splitting, PER1, FOS

Convergent lines of evidence establish that the circadian pacemaker in mammals is comprised of multiple oscillators. Several decades ago, a dual oscillator model was posited on the basis of behavioral observations that rhythm "splitting" of locomotor activity occurs in nocturnal and diurnal mammals after prolonged exposure to constant light (LL) or constant dark (DD), respectively, and that distinct rhythm components could free run simultaneously at different frequencies (Pittendrigh and Daan, 1976). Splitting is also measurable in several behavioral and physiological responses including LH secretion and body temperature (Pickard et al., 1984; Swann and Turek, 1982; Swann and Turek, 1985). The neural locus of the master circadian clock is the suprachiasmatic nucleus (Klein et al., 1991), and the demonstration of circadian rhythmicity in individual SCN neurons established the idea that the circadian pacemaker function

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derives from interactions among thousands of oscillating units (Welsh and Reppert, 1996; Yamaguchi et al., 2003). A key issue in understanding SCN function is how the individual oscillators are organized spatially and temporally to produce variations in behavioral rhythms such as splitting.

Insight into the interplay among oscillators within the SCN derives from the analysis of circadian pacemakers at the tissue level. In behaviorally split hamsters and mice, the left and right lobes of the SCN oscillate in antiphase (de la Iglesia et al., 2000; Ohta et al., 2005). Likewise, under a 22-h LD cycle, oscillators lying in 2 separate areas of rat SCN, here termed dorsomedial and ventrolateral, become temporally dissociated (de la Iglesia et al., 2004). These 2 compartments appear to mediate distinct activity components that are entrained or free running, respectively, and to contribute differentially to various circadian outputs (Cambras et al., 2007; de la Iglesia et al., 2004; Lee et al., 2009).

Further evidence for plasticity of oscillator organization within each of these broad anatomical divisions of the SCN and for mutual coupling between divisions derives from manipulation of operating conditions such as the photic input (Hamada et al., 2001; Yan et al., 2005). In the Syrian hamster, for example, the SCN contains a ventrocaudal core that receives direct retinal input via the retinohypothalamic tract (Bryant et al., 2000). In DD, the core region lacks detectable rhythms in clock gene expression, and some neurons lack circadian rhythms in electrical activity (Hamada et al., 2001; Jobst and Allen, 2002). In split hamsters housed in LL, however, neurons in the core region are reorganized and show robust rhythms in antiphase to that in the shell (Yan et al., 2005; Tavakoli-Nezhad and Schwartz, 2005). Following ablation of the core, the remaining SCN cannot sustain behavioral rhythms as measured by wheel-running activity, drinking, and gnawing (Kriegsfeld et al., 2004; LeSauter and Silver, 1999), indicating a specialized role of these cells in maintaining clock function (Antle et al., 2003; Antle et al., 2007).

An understanding of the multioscillator network of the SCN may be furthered through exploration of diverse entrainment paradigms, particularly those under strong experimental control. Under appropriate 24-h light:dark:light:dark (LDLD) cycles, hamsters can be induced to bifurcate their circadian activity such that robust wheel running is expressed in each of the 2 daily dark phases with complete inactivity in between (Gorman and Elliott, 2003; Gorman and Steele, 2006). Extensive behavioral characterization of this system suggests that the 2 daily activity bouts are mediated by circadian oscillators that are separately entrained by light. The 2 activity components can be entrained to a range of LDLD conditions (Gorman and Steele, 2006); the consequent bifurcated rhythms persist under "skeleton" photoperiods (Gorman and Elliott, 2003), but the 2 components quickly rejoin in constant darkness under the influence of mutual coupling (Evans et al., in press). Mice also exhibit a comparable, if much less stable, bifurcated activity pattern (Gorman and Elliott, 2003; Watanabe et al., 2007) associated with sustained bimodal mPer1 bioluminescence, in vitro, in both the left and right SCNs (Watanabe et al., 2007). Similarly, under forced desynchrony in LD11:11 clock gene expression in the rat dorsomedial (shell) and ventrolateral (core) compartments of the SCN, both appear to be unimodal but differentially phased with respect to one another (Cambras et al., 2007; de la Iglesia et al., 2004).

Because the bifurcated rhythms of hamsters under LDLD are readily induced under rigorous experimental control and afford extreme temporal separation of functional oscillator units (Gorman and Elliott, 2003; Gorman and Steele, 2006), it is an attractive model in which to examine the organization of SCN oscillators. In the present study, we characterize the patterns of PER1 and FOS protein expression to assess how the 2 light entrainable oscillators under LDLD map onto the oscillatory compartments of the SCN.

MATERIALS AND METHODS

Animals and Housing

Male Syrian hamsters (HsdHan: AURA, Harlan, Indianapolis, IN), 5 to 6 weeks at the beginning of the experiment, were housed individually in polypropylene cages (48 × 27 × 20 cm) equipped with 17-cm running wheels. Wheel-running activity was monitored by VitalView data acquisition package (Mini-mitter, Sun River, OR) and compiled into 6-min bins. Food and water were available *ad libitum*. Light during all photophases was generated by white fluorescent bulbs, producing an illumination of 80 to 200 lux at the level of the cage lid.

All dark phases were dimly illuminated (<0.1 lux) by green light-emitting diodes as previously described (Gorman and Steele, 2006). Studies were conducted in accordance with the regulations of the UCSD and Columbia University Institutional Animal Care and Use Committees.

Upon arrival in the laboratory, hamsters were housed in a 20-h light, 4-h dark condition (LD 20:4; lights-on: 0230-2230 h), which was modified 13 days later by adding a second 4-h dark phase to yield LDLD 8:4:8:4 (lights-on: 0230-1030 h, 1430-2230). Hamsters received a cage change, in darkness, during the first half hour of the first occurrence of the new dark phase to induce a bifurcated or "split" pattern of entrainment (Gorman et al., 2003). Hamsters were sacrificed 15 to 18 days later, at which time the stable state of the bifurcating behavior was established.

Brains were collected at 6 time points at 4-h (±15 min) intervals throughout the LDLD beginning at 0030 h (n = 4 at 0830 and 2030 h; n = 5 at other time points). To assess whether FOS expression at 0430 and 1630 h was endogenously driven or light induced, 2 additional groups of hamsters (n = 4/group) were maintained in dark for 2 h beyond the normal end of the morning or afternoon scotophase phase and sacrificed in darkness at 0430 and 1630 h, respectively.

Immunocytochemistry (ICC)

Animals were anesthetized (pentobarbital, 200 mg/kg, i.p.) and perfused intracardially with saline followed by fixative (4% paraformaldehyde in 0.1 M phosphate buffer). Brains were removed and post-fixed overnight in the same fixative, then cryoprotected in 30% sucrose for 2 days. Free-floating coronal sections (40 µm) were prepared using a cryostat.

Triple-label ICC for PER1, c-FOS, and CalB was performed using the following primary antibodies: PER1 (raised in goat, 1:4000; gift of Dr. Lehman, University of Western Ontario, London, Ontario, Canada), c-FOS (raised in rabbit, 1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA), and CalB (raised in mouse, 1:10,000; Sigma-Aldrich, St. Louis, MO). The specificity of the PER1 antibody has been characterized previously and was confirmed by preadsorption with the immunogen peptide (Yan et al., 2005; Yan and Silver, 2008). ICC on free-floating sections started with a 1-h blocking incubation in 2% normal donkey serum (NDS) in phosphate buffer with 0.1% Triton X-100 (0.1% PBT), followed by 48-h incubation in the primary antibodies (in 0.1% PBT plus NDS) at 4 °C. Sections were rinsed 3 times in 0.1 M PBT and then incubated in the following fluorescent secondary antibodies: Cy3-Donkey-anti-Goat, Cy2-Donkey-anti-Rabbit, and Cy5-Donkey-anti-Mouse (each at 1:1000; Jackson ImmunoResearch, West Grove, PA) overnight at 4 °C, then rinsed 3 times in 0.1 M PBT. After the ICC reaction, sections were mounted on gelatin-coated slides, dehydrated with alcohol rinses, cleared with xylene, air dried, and coverslipped with Krystalon. In all cases, the tissue was protected from light exposure once a fluorescent secondary was applied.

Data Analysis

Analysis of Behavioral Data

Actograms were prepared, and entrainment was assessed using ClockLab software (Actimetrics, Evanston, IL). Starting the second week of LDLD, daily activity onsets and offsets and total counts for each of the 2 daily activity bouts were determined for 5 days according to previously described criteria (Gorman and Elliott, 2003) and compared between bouts using paired t-tests.

Analysis of Immunostaining

Images were captured using a Nikon Eclipse E800 microscope (Nikon Co., Tokyo, Japan) equipped with the following filters: GFP, Texas Red, and CY5, which specifically pass the signal for CY2, CY3, and CY5, respectively. The microscope is fitted with a cooled CCD digital camera with SPOT software (Diagnostic Instruments, Sterling Heights, MI). Images were loaded into Photoshop 7.0 (Adobe Systems, San Jose, CA) for cell counting. The SCN subregions were delineated as previously described (Yan et al., 2005). Briefly, the region of calbindin-containing cells and the "cap" (Antle and Silver, 2005) immediately above it were termed "core," while the area surrounding this region, which is totally lacking in CalB cells and fibers, was termed shell (Yan et al., 2005; Yan and Silver, 2008) Two observers who were unaware of the experimental condition counted PER1- and c-FOS-positive cells bilaterally in the midcaudal SCN containing both the core and shell region. For the number of PER1-ir nuclei in the core and shell region, the results were analyzed by 2-way ANOVA (time × region). When there was a significant interaction between the 2 factors, 1-way ANOVA was performed within each region followed by a post hoc Tukey test.

RESULTS

Entrainment

Following transfer from LD to LDLD followed by the timed cage change, all 24 hamsters exhibited

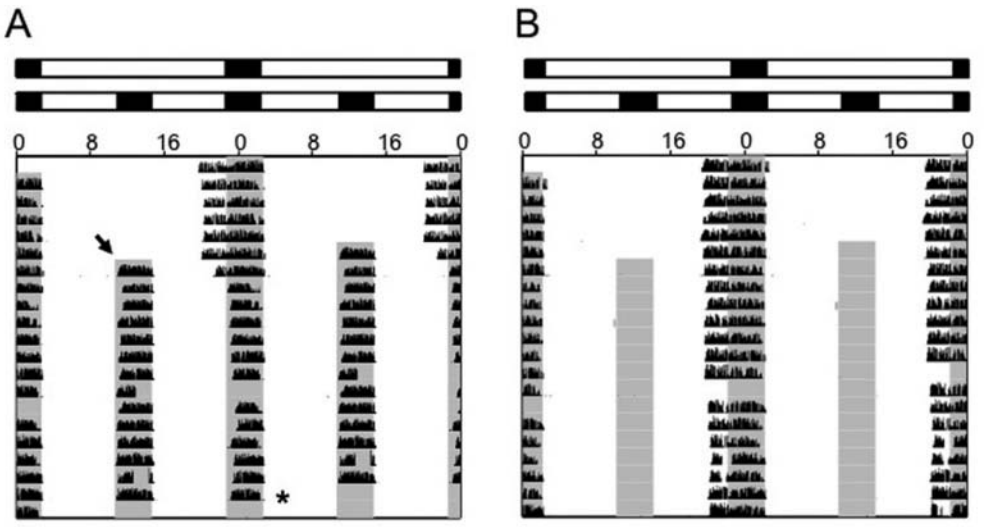

Figure 1. Double-plotted actograms of hamsters transferred from LD20:4 to LDLD8:4:8:4 to induce and maintain bifurcated wheel-running rhythms. Shading over actogram indicates intervals of darkness. In panel A, the arrow indicates a cage change at the onset of LDLD that triggered rapid bifurcation of the activity rhythm. In contrast, panel B illustrates an animal that remains conventionally entrained following LDLD that was not associated with a cage change. Too few animals maintained unbifurcated rhythms to be used in the current study. The asterisk indicates time of sacrifice for collection of brain tissue. In both actograms, a computer malfunction interrupted data collection for approximately 12 h 1 week before the end of the record.

unambiguously bifurcated activity rhythms evident within 1 to 2 circadian cycles. Figure 1 illustrates a representative actogram from the current experiment as well as one from a concurrently studied animal treated exactly as above except that it was not given a cage change upon transfer to LDLD and it did not bifurcate its rhythm. Too few animals were available from this condition to allow examination of their brains. During the week prior to collection of brains, the 2 activity bouts of these bifurcated rhythms remained entrained to LDLD but with significant differences between bouts. Specifically, compared to values for the nighttime activity component, the daytime bout showed a less negative phase angle of entrainment (–0.21 ± 0.10 v. –1.10 ± 0.07 h; $p < 0.001$), greater bout duration (3.83 ± 0.07 v. 2.95 ± 0.09 h; $p < 0.001$), and overall higher activity counts (25964 ± 1049 v. 20848 ± 948 counts; $p < 0.001$). The phase angle difference between the 2 activity bouts averaged 12.9 ± 0.1 h, which differs significantly from 12 h ($p < 0.001$).

Spatiotemporal Distribution of PER1, FOS, and CalB in SCN

Examination of the photomicrographs indicates that while some expression of PER1 in SCN was observed throughout the daily cycle, it was highest at 0030, 1230, 1630, and 2030 h, but localization varied as a function of time (Figure 2A, top row). In the shell region, PER1 was highest at 1230 h, less at 1630 h, and least at other time points. In the core SCN, most PER staining occurred at 0030 and 2030 h, with less at other time points. In contrast to PER1, FOS-ir was localized mostly in the core SCN and observed only at 0430 and 1630 h (Figure 2A, middle row). At other time points, only a few scattered FOS cells were observed in either core or shell SCN regions. CalB-ir cells were present at every time of day, with no time-dependent variations (Figure 2A, bottom row).

Quantitative analyses of these data are shown in Figure 2B. For PER1 (left panel), time-dependent changes were significant in both core and shell SCN regions (2-way ANOVA, time effect: $F_{5,44} = 8.5$; $p < 0.001$; region effect: $F_{1,44} = 67.3$; $p < 0.001$; interaction: $F_{5,44} = 17.1$; $p < 0.001$). In the shell SCN, the number of PER1-ir nuclei was highest at 1230 h (1-way ANOVA, $F_{5,22} = 13.5$; $p < 0.001$; post hoc Tukey test: $p < 0.05$). In the core SCN, the number of PER1-ir nuclei was highest at 0030 and 2030 h (1-way ANOVA, $F_{5,22} = 10.8$; $p < 0.001$; post hoc Tukey test: $p < 0.05$).

FOS-ir nuclei were mostly located in the core and absent in the shell region and, thus, were only counted in the core region of the SCN (Figure 2B, right panel). The quantitative analysis confirmed that the number of FOS-ir nuclei was high at 0430 and 1630 h (1-way ANOVA, $F_{5,22} = 42.9$; $p < 0.001$; post hoc Tukey test: $p < 0.05$).

Influence of Light on FOS Expression

We next tested whether the high FOS expression at 0430 and 1630 h (Figure 2B) was endogenously driven or induced by light during photophase (Figure 3). Two groups of behaviorally bifurcated hamsters

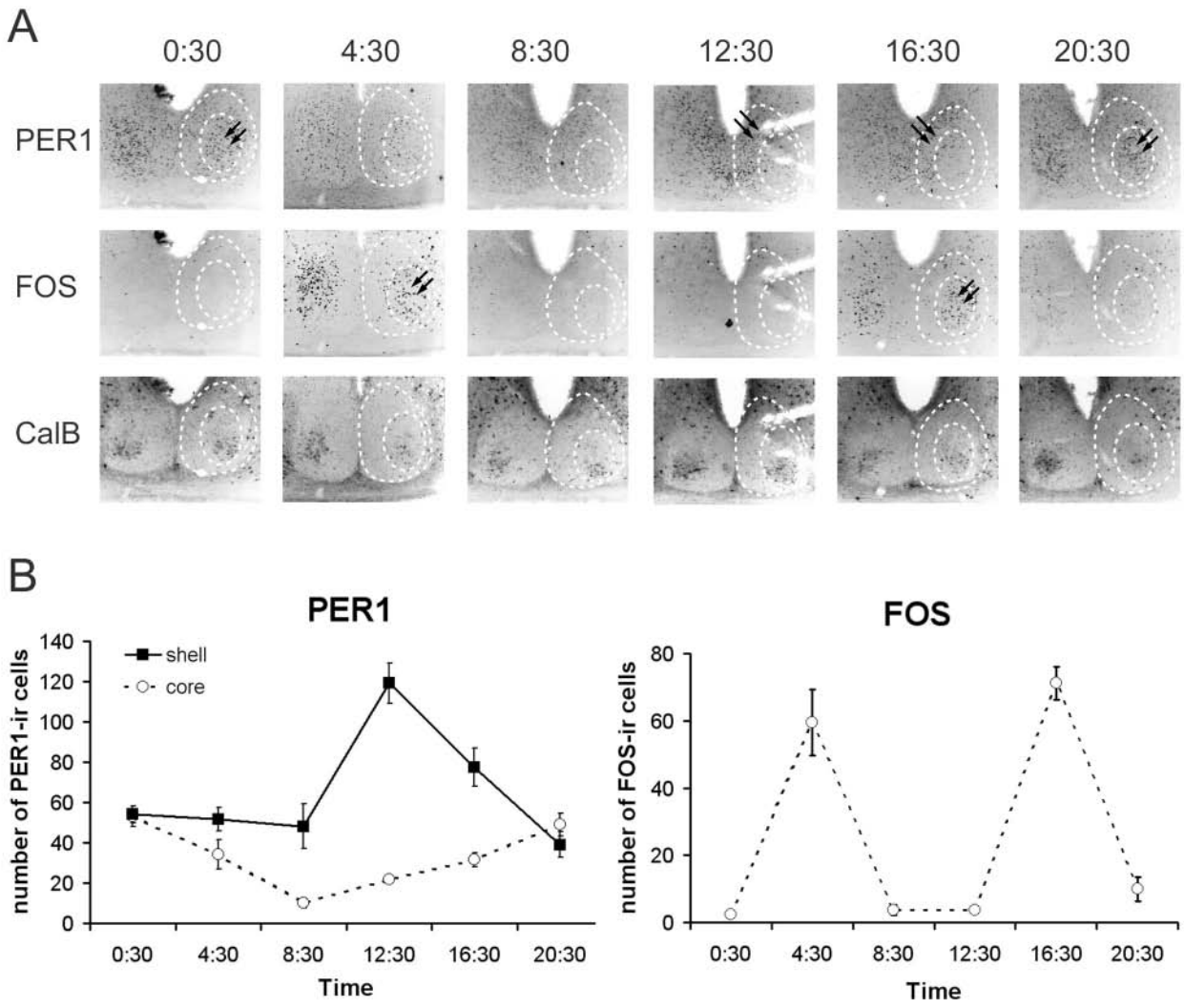

Figure 2. PER1 and FOS expression in the SCN of behaviorally bifurcated hamsters induced by LDLD condition. (A) Representative photomicrographs showing PER1 (top), FOS (middle), and CalB (bottom) in the SCN of behaviorally bifurcated hamsters through a daily cycle. Numbers on the top indicate the time when animals were sacrificed. White dashed lines show the outlines of the SCN and the core region. Arrows indicate the SCN subregions showing higher PER1 or FOS expression. Images for PER1, FOS, and CalB at each time point were taken from the same sections that were triple labeled. (B) Quantitative analysis of PER1-ir (left) and FOS-ir (right) cells. The results are presented as mean ± SEM.

were kept in the dark after either the morning or afternoon scotophase and were sacrificed at 0430 or 1630 h, respectively (Figure 3A). Irrespective of whether they were sacrificed at 0430 or 1630 h, the number of FOS-ir nuclei in the SCN of animals placed in the dark decreased significantly (2-way ANOVA, time effect: $F_{1,12} = 0.6$; $p > 0.05$; light effect: $F_{1,12} = 79.0$; $p < 0.001$; interaction: $F_{1,12} = 0.2$; $p > 0.05$).

DISCUSSION

The present results confirm the findings that with permissive LDLD cycles, the locomotor activity rhythms of hamsters bifurcate into 2 components that entrain with a stable phase angle to the 2 respective dark phases (Figure 1). Prior studies argue that these 2 activity components reflect outputs of separate underlying oscillators (Gorman and Elliott, 2003). The present study suggests a neural mapping of these oscillators in the hamster SCN. The core and shell exhibit dissociated rhythms of PER1 indicative of a temporal reorganization of the central circadian pacemaker (Figure 2). Specifically, SCN oscillators (marked by PER1-ir) in the core and shell region are dissociated in LDLD corresponding to the bifurcated activity. Furthermore, cells in the core region are activated in each photophase, revealed by FOS-ir (Figure 2).

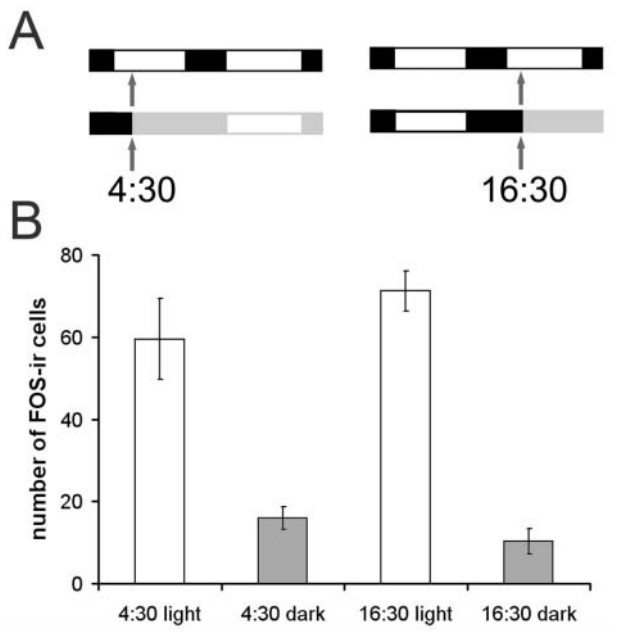

Figure 3. FOS expression in the core SCN of bifurcated hamsters is light dependent. (A) Schematics showing the time when the LDLD and the control animals were sacrificed. For the LDLD animals at 0430 h, the first photophase was replaced with darkness. For the LDLD animals at 1630 h, the second photophase was replaced with dark. (B) Quantitative results for FOS-ir cells in the core SCN of LDLD and dark-housed animals at 0430 and 1630 h. The data for LDLD animals were replotted from Figure 2. The results are presented as mean ± SEM.

Finally, the activation of SCN neurons in the LDLD condition seems to be light dependent and decreases dramatically when the light is turned off (Figure 3). Unlike the mechanism thought to mediate splitting in hamsters (de la Iglesia et al., 2000), the present results suggest that the bifurcated behavioral components seen in LDLD animals are a product of separate groups of oscillators within each SCN. Although the LDLD light cycle does not occur in nature, this bifurcated entrainment model affords the opportunity to explore the network properties of the SCN and its separate oscillating clusters.

The lighting conditions employed in the present study reflect the gradual optimization of a dissociation protocol initially reported (Mrosovsky and Janik, 1993) using repeated exposure to novel wheel running (NWR). Following that initial report, we explored several different paradigms including the entrainability of NWR-bifurcated rhythms to LDLD cycles (Gorman et al., 2001), the sufficiency of LDLD cycles without NWR for induction of bifurcated rhythms (Gorman, 2001), the facilitating role of dim (<0.1 lux) nighttime illumination for this entrainment configuration (Gorman and Elliott, 2003), and robust entrainment to varying LDLD conditions (Gorman and Steele, 2006). In comparison, the specific protocol used in the present study represents the most efficient method to date in inducing bifurcated activity pattern with 100% of animals adopting the desired entrainment patterns within only 1 to 2 circadian cycles of exposure. Although it remains to be determined whether the alternative methods for bifurcating activity rhythms generate a common configuration of SCN activity, the behavioral outputs under these protocols are largely similar and are characterized by stable and negative phase angles of entrainment for both bouts, a complete lack of any activity between bouts, and in constant conditions, expression of 2 distinct bouts for a few to several days before rejoining occurs.

Four different models have some precedent in the circadian literature with regard to how functionally defined oscillations map onto SCN organization. First, the bifurcated bouts could derive from oscillators within versus outside the SCN (Abe et al., 2001), similar to those involved in food- and light-entrained oscillators (Mendoza, 2007; Stephan, 2002); alternatively, comparable to LL-induced split rhythms, the left and the right SCN may become temporally dissociated (de la Iglesia et al., 2000; Yan et al., 2005). Third, functional compartments delineated by dorsal/ventral or core/shell of the SCN may become temporally dissociated, as in free-running and entrained oscillations in T22 rats (de la Iglesia et al., 2004), or the LL-induced split hamsters (Tavakoli-Nezhad and Schwartz, 2005; Yan et al., 2005). Finally, cells even within SCN subregions may cycle with a distribution of peak phases to produce bimodality at the population level. Corroborating conclusions from alternative LDLD bifurcation protocols (Edelstein et al., 2003; Watanabe et al., 2007), we must reject the first 2 hypotheses, as there is clear evidence of temporal reorganization within the SCN and no hint of lateral asymmetries in this paradigm.

The most parsimonious interpretation is that the bifurcated rhythm derives from the dissociation of clock gene oscillations in the SCN core versus the shell as reflected by PER1 expression profiles. Our hypothesis regarding the relationship is that the PER1 expression in the shell is responsible for the part of activity during the dark phase that continues uninterrupted when animals are shifted from LD to the LDLD condition (Figure 1). We further suggest that the core region is responsible for the newly emergent activity bout that occurs in the other dark phase (Figure 4). The provisional assignment of core and shell rhythmicity to daytime and nighttime activity bouts, respectively, provides a framework to understand a key functional difference between the

2 oscillator groups in how they rejoin over time into a typical unimodal activity rhythm following transfer from LDLD to DD or to LD (Evans et al., in press; Gorman and Steele, 2006). It is likely that the antiphase PER1 oscillation in the core SCN, seen in the bifurcated hamsters, is dependent on lighting condition. When hamsters were housed in DD, there were no detectable rhythms in the core in either *Per1* mRNA or PER1 protein (Hamada et al., 2001; Yan and Silver, 2008). Thus, it is reasonable to speculate that when the bifurcated animals are released from LDLD to DD, and the 2 activity bouts become consolidated, the rhythm of PER1 in the core SCN regions will no longer be detected. Whereas the core/shell distinction provides an explanation for differences between bifurcated bouts, it leaves unexplained the mechanism whereby the system produces 2 daily oscillations that otherwise appear generally more similar than different (i.e., in terms of effects on body temperature, phase shifting, melatonin secretion, etc.) (Gorman et al., 2001; Rosenthal et al., 2005).

It would be of interest to know how the oscillator cells are organized in the SCN of hamsters housed in LDLD prior to the occurrence of behavioral bifurcation. In the present study, the LDLD was combined with a cage change to hasten the bifurcation, which then occurred within 2 days. We have previously shown that LDLD alone is sufficient to induce bifurcation, although its emergence was more prolonged and variable between individuals than in the present study (Gorman, 2001). Evidence from split hamsters housed in LL suggests that their antiphase oscillation in the left versus right SCN antecedes behavioral splitting (Tavakoli-Nezhad and Schwartz, 2005; Yan et al., 2005). Although direct evidence is not available, it is likely that the central nervous system change in the LDLD condition also antecedes the behavioral change.

In addition to temporal changes in clock gene rhythms in the core versus the shell, the LDLD bifurcated hamster exhibits functional bimodality within the SCN core. High FOS expression was observed twice daily, at the beginning of each light phase, 0430 and 1630 h. When the photophase was replaced with darkness, FOS-ir in the core SCN decreased significantly at both time points, suggesting the high FOS expression is light induced and not endogenously driven (Figure 3). However, as the level of FOS expression at 0430 and 1630 h in hamsters maintained in darkness still seems to be higher than those at other time points (Figure 2), we cannot rule out the possibility of a dampened oscillation of FOS that is endogenously driven and is superimposed with the light-induced expression. A similar bimodal induction of FOS by light has been noted in hamsters bifurcated by NWR (Gorman et al., 2001). Prior work establishes a critical role for entrainment of light pulses before and/or after each of the bifurcated activity bouts (Gorman and Elliott, 2003) and shows that bifurcated rhythms can be entrained to a variety of LDLD conditions (Gorman and Steele, 2006). The induction of FOS expression in the retinorecipient core suggests that at least the early stages of light entrainment are comparable for the 2 oscillators and similar to that of the typical unimodal pacemaker.

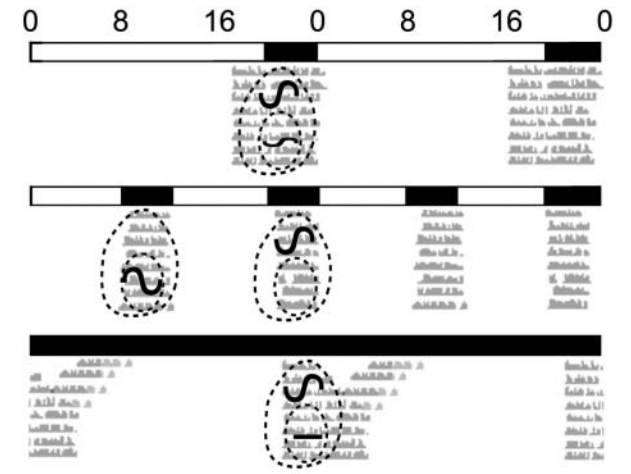

Figure 4. Schematics showing the hypothesized reconfiguration of the SCN oscillations when the animals are moved from LD (top) to LDLD (middle) and then back to DD (bottom). When the hamsters are housed in 20:4 LD condition, the oscillators in the shell SCN region have coherent circadian oscillation, while the oscillators in the core region show low-amplitude oscillation in phase with that in the shell. In LDLD, the oscillators in the core region are reorganized and produce a rhythm out of phase with that in the shell. The newly emergent rhythm in the core is associated with new activity bout in the dark phase of the LDLD cycle. When the animals are moved to DD and environmental (photic or nonphotic) stimuli are absent, SCN networks return to steady state with the shell being highly rhythmic and the core lacking detectable rhythms. The numbers on top indicate the time (h) of day. The white-black bars represent the light-dark conditions.

Sensitivity of the circadian system to light is temporally gated. In LDLD, the bimodal FOS expression induced by light suggests that these cells are gated by multiple oscillating groups, perhaps including the synchronized oscillators of the core. The phenotype of the photosensitive cells remains unknown, but they show very little colocalization with CalB as those seen in the hamsters housed in long photoperiod (Yan and Silver, 2008). It is also unclear whether these cells are distinct from those expressing PER1 in the core region because the peak expression of FOS and PER1 in the core occurred at different times. Cells that show FOS-ir may be a separate population from

the PER1-ir oscillators in the core SCN revealed here. Alternatively, it may be that the oscillators in the core region are also directly light sensitive, and their sensitivity to light may be controlled by their own cellular clock and/or by oscillators in the shell. These possibilities can be explored in future experiments using specific gene or neuropeptide markers. In either case, the bimodality of function in the core identifies a site where multiple oscillations can be simultaneously expressed. Determination of the precise source of each oscillation and their integration at the tissue and cellular level awaits further study.

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