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Calcium dynamics and circadian rhythms in suprachiasmatic nucleus neurons

by

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Summary

The hypothalamic suprachiasmatic nucleus (SCN) has a pivotal role in the mammalian circadian clock. SCN neurons generate circadian rhythms in action potential firing frequencies and neurotransmitter release, and the core oscillation is thought to be driven by "clock gene" transcription-translation feedback loops. Cytosolic Ca^{2+} mobilization followed by stimulation of various receptors has been shown to reset the gene transcription cycles in SCN neurons, whereas contribution of steady-state cytosolic Ca^{2+} levels to the rhythm generation is unclear. Recently, circadian rhythms in cytosolic Ca^{2+} levels have been demonstrated in cultured SCN neurons (Ikeda and others *Neuron* 38:252-263, 2003). The circadian Ca^{2+} rhythms are driven by the release of Ca^{2+} from ryanodine-sensitive internal stores and resistant to the blockade of action potentials. These results raise the possibility that gene translation/transcription loops may interact with autonomous Ca^{2+} oscillations in the production of circadian rhythms in SCN neurons.

Introduction

Daily temporal patterns from cellular activities to animal behaviors are largely governed by the endogenous clock system, which has been proposed to be driven by gene transcription-translation feedback loops. The circadian oscillations in the "clock genes" have been observed in a wide range of systems, from unicellular organisms to mammalian cells and from peripheral organs to the central nervous system, although their DNA sequences are variable across species (Kondo and Ishiura 1999; Yamazaki and others 2000; Panda and others 2002a). Cytosolic free Ca^{2+} is a general intracellular messenger that regulates diverse cellular processes, including enzymatic activities, membrane potentials, secretions, and gene expressions, and is one of the candidate intracellular messengers involved in circadian rhythms. Although the contribution of cytosolic Ca^{2+} signaling in the regulation of the circadian clock has been extensively studied in diverse organisms, including unicellular organisms and plants (Goto and others 1985; Johnson and others 1995; Hasegawa and others 1999), no commonalities in the Ca^{2+}-mediated signaling processes have been described among the various circadian clock systems.

In mammals, the hypothalamic suprachiasmatic nucleus (SCN) is known to have a core role in the generation of circadian rhythms because lesions of the SCN result in arrhythmic circadian behaviors under time cue-free conditions (Moore and Eichler 1972; Stephan and Zucker 1972). In mice SCN, the clock gene products BMAL1 and CLOCK are basic helix-loop-helix PAS transcription factors that form heterodimers and bind to the E-box enhancers upstream of *Per* and *Cry* genes to activate their transcription (Antoch and others 1997; King and others 1997; Tei and others 1997; Honma and others 1998; Sangoram and others 1998; van der Horst and others 1999; Jin

and others 1999; Kume and others 1999). The protein products PER and CRY form heterodimers and translocate into the nucleus to inhibit transactivation by CLOCK/BMAL1. A protein product of *Dec* gene families recently found in the SCN, is also a basic helix-loop-helix PAS transcription factor, and thus is hypothesized to organize an additional molecular loop in the SCN clock (Honma and others 2002). One cycle of the molecular loop is assumed to generate a circadian cycle, called a "core loop" (Shearman and others 2000; Reppert and Weaver 2002; Honma and Honma 2003).

The SCN receives glutamatergic projections from retinal ganglion cells, and thus the core loop is synchronized to environmental light-dark cycles (12:12 hour cycles under common breeding conditions). In animals maintained in constant darkness or even in the *in vitro* SCN, the core loop maintains a circadian oscillation, demonstrating the self-sustained circadian nature of these gene transcription-translation cycles. A light pulse given during the active phase of nocturnal rodents kept in constant darkness or glutamate stimulation in the *in vitro* SCN during the corresponding circadian timing (i.e., the subjective night) can reset the core loop oscillations. Because Ca^{2+} influx is an initial cellular event in response to glutamate stimulation, cytosolic Ca^{2+} signaling has been proposed to be one of the intracellular messengers conveying environmental time signals to the endogenous clock, although the mechanism underlying the regulation of clock genes by cytosolic Ca^{2+} is not fully understood.

The core loop in the SCN may regulate diverse physiological events such as action potential firing rhythms (Inouye and Kawamura 1979; Green and Gillette 1982) and rhythmic secretion of arginine-vasopression and vasoactive intestinal polypeptide (Earnest and Sladek 1986; Shinohara and others 1995). These electrical and humoral

outputs from SCN neurons may ultimately synchronize circadian behaviors (Silver and others 1996a; Aston-Jones and others 2001). Despite the successful cloning of several clock genes, the link between the gene transcription-translation cycles and oscillations in physiological activities of SCN neurons is still unclear. Recent findings that cytosolic Ca^{2+} concentrations oscillate with a circadian profile in SCN neurons (Ikeda and others 2003a) raise the possibility that mechanisms underlying Ca^{2+} homeostasis in SCN neurons may be principal targets for clock gene regulation (Honma and Honma 2003). If so, cytosolic Ca^{2+} may be a key intracellular messenger of both input to and output from the core of the mammalian circadian clock.

In this article, recent studies concerning intracellular Ca^{2+} signaling in SCN neurons are reviewed and the technical background underlying the recent findings of circadian Ca^{2+} rhythms in SCN neurons (Ikeda and others 2003a) is described. Based on these, hypothetical interactions between Ca^{2+} signaling and the core loop are discussed with regard to the importance of Ca^{2+} signaling cascades in the organization of circadian rhythms in SCN neurons.

1. Nocturnal light causes changes in cytosolic calcium levels via retinohypothalamic transmission: a key intracellular signal for the resetting of the circadian clock

SCN rhythms are tightly coupled to environmental light-dark cycles via the mono-synaptic projection from melanopsin-positive retinal ganglion cells (Gooley and others 2001; Berson and others 2002; Hattar and others 2002; Provencio and others 2002), the retinohypothalamic tract (RHT; Rusak and Zucker 1979; Fig. 1). The major neurotransmitter conveying photic information to the SCN is glutamate (Cahill and

Menaker 1989a,b; de Vries and others 1993) although the pituitary adenylate cyclase-activating peptide also functions as a secondary neurotransmitter to the SCN (Hannibal and others 1997; von Gall and others 1998; for review Hannibal 2002). All three subtypes of glutamate receptors, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), kainate, and *N*-methyl-D-aspartate (NMDA) receptors, are expressed on SCN neurons and may function in RHT transmission (Takeuchi and others 1991; Gannon and Rea 1994; Jiang and others 1997; Schurov and others 1999; Michel and others 2002; Ikeda and others 2003b; Moriya and others 2003). The postsynaptic membrane of glutamate-receptive neurons such as this is generally depolarized by Na^{+}/Ca^{2+} influx via non-NMDA receptors, and subsequently depolarized further by Ca^{2+} influx through NMDA receptor-linked channels followed by the glutamate stimulation. The Ca^{2+} influx through NMDA receptor-linked channels in SCN neurons may be the most critical step for clock regulation via RHT transmission, because NMDA receptor antagonists block light-pulse-induced phase shifts in locomotor activity rhythms, application of NMDA to brain slices containing the SCN during early subjective night produces a delay-shift, and application of NMDA during late subjective night produces an advance-shift, similar to the circadian profile of light-pulse-induced behavioral phase shifts (Ding and others 1994).

SCN neurons also express various other receptors that may interact with the glutamatergic system and modulate cytosolic Ca^{2+} levels. For example, serotonin (5-HT) 1B receptors expressed presynaptically on RHT terminals and 5-HT 1A/7 receptors expressed postsynaptically on SCN neurons both inhibit RHT transmission (Pickard and others 1996, 1999; Jiang and others 2000; Smith and others 2001). The

Ca^{2+} transient caused by RHT stimulation in acute SCN slices (Flett and Colwell 1999) and glutamate-induced Ca^{2+} influx in cultured SCN neurons (Quintero and McMahon 1999) are significantly reduced by 5-HT agonists. Therefore, a 5-HT-induced reduction in glutamate-induced Ca^{2+} responses in SCN neurons may be the mechanism by which 5-HT inhibits light-pulse-induced behavioral phase shifts (Pickard and others 1996; Ying and Rusak 1997; for review, Rea 1998). Similar to postsynaptic 5-HT receptors, stimulation of orphanin-FQ/nociceptin receptors, which are expressed on the majority of SCN neurons, may activate K^+ currents and inhibit NMDA-induced Ca^{2+} influx (Allen and others 1999). Again, the inhibitory effects of orphanin-FQ/nociceptin on NMDA receptors may be the mechanism by which orphanin-FQ/nociceptin inhibits light-pulse-induced behavioral phase shifts (Allen and others 1999). These data suggest that changes in glutamate-induced Ca^{2+} mobilization in SCN neurons due to interactions with other receptor systems determine the total potency of RHT transmission and magnitude of the circadian phase shifts.

The majority of SCN neurons synthesize GABA and receive GABA projections, and thus GABA is thought to be a principal neurotransmitter within the SCN (Perez de la Mora and others 1981; Okamura and others 1986; Decavel and van den Pol 1990; Moore and Speh 1993; Buijs and others 1994). In addition, an indirect retinal input onto SCN neurons is mediated via the geniculo-hypothalamic tract (GHT; Harrington and Rusak 1989; Zhang and Rusak 1989; Edelstein and Amir 1999), which also releases GABA onto SCN neurons (Harrington and others 1987; Moore and Speh 1993). The dominant GABA receptor subtypes in SCN neurons are Cl^- permeable ionotropic receptors, the $GABA_A$ receptors ($GABA_A$Rs). Stimulation of $GABA_A$Rs generally triggers influx of Cl^- into the cells and membrane hyperpolarization, which inhibits

neuronal excitation. The function of $GABA_A$Rs in SCN neurons may not be this simple, however, because Wagner and others (1997) have observed that activation of $GABA_A$Rs produce depolarization during the day and hyperpolarization during the night in adult rat SCN neurons. This difference in $GABA_A$Rs function has been suggested to be due to a day-night difference in intracellular Cl^- concentration that may produce $GABA_A$R-mediated Cl^- efflux (i.e., depolarization) or Cl^- influx (i.e., hyperpolarization) in SCN neurons (Wagner and others 1997, 2001). In dispersed cultures of SCN neurons, daily stimulation of $GABA_A$Rs synchronizes the dissociated circadian rhythms in each individual SCN neuron (Liu and Reppert 2000). This effect is not due to an inhibitory action (i.e., decreasing of firing frequency) via $GABA_A$Rs, because the metabotropic GABA receptor ($GABA_B$R) agonist baclofen inhibits action potential firing both during the subjective day and night without causing a significant phase shift (Liu and Reppert 2000). Our group found that, at least at a particular developmental stage, stimulation of $GABA_A$R increases cytosolic Ca^{2+} during the day and decreases cytosolic Ca^{2+} during the night via an interaction with NMDA receptors and/or voltage-sensitive Ca^{2+} channels (Ikeda and others, 2003b). Therefore, $GABA_A$R-mediated neuronal signaling may also involve cytosolic Ca^{2+} signaling in the regulation of the circadian clock, although further studies are needed to characterize the link between $GABA_A$R and cytosolic Ca^{2+} signaling.

Ca^{2+} influx through NMDA receptor-linked channels in SCN neurons may activate diverse downstream intracellular signaling cascades that are more closely involved in the gene transcription-translation cycles in the SCN. Light or glutamate receptor activation during early subjective night induces circadian phase delay, which is proposed to be mediated by ryanodine receptors (Ding and others 1998). Ryanodine

receptors are known to contribute to the amplification of cytosolic Ca^{2+} signals via the release of Ca^{2+} from ryanodine-sensitive internal Ca^{2+} stores, called Ca^{2+}-induced Ca^{2+} release (CICR). Therefore, this cascade is highly Ca^{2+} dependent. Rapid gene transcription of *Per1* following Ca^{2+}/calmodulin-dependent protein kinase (CaMK) II-dependent phosphorylation of the cAMP response element binding protein (CREB) is the proposed downstream signaling pathway for the circadian phase shifts (Ginty and others 1993; Ding and others 1997; von Gall and others 1998; Yokota and others 2001). Therefore, an increase in cytosolic Ca^{2+} and CaMK II activation during early subjective night may be the signal that produces phase-delays in SCN neurons.

Although day-night or circadian variations in the magnitude of NMDA-induced Ca^{2+} responses have been proposed in SCN neurons (Colwell 2001; Pennartz and others 2001; Ikeda and others 2003b), no differences have been found in the responsiveness during the night. Consistently, CREB phosphorylation in SCN neurons is triggered by light or glutamate stimulation during both early and late subjective night (von Gall and others 1998). The response to light or glutamate, however, is opposite (i.e., delay versus advance) for stimulation during early or late subjective night. This difference may be explained in part at the level of intracellular messengers, because phase-advance due to late subjective night stimulation is insensitive to ryanodine receptor blockade but is sensitive to activation of cyclic GMP-dependent protein kinase (PKG; Prosser and others 1989; Weber and others 1995; Mathur and others 1996; Ding and others 1998; Tischkau and others 2003). PKG can be activated by nitric oxide (NO), the production of which is upregulated by NMDA-induced cytosolic Ca^{2+} mobilization (Garthwaite and others 1988; Knowles and others 1989; Bredt and Snyder 1989, 1990), thus, the PKG cascades may be activated as “secondary” or “indirect” signaling cascades downstream

from glutamate-induced Ca^{2+} mobilization. The difference in the downstream signaling sequences followed by the cytosolic Ca^{2+} mobilization may produce differential clock responses depending on the particular circadian time window (for review, Gillette and Tischkau 1999; Gillette and Mitchell 2002).

2. Technical issues in the estimation of the circadian dynamics of cytosolic Ca^{2+} levels in SCN neurons

Despite the large number of reports suggesting the involvement of intracellular Ca^{2+} responses (i.e., receptor-mediated changes in intracellular Ca^{2+} concentration) in the regulation of the SCN clock, information is still limited about steady-state intracellular Ca^{2+} levels in SCN neurons. Whether or not the basal level of intracellular Ca^{2+} oscillates with a circadian profile is a fundamental question, but is technically more difficult to address.

The circadian time-dependent cytosolic Ca^{2+} levels in SCN cells were first estimated using fura-2 acetoxymethyl ester (AM)-based Ca^{2+} imaging in acute hypothalamic slices from rats, and the population mean cytosolic Ca^{2+} in SCN cells was found to be higher during the day than during the night (Colwell 2000). It was proposed that action potentials and voltage-gated Ca^{2+} channel activation drive the circadian variations in cytosolic Ca^{2+} levels because the day-night or circadian difference in cytosolic Ca^{2+} was blocked by the voltage-gated Na^{+}-channel blocker tetrodotoxin (TTX) and the voltage-sensitive Ca^{2+}-channel blocker methoxyverapamil (Colwell 2000). Using a similar experimental strategy, however, our group observed neither the day-night difference in the population mean cytosolic Ca^{2+} nor the effect of TTX on the cytosolic Ca^{2+} levels in SCN cells (Ikeda and others 2003b). In contrast,

we observed significant effects of extracellular Mg^{2+} on baseline cytosolic Ca^{2+} levels both during the day and night (Ikeda and others 2003b). We observed later, however, that the effect of Mg^{2+} was caused by the differential dissociate constant of fura-2 to Ca^{2+} in the presence or absence of Mg^{2+}, not physiological processes (Ikeda, unpublished data). Both of these Ca^{2+} imaging experiments involved staining hypothalamic slices with membrane permeable fura-2 AM, which does not distinguish neuronal and glial Ca^{2+} responses (Colwell 2000; Ikeda and others 2003b). In addition, both of these studies compared different SCN cells sampled in different slices during either the day or night, because fura-2 can not be used to follow cytosolic Ca^{2+} of single cells over a complete circadian cycle. Therefore, a part of the controversy may be due to these experimental difficulties.

More fundamental and theoretical problems, however, may underlie the analysis of steady-state cytosolic Ca^{2+} levels using conventional Ca^{2+} imaging techniques. In general, high-affinity Ca^{2+} dyes, such as fura-2 (K_d = 135 nM in Mg^{2+}-free buffer and K_d = 224 nM in 1 mM Mg^{2+} buffer), if the cells are thoroughly loaded (bath application at 3-10 mM), may reduce the baseline cytosolic Ca^{2+} levels because their affinity range is similar to that of the commonly used Ca^{2+} chelator BAPTA (K_d = 160 nM in Mg^{2+}-free buffer and K_d = 700 nM in 1 mM Mg^{2+} buffer) and is higher than the physiological baseline cytosolic Ca^{2+} concentration. Reduction of the dye concentration and observation of dimmer cells with a more sensitive imaging setup may increase the accuracy of the estimated baseline Ca^{2+} levels, but such precise control of intracellular dye concentrations is theoretically difficult due to the unknown dye-leakage rate from the cells and dye-uptake rate into the organelles. Alternatively, use of low-affinity Ca^{2+} dyes, such as BTC (K_d = 7 μM), may reduce the Ca^{2+} chelating artifacts, but these

dyes are not sensitive enough to be used for detection of nanomolar levels of cytosolic Ca^{2+}. Therefore, conventional Ca^{2+} dyes may not be useful in the estimation of the steady-state levels of Ca^{2+} in SCN neurons.

Nevertheless, circadian rhythmic changes in cytosolic Ca^{2+} have been successfully observed in tobacco and *Arabidopsis* plants (Johnson and others 1995; Wood and others 2001) using the Ca^{2+}-sensing protein sensor aequorin. Aequorin is a jellyfish protein used to detect a wide range of cytosolic Ca^{2+} concentrations (0.1-100 μM) via its chemi-luminescence. Aequorin is not exported, secreted, compartmentalized, nor sequestered within cells, and is thus suitable for the long-term measurement of cytosolic Ca^{2+}. Also, photon-counting analysis of aequorin chemi-luminescence enables cytosolic Ca^{2+} measurement using a smaller number of sensor molecules and may avoid Ca^{2+} chelating problems such as those described with conventional dye imaging. Despite the advantageous characteristics of aequorin for the long-term monitoring of cytosolic Ca^{2+} in tobacco and *Arabidopsis* seedlings (millimeter to centimeter in length), it has not been successfully used in SCN neurons (the cell body is less than 10 μm diameter), presumably due to the limited spatial resolution of chemi-luminescence signals.

To examine progressive changes in free Ca^{2+} concentration in single SCN neurons over multiple circadian cycles, our group used the Ca^{2+}-sensitive fluorescent protein cameleon (Miyawaki and others 1997, 1999). The cameleon used (YC2.1) was constructed with cyan-shifted and yellow-shifted green fluorescent proteins linked to opposite ends of the Ca^{2+} binding region of calmodulin. Cytosolic Ca^{2+} levels are estimated from the reciprocal emission intensity changes at 480 nm and 535 nm (i.e., F535 nm/F480 nm ratio) produced by the fluorescent resonance energy transfer (FRET)

in the Ca^{2+}-bound cameleon molecule. Although the primary dissociation constant of YC2.1 (K_{d1} = 100 nM) is higher than that of fura-2, a single YC2.1 molecule is much brighter. This enables the visualization of single neurons using a smaller number of sensor molecules, and may avoid Ca^{2+} chelating artifacts. YC2.1 has been successfully used in primary cultures of hippocampal and cortical neurons (Miyawaki and others 1999; Tsuchiya and others 2002). Also, YC2.1 has been stably expressed in *C. elegans* neurons (Kerr and others 2000), demonstrating its applicability for long-term monitoring of neuronal cytosolic Ca^{2+}. Using SCN slice cultures transfected with YC2.1 cDNA linking to a neuron-specific enolase promoter (Sakimura and others 1995), our group finally succeeded in the continuous monitoring of steady-state cytosolic Ca^{2+} levels in single SCN neurons over entire circadian periods (Ikeda and others 2003a: Fig. 2).

3. Circadian rhythms in cytosolic calcium concentration in SCN neurons: what the message tells us.

Circadian rhythms are observed in cytosolic Ca^{2+} concentration in approximately two thirds (64%) of the population of cultured SCN neurons with a trough at approximately 120 nM and peak at approximately 440 nM (Ikeda and others 2003a). The circadian periods are generally synchronized among cells in the organotypic culture, but the phases are slightly advanced or delayed (Fig. 2). The average peak in Ca^{2+} is 4-5 hours before the peak in action potential firing, recorded simultaneously using a multiple-electrode-array dish. The circadian cytosolic Ca^{2+} rhythm is specific to SCN neurons because neurons and glial cells located outside of the SCN exhibit no changes in cytosolic Ca^{2+} levels. The circadian cytosolic Ca^{2+} rhythm is driven by the release

of Ca^{2+} from ryanodine-sensitive Ca^{2+} stores because ryanodine and 8-bromo-cyclic ADP ribose significantly damp the cytosolic Ca^{2+} rhythm. Inositol (1,4,5)-trisphosphate (IP_3)-sensitive Ca^{2+} stores are present in SCN neurons (Kopp and others 1999; Ikeda and others 2000), but the contribution of this Ca^{2+} store in the generation of the circadian cytosolic Ca^{2+} rhythm appears to be small, because the Ca^{2+}-ATPase inhibitor, thapsigargin, has little effect on the circadian cytosolic Ca^{2+} rhythm. The circadian cytosolic Ca^{2+} rhythm is also resistant to TTX, which blocks action potential firing in SCN neurons. Moreover, the circadian cytosolic Ca^{2+} rhythm is resistant to nimodipine, which blocks L-type Ca^{2+} channels and is reported to reduce action potential firing in SCN neurons (Pennartz and others 2002). These findings suggest that Ca^{2+} influx through the plasma membrane is not the primary mechanism underlying the generation of the circadian cytosolic Ca^{2+} rhythm.

The observation that the circadian cytosolic Ca^{2+} rhythm is TTX-resistant raises the possibility that some of the clock genes regulate the circadian cytosolic Ca^{2+} rhythm (Fig. 3A). Therefore, screening of gene function in this regard is a critical next step toward a more complete understanding of the mechanisms underlying the circadian clock. An important aspect to remember in this process of discovery, is that for many genes, the converse will be true; the Ca^{2+} rhythms regulate the gene transcription cycles, because cytosolic Ca^{2+} is a transcriptional regulator for numerous genes. Using DNA microarrays, not only the known clock genes but also hundreds of other genes have been shown to oscillate with a circadian profile in the SCN (Panda and others 2002b; Ueda and others 2002; for review, Delaunay and Laudet 2002). It is unlikely that all these genes oscillate by their transcription-translation feedback loops. Rather, it is more plausible that many of these gene transcription rhythms are driven by the circadian

cytosolic Ca^{2+} rhythm (Fig. 3B).

Consequently, this raises the specific question as to whether the known clock gene oscillations are under the influence of circadian cytosolic Ca^{2+} rhythms or vice versa. As described above, glutamate-induced Ca^{2+} influx together with ryanodine-receptor mediated amplification of Ca^{2+} signals and resultant CREB phosphorylation by CaMKII have been proposed to be the primary signaling pathway for resetting the clock gene (Ginty and others 1993; Ding and others 1997, 1998; von Gall and others 1998; Yamaguchi and others 2000; Yokota and others 2001). Despite the evidence for cytosolic Ca^{2+}-mediated control of clock gene transcription, the current model explaining the core circadian oscillations is based primarily on the feedback regulations of clock gene transcriptions by their own translational products with reciprocal interactions of different clock gene products (Shearman and others 2000; Reppert and Weaver 2002). The model is reasonable because the core SCN oscillation was proposed to be TTX-resistant (Schwartz and others 1987) and the circadian rhythms in cytosolic Ca^{2+} estimated in acute SCN slices using conventional fura-2 imaging were proposed to be TTX-dependent (Colwell 2000). Based on these earlier data, the involvement of TTX-sensitive Ca^{2+} levels in the regulation of the core circadian loop would be ruled out. Nevertheless, based on the recent demonstration of a TTX-resistant circadian cytosolic Ca^{2+} rhythm in cultured SCN neurons (Ikeda and others 2003a), and the fact that this rhythm is driven by the Ca^{2+} release from ryanodine-sensitive internal Ca^{2+} stores (i.e., the same machinery that activates *Per1* transcription following glutamate stimulation) and phase-advanced to the luciferase-reported circadian rhythms of *Per1* transcriptions (Yamaguchi and others 2001), it is likely that circadian oscillations in at least one known clock gene are under

the strong influence of circadian cytosolic Ca^{2+} rhythms.

4. Sedative nuclear Ca^{2+} levels in SCN neurons: a characteristic common to all clock cells?

One of the advantages of protein sensor-based Ca^{2+} imaging is that the sensor can be designed to travel into specific organelles by fusion of the sensor protein to organelle-transferring proteins. Using nucleus-targeted cameleon, nuclear Ca^{2+} dynamics were studied in SCN neurons (Ikeda and others 2003a). We found that nuclear Ca^{2+} is mobilized as well as cytosolic Ca^{2+} after pharmacological stimulation of NMDA receptors in SCN neurons, suggesting that the nuclear targeted YC2.1 is functional in SCN neurons. There is no evidence, however, of circadian rhythms in nuclear Ca^{2+} concentration. The nuclear Ca^{2+} concentration is steady at approximately 35 nM, suggesting a sedative nature of nuclear Ca^{2+} in SCN neurons. In general, nuclear Ca^{2+} levels have been described to parallel cytosolic Ca^{2+} levels, due to Ca^{2+} permeable pores and an absence of an active Ca^{2+} transport system on the nuclear membrane (Brini and others 1993, 1994). The compartmentalization of cytosolic and nuclear Ca^{2+} has been described in several cell types, however. For example, using nucleus-targeted and untargeted aequorin, Badminton and others (1995) demonstrated a smaller Ca^{2+} response in the nucleus than that in the cytosol of COS7 cells, suggesting "theoretical" nuclear Ca^{2+} barriers.

One possible mechanism underlying the reduced nuclear Ca^{2+} levels in SCN neurons may be Ca^{2+} buffering proteins, such as calbindin-D28k (CB) and calretinin (CR), that are expressed on SCN neurons in a species-specific manner (Silver and others 1996b, 1999; Mahoney and others 2000). The hypothalamic cultures used for

the cameleon Ca^{2+} imaging experiments had significant levels of CB and CR expression in the SCN (Ikeda and Allen 2003; Ikeda and others 2003a). Both of these Ca^{2+}-binding proteins have high affinity for Ca^{2+} (K_d = 300 nM for CB and 250 nM for CR; Cheung and others 1993) close to the range of circadian cytosolic Ca^{2+} variations in SCN neurons. The molecular sizes of CB and CR are small enough to distribute in the nucleus as well as in the cytosol in SCN neurons. Therefore, these Ca^{2+}-binding proteins may buffer both cytosolic and nuclear Ca^{2+}. More importantly, CB may not be evenly distributed inside the SCN neurons throughout the circadian cycles, because the number of nuclear CB-positive SCN neurons is greater during the subjective day than during the subjective night (Hamada and others 2003). Thus, it is likely that a Ca^{2+}-buffering system may contribute to the circadian dynamics of cytosolic Ca^{2+} concentrations and/or stabilization of nuclear Ca^{2+} levels in SCN neurons. A high-capacity Ca^{2+}-buffering system in SCN neurons may prevent undesirable phase shifts via general levels of neuronal excitation and contribute to the stabilization of circadian oscillations.

The intracellular Ca^{2+} dynamics described in tobacco seedlings by Wood and others (2001) are similar to the intracellular Ca^{2+} dynamics in SCN neurons; there are circadian rhythms in cytosolic Ca^{2+}, but not in nuclear Ca^{2+} concentration. Although Ca^{2+} homeostasis in plant cells is different from that in mammalian cells, the presence of circadian rhythms in cytosolic Ca^{2+} and a stable nuclear Ca^{2+} concentration across circadian cycles may be a common characteristic for a wide variety of cells with a critical role in circadian clock regulation.

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Figure legends

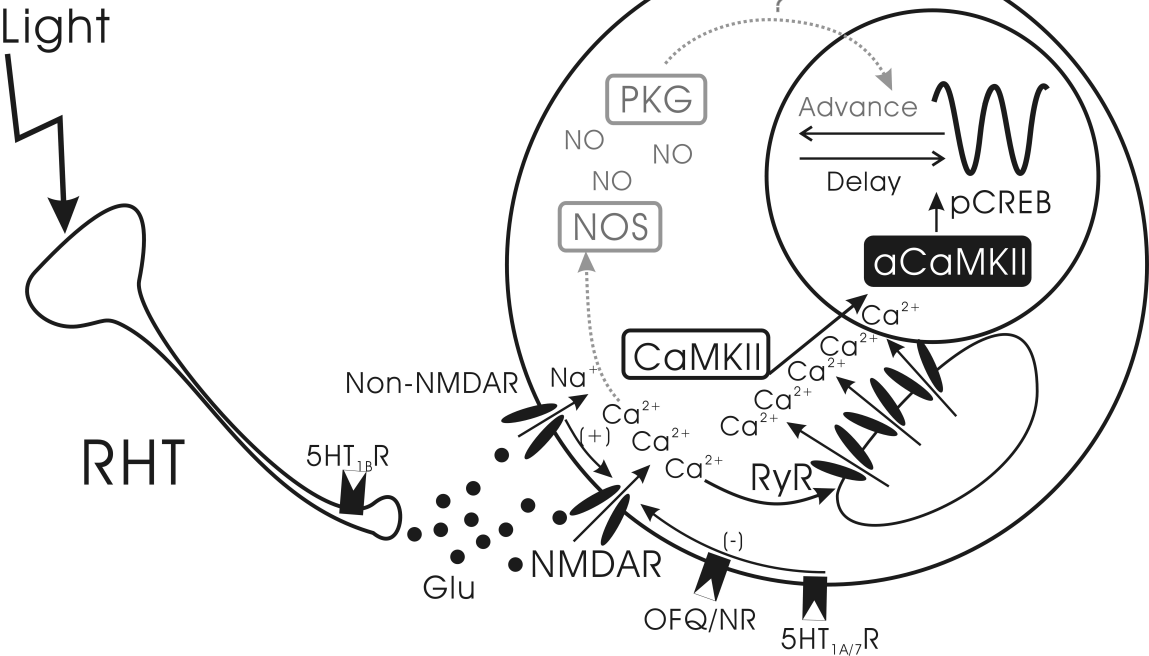
Figure 1. A schematic illustration of the cellular signaling processes underlying circadian phase-shifts (i.e., resetting) caused by nocturnal light. Environmental light stimulation of retinal ganglion cells activates SCN neurons via the glutamatergic retinohypothalamic tract (RHT). Glutamate (Glu) released at the pre-synaptic terminus may be negatively regulated by serotonin 1B receptors ($5HT_{1B}R$). Both AMPA/kinate glutamate receptors (non-NMDAR) and NMDA receptors (NMDAR) are expressed on SCN neurons, and Ca^{2+} influx through NMDAR may have a critical role in RHT-SCN transmission. NMDAR-mediated Ca^{2+} responses at the post-synaptic membrane are also negatively regulated by $5HT_{1A/7}R$ and orphanin-FQ/nociceptin receptors (OFQ/NR). The intracellular Ca^{2+} activation of ryanodine receptors (RyR) triggers further Ca^{2+} release from ryanodine-sensitive internal Ca^{2+} stores. This process may activate Ca^{2+}/calmodulin-dependent protein kinases II (CaMKII). The translocation of activated CaMKII (aCaMKII) or direct activation of CaMKII by nuclear Ca^{2+} mobilization triggers phosphorylation of the cAMP response element binding protein (CREB). Phosphorylated CREB (pCREB) is a potent transcription factor for the clock genes, such as *Per1*. Increase of cytosolic Ca^{2+} late in the subjective night may activate additional Ca^{2+}-dependent signaling cascades (grey) via nitric oxide synthase (NOS), nitric oxide (NO), and protein kinase G (PKG) that has been proposed to produce advance shifts in the circadian clock.

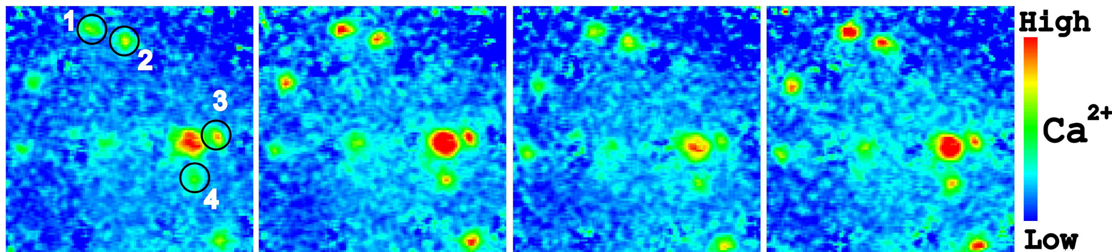
Figure 2. An example of a recording of the cytosolic Ca^{2+} concentration in an organotypic slice culture of SCN. Upper panels denote virtual color cytosolic Ca^{2+} levels. Yellow-green spots on the first panel indicate the approximate location of cell

bodies of SCN neurons that expressed cameleon Ca^{2+} sensors. Temporal changes in cytosolic Ca^{2+} levels in four neurons (circled in the top left panel) were plotted in the bottom panel. Synchronous circadian oscillations were observed in the cytosolic Ca^{2+} concentrations in these neurons, although the circadian phases among them were slightly advanced or delayed.

Figure 3. A. A schematic illustration of the cellular output processes for the autonomous circadian cycles of clock genes. The oscillations in gene transcription/translation cycles may generate circadian rhythms in cytosolic Ca^{2+} concentrations presumably via the effects on release of Ca^{2+} from ryanodine-sensitive internal Ca^{2+} stores. The hypothetical targets for clock gene regulation are located at ryanodine receptors (RyR) and mitochondria (Mt), which indirectly modulate RyR activity via cyclic ADP ribose (cADPR). The circadian rhythms in cytosolic Ca^{2+} concentrations may cause rhythmic neurotransmitter release and action potential firing rhythms in SCN neurons, although several other intermediate steps may be involved. **B.** The circadian cytosolic Ca^{2+} rhythms may be involved in the generation of gene transcriptional cycles in SCN neurons, because Ca^{2+} release from ryanodine-sensitive internal Ca^{2+} stores is a principal signaling process for the resetting of clock gene oscillations by nocturnal light (see Fig. 1 for details). This raises the possibility that circadian cytosolic Ca^{2+} rhythms may mediate not only the output signaling processes from the clock gene oscillations, but also the input signaling processes that generate clock gene oscillations in SCN neurons.

SCN neuron




1
2
3
4
High
Ca^{2+}
Low

Ratio (F535 nm / F480 nm)
3.0
2.9
2.8
2.7
2.6
2.5
2.4
2.3
2.2
0
720
1440
2160
2880
Time (min)
Neuron #1
Neuron #2
Neuron #3
Neuron #4

A

The clock gene oscillations

?

?

?

Mt

cADPR

Ca^{2+} Ca^{2+}

Ca^{2+} Ca^{2+}

Secretory rhythms

RyR

Action potential firing rhythms

B

The clock gene oscillations

pCREB

aCaMKII

CaMKII

Ca^{2+} Ca^{2+}

Ca^{2+} Ca^{2+}

RyR