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Day-length Encoding Through Tonic Photic Effects in the Retinorecipient SCN Region

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Abstract

The circadian clock in the suprachiasmatic nucleus (SCN) plays a critical role in seasonal processes by sensing ambient photoperiod. To explore how it measures daylength, we assessed the state of SCN oscillators using markers for neuronal activity (c-FOS) and the clock protein (PER1) in Syrian hamsters housed in long (LD, 16:8hr light:dark) versus short days (SD, 8:16hr light:dark). During SD, there was no detectable phase dispersion across the rostro-caudal extent of the nucleus. In contrast, during LD, rhythms in the caudal SCN phase led those in the mid- and rostral SCN by 4–8 h and 8–12 h, respectively. Importantly, some neurons in the retinorecipient core SCN were unique in that they were FOS-positive during the dark phase in LD, but not SD. Transfer of LD animals to constant darkness or skeleton photoperiod revealed that dark phase FOS expression depends on tonic light exposure rather than on intrinsic clock properties. By transferring animals from SD to LD, we next discovered that there are two separate populations of SCN cells one responding to acute and the other to tonic light exposure. The results suggest that the SCN's seasonal encoding of day-length entails reorganization of its constituent oscillators by a subgroup of neurons in the SCN core that respond to tonic photic cues.

Keywords

Syrian hamster; PER1; c-FOS; circadian rhythms; photoperiod

Introduction

Accumulating evidence exploring responses to photic stimuli, clock gene expression and neuronal activity has established that the suprachiasmatic nucleus (SCN) serves not only as a daily clock but also as a seasonal timer, by encoding day-length (Schwartz *et al.*, 2001; Sumova *et al.*, 2004; Meijer *et al.*, 2007). The SCN is a bilaterally symmetrical hypothalamic nucleus bearing ~10,000 neurons on each side. In dispersed cell culture, individual SCN neurons express circadian oscillations in electrical activity and in expression of core clock genes (Welsh *et al.*, 1995). The *period* gene, shows robust circadian oscillation in the expression of both mRNA (*Per1*, *Per2*) and protein (PER1, PER2), thus serving as useful markers for clock phase (Hamada *et al.*, 2001; Yan & Okamura, 2002; Yamaguchi *et al.*, 2003; Yoo *et al.*, 2004).

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The SCN is a heterogeneous structure and is organized into anatomically and functionally distinct sub-regions, termed the shell and the core (Moore, 1996; Antle & Silver, 2005). The shell SCN region shows robust circadian rhythms, while the core region shows only low amplitude or nondetectable rhythms (Hamada *et al.*, 2001; Jobst & Allen, 2002; Yan & Okamura, 2002; Karatsoreos *et al.*, 2004). The core SCN region receives direct retinal input (Moga & Moore, 1997; Abrahamson & Moore, 2001; Muscat *et al.*, 2003), and light-induced *Per1* and *Per2* gene expression initially occurs in the core SCN, then spreads into the rest of the nucleus (Yan *et al.*, 1999; Yan & Silver, 2002). In addition, the core SCN appears to be critical in maintaining synchrony among the oscillators in the shell SCN (Antle *et al.*, 2003; Yamaguchi *et al.*, 2003) and in maintaining rhythmicity in behavior and physiology (LeSauter & Silver, 1999; Kriegsfeld *et al.*, 2004). In hamsters, the core SCN region contains a group of neurons expressing neuropeptide Calbindin D28 (CalB). CalB also occurs in the area outside the SCN but is absent in the shell SCN, thereby providing a good marker for SCN subregions (Antle & Silver, 2005).

While circadian timing by the SCN has been amply examined, the mechanisms underlying seasonal timing are less well understood, especially in photoperiodic species. Several hypotheses have been put forth to account for day-length measurement by a circadian timer, ranging from mathematical models of external and internal coincidence to physiological models of multioscillator networks and a circannual rhythm generator centered in the pituitary gland (Goldman, 2001; Lincoln *et al.*, 2003; Duncan, 2007; Meijer *et al.*, 2007; Macgregor & Lincoln, 2008). Previously, we have shown that long-term exposure to constant light can fundamentally alter responses of the SCN (Yan *et al.*, 2005). These photic effects are mediated by changes in dynamic interactions among cells in the core and shell SCN. In the present study, we explored how changes in day-length affect discrete SCN regions so as to encode seasonal information. Using *PER1*, the protein product of clock gene *Per1*, and the neural activity marker c-FOS, double-labeled with CalB, we assessed the phase and status of SCN oscillators under long- and short-photoperiod. Next we used various lighting conditions to investigate the contribution of cells in the retinorecipient SCN core.

Methods

Animals

Male Syrian hamsters (n=50, Charles River Laboratories, Kingston, NY), 4 weeks old at the time of purchase, were group housed (n=3/cage) in long days (LD: 16 hr light:8 hr dark). Food and water were available *ad libitum*. The room was equipped with a white noise generator (91 dB spl) to mask environmental noise. After 6 weeks of LD housing, half of the animals were transferred to short day (SD: 8hr light:16hr dark) by advancing dark onset while the rest remain in LD. Clock time 0 (T 0) was defined as the time of lights on in both LD and SD conditions. Animals were used for experiments after two or more weeks of LD or SD housing.

In the experiments evaluating *PER1* and *FOS* expression in LD and SD, hamsters were sacrificed every 4 hr throughout the day starting from T 2 (2hr after light onset, n=4/time point). In the experiments investigating the effect of light on *FOS* expression in the dark phase, 4 groups of animals were used. In group 1, hamsters were transferred from LD to constant darkness (DD) for 1 day (n=3) or 3 days (n=3). Animals in group 2 (n=3) were transferred from LD to a skeleton photoperiod (SK, LDLD 1:14:1:8) for 1 week. In group 3 (n=3) animals were transferred from LD to DD for 3 days then moved back to LD for 1 day. In the last group, animals were moved from SD to LD for 1 day (n=3) or 3 days (n=3). Animals from all 4 groups were sacrificed at T 18. Studies were conducted in accordance

with the regulations of the Columbia University Institutional Animal Care and Use Committee.

Immunocytochemistry (ICC)

Animals were anesthetized (pentobarbital, 200 mg/kg, ip) and perfused intracardially with saline followed by fixative (4% paraformaldehyde in 0.1M phosphate buffer, PB). Brains were removed and post-fixed overnight in the same fixative, then cryoprotected in 20% sucrose for 2 days. Alternate coronal sections (40 μ m) were incubated with CalB (raised in mouse, 1:10,000, Sigma) and either PER1 (raised in goat, 1:4,000, gift of Dr. Lehman, University of Western Ontario, Ontario, CA), or c-FOS (raised in rabbit, 1:5,000, Santa Cruz Biotechnology, Santa Cruz, CA) for 48 hr at 4°C. Sections were rinsed 3 $\times$ in 0.1 M PB containing 0.1% Triton (PBT), incubated for 2 hr at room temperature in the following fluorescent secondary antibodies: Cy2-Donkey-anti-Mouse and either Cy3-Donkey-anti-Goat or Cy3-Donkey-anti-Rabbit (each at 1:200; Jackson ImmunoResearch, West Grove, PA), and then finally rinsed 3 $\times$ in 0.1M 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.

Image analysis

Light microscopy—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. PER1 -positive cells were counted in sections at five levels along the rostro-caudal (R-C) axis from each animal. Within each alternate set of SCN sections, two sections contained the CalB sub nucleus, and those two were always designated as R-C 3 and 4. Two sections immediately rostral to R-C 3 and one section immediately caudal to R-C 4 were selected as R-C 1, 2 and 5, respectively. The sections of R-C 1, 2 and 5 do not have core region, thus the PER1 or c-FOS-ir cell counts in these sections were attributed to the shell SCN. At R-C 3 and 4, where both core and shell regions of the SCN were evident, the cell counts were assigned to each sub-region. FOS-ir cells were counted in R-C 3 and 4 sections only. Cells outside the SCN were not counted. CalB-ir cells were used as regional markers and were not counted.

The core and shell region were defined as previously described (Yan *et al.*, 2005, Antle and Sivler 2005). Briefly, the core includes the area of CalB-containing cells and the “cap” region above it, while the shell is the rest of the SCN (Figure 1). Data were analyzed with two-way and one-way ANOVA followed by Tukey’s post hoc tests, as appropriate. All results are reported as mean $\pm$ SEM.

Confocal microscopy—Colocalization of FOS with CalB in the sections at R-C 3 and 4 was assessed using a Zeiss LSM 510 (Zeiss, Thornwood, NY). Sections were excited sequentially with an argon-krypton laser using the standard excitation wavelength for Cy2, Cy3 or Cy5. Stacked images were collected as 1 μ m multi-tract optical sections. LSM 3.95 software (Zeiss) was used to superimpose and analyze images of the sections.

Results

Spatiotemporal basis of day-length encoding in SCN

The effect of day-length on SCN oscillators is seen in differences in the spatiotemporal organization of PER1 expression patterns between animals housed in long days (LD) and short days (SD). In LD, PER1-ir cells are seen in the caudal SCN at T6, through the entire SCN at T 10–14, in the rostral SCN at T18–22, and are not detectable at T2 (Figure 1A). At the rostral pole (Figure 1A top row, R-C 1), the highest PER1 expression occurs at T 18, while at the caudal pole (Figure 1A bottom row, R-C 5), the highest PER1 expression occurs at T 6. In SD (Figure 1B), PER1-ir cells are concentrated at T 6–10, and there is not detectable rostro-caudal difference in the circadian profile of PER1 expression. Using CalB as a second label on the same sections, we delineated the retinorecipient core SCN region (Figure 1, left column, see definition in method section). PER1-ir rhythms appear to be in phase in the core and shell regions.

To quantify the foregoing spatial and temporal expression patterns of SCN oscillators, the numbers of PER1-ir cells were counted in the functionally distinct core and shell regions and at each level along the rostro-caudal axis (Figure 2). In LD, there were time-dependent fluctuations in the numbers of PER1-ir cells in both core and shell, but the amplitude of PER1-ir rhythm was higher in the shell than in the core region (Figure 2A; two-way ANOVA: time factor, $p<0.001$; region factor, $p<0.001$; interaction, $p<0.001$). A similar pattern was observed in SD (Figure 2B; two-way ANOVA: time factor, $p<0.01$; region factor, $p<0.01$; interaction, $p<0.01$). However, the distribution of PER1-ir cells is different between groups, with a broader temporal distribution in LD than in SD (Figure 2A, B). In LD, PER1-ir is high at T 10–18 in the both shell and core SCN (post-hoc Tukey test, $p<0.05$). In SD, PER1-ir peaks at T 6–10 in the shell, and T 6–14 in the core SCN ($p<0.05$). PER1 expression in the shell and core SCN are in phase in both LD and SD animals.

Analysis of the number of PER1-ir cells indicates a clear difference between the poles of the SCN in the phase distribution of oscillators as a function of day-length, with an orderly change in the oscillator phases from the caudal to rostral aspects of the nucleus (Figure 2C). In LD, there are time and R-C level dependent changes in PER1-ir cell counts (Two-way ANOVA, time: $p<0.001$; R-C: $p<0.001$; interaction: $p<0.001$). The number of PER1-ir cells first peak in the caudal SCN at T 6–14 (R-C 4 and 5, post hoc Tukey test, $p<0.05$, (here and below, each of these time point is higher than the rest of the time points studied)), next in the mid SCN at T10–14 (R-C 3), then in the mid rostral SCN at T 10–18 (R-C 2), and lastly in the most rostral region at T14–22 (R-C 1). Two weeks after the animals were shifted from LD to SD, the phase distribution of the oscillators was compressed (two-way ANOVA, time: $p<0.001$; R-C: $p<0.001$; interaction: $p=0.032$), with PER1 peaking at T6–10 in mid-SCN (R-C 2, 3 and 4) while the rostral (R-C 1) and caudal (R-C 5) SCN peaking at T 6–18 and T 6–14 respectively (Figure 2D). When day-length was shortened by advancing dark onset 8 h, the phase of the most caudal region remained unchanged, while other SCN regions shifted earlier with the greatest advance in the most rostral part of the nucleus.

Long photoperiods activate neurons in the core retinorecipient region

Using c-FOS as a marker, we examined the activity of SCN neurons in the core and shell regions of hamsters housed in LD and SD, as above. In shell SCN, FOS-ir cells were moderately labeled in both groups (Figure 3A) with the number of FOS-ir cells peaking at T 6 in LD and T2–6 in SD (Tukey test, $p<0.05$, Figure 3B). In the core SCN, FOS-ir cells were seen in early morning and dark phase in the LD, and only in early morning in the SD (Figure 3A). The quantification shows the number of FOS-ir cells peak at T 2, 18 and 22 in LD and at T 2 in SD (Tukey test, $p<0.05$, Figure 3B). While the temporal profile of FOS is different

in both shell and core region, the most striking difference between the LD and SD animals is the high FOS expression in the core region during the dark phase in LD.

We next asked whether the dark phase FOS expression in the core region is endogenously controlled or dependent on prior light exposure in LD (Figure 4). For this, we transferred animals from LD to DD and examined FOS expression at T 18 on the first day of DD (Figure 4A). The number of FOS-ir cells decreased significantly ($p < 0.01$), indicating that FOS expression in the core in LD housed animals is dependent on exposure to light (Figure 4A, C). We then asked whether this light effect is phasic or tonic. To test this question, we transferred LD animals to a skeleton photoperiod, where 1 h of light is present at each transition time (Figure 4B). After one week housed in skeleton photoperiod, the animals show decreased FOS level in the core SCN comparable to those in DD (Figure 4B, C).

Next, we investigated whether the activation of core neurons is an acute or a gradual response to light in two groups of animals (Figure 5A, B). We put the LD animals in DD for 3 days and then back to LD (Figure 5A). On the 3rd day of DD, FOS-ir cell number is significantly lower than that in LD ($p < 0.01$ Figure 5C). Once back in LD, the number of FOS-ir cells increased to the same level as in the prior LD (Figure 5C). To further distinguish between acute and gradual FOS induction of core cells by light, we next shifted animals from SD to LD by delaying dark onset by 8 hr (Figure 5B). On the first day of LD, there was a substantial induction in FOS expression in the retinorecipient region, and the number of FOS-ir cells was higher than seen in LD in both core and shell region ($p < 0.01$, Figure 5C). On the third day, the number of FOS-ir cells decreased compared to the first day, but were still higher compared to LD ($p < 0.01$, Figure 5C).

In the final experiment, we examined the phenotype of FOS-ir cells (Figure 5D). In LD, even though the distribution of FOS-ir cells in the dark phase of the LD cycle overlaps with that of CalB cells, there is little co-localization of the two proteins. In LD, only $8.1 \pm 1.3\%$ of CalB cells contain FOS. In contrast, when the animals were shifted from SD to LD, on the first day most CalB cells ($91.6 \pm 2.2\%$) showed FOS-ir, while FOS-positive CalB cells decreased to $45.5 \pm 4.5\%$ by the third day.

Discussion

Using PER1 as a phase marker, we show that changes in day-length cause altered spatiotemporal organization of the SCN. Our data are consistent with the proposal, based on gene expression and electrical activity measures in several species, that day-length is encoded in the SCN (Messenger *et al.*, 1999; Schwartz *et al.*, 2001; Sumova *et al.*, 2004; Johnston, 2005). What remains under debate is whether encoding of day-length is a function of the molecular components of the clock within individual oscillator cells, and/or is a network property requiring reorganization of multiple oscillators (Daan *et al.*, 2001; Lincoln *et al.*, 2003; Johnston, 2005; Inagaki *et al.*, 2007). In vitro electrophysiological recording and real-time imaging are very powerful tools for analysis of the network organization of circadian clocks (Yamaguchi *et al.*, 2003; VanderLeest *et al.*, 2007; Inagaki *et al.*, 2007; Naito *et al.*, 2008). The SCN slice preparation however, may alter the SCN oscillator networks by changing coupling strength among cells following fiber transection or by resetting the phases of the oscillators depending on the time of day that the tissue is harvested (Jagota *et al.*, 2000; Yoshikawa *et al.*, 2005). Thus *in vivo* results are necessary to confirm and strengthen hypotheses derived from *in vitro* work. In the present studies, we explored this question by analyzing the phase of oscillators at five spatial levels from the rostral to the caudal SCN. The results are consistent with previous studies of electrical activity and with those monitoring clock gene expression in mouse SCN (Inagaki *et al.*, 2007; Naito *et al.*, 2008), and provide *in vivo* evidence from an photoperiodic species

supporting the hypothesis of day-length encoding by re-organization of the SCN oscillator networks (Figure 1, Figure 2). Furthermore, the present methods enable both temporal and spatial changes in circadian phase of oscillators within the nucleus and reveal an orderly phase distribution of SCN oscillators along the rostral-caudal axis corresponding to day-length. This rostra-caudal arrangement is particularly interesting compared to the ventral/core and dorsal/shell dissociation previously reported (Yan & Silver, 2005; de la Iglesia *et al.*, 2004; Watanabe *et al.*, 2007), suggesting substantial flexible and plastic changes in SCN networks under different lighting conditions.

The PER1 data indicates the phase distribution of SCN oscillators and differences between LD and SD animals. The FOS data on the other hand, provides insight into a different aspect of day-length encoding by the SCN. The FOS results show that long-duration light exposure activates a group of neurons in the core SCN of the LD animals. We propose that this is the mechanism mediating the SCN oscillator reorganization following changes in day-length. As we discussed earlier, the SCN is heterogeneous, the densest retinorecipient region lies in the core SCN (Abrahamson & Moore, 2001; Muscat *et al.*, 2003). Both acute and tonic light exposure induce expression of clock genes primarily in the core SCN (Shigeyoshi *et al.*, 1997; Yan *et al.*, 1999; Hamada *et al.*, 2001; Yan & Silver, 2002; Yan *et al.*, 2005). The neurons in the core SCN not only relay the light information to the rest of the SCN, but also are critical for setting the phase of the oscillators and keeping them in synchrony (LeSauter & Silver, 1999; Antle *et al.*, 2003; Yamaguchi *et al.*, 2003). As seen in behaviorally “split” hamsters, the accumulated effect of exposure to light seems to occur first in the core region and then in the rest of the SCN (Yan *et al.*, 2005). Thus, in the present study, we explored what roles neurons in the core region play in re-organizing SCN cell groups in LD versus SD.

In a previous study of the “split” hamster, we found that exposure to constant light resulted in a high amplitude PER1 and FOS rhythm in the core in antiphase to that in shell (Yan *et al.*, 2005). We hypothesized that is one of the mechanisms mediating tonic light effects. While PER1-ir rhythms are in phase in the core and shell regions in animals in the present conditions, we did find high amplitude FOS rhythm in the core in antiphase to that in shell in the LD animals. Restated, in long photoperiod condition, FOS is high in the light phase in the shell, but high in the dark phase in the core (Figure 3). To our knowledge, this is the first report of high FOS expression in the core region in the dark phase of a light-dark cycle in the absence of a light pulse. FOS expression in the SCN of animals has been thoroughly studied under many different photoperiod conditions (Kornhauser *et al.*, 1990; Colwell & Foster, 1992; Vuillez *et al.*, 1996; Sumova *et al.*, 1998; Sumova *et al.*, 2000). In the absence of acute light exposure, FOS expression in the retinorecipient SCN region is typically low in the dark phase (Colwell & Foster, 1992; Vuillez *et al.*, 1996; Sumova *et al.*, 2000). The difference between our results and previous findings may be attributable to species differences (photoperiodic vs nonphotoperiodic species), to differences in experimental conditions (such as light intensity and duration), or to the fact that the retinorecipient area is small and may be missed in studies relying on geographical rather than peptidergic markers. Indeed, the species difference in photoperiodic response of the SCN has been reported in electrical activity Using horizontal brain slice, Jagota *et al.* (2000) reported two-peak activity pattern in the SCN of hamsters in long photoperiods. While Burgoon *et al.* (2004) using same preparation confirmed the two-peak pattern in hamster SCN, but only detected one peak in the SCN of mice and rats housed in same long photoperiods.

Interestingly, the high FOS expression in the dark phase decreased once the animals were transferred from LD to DD or to a skeleton photoperiod. It is well established that in a skeleton photoperiod hamsters entrain as in the preceding long photoperiod (Stephan, 1983; Gaten *et al.*, 2005). Thus, the results suggest that FOS expression is light dependent, and

that the 1 h of light at the transition time is not sufficient to maintain FOS expression in the core. Rather, our results indicate that core FOS expression depends on tonic light or long duration of the antecedent light exposure.

We also explored whether the FOS in the core SCN of LD animals is an acute or gradual response. This is important because in nature, while there are systematic day to day changes in the timing of sunrise or sunset, routine variations in weather conditions, e.g. an evening storm can produce significant variations in dawn or dusk. Thus, animals would need to compute accumulated day-length information over at least several days to determine if the days are getting longer or shorter. In this view, the mechanisms mediating seasonal light effect respond to changes of day-length gradually. In the first experiment, LD animals were housed in DD for 3 days, then were placed back in LD. On the first day back in LD, we detected high FOS signals in the retinorecipient region, equivalent to that in LD group. This result may point to an immediate response to long days, as in the "first-day release" model in which LH and FSH secretion occurs on the first day after release to long photoperiods (Nicholls *et al.*, 1983). Or as observed in Siberian hamsters, a single long-day exposure prevented testicular regression in subsequent short-photoperiod (Finley *et al.*, 1995). This conclusion is constrained however by the fact that the animals had been previously in LD for 8 weeks, and that history may contribute to the acute response to the long day. To further distinguish the acute and gradual response, we transferred the SD animal to LD. We found that lengthening of the day causes activation of cells in the retinorecipient region, but importantly it takes more than 3 days to reach steady-state (Figure 5). This result reveals that the high FOS in the core region observed in LD animals is a gradual response, and supports the idea that the activation of a subgroup of SCN cells mediating changes of day-length.

It is of interest to characterize the cells that show steady state FOS activation in LD. They are predominantly located in the core SCN region, in the CalB-containing region and the "cap" region above it (Antle & Silver, 2005). This core region includes most of the retinorecipient region, and maps particularly well to the area densely innervated by the contralateral retina in hamster (Muscat *et al.*, 2003). CalB cells in the hamster SCN receive retinal input directly (Bryant *et al.*, 2000), and express light-induced FOS or clock genes after a brief light exposure (Silver *et al.*, 1996; Hamada *et al.*, 2001). However, in LD we found very little co-localization of FOS and CalB. In contrast, on the first day from SD to LD, most CalB cells showed FOS-ir, while on the third day only half of the CalB cells co-localized with FOS. Taken together, these results suggest that the activation of CalB cells is linked to acute light response, and the neural activation revealed by FOS-ir in core non-CalB cells are the result of tonic parametric light effects.

The mechanisms by which animals entrain to the light-dark cycle have long been argued (Daan, 2000). One view is that the entrainment is through parametric tonic light effect, while the other view is through non-parametric phasic light effects. Non-parametric light effects have been intensively studied at behavioral (phase-response curve), cellular and molecular levels (Kornhauser *et al.*, 1990; Schwartz & Zimmerman, 1990; Shigeyoshi *et al.*, 1997; Yan *et al.*, 1999). On the other hand, parametric light effects have received less attention. Our results show that there is a unique population of cells responding to tonic parametric photic stimulation; these cells are located in the core SCN, but are non-CalB cells. When the days get longer or shorter, this group of cells is either activated or inactivated. This activation or inactivation will alter the strength of intercellular connections in the SCN network, and subsequently lead to reorganization of the SCN oscillators.

Accurate prediction of seasonal changes in food availability, temperature, weather, or predator activity is crucial for survival of many species. Changes in day-length are one of the best predictors of environmental seasonal change, and are used by organisms to

coordinate the timing of seasonally important changes in physiology and behavior, e.g. reproduction, hibernation and migration (Prendergast *et al.*, 2002). Our results support the idea that the day-length is encoded in the SCN through the reorganization of groups of oscillators, suggest a potential mechanism mediating SCN network reorganization under changing day-length, and point to a subtype of the neurons in the core SCN mediating such re-organization through a parametric effect of light.

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Figure 1.

Representative photomicrographs depicting PER1 expression in different subregions of the SCN of Syrian hamsters under LD (A) and SD (B). Numbers on the top row of the images indicate the time (T, T 0 defined as lights on). Images of CalB labeling were captured from the sections shown for T2, as the sections were double-labeled. The CalB labeling was used to define the core region, and to determine the rostral caudal (R-C) level as well. In LD (A), the PER1-ir cells are absent at T2, first appear in the caudal portion of the SCN at T 6, then spread through the entire rostral-caudal extent at T10 and 14, and finally disappear in the caudal SCN at T18 and 22 despite remaining abundant in the rostral SCN. In SD (B), the

PER1-ir cells are absent at T 2, appear at T 6 throughout the SCN, peak at T 10, then start to decrease at T 14, becoming low or absent from T 18 to 22. Scale bar, 300 μ m.

Figure 2. PER1-ir cells in SCN sub-regions in LD and SD

(A and B) Number of PER1-ir cells in the core and shell regions. In LD (A), the number of PER1-ir cells is high at T 10–18 in both the core and shell of the SCN (post-hoc Tukey test, $p < 0.05$). In SD (B), the number of PER1-ir cells peaks at T 6 and 10 in the shell, and at T 6–14 in the SCN core (post-hoc Tukey test, $p < 0.05$).

(C and D) Number of PER1-ir cells at 5 levels from the rostral to caudal (R-C) SCN. In LD (C), number of PER1-ir cells in the R-C 4 and 5 peak the earliest at T6–14, then in the R-C 3 peaks at T10–14, next in the R-C 2 peaks at 10–18, at last the R-C 1 peaks at T14–22. In SD (D), number of PER1-ir cells is high in three middle levels R-C 2, 3 and 4 at T 6–10, in R-C 1 at T6–18, and in R-C 5 at T6–14.

The results are presented as mean $\pm$ SEM ($n=5$).

Figure 3. Elevated FOS expression in the SCN core during the dark phase of LD compared to SD
 (A) Representative photomicrograph of double-labeled c-FOS (red) and CalB (green) in the SCN of LD (upper panel) and SD (lower panel) hamsters. The number at the upper right corner indicates the time. At T18 and 22, intensely labeled FOS-ir cells are observed in the core SCN in LD, but not in SD animals. Scale bar, 300 μ m.
 (B) Quantitative analysis of FOS-ir in LD (left panel) and SD (right panel). The results are presented as mean $\pm$ SEM (n=4).

Figure 4. FOS expression at T18 of LD is dependent on the parametric light effect

(A, B) Experimental paradigms and representative photomicrographs of double-labeled c-FOS (red) and CalB (green) in the SCN at T18 after the animals were transferred to DD (A) or to skeleton photoperiod (SK, B). Scale bar, 300 μ m.

(C) Quantitative analysis of FOS-ir cells at T18 in the core and shell SCN. The data for LD is re-plotted from figure 2B. The results are presented as mean $\pm$ SEM (n=3 for the LD-DD and LD-SK groups).

Figure 5. FOS expression at T18 of LD is a gradual response to light

(A, B) Experimental paradigms and representative photomicrograph of double-labeled c-FOS (red) and CalB (green) in the SCN at T18. Hamsters were either transferred from LD to DD for 3 d and then back to LD (A), or were transferred from SD to LD (B). Scale bar, 300 μ m.

(C) Quantitative analysis of FOS-ir at T18 in the core and shell SCN. The data for LD is re-plotted from figure 2B. The results are presented as mean $\pm$ SEM (n=3 for each group).

(D) Confocal scan images showing co-localization of FOS and CalB in the core SCN. In LD, few FOS-ir cells are localized with CalB cells. When the hamsters were released from

SD to LD, on the first day, the majority of CalB cells expressed FOS, but on the third day, the FOS positive CalB cells decreased to about half. Scale bar, 100 μ m.