Expression of *Period* Genes: Rhythmic and Nonrhythmic Compartments of the Suprachiasmatic Nucleus Pacemaker

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The mammalian circadian clock lying in the suprachiasmatic nucleus (SCN) controls daily rhythms and synchronizes the organism to its environment. In all organisms studied, circadian timekeeping is cell-autonomous, and rhythmicity is thought to be generated by a feedback loop involving clock proteins that inhibit transcription of their own genes. In the present study, we examined how these cellular properties are organized within the SCN tissue to produce rhythmicity and photic entrainment. The results show that the SCN has two compartments regulating *Period* genes *Per1*, *Per2*, and *Per3* mRNA expression differentially. One compartment shows endogenous rhythmicity in

Per1, *Per2*, and *Per3* mRNA expression. The other compartment does not have rhythmic mRNA expression but has gated light-induced *Per1* and *Per2* and high levels of endogenous nonrhythmic *Per3* mRNA expression. These results reveal the occurrence of differential regulation of clock genes in two distinct SCN regions and suggest a potential mechanism for producing functional differences in distinct SCN subregions.

Key words: suprachiasmatic nuclei; circadian rhythms; clock gene; *Per1*; *Per2*; *Per3* Bma1; calbindin_{D28K}; vasopressin; *Fos*; light pulse; oscillator; pacemaker

Circadian regulation of physiology and behavior has been observed in organisms from cyanobacteria to mammals. The discovery of mammalian homologs of nonmammalian clock genes has focused attention on the conservation of circadian mechanisms in evolution (Dunlap, 1999). Molecular models of circadian oscillators in mammals are based on findings in *Drosophila* and *Neurospora* (for review, see Young 1998; Dunlap 1999). In these models, rhythmicity is produced by a cell-based negative transcriptional feedback loop, in which expression of the putative clock genes is suppressed by their own protein products, and entrainment results from their modification by light-induced signals. Although many clock genes are conserved, there are distinct species differences, as noted in the reviews cited above. Mice have three *Period* genes (*mPer1*, *mPer2*, and *mPer3*), whereas *Drosophila* has one (*dPer*). Although the expression of *dPer* is rhythmic, *dPer* is not acutely responsive to light, as are *mPer1* and *mPer2* mRNAs. These data suggest that certain features of entrainment and oscillation may be unique to mammals.

A model explaining the mammalian circadian system will require understanding the circadian clock in the suprachiasmatic nucleus (SCN) at both the cellular and tissue levels. There is substantial evidence that SCN cells are autonomous oscillators. Individual dispersed SCN cells exhibit a circadian rhythm of electrical activity (Welsh et al., 1995; Liu et al., 1997; Herzog et al., 1998), rhythmically secrete vasopressin (VP; Murakami et al.,

1991; Watanabe et al., 1993), and restore rhythmicity in locomotor activity to SCN-ablated hamsters (Ralph et al., 1990; Silver et al., 1990). These results demonstrate the cell-autonomous nature of the SCN oscillators but cannot account for entrainment. Mechanisms of entrainment in the mammalian SCN have been addressed only at the tissue level. Photic cues are the most important entraining signals, and it is well established that the greatest density of retinal fibers travels to an area of the SCN termed the “ventrolateral” or “core” region and are more sparse in the area called the “dorsomedial” or “shell” region (Leak and Moore, 1996; Miller et al., 1996; Moore, 1996). These reports indicate that SCN cells are neither functionally nor regionally homogeneous.

The present study explores how cell-autonomous oscillators are organized within SCN tissue to produce rhythmicity and photic entrainment. We examine the localization of light-induced and endogenously rhythmic expression of *Per1*, *Per2*, and *Per3* mRNAs using calbindin_{D28K} (CalB), FOS, and VP mRNA or protein as markers. The results show that the regulation of clock genes is not uniform among SCN cells. Expression of rhythmic and light-induced *Per* mRNA is regionally specific. Endogenous rhythmicity in *Per1* and *Per2* mRNA expression was primarily restricted to the VP region of the SCN, whereas light-induced *Per1* and *Per2* mRNA expression occurred primarily in the CalB region, where rhythmic *Per1* and *Per2* mRNA expression was not detectable. Interestingly, *Per3* mRNA was strongly expressed in the CalB region but was not rhythmic in this area. In the VP region, *Per3* mRNA expression showed low-amplitude rhythmicity. These data suggest that two compartments, one rhythmic and the other nonrhythmic, constitute the SCN pacemaker and form the basis of functional differences within the SCN.

Parts of this paper were presented previously at the seventh meeting of the Society for Research in Biological Rhythms, 2000 (Amelia Island, FL).

Received March 20, 2001; revised July 23, 2001; accepted July 23, 2001.

This research was supported by National Institutes of Health Grant NS37919 (R.S.) and a grant from the Japan Society for the Promotion of Science (T.H.). We thank Drs. Paul Hardin, Lance Kriegsfeld, and William J. Schwartz for comments on drafts of this manuscript and Honor Kirwan for technical assistance.

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MATERIALS AND METHODS

Animals and housing. Adult male hamsters (*Mesocricetus auratus*) were given food and water *ad libitum*. The animal colony room was kept on a 12 hr light/dark cycle (LD), with light intensity of 600 lux. The testing rooms were equipped with a white noise generator (91 dB sound pressure level) to mask environmental noise. For animals housed in constant darkness (DD), a dim red light (<1 lux; Delta 1, Dallas, TX) allowed for maintenance.

For studies of zeitgeber time (ZT), animals were maintained in LD for at least 2 weeks before being killed. For studies of circadian time (CT), animals were housed in LD and then placed in DD for at least 1 week before being killed. In this case, hamsters transferred to DD were placed in cages equipped with running wheels (diameter, 16 cm), and locomotor activity was monitored continuously using a computer-based data acquisition system (Dataquest; Data Sciences, St Paul, MN).

All handling of animals was done in accordance with the Institutional Animal Care and Use Committee guidelines of Columbia University.

Free-floating digoxigenin in situ hybridization. Hamster *Per1*, *Per2*, and *Per3* cDNA fragments were PCR-amplified using the following oligonucleotides: 5'-CGAGATGTGTTTCGGGGTG-3' and 5'-AGAGTGGTCAAAGGGCTGC-3' for *Per1*, 5'-TGCCGTGTACGCTTGGA-3' and 5'-CGCTGGATGATGTCTGGCT-3' for *Per2*, and 5'-GAA-GAAGCCAAGCAGAGCC-3' and 5'-GGGAGAGCAGACAACA-GAG-3' for *Per3*. Hamster CalB, VP, and *Bmal1* cDNA fragments were amplified by PCR using the following oligonucleotides: 5'-CTGGAAG-GAAAGGAGCTG-3' and 5'-GTATCCGTTGCCATCCTG-3' for CalB, 5'-AGTGTCTCCCCTGCGGCC-3' and 5'-CAGCTGCGTGGCGTT-GCTC-3' for VP, and 5'-GCAACCGCAAGAGGAAAGG-3' and 5'-AACAGGTGGAGGCGAAGTC-3' for *Bmal1*. These PCR products were then cloned into the pGEM-T Easy vector (Promega, Madison, WI) and sequenced to verify their identity. Cloned partial hamster *Per1* and *Per3* show 93 and 75% homology to mouse *Per1* and *Per3*, respectively. Hamster *Per2* was 90% homologous to rat *Per2*. Hamster *Bmal1*, CalB, and VP were 96, 94, and 90% homologous to rat *Bmal1*, CalB, and VP, respectively. Antisense and sense cRNA probes (digoxigenin-labeled) were generated using the MEGAscript *in vitro* transcription kit (Ambion, Austin, TX). For *in situ* hybridization using digoxigenin (DIG) cRNA probes in free-floating tissue, we examined 50–100 serial sections (20–30 μ m) through the hypothalamic region, including the entire extent of the SCN, for each animal reported here. *In situ* hybridization was performed as described previously (Hamada et al., 1999), except for the use of free-floating sections in the present study. Sections were photographed on Fuji 35 mm film, and color prints were made. For quantification of optical density, images of brain sections were captured using a CCD video camera (Sony XC77) attached to a light microscope (BH-2; Olympus Optical, Tokyo, Japan). mRNA expression was quantified by measuring stain density using the NIH Image program version 1.61.

Measurements of relative optical density (ROD), assessing the mean gray value per pixel of the measured area, were used to quantify the intensity of the signal. Optical density of staining for *Per1*, *Per2*, *Per3*, and *Bmal1* mRNA were assessed in the CalB and VP regions, as defined in alternate sections stained for these peptides. To calculate the ROD, the background intensity of staining was subtracted from the intensity of staining in the SCN region of interest. Background OD for *Per1*, *Per2*, *Per3*, and *Bmal1* mRNA was measured in the lateral hypothalamic area. Background OD for CalB mRNA was measured in the VP area of the SCN. This was done because that CalB immunoreactivity occurs in the extra-SCN hypothalamic region, whereas the VP region of the SCN is devoid of CalB mRNA and protein (Fig. 1*A,B*; Silver et al., 1996).

Immunohistochemistry. Hamsters were heavily anesthetized (pentobarbital, 200 mg/kg), and perfused intracardially with 150 ml of 0.9% saline followed by 300–400 ml of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3. Brains were post-fixed for 18–24 hr at 4°C and cryoprotected in 20% sucrose in 0.1 M phosphate buffer overnight. For immunocytochemistry, sections (20–30 μ m) were processed using the avidin-biotin-immunoperoxidase method (Silver et al., 1996; LeSauter et al., 1999a). The primary antibodies used were rabbit polyclonal FOS (1:5000; Calbiochem, Cambridge, MA) and mouse monoclonal CalB (1:20,000; Sigma, St. Louis, MO.).

Delineation of distinct SCN regions. We used immunoreactivity or *in situ* hybridization for CalB, VP, and light-induced FOS to demarcate distinct regions of the SCN. To examine the localization of *Per1*, *Per2*, *Per3*, CalB, and VP mRNA, alternate free-floating sections were processed for DIG *in situ* hybridization and immunocytochemistry.

Various terms have been used previously to delineate distinct SCN

regions. We use the descriptive terms “CalB region” and “VP region” to demarcate nonoverlapping regions of the hamster SCN (Fig. 1*A*), with explicit recognition that SCN regions delineated by these markers contain other cell types. We concur with the characterization of the rodent SCN as heterogeneous on the basis of the peptidergic content of its neuronal populations and their projections. Further analysis will be required to determine how the SCN regions characterized by their clock gene expression in the present study correspond to those previously defined as ventrolateral and dorsomedial or core and shell in hamster, mouse, and rat (Miller et al., 1996; Moore, 1996; Moore and Silver, 1998; Leak et al., 1999).

RESULTS

Rhythmic expression of *Per1* and *Per2* mRNA occurs outside the calbindin_{D28K} region

By examining alternate sections by *in situ* hybridization and immunocytochemistry, we characterized the regional distribution of *Per1*, *Per2*, and VP mRNA and CalB protein in coronal sections from the rostral through the central (anterior and posterior portions) and caudal quadrants of the SCN. Peak expression of *Per1* mRNA in whole SCN occurs around CT4 and ZT4, whereas *Per2* mRNA in whole SCN peaks at CT8–10 and ZT10–12 in the hamster (Maywood et al., 1999; Horikawa et al., 2000; Moriya et al., 2000; Yokota et al. 2000). Figure 1*A* shows photomicrographs of brain sections harvested at CT4 and stained for CalB taken through the extent of the SCN. As previously reported (Silver et al., 1996), CalB is highly restricted to the central posterior SCN. Photomicrographs of *Per1* mRNA at CT4 and CalB protein in adjacent sections indicate that *Per1* mRNA is expressed throughout most of the SCN but not in the CalB-positive region. This point is highlighted in an enlarged view showing an overlay of *Per1* mRNA and CalB taken from adjacent sections at ZT4 (Fig. 1*B*). There is little or no detectable overlap in the distribution of cells expressing *Per* mRNA and CalB protein (Fig. 1*B*). Photomicrographs from animals killed at ZT4 indicate that the expression and distribution of *Per1* mRNA is similar under both LD and DD. The localization of *Per2* mRNA does not differ from that of *Per1* mRNA under LD (data not shown) and DD (Fig. 1*A*), although the expression level of *Per2* mRNA is much higher.

Expression of VP mRNA was examined at CT8, when it peaks (Jin et al., 1999). Some VP mRNA can be seen in each quadrant of the SCN, but its distribution does not overlap with that of CalB protein (Fig. 1*A*). Comparison of mRNA expression for *Per1* and *Per2* in sections adjacent to those showing VP mRNA at two circadian times indicates that rhythmic *Per1* (Fig. 1, *A* vs *C*) and *Per2* (Fig. 1, *A* vs *D*) mRNAs are expressed in the VP region.

Quantitative analysis (Fig. 2*A*) of the ROD in each quadrant of the SCN outside the CalB area shows marked differences in *Per1* and *Per2* mRNA expression during subjective day and night (Fig. 2*A*). In contrast, within the CalB area, expression of *Per1* and *Per2* mRNA is at low, background levels evident at all circadian times (Fig. 2*B*).

Light-induced and rhythmic *Per1* and *Per2* mRNA are expressed in different SCN compartments

The hamster SCN receives photic input primarily in the CalB region. This is based on both tract-tracing (Moore and Silver, 1998) electron microscopic studies (Bryant et al., 2000) and FOS induction after a light pulse (Hastings et al., 1996; Silver et al., 1996). The present results show that rhythmic *Per1* and *Per2* mRNAs are not detectable in this region (Fig. 1*A,B*). In the mouse, however, *Per1* and *Per2* are reported to have an important

Figure 1. Photomicrographs depicting the localization of endogenously rhythmic (*A*, *B*) and light-induced (*C–F*) expression of *Per1*, *Per2*, and *Per3* mRNA with respect to other known markers of the SCN, namely, CalB, VP, and FOS. Asterisks denote adjacent sections. *A*, The columns show coronal sections of each SCN quadrant from rostral to caudal from the same animal. At circadian times when *Per1* and *Per2* mRNA expression peaks (CT4, CT8, respectively), both signals are localized to the VP region of the SCN, and there is little expression (background level) in the CalB region of the central SCN, posterior aspect. This is highlighted in higher-power photomicrographs (*B*) in which the image of the *Per1* mRNA at ZT4 is captured in Adobe Photoshop, converted to a red signal, and superimposed in an overlay on the image of the adjacent section immunoreacted for CalB. The last two columns of *A* show *Per3* mRNA at two circadian times, CT5 and CT20.5. Comparison of expression in each SCN quadrant at these times reveals weak *Per3* rhythmic expression outside the CalB region. In contrast to *Per1*, *Per2*, and VP mRNA, *Per3* mRNA is strongly expressed in the CalB region at both CT5 and CT20.5 but is not rhythmic. *C*, *D*, Comparison of responses in the presence and absence of light [*Light*(+) vs *Light*(–)] indicates that photic input induces *Per1* (*C*) and *Per2* (*D*) mRNA expression in the CalB region. The light-induced response can be seen during subjective night (CT20.5, *Per1*; CT21, *Per2*) but not during subjective day (CT5.5, *Per1*; CT10, *Per2*). Note that light-induced *Per1* and *Per2* mRNA and rhythmic *Per1* and *Per2* mRNA are expressed in different SCN compartments. *E*, Data shown in *C* and *D* highlighted in a high-power photomicrograph and overlay in which the image of the *Per2* mRNA is captured in Adobe Photoshop, converted to a red signal, and superimposed in an overlay on the image of the adjacent section immunoreacted for CalB protein. The time of killing is given at the top of each column. *Light*(+), Presentation of a light pulse (600 lux for 30 min); *light*(–), no light pulse. Each column shows sections from two central SCN quadrants [anterior (*Ant*) and posterior (*Post*)] of the same animal. *F*, Photomicrographs showing the localization of light-induced FOS, *Per3*, and CalB mRNA in the central anterior and posterior aspects of the SCN. It is clear that light induces FOS in the CalB region of the SCN. Comparison of *Per3* at CT5 in *A* and at CT20.5 in *F* indicates that light does not affect the expression of either *Per3* or CalB mRNA.

role in light-induced phase shifts (Shigeyoshi et al., 1997; Akiyama et al., 1999; Wakamatsu et al., 2001), and *Per1* is induced by light in the ventromedial SCN (Shigeyoshi et al., 1997). We therefore examined the regional distribution of light-induced *Per1* and *Per2* mRNAs. Hamsters were exposed to a 30 min light pulse at suitable circadian times and killed 1.5–2 hr later. Control animals were not exposed to light but were otherwise treated identically. Photomicrographs through the two central quadrants of the SCN reveal that a light pulse during the subjective day did not induce *Per1* or *Per2* mRNA expression (Fig.

1*C,D*). Control animals not exposed to a light pulse are shown in Figure 1*A*. In contrast, during late subjective night, there was strong light induction of *Per1* and *Per2* mRNA, highly concentrated in the CalB region. Further evidence of the overlap in their distribution is shown in adjacent sections of *Per2* mRNA and CalB protein at high power (Fig. 1*E*). This is different from the area that expresses endogenously rhythmic *Per1* and *Per2* mRNAs. Quantification of the results shows significant light-induced *Per1* and *Per2* mRNA during subjective night but not during subjective day in the CalB region (Fig. 2*B*).

Figure 1. Continued

***Per3* mRNA expression is high but is neither rhythmic nor affected by a light pulse in the calbindin_{D28K} region**

The pattern of *Per3* mRNA expression differs substantially from that of *Per1*, *Per2*, and VP mRNA (Figs. 1A, 2C,D). Endogenous *Per3* mRNA is expressed in both the VP and CalB regions (Fig. 1A). There is circadian expression of *Per3* mRNA in the rostral and central VP region of the SCN (CT4–5 vs CT20.5–22). The amplitude of the *Per3* mRNA rhythm is less than that of *Per1* and *Per2* mRNA in this area. In contrast, there is no detectable circadian expression of *Per3* mRNA within the CalB region, where rhythmic *Per1*, *Per2*, and VP mRNA expression is also not detectable (Fig. 1A,B). *Per3* mRNA expression in the CalB region

is constant and high (Fig. 2C). Notably, this is the region where light induces *Per1* and *Per2* mRNA (Fig. 1C–E). *Per3* mRNA, however, is not detectably induced by light during subjective night in any part of the central SCN (Figs. 1F, 2D). Quantification confirms the weak but significant ($p < 0.05$, Student's t test) rhythmic expression of *Per3* mRNA in the VP region (Fig. 2D).

***Bmal1* mRNA expression occurs outside the calbindin_{D28K} region**

To further define the regulatory mechanisms that underlie the differential regulation of the *Per* genes in the two SCN regions, we used the expression of the clock gene *Bmal1* as a marker to study

Figure 2. Quantification of the expression of *Per* genes in distinct SCN regions for each quadrant in the presence (+) and absence (–) of light. Values shown are mean $\pm$ SEM; $n = 3$ –4 hamsters per time point. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, Student's *t* test). **A**, In the VP area, ROD measurements of *Per1* and *Per2* mRNA at times of peak and trough expression reveal rhythmicity in each SCN quadrant. **B**, In the CalB region, comparison of circadian day and night for *Per1* and *Per2* mRNA in the absence of light indicates no detectable change in either mRNA. On the other hand, marked light-induced *Per1* and *Per2* mRNA occurs in the CalB region of the SCN. **C**, ROD measurements for *Per3* mRNA in each SCN region indicate differences between subjective day and night in the VP region of the rostral and central SCN. Note that *Per3* mRNA is strongly expressed but is not rhythmic in the CalB region of the central posterior SCN in both subjective day and night. **D**, Expression of *Per3* and CalB mRNA is not affected by a light pulse given during the subjective night. Ant, Anterior; Post, posterior.

its relation to light-induced FOS protein expression with the following rationale: Most light-induced expression of FOS is concentrated in the CalB area rather than in the VP region of the SCN (Fig. 1*F*; Hastings et al., 1996; Silver et al., 1996). BMAL1 and CLOCK are important regulators of rhythmic VP and *Per1* gene expression in the SCN (Jin et al., 1999). To determine whether there is differential expression of the regulatory gene *Bmal1* in distinct SCN regions, we compared *Bmal1*, *Per3*, and CalB mRNA expression in each SCN quadrant. Results indicate that at its peak, the *Bmal1* mRNA signal is strong in the VP region in the rostral and central anterior SCN, but it is very weakly expressed in the central posterior CalB region (Fig. 3). In contrast, *Per3* mRNA is very highly expressed in the CalB region at CT16. Light-induced FOS also occurs here (Fig. 1*F*).

DISCUSSION

The identification and analysis of clock genes have been very successful in demonstrating how a cellular oscillator can be built both mechanistically (Young, 1998; Dunlap, 1999; King and Takahashi, 2000) and formally (Goldbeter, 1995). In mammals, the mechanism of cellular oscillation is likely to be similar to the *Drosophila* model, although some components of the oscillator are different (Shearman et al., 2000b). The *Drosophila* model, however, does not speak to the mechanism of photic entrainment in mammals. The present analysis uses the information derived from molecular events to understand SCN organization at the tissue level. The results indicate that understanding mechanisms of entrainment in mammals will require knowledge of SCN circuitry. The present studies of cellular organization of the SCN reveal novel aspects of the mammalian circadian clock organization that set the parameters for further studies of mechanisms at the level of the cell, the tissue, and the organism as a whole.

First, cells in one subregion of the hamster SCN do not oscillate with respect to *Per* production. The relationship between

nonoscillating and oscillating cells is shown schematically in Figure 4. The commonly used tripartite organization of the SCN, with an input, clock, and output, is retained. The key feature is the segregation of the SCN clock into two distinct elements, shown as a small nonrhythmic region and a larger rhythmic region expressing the clock genes. It is noteworthy that cells in the nonrhythmic CalB region are easily missed, because they form a small proportion of the SCN population. In the rat, each SCN has ~8000–10,000 cells (van den Pol, 1980); assuming that the hamster SCN has a comparable number of cells, the CalB subregion constitutes no more than 10% of the nucleus (Silver et al., 1996). In the hamster the CalB region is restricted to the posterior aspect of the central region of the nucleus (Fig. 1). In other species, cells with comparable properties may be more dispersed among other SCN neurons and difficult to localize.

As shown in the model, clock gene oscillation is a property of some but not all SCN cells. One SCN region, marked by VP mRNA in this study, shows rhythmic expression of *Per1*, *Per2*, and *Per3* mRNA. It has been reported that spontaneous expression of Jun-B and FOS occur in the dorsomedial region of hamster SCN (Guido et al., 1999). The model also shows that the other SCN region, marked by CalB cells, has time-gated light-induced *Per1* and *Per2* mRNA and FOS protein and constant high expression of *Per3* mRNA. The key concept emerging from the present results is this separation of the nonrhythmic and rhythmic compartments of the SCN expressing clock genes, along with the demonstration that responses of nonrhythmic cells are themselves gated by the circadian system (Fig. 4*A,B*). Photic information from the retina reaches the CalB region of the SCN directly (Bryant et al., 2000). This SCN compartment is not rhythmic in *Per* mRNA (Fig. 2), CalB mRNA (data not shown), or CalB protein expression (LeSauter et al., 1999a). At appropriate times of day, in the presence of a light pulse, photic input induces *Per1* and *Per2* mRNA (Fig.

Figure 3. Expression of *Bmal1* and *Per3* mRNA occur in different regions. Photomicrographs show the localization of *Bmal1* mRNA with respect to *Per3* and *CalB* mRNA in each SCN quadrant of the SCN. (For *CT16*, each row shows serial sections from the same animal.) Expression of *Bmal1* mRNA is stronger outside the *CalB* region of the SCN, whereas *Per3* mRNA expression is high within this region. Comparison between *CT4* and *CT16* shows that *Bmal1* mRNA is expressed rhythmically outside the *CalB* region. *Ant*, Anterior; *Post*, posterior.

4*B*). These results are consistent with many reports in hamsters of light-induced responses concentrated in the *CalB* region, including induction of *FOS* (Silver et al., 1996; Fig. 3*D*), *vgl* (Wisor and Takahashi, 1997), and *egr-3* (Morris et al., 1998). An important question is how (and whether) phase-setting information, reflected in high expression of light-induced *Per1* and *Per2*, travels from the *CalB* compartment to other SCN regions. One possibility is that the *VP* region itself receives direct retinal input. Tract-tracing studies, which label retinofugal pathways, indicate that retinal fibers occupy a large region of the SCN, although they are most dense in the *CalB* (i.e., ventrolateral or core) region (Pickard and Silverman 1981; Johnson et al., 1988; Youngstrom et al., 1991; Miller et al., 1996; Silver et al., 1996). Synaptic connections made by retinal fibers in the hamster have been documented only for *CalB* (Bryant et al., 2000) and gastrin-related peptide, which is located in the *CalB* region (Aioun et al., 1998). It seems unlikely that photic input directly resets rhythmic *Per1* and *Per2* expression in the *VP* region, given the sparse expression of these mRNAs after a light pulse (Fig. 1*C,D*). The relatively sparse induction of *FOS* in this region after photic input also argues against this possibility (Kornhauser et al., 1990; Rea, 1992; but see Guido et al., 1999). The alternative possibility is that *Per1* and *Per2* mRNA expression in the *VP* region are produced indirectly, via information relayed from the *CalB* region. It has been shown in anatomical studies of rats that information travels selectively within the SCN, from the retinorecipient to the non-retinorecipient regions (Moore 1996; Leak et al., 1999), even though available evidence on intra-SCN circuitry suggests that SCN neurons make hundreds of intranuclear synapses and appositions in rats and hamsters (Guldner 1976; van den Pol and Gorcs, 1986; Daikoku et al., 1992; Romijn et al., 1997; Jacomy et

Figure 4. Model of rhythmicity and photic entrainment at the tissue level, comprising two functionally different SCN compartments. As is widely accepted, the circadian system has three primary components: input, SCN, and output. *A*, Model of rhythmicity. In both *Light:dark* and *Constant dark* conditions, there are two distinct regions of the SCN showing the same pattern of *Per* gene expression. In the hamster SCN, these regions can readily be delineated by cells containing *CalB* and *VP* respectively, as indicated in Materials and Methods. These two compartments constitute the SCN pacemaker. In the *CalB* region, rhythmic expression of *Per1* and *Per2* mRNA are not detectable (*SCN*, left). Another characteristic of this area is that *CalB* and *Per3* mRNA are highly expressed but not rhythmic. Endogenous rhythmic expression of clock genes (*Per1*, *Per2*, *Per3*, and *Bmal1* mRNA) and *VP* mRNA occurs in a large number of SCN cells restricted to the *VP* region (*SCN*, right). *B*, Model of photic entrainment. Gated light induced *Per1* and *Per2* mRNA and *FOS* expression. A light pulse during the day (*top*) has no effect on the expression of *Per1* and *Per2* mRNA or on *FOS*. A light pulse during the night (*bottom*) induces *Per1* and *Per2* mRNA and *FOS* expression in the *CalB* region. Importantly, some genes in this region are not induced by light pulse during the night (*Per3*, *CalB*). See Discussion for the proposed mechanism of entrainment. *Ant*, Anterior; *Post*, posterior.

al., 1999; LeSauter et al., 1999b). In support of this hypothesis, in SCN cell culture, vasoactive intestinal polypeptide (VIP) induces phase shifts of VP secretion in a light-like phase shift (Watanabe et al., 2000). Importantly, gastrin-related peptide (Moore and Silver, 1998) strongly induces a light pulse-type phase shift in hamster locomotor activity (Piggins et al., 1995).

The absence of rhythmic expression of clock genes in the CalB region begs the question of why lesions of the CalB region, which spare other compartments of the SCN, result in loss of circadian rhythms in hamsters (LeSauter and Silver, 1999). One possibility is that there is a population of pacemaker cells, yet to be identified, lying near but not in the CalB region, with rhythmic *Per* expression. An alternative possibility is that in the absence of the cell group with gated expression of clock genes, oscillators in extra-SCN brain areas drift out of synchrony. One puzzling feature of SCN organization is the fact that efferents from the VP region and CalB regions both reach common target areas in the hamster brain (LeSauter et al., 1999b). Although the function of these overlapping pathways remains to be examined directly, such results indicate how phase information might travel.

Another feature suggested by our data, that the regulation of clock genes is not uniform among SCN cells, is counter to current models of circadian regulation of *Per* genes (Dunlap 1999; Shearman et al., 2000b). In rat and mouse, the *Per1* promoter contains both an E-box (CACGTG) and a cAMP response element (CRE; Hida et al., 2000; Yamaguchi et al., 2000). VP also has an E-box and CRE sequence in its promoter, and circadian expression of VP is thought to be controlled by BMAL1 and CLOCK protein binding to the E-box (Jin et al., 1999). *Bmal1* has an important role in activating E-box-dependent rhythmic *Per1* and VP mRNA expression. Although the underlying mechanism is not yet known, rhythmicity in FOS and in CRE-mediated gene expression (using CRE- β -galactosidase transgenic mice) has also been reported (Obrietan et al., 1999; Schwartz et al., 2000). The present studies show that the VP area is rich in *Bmal1* mRNA (Fig. 3), suggesting that the circadian expression of *Per1*, *Per2*, *Per3*, and VP mRNA in this region occurs through the activation of E-boxes. In contrast, in the CalB region, where the expression of BMAL1 is low, *Per1* and *Per2* mRNA and FOS protein are strongly light-induced. This suggests that *Per1* and *Per2* mRNA regulation in this region may be achieved through a light response element such as the CRE. In further support of the notion that light induction involves the CRE, expression of phosphorylated CRE binding protein, a positive regulator of the *c-fos* gene, also occurs in the region where light induces FOS protein (Schurov et al., 1999). Last, *Per3* mRNA is strongly expressed in the CalB region but is not induced by light, suggesting that *Per3* regulation in this region is achieved by a distinct transcriptional system from that of *Per1* and *Per2*. These results suggest that cells expressing rhythmic *Per1*, *Per2*, *Clock*, and *Bmal1* mRNA in the VP region of the SCN are candidates for principal oscillator cells.

Many previous reports indicate that the SCN may have functionally distinct regions. In the rat and hamster, a substantial population of VP-containing cells lies in the dorsomedial SCN, whereas VIP is located mainly in the ventrolateral SCN (Card and Moore, 1984; Inouye and Shibata, 1994; Miller et al., 1996; Moore, 1996; Moore and Silver, 1998). The ventrolateral region of the rat SCN has a dense region of CalB expression (Arvanitogiannis et al., 2000), although these are not as densely packed as in the hamster. The VP content of the SCN shows circadian rhythmicity in both LD and DD and is not affected by a light pulse (Inouye and Shibata, 1994). In contrast, the VIP content of the

SCN has diurnal variation in LD but not in DD *in vivo*. Light-induced *fos* family genes are concentrated in the ventrolateral region, whereas circadian rhythms of *fos* occur primarily in the dorsomedial subdivision of the rat and hamster SCN (Chambille et al., 1993; Guido et al., 1999; Schwartz et al., 2000). In support of the notion that functional retinal input is highly localized to part of the SCN, electrical stimulation of the optic nerve evokes fast positive and late large negative waves in the ventrolateral but not the dorsolateral SCN in a horizontal slice preparation of the rat SCN (Shibata et al., 1984). These reports suggest that the SCN has two functionally distinct regions, wherein one receives light information and the other does not. Importantly, daily rhythms driven by the LD cycle, such as SCN VIP content, do not involve an E-box-dependent negative feedback loop. This is consistent with the present results showing that there is no detectable rhythm in expression of *Per1* and *Per2* mRNA in the CalB region. Furthermore, in this region, *Per1* and *Per2* mRNA expression are the same in both LD and DD (Figs. 1A,B, 4A). In support of this idea, individual SCN neurons in homozygous *Clock* mutant mice are arrhythmic in electrical activity, paralleling the effects on locomotor activity in these animals (Herzog et al., 1998) and suggesting a role for E-box-dependent mechanisms in SCN pacemaker function. The occurrence of regional differences in E-box-dependent negative feedback loops of clock genes and their products, shown in the present study, seems to play an important role in regional differences within the SCN.

Our results also explain some of the behavioral phenotypes of various circadian mutants. *Per2* mutant mice, characterized by a deletion mutation in the PAS domain of the *Per2* gene, show arrhythmic responses in constant darkness (Zheng et al., 1999). This is consistent with our results showing that rhythmicity in *Per2* occurs in SCN pacemakers (located in the VP region). Our results suggest that regulation of light-induced clock genes is distinct from that of endogenously occurring clock gene expression in the VP region. In this context, it is interesting that *Clock* mutant mice (Vitaterna et al., 1994) and *Mop3* (also known as *Bmal1*) knock-out mice (Bunger et al., 2000) entrain but show disrupted circadian rhythms of locomotor activity in DD. As might be predicted from the present results, *Clock* mutant mice express light-induced *Per1* and *Per2* mRNA (Shearman and Weaver, 1999). *Bmal1* and *Clock* are both important for the regulation of rhythmicity (King and Takahashi, 2000). On the other hand, very low expression of CLOCK protein (van Esseveldt et al., 2000) and *Bmal1* mRNA (Fig. 4) in the CalB region bring into question the role of these genes in light-induced *Per1* and *Per2* mRNA expression. Finally, mice with targeted disruption of the *mPer3* gene had normal activity rhythms, with a slightly shorter free-running period than wild-type animals (Shearman et al., 2000a), consistent with our finding of low *Per3* mRNA expression in the rhythmic compartment.

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