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Circadian Trafficking of Calbindin-ir in Fibers of SCN Neurons

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Abstract Calbindin-D28K (CalB)–containing cells form a distinct cluster within the core of the hamster suprachiasmatic nucleus (SCN). These cells are directly retinorecipient but lack detectable rhythms in clock gene expression or electrical activity. In studies exploring SCN connectivity using double-label immunochemistry, we previously reported an absence of contacts among CalB fibers and vasopressin (VP) cells in animals sacrificed during the day. Here, we explored circadian variations in CalB-immunoreactivity (-ir) and re-examined the connections between CalB and other SCN cell types at zeitgeber times (ZT) 4 and 14. The results reveal a circadian rhythm of CalB-ir in fibers of SCN cells with high expression during the night and subjective night and low expression during the day and subjective day. This circadian difference is not seen in the other brain regions studied. Significantly more appositions were detected between CalB fibers and VP cells during the night than during the day, while circadian variation in numbers of contacts was not seen between CalB fibers and vasoactive intestinal polypeptide (VIP), cholecystokinin (CCK), or gastrin-releasing peptide (GRP) cells. There was no detectable variation in appositions from any peptidergic fiber type onto CalB cells. The present findings suggest that CalB cells relay photic information to VP oscillator cells of the SCN shell in a temporally gated manner.

Key words suprachiasmatic, calbindin-D28K, hamster

Calbindin-D28K (CalB)–containing cells are localized to the core region of the suprachiasmatic nuclei (SCN) of hamsters. These cells are c-FOS positive in response to a light pulse (Silver et al., 1996), and double-label electron-immunochemistry studies indicate that they receive direct retinal input via the retinohypothalamic tract (Bryant et al., 2000). In previous work, we suggested that the SCN is comprised of 2 fundamentally different cell types with regard to circadian rhythmicity. One population, including CalB-containing cells of the hamster SCN, expresses *Per1* and *Per2* mRNA and their proteins in response to a light pulse but lacks a detectable rhythm in endogenous *Per1* and *Per2* mRNA expression or in electrical activity (Hamada et al., 2001; Jobst and Allen, 2002). The second population oscillates on a circadian basis with respect to clock genes and electrical activity and is not directly retinorecipient. The latter also differs from CalB-containing cells in that they do not express *Per* genes immediately after a light pulse (Hamada et al., 2001; Jobst and Allen, 2002). A gate-oscillator model explores how this 2-compartment SCN can

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sustain rhythmicity (Antle et al., 2003; Antle et al., 2007). In this model, non-oscillating gate cells provide a signal that maintains coherence among a population of independent oscillators, which themselves are reset by signals from gate cells. Feedback is required to control the state of the gate.

To understand the circuit organization of the SCN, it is necessary to determine the relationship between oscillators and directly retinorecipient cells. Because CalB cells are on the input pathway from the retina to SCN oscillators, we were surprised at the absence of appositions between CalB and vasopressin (VP) cells (LeSauter et al., 2002). In the present study, we took into account the fact that we had previously examined efferent connections at only one time of day, zeitgeber time (ZT) 2 to 8 (LeSauter et al., 2002). In view of evidence of a circadian rhythm in the localization of CalB protein within the soma of SCN neurons (Hamada et al., 2003), we sought here to explore the distribution of CalB in projections of these cells. The results indicate a diurnal and a circadian rhythm of CalB-immunoreactivity (-ir) within the fibers of CalB-containing SCN cells and also reveal numerous appositions between CalB fibers and VP cells at times when fibers contain high levels of CalB.

MATERIALS AND METHODS

Animals and Housing

Adult male LVG hamsters (*Mesocricetus auratus*) were purchased from Charles River Laboratories (Wilmington, MA) or from Kyudo Company (Tosu, Japan) at age 4 to 5 weeks. Circadian changes in fiber-ir and diurnal changes in appositions were analyzed at Columbia University. Diurnal changes in fiber-ir were analyzed at Kyushu University. Animals were housed in translucent propylene cages (48 × 27 × 20 cm) and provided with ad libitum access to food and water. The rooms were kept at 22 ± 1 °C. Animals were either kept in a 12:12 light-dark (LD) cycle or transferred to constant darkness (DD) for 7 to 8 days. A dim red light that generates less than 1 lux (Delta 1, Dallas, TX) allowed for animal maintenance. The room was equipped with a white noise generator (91 dB spl) to mask environmental noise. Animals housed in DD were sacrificed at 4-h intervals, and those housed in LD cycle were sacrificed at ZT4 or 14. All handling of animals was done in accordance with Institutional Animal Care and Use Committee guidelines of Columbia University and by the Committee of Animal Care of Kyushu University.

Immunocytochemistry

Hamsters were heavily anesthetized (pentobarbital: 200 mg/kg) and perfused intracardially with 150 mL 0.9% saline followed by 300 to 400 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3). Brains were postfixed for 18 to 24 h at 4 ºC and cryoprotected in 20% sucrose in 0.1 M phosphate buffer overnight. For quantification of CalB fibers, serial 20-µm coronal sections from animals in DD (N = 4-5 per time point) or in LD (N = 4 per time point) were cut and collected in individual wells. To control for possible variations in immunochemical runs, sections from animals at each experimental time point were processed simultaneously. The sections were processed with mouse monoclonal anti-CalB (1:20,000; Sigma, St. Louis, MO); using a modified avidin-biotin-immunoperoxidase technique, the chromogen used was either DAB or the SG substrate (Vector, Burlingame, CA). The sections were coverslipped with Permount. For fiber distribution, serial 40-µm coronal and sagittal sections from ZT14 animals (N = 4) were stained for CalB (1:20,000) using DAB. For appositions onto other cells, serial 50-µm sections from ZT4 (N = 9) and ZT14 (N = 15) animals were stained for double-label fluorescence for CalB (1:20,000) and VP (1:5000, made in guinea pig; Peninsula Laboratories, San Carlos, CA), vasoactive intestinal polypeptide (VIP) (1:5000, made in guinea pig; Peninsula), gastrin-releasing peptide (GRP) (1:2000, made in rabbit; DiaSorin, Stillwater, MN), or cholecystokinin (CCK) (1:3000, made in rabbit; DiaSorin). Sections were coverslipped with Krystalon (EMD Chemicals, Gibbstown, NJ) and coverglass Nº 1 1/2.

Quantification of CalB Fibers

CalB fibers were observed under a light microscope (Olympus BH-2, Tokyo, Japan) by 2 independent investigators blind to the experimental conditions**.** In the SCN, fiber staining was assessed in the Columbia University laboratory by counting fibers crossing the lines of 32 squares (8 × 4) on a 180 × 90-µm grid. The grid was placed on the SCN region dorsal to the CalB subnucleus in 4 adjacent sections (8 SCN per animal). (Similarly, the Kyushu University laboratory used a 32 square grid [8 × 4 squares] measuring 200 × 100 µm.) We also determined whether differences in CalB-ir occurred in other brain regions at CT4 and CT14. In

the paraventricular nucleus (PVN) and in the amygdaloid nucleus, CalB-ir fiber staining was quantified by relative optical density (ROD), measured as optical density of staining within the region of CalB fiber-ir minus optical density of the background. The area to be measured was determined using the free-hand tracing tool in NIH image. Care was taken not to include cell bodies in the area analyzed. The staining for each animal was expressed as the average ROD in 2 brain sections for each nucleus. The time of day effects were analyzed by ANOVA followed by the Fisher protected least significant difference or by an unpaired *t*-test.

Tracing of CalB Fibers

Images were captured using a Nikon Eclipse 800 (Tokyo, Japan) epifluorescent microscope and a cooled CCD digital camera using Spot software (Diagnostic Instruments, Sterling Heights, MI). Images were loaded into Photoshop (Adobe, San Jose, CA), and tracings of fibers were made of the ZT14 DAB-stained sections.

Appositions

Double-labeled sections were observed under a confocal microscope (Zeiss Axiovert 100TV, Carl Zeiss, Thornwood, NY) with an argon-krypton LSM 510 laser (Carl Zeiss). The images were collected with a 63x Zeiss C-apochromat water immersion objective and digital image resolution of 1024 × 1024, as 1-µm multitract optical sections, with sequential excitation by each laser to avoid cross-talk between the two wavelengths. Red and green images were superimposed using LSM 3.95 software (Zeiss). Each cell was examined through its entirety in 1-µm steps to estimate the number of axosomatic and/or dendrosomatic appositions. Appositions among fibers were not evaluated. Each yellow profile (corresponding to signal from both red and green fluorescent dyes) was counted as one apposition regardless of its depth and area. Caveats to the present methods include the following: 1) dendrites and axons cannot be differentiated, 2) synapses cannot be evaluated and 3) overestimation or underestimation of the number of appositions due to immunocytochemistry procedure, or 4) fibers may be cut during tissue preparation, leading to underestimation of appositions. These caveats do not, however, affect comparison between time points, germane to the present study.

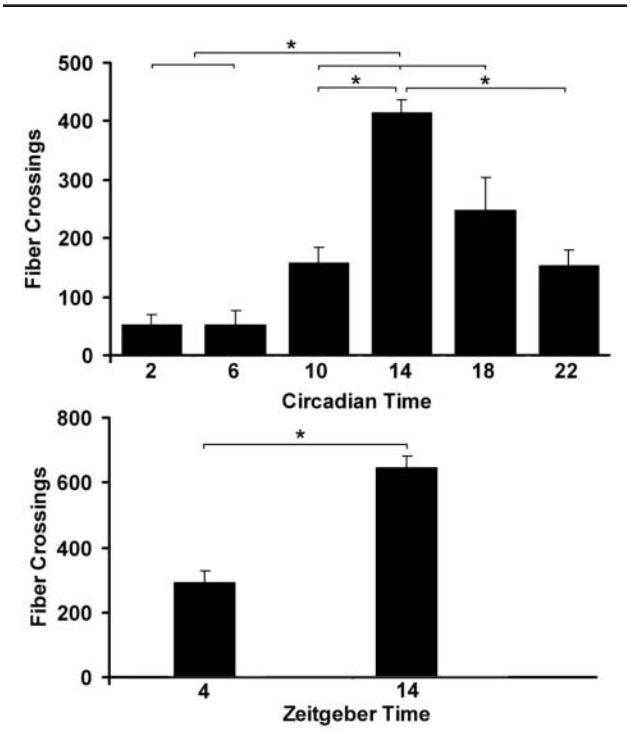

Figure 1. Bar graphs describe the circadian rhythm in the number of grid crossings of CalB-positive fibers in the SCN region dorsal to the CalB subnucleus in animals maintained either in constant darkness (Upper panel: *$p < 0.0001$) or in a light-dark cycle (Lower panel: *$p < 0.005$).

RESULTS

Circadian Fluctuations in CalB-ir

Quantification of SCN fibers indicates a circadian rhythm in immunoreactivity with few fibers detected in the first half of subjective day and an elevation at the beginning of subjective night ($F_{5\text{-}17} = 19.8$; $p < 0.0001$) (Fig. 1A). Similar changes were seen in the SCN of animals housed in LD, with more fibers at ZT14 than ZT4 ($t(6) = -4.42$; $p < 0.005$) (Fig. 1B). In the PVN and amygdaloid nucleus, no differences in fiber staining were seen between CT4 and CT14 ($t(7) = 0.006$; $p = 0.99$ and $t(7) = 0.34$; $p = 0.74$, respectively).

Fiber Distribution

Representative photomicrographs of coronal sections stained for CalB show that more CalB fibers are detected at CT14 than at CT4 (Fig. 2A and B, respectively). Schematics of rostral to caudal coronal

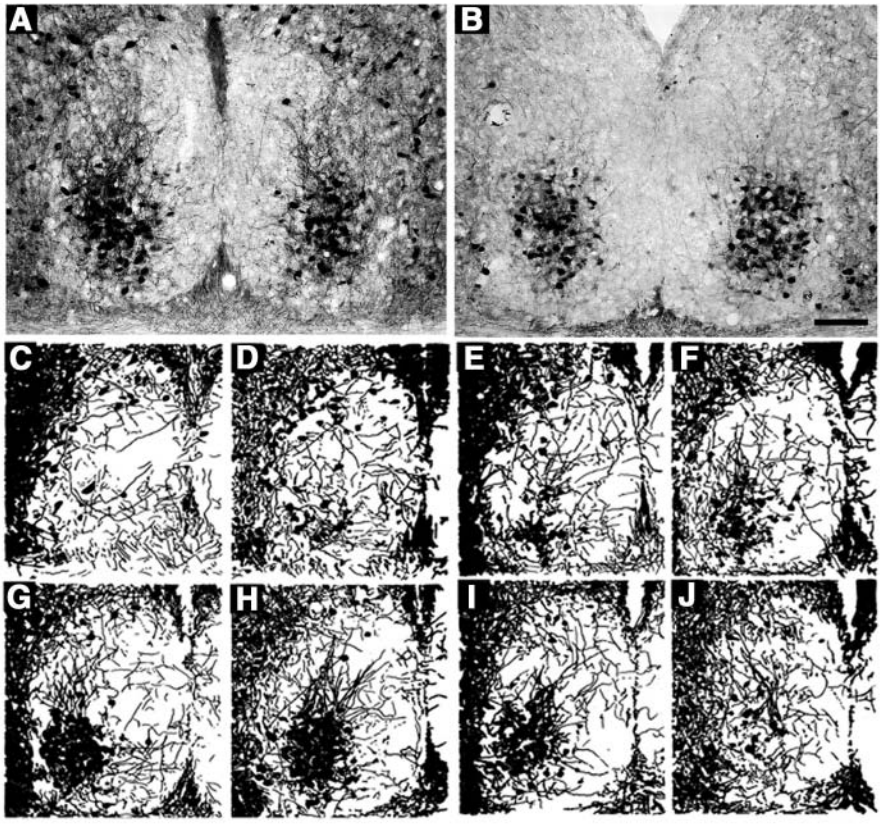

Figure 2. Photomicrographs show the difference between (A) subjective night at CT14 and (B) subjective day at CT4 in detectable CalB-ir fibers in coronal SCN slices (20 µm). Exposure time was set identically for both images but overexposed to optimize visualization of fibers. A dense fiber plexus can be seen extending dorsally from the CalB-containing cells at CT14. Scale bar: 100 µm. (C-J) Tracings of CalB-positive neurons showing the distribution of immunoreactivity in rostral to caudal coronal sections at ZT14.

sections show the distribution of CalB-containing neurons and their fibers at ZT14 (Fig. 2C-J). The majority of fibers emanating from the CalB subnucleus extend dorsally within the SCN (Fig. 2D-I). Some fibers course toward the dorsomedial region or the ventral SCN (Fig. 2G and H). A few fibers are detected within the medial SCN (Fig. 2E-J), where some are seen crossing the midline (Fig. 2D-F). Few CalB fibers are seen in the rostral-most aspect of the SCN (not shown).

In sagittal sections, more fibers are seen extending further from the CalB subnucleus at ZT14 than at ZT4 (Fig. 3A and B, respectively). In schematics of lateral to medial sections (Fig. 3C-F), few fibers are detected in the most lateral and most medial aspects of the SCN (Fig. 3C and F). At the level of the mid-SCN where CalB cells are densest, most fibers are seen coursing dorsally and dorsorostrally within the shell (Fig. 3E).

Appositions among SCN Cell Types

We had previously studied contacts of CalB fibers onto other SCN cell types at ZT2 to 8 and found few appositions with VP cells (LeSauter et al., 2002). In view of the present observations of a circadian rhythm in CalB localization in fibers of SCN neurons, we re-examined contacts among SCN neurons of various peptidergic phenotypes at ZT4 and 14 (Table 1 and Fig. 4).

We counted appositions by evaluating separately those with 1 to 2 appositions, which might be false positives, and those with more than 3 appositions, which were unlikely to occur by chance, as has been done previously (LeSauter et al., 2002). The quantitative analysis of CalB fiber appositions on SCN somata indicates that the number of cells bearing ≥3 CalB-positive appositions is greater at ZT14 than at ZT4 and differs among cell types (2-way ANOVA: time [df 1; $F = 10.7$; $p = 0.004$] × peptide [df 3; $F = 5.2$; $p = 0.01$]). This time of day effect is significant for appositions onto VP cells (Tukey test, $p = 0.003$) but not onto VIP, CCK, and GRP cells. Similar to the previous report (LeSauter et al., 2002), CalB fibers make 1 to 2 appositions with few VP cells.

The quantitative analysis of ≥3 appositions from VP, VIP, CCK, or GRP fibers onto CalB somata indicates no effect of time but a difference between the different cell types (2-way ANOVA: time [df 1; $F = 0.02$; ns] × peptide [df 3; $F = 59.9$; $p < 0.001$]). The VIP fibers appose more CalB cells than do VP, CCK, or GRP fibers at both time points ($p < 0.008$), and GRP fibers contact more CalB cells than do VP or CCK fibers at both times ($p < 0.008$). The VP and CCK fibers contact a similar number of CalB cells. In summary, there is dense innervation of the CalB subnucleus by VIP fibers, somewhat less dense GRP innervation, and sparse VP and CCK innervation (Table 1).

The top row of Figure 4 shows photomicrographs of sections (50 µm) double-labeled for CalB (red) and VP, VIP, CCK, or GRP (green), taken at the level of the

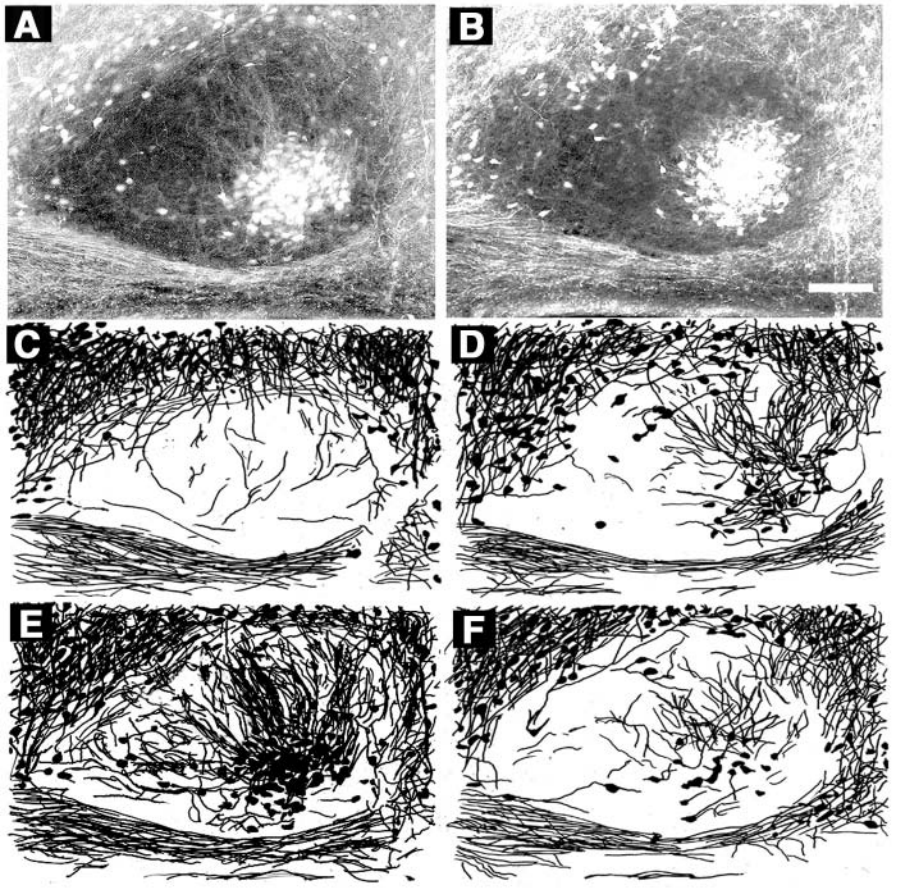

Figure 3. Photomicrographs show the difference between (A) subjective night at CT14 and (B) subjective day at CT4 in detectable CalB-ir fibers in sagittal SCN slices (40 µm). Exposure time was set in the same manner as in Figure 2. As in the coronal sections, a dense fiber plexus can be seen extending dorsally from the CalB-containing cells at ZT14. Scale bar: 100 µm. Tracings of CalB-containing cells show the distribution pattern of fibers at ZT14 in sagittal (C-F: lateral to medial) sections of the SCN.

mid-SCN where CalB cells are densest, from animals sacrificed at ZT14. The middle row of confocal optical slices (2 µm) shows CalB fiber appositions onto VP, VIP, CCK, or GRP cells. The bottom row of confocal slices shows VP, VIP, CCK, or GRP contacts onto CalB cells. In the first column, CalB fibers are seen extending dorsally and dorsomedially toward the region of VP cells (top panel) and make numerous appositions onto VP cells (middle panel). The VP fibers are detected within the CalB subnucleus and make a few appositions onto CalB cells (bottom panel). The second column shows the interrelationship of VIP and CalB. The ventral VIP cells send fibers throughout the SCN (top panel). In the ventral SCN, CalB fibers make appositions onto VIP cells (midpanel). Many VIP fibers make numerous appositions onto the CalB cells (bottom panel). The third column reveals the distribution of CCK and CalB cells and fibers. The CCK cells form a ring within the borders of the SCN (top panel). Most CCK cells receive a few appositions from CalB fibers (midpanel). Some CCK fibers contact CalB cells, making few appositions in most instances (bottom panel). The fourth column shows the distribution of GRP and CalB cells and fibers. The CalB cells and GRP fibers are dense within the SCN core (top panel). The CalB-ir fibers make many appositions onto the GRP cells and vice versa (midpanel and bottom panel). A schematic representing the connections to and from the CalB-ir cells to other SCN peptidergic cells is shown at the bottom of Figure 4 for both ZT4 and ZT14.

DISCUSSION

Light is the most salient stimulus adjusting the circadian clock to the local light-dark cycle, although resetting occurs only at specific times of the day. Light-induced phase shifts of behavior occur during the night (or subjective night) and not during the day (or subjective day). The mechanisms mediating such gating of photic effects are not well understood. The retinorecipient SCN contains CalB-expressing cells, and the protein appears to influence the responses to photic cues as indicated by studies using CalB-antisense oligonucleotides (Hamada et al., 2003). The CalB protein is contained within SCN cells that are directly retinorecipient (Bryant et al., 2000), and cells of this core region are thought to be important for relaying photic signals to those SCN cells that are not directly retinorecipient. Thus, it had been a surprise to find, in a previous study, that CalB cells did not make contacts with VP cells (LeSauter et al., 2002). The present findings go a long way to explaining that puzzle.

While overall CalB protein is not rhythmic in the SCN (Cayetanot et al., 2007; LeSauter et al., 1999), the results show a marked circadian rhythm of CalB-ir in fibers of SCN cells. The number of detectable fibers peaks at night around ZT/CT 14 and is low at ZT/CT

Table 1. Quantification of appositions among peptidergic cell types during night and day.

	ZT 14				*ZT 4*			
	Cells with ≥3 Appositions, %	*Cells with 1-2 Appositions, %*	*Animals, N*	*Cells, N*	*Cells with ≥3 Appositions, %*	*Cells with 1-2 Appositions, %*	*Animals, N*	*Cells, N*
CalB to VP	64.1 ± 8.2	29.4 ± 4.6	4	306	0	12.9 ± 0.3	2	62
VP to CalB	24.7 ± 7.7	62.5 ± 12.9	4	291	18.8 ± 0.9	71.8 ± 6.8	2	117
CalB to VIP	32.5 ± 2.5	53.3 ± 3.8	3	77	29.2 ± 1.2	56.9 ± 2.1	2	65
VIP to CalB	91.4 ± 2.8	8.2 ± 2.7	4	233	95.1 ± 1.5	4.9 ± 1.5	3	183
CalB to GRP	61.1 ± 5.7	27.8 ± 3.4	4	36	50.0 ± 3.9	35.0 ± 4.3	2	20
GRP to CalB	59.1 ± 7.6	25.1 ± 4.3	4	291	53.7 ± 5.4	33.9 ± 8.3	2	121
CalB to CCK	26.7 ± 2.9	57.3 ± 10.5	5	75	20.0 ± 4.7	62.9 ± 9.0	3	36
CCK to CalB	19.3 ± 3.3	80.0 ± 3.2	5	290	19.8 ± 3.9	77.5 ± 2.3	3	182

CalB = calbindin-D28K; VP = vasopressin; VIP = vasoactive intestinal polypeptide; GRP = gastrin-releasing peptide; CCK = cholecystokinin.

2 to 6. This rhythm is associated with spatial localization of CalB in the soma, with nuclear content highest during the day and lowest at night (Hamada et al., 2003 [hamster]; Cayetanot et al., 2007 [mouse lemur]). The CalB-ir fibers make numerous appositions onto VP cells at night, while few such contacts are detected during the day. In contrast, circadian changes in the numbers of contacts were not seen between CalB fibers and VIP, CCK, and GRP cells. The absence of differences in CalB fiber-ir in the PVN and in the amygdala suggests that the circadian expression of CalB in fibers is specific to the SCN.

The cause of changes in CalB-ir in fibers may reflect local variation in the amount of CalB protein or may be the result of changes in the amount of bound versus unbound CalB or of conformational changes in the CalB protein and associated variation in antibody recognition (Winsky and Kuznicki, 1996). Whatever the cause, the present findings suggest a temporally gated signaling pathway between the retinorecipient CalB cells and the oscillators of VP cells. As the present study cannot differentiate between axons and dendrites, such gating may modulate the axonal release of neurotransmitters from the CalB cells by changing the amount of free calcium ions, or it may modify dendritic signaling from oscillator cells to CalB cells. In biocytin-filled cells in mouse SCN, we have shown that both axons and dendrites of GRP cells extend into the shell and contact VP cells (Drouyer, in press).

Effect of Changes in CalB

CalB is a high-affinity calcium-binding protein and is implicated in the regulation of Ca^{2+} homeostasis by acting as a cytosolic Ca^{2+} buffer and possibly as a Ca^{2+} sensor (Berggard et al., 2002; Schmidt et al., 2005; Schwaller, 2009; Schwaller et al., 2002). This function suggests several possible mechanisms whereby CalB cells of the SCN might act to modulate the gating of photic input. In axon terminals of the caudate-putamen nuclei, CalB contributes to the immobilization of Ca^{2+} and has been implicated in terminating GABA release (Pickel and Heras, 1996). In contrast, in postsynaptic spines, CalB acts as a shuttle, allowing Ca^{2+} ions to travel greater distances (Schmidt and Eilers, 2009; Schmidt et al., 2007). Another possible mechanism for the gating effect of CalB could be through voltage-dependent Ca^{2+} currents (reviewed in Schwaller, 2009). The absence of CalB is linked to an increase in Ca^{2+}-dependent inactivation of voltage-dependent Ca^{2+} currents in CalB knockout animals (Klapstein et al., 1998) and in patients with Ammon horn sclerosis (Nagerl and Mody, 1998). Similar mechanisms may apply in the SCN. Voltage-gated calcium channels play a crucial role in phase shifting (Kim et al., 2005). The presence of CalB in fibers at night may cause a decrease in Ca^{2+}-dependent inactivation of voltage-dependent Ca^{2+} current. During the day, on the other hand, when CalB is absent from the terminals, there would be inactivation of voltage-dependent Ca^{2+} currents.

Diversity of CalB-Containing Cells

CalB cells coexpress other peptides, including GRP and substance P (SP) that can themselves phase-shift oscillators. About 40% of CalB cells contain GRP (LeSauter et al., 2002). GRP induces phase shifts in locomotor (Albers et al., 1995; Piggins et al., 1995a) and electrical activity (McArthur et al., 2000). GRP induces c-FOS, MAPK, *Per1*, and *Per2* mRNA in cells of the SCN shell, and impairing the MAPK pathway by inhibiting ERK1/2 phosphorylation attenuates GRP-induced phase shifts (Antle et al.,

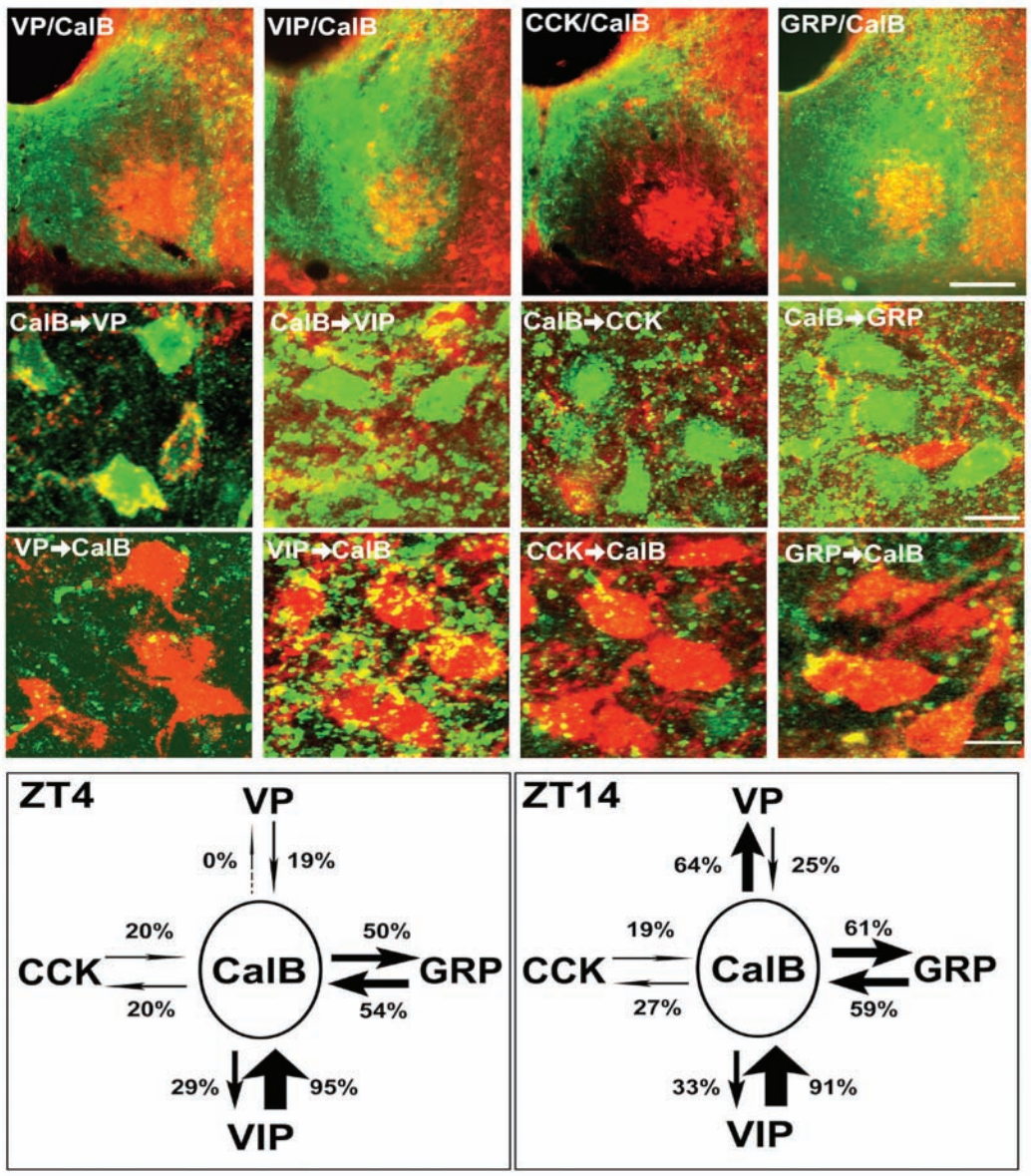

Figure 4. Upper panels: Photomicrographs taken at ZT14 of double-labeled coronal SCN sections. The top row of photomicrographs shows the distribution of CalB-containing cells in relationship to vasopressin (VP)–, vasoactive intestinal polypeptide (VIP)–, cholecystokinin (CCK)–, and gastrin-releasing peptide (GRP)–immunoreactivity (-ir) (left to right, respectively) in sections of the caudal SCN (50 µm). The CalB cells form a dense cluster in the ventrolateral region, and some scattered cells are seen in the dorsal and the peri-SCN region. Scale bar: 100 µm. The second and third rows are confocal sections (2-µm optical slice). The second row shows CalB fibers within the region of VP- (dorsomedial SCN), VIP- (ventral SCN), CCK- (medial SCN), and GRP- (ventromedial SCN) immunoreactivity. CalB fibers make numerous appositions with VP and VIP cells. Sparse CalB-ir fibers are seen among the CCK-ir cells with some contacts on soma. CalB-ir fibers make appositions onto GRP-ir cells. Scale bar: 10 µm. The third row shows VP-, VIP-, CCK-, and GRP-ir within the CalB subregion. As can be seen, VIP makes numerous appositions with the CalB cells, while VP and CCK make few appositions. The GRP cells are distributed among the CalB cells, and GRP fibers contact CalB cells. Scale bar: 10 µm. The bottom row is a schematic representation of the connections between the CalB cells and other peptidergic cell types. The arrow thickness and the percentages indicate the proportion of cells receiving 3 or more appositions.

2005). About 65% of the CalB cells contain SP, and more than 90% SP cells are CalB positive (LeSauter et al., 2002). SP induces phase shifts in electrical activity in rat SCN cells at night (Shibata et al., 1992). The SP increases the firing rate in most hamster SCN cells in vitro (Piggins et al., 1995b) but does not phase-shift hamster activity rhythms (Piggins and Rusak, 1997). The SP receptor neurokinin-1 is located mostly at the dorsal and dorsolateral border and outside of the SCN. Administration of the SP receptor antagonist spantide attenuates light-induced c-FOS in most of the SCN, including the dorsomedial region of VP cells, but not in the region of CalB cells (Abe et al., 1996).

CalB and the Maintenance of Circadian Rhythms

CalB has an important role in the generation/maintenance of rhythmic circadian outputs. While CalB cells lack detectable rhythms in electrical activity (Jobst and Allen, 2002) or gene expression (Hamada et al., 2001), they may be essential for maintaining synchronicity among the oscillator cells in order to

generate a rhythmic output. Mice lacking CalB show low-amplitude behavioral rhythms or become arrhythmic in constant conditions (Kriegsfeld et al., 2008). The SCN of CalB knockout mice has reduced levels of PER2 and VP (Kriegsfeld et al., 2008). The CalB subregion of the hamster SCN is essential for the maintenance of rhythmicity as its ablation results in arrhythmicity even if large parts of the shell remained intact (Kriegsfeld et al., 2004; LeSauter and Silver, 1999).

The role of core cells in the synchronization of SCN oscillators has been demonstrated. Mice lacking the VIP receptor VPAC2 show low-amplitude rhythms or arrhythmicity, abnormal entrainment (similar to that seen in mice lacking CalB), and desynchronization among oscillators. Synchronization is restored by VIP agonists (Aton et al., 2005; Aton et al., 2006) or by GRP administration (Brown et al., 2005; Maywood et al., 2006). This could occur through axosomatic signaling or through dendritic release as has been described in other hypothalamic regions (Ludwig and Leng, 2006). The SCN oscillator cells may also contribute to temporal regulation of CalB cells through somatodendritic communication.

Hamster-Mouse Comparison

Species comparisons can reveal general principles of SCN function, although these give rise to terminological and perhaps conceptual problems. The terms "core" and "shell" were initially introduced by Miller, Morin, Schwartz, and Moore (Miller et al., 1996) to characterize the hamster SCN, and their use has been extended even though the precise localization of these compartments differs in other species (Morin and Allen, 2006). In contrast to the hamster, the adult mouse SCN lacks a cluster of CalB in the SCN core (Ikeda and Allen, 2003; Kriegsfeld et al., 2008; Silver et al., 1996). However, we have recent evidence of coexpression of CalB and GRP in the perinatal mouse (Drouyer, in press), and like the hamster, these core cells respond to light at night but are not rhythmic in gene expression (Karatsoreos et al., 2004) or electrical activity (LeSauter and Silver, 2005). These findings suggest the possibility of topographical and functional similarity in SCN organization among species.

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REFERENCES

Abe H, Honma S, Shinohara K, and Honma K (1996) Substance P receptor regulates the photic induction of Fos-like protein in the suprachiasmatic nucleus of Syrian hamsters. Brain Res 708:135-142.

Albers HE, Gillespie CF, Babagbemi TO, and Huhman KL (1995) Analysis of the phase shifting effects of gastrin releasing peptide when microinjected into the suprachiasmatic region. Neurosci Lett 191:63-66.

Antle MC, Foley DK, Foley NC, and Silver R (2003) Gates and oscillators: a network model of the brain clock. J Biol Rhythms 18:339-350.

Antle MC, Foley NC, Foley DK, and Silver R (2007) Gates and oscillators II: zeitgebers and the network model of the brain clock. J Biol Rhythms 22:14-25.

Antle MC, Kriegsfeld LJ, and Silver R (2005) Signaling within the master clock of the brain: localized activation of mitogen-activated protein kinase by gastrin-releasing peptide. J Neurosci 25:2447-2454.

Aton SJ, Colwell CS, Harmar AJ, Waschek J, and Herzog ED (2005) Vasoactive intestinal polypeptide mediates circadian rhythmicity and synchrony in mammalian clock neurons. Nat Neurosci 8:476-483.

Aton SJ, Huettner JE, Straume M, and Herzog ED (2006) GABA and Gi/o differentially control circadian rhythms and synchrony in clock neurons. Proc Natl Acad Sci U S A 103:19188-19193.

Berggard T, Miron S, Onnerfjord P, Thulin E, Akerfeldt KS, Enghild JJ, Akke M, and Linse S (2002) Calbindin D_{28k} exhibits properties characteristic of a Ca^{2+} sensor. J Biol Chem 277:16662-16672.

Brown TM, Hughes AT, and Piggins HD (2005) Gastrin-releasing peptide promotes suprachiasmatic nuclei cellular rhythmicity in the absence of vasoactive intestinal polypeptide-VPAC2 receptor signaling. J Neurosci 25: 11155-11164.

Bryant DN, LeSauter J, Silver R, and Romero MT (2000) Retinal innervation of calbindin-D28K cells in the hamster suprachiasmatic nucleus: ultrastructural characterization. J Biol Rhythms 15:103-111.

Cayetanot F, Deprez J, and Aujard F (2007) Calbindin D28K protein cells in a primate suprachiasmatic nucleus: localization, daily rhythm and age-related changes. Eur J Neurosci 26:2025-2032.

Drouyer ELJ, Hernandez AL, and Silver R (In press) Specializations of gastrin releasing peptide cells of the mouse suprachiasmatic nucleus. J Comp Neurol.

Hamada T, LeSauter J, Lokshin M, Romero MT, Yan L, Venuti JM, and Silver R (2003) Calbindin influences response to photic input in suprachiasmatic nucleus. J Neurosci 23:8820-8826.

Hamada T, LeSauter J, Venuti JM, and Silver R (2001) Expression of Period genes: rhythmic and nonrhythmic compartments of the suprachiasmatic nucleus pacemaker. J Neurosci 21:7742-7750.

Ikeda M and Allen CN (2003) Developmental changes in calbindin-D_{28k} and calretinin expression in the mouse suprachiasmatic nucleus. Eur J Neurosci 17:1111-1118.

Jobst EE and Allen CN (2002) Calbindin neurons in the hamster suprachiasmatic nucleus do not exhibit a circadian variation in spontaneous firing rate. Eur J Neurosci 16:2469-2474.

Karatsoreos IN, Yan L, LeSauter J, and Silver R (2004) Phenotype matters: identification of light-responsive cells in the mouse suprachiasmatic nucleus. J Neurosci 24:68-75.

Kim DY, Choi HJ, Kim JS, Kim YS, Jeong DU, Shin HC, Kim MJ, Han HC, Hong SK, and Kim YI (2005) Voltage-gated calcium channels play crucial roles in the glutamate-induced phase shifts of the rat suprachiasmatic circadian clock. Eur J Neurosci 21:1215-1222.

Klapstein GJ, Vietla S, Lieberman DN, Gray PA, Airaksinen MS, Thoenen H, Meyer M, and Mody I (1998) Calbindin-D_{28k} fails to protect hippocampal neurons against ischemia in spite of its cytoplasmic calcium buffering properties: evidence from calbindin-D_{28k} knockout mice. Neuroscience 85:361-373.

Kriegsfeld LJ, LeSauter J, and Silver R (2004) Targeted microlesions reveal novel organization of the hamster suprachiasmatic nucleus. J Neurosci 24:2449-2457.

Kriegsfeld LJ, Mei DF, Yan L, Witkovsky P, Lesauter J, Hamada T, and Silver R (2008) Targeted mutation of the calbindin D_{28K} gene disrupts circadian rhythmicity and entrainment. Eur J Neurosci 27:2907-2921.

LeSauter J, Kriegsfeld LJ, Hon J, and Silver R (2002) Calbindin-D(28K) cells selectively contact intra-SCN neurons. Neuroscience 111:575-585.

LeSauter J and Silver R (1999) Localization of a suprachiasmatic nucleus subregion regulating locomotor rhythmicity. J Neurosci 19:5574-5585.

LeSauter J and Silver R (2005) Phenotype matters: membrane properties of gastrin-releasing cells of the SCN. Soc Neurosci Abstract 312.8.

LeSauter J, Stevens P, Jansen H, Lehman MN, and Silver R (1999) Calbindin expression in the hamster SCN is influenced by circadian genotype and by photic conditions. Neuroreport 10:3159-3163.

Ludwig M and Leng G (2006) Dendritic peptide release and peptide-dependent behaviours. Nat Rev Neurosci 7:126-136.

Maywood ES, Reddy AB, Wong GK, O'Neill JS, O'Brien JA, McMahon DG, Harmar AJ, Okamura H, and Hastings MH (2006) Synchronization and maintenance of timekeeping in suprachiasmatic circadian clock cells by neuropeptidergic signaling. Curr Biol 16:599-605.

McArthur AJ, Coogan AN, Ajpru S, Sugden D, Biello SM, and Piggins HD (2000) Gastrin-releasing peptide phase-shifts suprachiasmatic nuclei neuronal rhythms in vitro. J Neurosci 20:5496-5502.

Miller JD, Morin LP, Schwartz WJ, and Moore RY (1996) New insights into the mammalian circadian clock. Sleep 19:641-667.

Morin LP and Allen CN (2006) The circadian visual system: 2005. Brain Res Brain Res Rev 51:1-60.

Nagerl UV and Mody I (1998) Calcium-dependent inactivation of high-threshold calcium currents in human dentate gyrus granule cells. J Physiol 509(Pt 1):39-45.

Pickel VM and Heras A (1996) Ultrastructural localization of calbindin-D_{28k} and GABA in the matrix compartment of the rat caudate-putamen nuclei. Neuroscience 71:167-178.

Piggins HD, Antle MC, and Rusak B (1995a) Neuropeptides phase shift the mammalian circadian pacemaker. J Neurosci 15:5612-5622.

Piggins HD, Cutler DJ, and Rusak B (1995b) Ionophoretically applied substance P activates hamster suprachiasmatic nucleus neurons. Brain Res Bull 37:475-479.

Piggins HD and Rusak B (1997) Effects of microinjections of substance P into the suprachiasmatic nucleus region on hamster wheel-running rhythms. Brain Res Bull 42:451-455.

Schmidt H and Eilers J (2009) Spine neck geometry determines spino-dendritic cross-talk in the presence of mobile endogenous calcium binding proteins. J Comput Neurosci 27(2):229-243.

Schmidt H, Kunerth S, Wilms C, Strotmann R, and Eilers J (2007) Spino-dendritic cross-talk in rodent Purkinje neurons mediated by endogenous Ca^{2+}-binding proteins. J Physiol 581:619-629.

Schmidt H, Schwaller B, and Eilers J (2005) Calbindin D_{28k} targets myo-inositol monophosphatase in spines and dendrites of cerebellar Purkinje neurons. Proc Natl Acad Sci U S A 102:5850-5855.

Schwaller B (2009) The continuing disappearance of "pure" Ca^{2+} buffers. Cell Mol Life Sci 66:275-300.

Schwaller B, Meyer M, and Schiffmann S (2002) 'New' functions for 'old' proteins: the role of the calcium-binding proteins calbindin D-28k, calretinin and parvalbumin, in cerebellar physiology. Studies with knockout mice. Cerebellum 1:241-258.

Shibata S, Tsuneyoshi A, Hamada T, Tominaga K, and Watanabe S (1992) Effect of substance P on circadian rhythms of firing activity and the 2-deoxyglucose uptake in the rat suprachiasmatic nucleus in vitro. Brain Res 597:257-263.

Silver R, Romero MT, Besmer HR, Leak R, Nunez JM, and LeSauter J (1996) Calbindin-D28K cells in the hamster SCN express light-induced Fos. Neuroreport 7:1224-1228.

Winsky L and Kuznicki J (1996) Antibody recognition of calcium-binding proteins depends on their calcium-binding status. J Neurochem 66:764-771.