Published in final edited form as:

J Neurophysiol. 2004 July ; 92(1): 311–319. doi:10.1152/jn.01078.2003.

Circadian Rhythm in Inhibitory Synaptic Transmission in the Mouse Suprachiasmatic Nucleus

Jason Itri, Stephan Michel, James A. Waschek, and Christopher S. Colwell

Mental Retardation Research Center, Department of Psychiatry and Biobehavioral Sciences, University of California, Los Angeles, California 90024-1759

Abstract

It is widely accepted that most suprachiasmatic nucleus (SCN) neurons express the neurotransmitter GABA and are likely to use this neurotransmitter to regulate excitability within the SCN. To evaluate the possibility that inhibitory synaptic transmission varies with a circadian rhythm within the mouse SCN, we used whole cell patch-clamp recording in an acute brain slice preparation to record GABA-mediated spontaneous inhibitory postsynaptic currents (sIPSCs). We found that the sIPSC frequency in the dorsal SCN (dSCN) exhibited a TTX-sensitive daily rhythm that peaked during the late day and early night in mice held in a light:dark cycle. We next evaluated whether vasoactive intestinal peptide (VIP) was responsible for the observed rhythm in IPSC frequency. Pretreatment of SCN slices with VPAC₁/VPAC₂- or VPAC₂-specific receptor antagonists prevented the increase in sIPSC frequency in the dSCN. The rhythm in sIPSC frequency was absent in VIP/peptide histidine isoleucine (PHI)-deficient mice. Finally, we were able to detect a rhythm in the frequency of inhibitory synaptic transmission in mice held in constant darkness that was also dependent on VIP and the VPAC₂ receptor. Overall, these data demonstrate that there is a circadian rhythm in GABAergic transmission in the dorsal region of the mouse SCN and that the VIP is required for expression of this rhythm.

Introduction

Most circadian rhythms in mammals are generated by a pair of nuclei in the anterior hypothalamus known as the suprachiasmatic nuclei (SCN). An understanding of the molecular machinery that drives circadian rhythmicity has been emerging rapidly, and it is believed to involve interacting positive and negative transcriptional feedback loops (Allada et al. 2001; Reppert and Weaver 2001). These molecular feedback loops function at the level of individual cells, and to date, most evidence suggests that single SCN neurons function as independent oscillators (Herzog and Schwartz 2002). This is not to imply that all SCN neurons are the same; in fact, a wide range of evidence is emerging for distinct cell populations within the SCN (e.g., Hamada et al. 2001; Kuhlman et al. 2003; Lee et al. 2003; Yan and Silver 2002). Anatomical evidence supports the broad division of the SCN into distinct core (ventrolateral) and shell (dorsomedial) subdivisions (Abrahamson and Moore 2001). Neurons in the core are innervated by visual inputs, and in many cases, express the neuropeptide vasoactive intestinal polypeptide (VIP). The mechanisms by which SCN neurons maintain synchrony with each other within a subdivision or between the two subdivisions are not yet known.

Most SCN neurons express the classical neurotransmitter GABA and are likely to use this neurotransmitter to regulate neuronal excitability and synchronization of spontaneous activity

within the nucleus. Glutamic acid decarboxylase (GAD), the enzyme responsible for synthesizing GABA, is found in nearly all neurons of the SCN (Moore and Speh 1993), while both GABA_A and GABA_B receptors have been identified in the SCN using autoradiographic and electrophysiological techniques (Francois-Bellan et al. 1989; Liou and Albers 1990). Electrophysiological analysis indicates that SCN neurons receive a tonic input of GABA_A-mediated postsynaptic currents that, at least partly, originate within the SCN itself (de Jeu and Pennartz 2002; Jiang et al. 1997; Kim and Dudek 1992; Strecker et al. 1997). Other sources of GABAergic activity include the contralateral SCN and other hypothalamic nuclei (e.g., Morin and Blanchard 2001; Saeb-Parsy et al. 2000). Although the effects of GABA on spontaneous firing are currently under debate, there is no question that this transmitter plays a critical role in regulating neuronal activity and excitability in the SCN (see de Jeu and Pennartz 2002; Gribkoff et al. 1999; Liu and Reppert 2000; Shimura et al. 2002; Shirakawa et al. 2000; Wagner et al. 1997). Importantly, it has been shown in culture that GABA, acting through the GABA_A receptor, can both phase-shift and synchronize the electrical activity of SCN neurons (Liu and Reppert 2000; Shirakawa et al. 2000; Tominaga et al. 1994). Thus the synaptic release of GABA is likely to play a critical role in the coupling of the neural activity of individual SCN oscillators.

In this study, whole cell patch electrophysiological techniques were utilized to record spontaneous inhibitory postsynaptic currents (sIPSCs) in SCN neurons. Comparisons were made between inhibitory currents recorded in the day and night as well as ventral and dorsal regions of the SCN. Next, the possible role of the neuropeptide vasoactive intestinal peptide (VIP) in driving a daily rhythm in sIPSC was examined. In addition, the possibility that VIP's actions on GABAergic sIPSCs are mediated by the cAMP/protein kinase A (PKA)-dependent pathway was evaluated. Finally, experiments determined whether any daily variation would remain when animals were held in constant darkness (DD), a hallmark feature of a circadian rhythm.

Methods

Animals and brain slice preparation

The UCLA Animal Research Committee approved the experimental protocols used in this study. Mice were received at 21 days of age and housed in light:dark (LD; 12:12) for ≥ 1 wk prior to performing electrophysiological experiments. Brain slices were prepared using standard techniques from C57 BL/6 and VIP/PHI-deficient mice back-crossed for eight generations between 3 and 8 wk of age. The VIP/PHI-deficient mice used in this study are described in detail by Colwell et al. (2003). Animals were killed by decapitation, and brains were dissected and placed in cold oxygenated artificial cerebral spinal fluid (ACSF) containing (in mM) 130 NaCl, 26 NaHCO₃, 3 KCl, 5 MgCl₂, 1.25 NaH₂PO₄, 1.0 CaCl₂, and 10 glucose (pH 7.2–7.4). After cutting slices (Microslicer, DSK Model 1500E) from areas to be analyzed, coronal sections (350 μ m) were placed in ACSF (25–27°C) for ≥ 1 h (in this solution, CaCl₂ is increased to 2 mM, MgCl₂ is decreased to 2 mM). Slices were constantly oxygenated with 95% O₂–5% CO₂ (pH 7.2–7.4, osmolality 290–300 mOsm).

Whole cell patch-clamp electrophysiology

Methods are similar to those described previously (Colwell 2001; Itri and Colwell 2003; Michel et al. 2002). Briefly, slices were placed in a recording chamber (PH-1, Warner Instruments) attached to the stage of a fixed-stage upright microscope equipped with infrared differential interference contrast (IR-DIC) optics and a camera. The standard solution in the patch pipette contained (in mM) 112.5 K-gluconate, 1 EGTA, 10 Hepes, 5 MgATP, 1 GTP, 0.1 leupeptin, 10 phosphocreatine, 4 NaCl, 17.5 KCl, 0.5 CaCl₂, and 1 MgCl₂. The pH was adjusted to 7.25–7.3 and the osmolality between 290–300 mOsm. Whole cell recordings were obtained with an

Axon Instruments 200B amplifier and monitored on-line with pCLAMP (Ver. 8.0, Axon Instruments). To minimize changes in offset potentials, the ground path used a KCl agar bridge to an Ag/AgCl ground well. Cells were approached with slight positive pressure (2–3 cm · H₂O), and offset potentials were corrected. After forming a high-resistance seal (>10 GΩ) by applying negative pressure, a second pulse of negative pressure was used to rupture the membrane. Data were not collected if access resistance was >40 MΩ or if the value changed significantly (>20%) during the course of the experiment. The standard extracellular solution used for all experiments was ACSF. Drug pretreatments were performed by dissolving antagonists in the ACSF used to incubate the slices for ≥1 h and continuing to bathe the slices with antagonist in the recording chamber throughout the experiment. Solution exchanges within the slice were achieved by a rapid gravity feed delivery system. In our system, the effects of bath applied drugs began within 15 s and were typically maximal by 3–5 min.

Spontaneous currents were recorded with pClamp in the gap-free mode and analyzed using MiniAnalysis software (Ver. 5.2.12, Synaptosoft). Events were detected using the following criteria: threshold, 5 pA; period to find local maximum, 5 ms; time before peak for baseline, 6 ms; period to search decay time, 50 ms; fraction of peak to find decay time, 0.38; period to average baseline, 2.5 ms; area threshold, 15; and detect complex peak enabled. Threshold was determined by measuring noise and setting the minimum threshold at 2 SD above the noise. Events were excluded if the decay time was <6 ms (Michel et al. 2002). The same criteria were used for evaluating the frequency of miniature IPSCs in the presence of TTX. IPSC frequency was determined by counting the number of events during a 1- to 2-min time bin and reporting this number as events per second or Hertz. Baseline frequency was determined 3 min after entering the whole cell configuration and treatment frequency was determined 5 min after the beginning of drug perfusion. All recordings were completed within 12 min to avoid cell dialysis (Schaap et al. 1999). Using these criteria in untreated cells, baseline sIPSC frequency typically did not vary significantly over the 12-min time span. Neurons that demonstrated at least a 5% change in the frequency of sIPSCs after drug treatment were considered “responders” and included in the appropriate treatment group.

Lighting conditions

Male mice, ≥21 days of age, were housed individually, and their wheel-running activity was recorded as revolutions per 3-min interval. The running wheels and data acquisition system were obtained from Mini Mitter (Bend, OR). To evaluate diurnal variations in sIPSCs and VIP-mediated modulation of these GABA currents, animals were killed ~1 h before the beginning of the phase of interest. When taking animals from the dark portion of the LD cycle, an infrared (IR) viewer was used to avoid light exposure. Zeitgeber time (ZT) is used to describe the projected time of the circadian clock within the SCN based on the previous light cycle, with lights-on defined as ZT 0. Data were pooled either in 1-h time bins (Fig. 2) or in groups to compare phases termed “day” (ZT 4–8), “early night” (ZT 13–15), and “late night” (ZT 20–22).

To evaluate the effects of constant darkness on IPSC frequency and VIP release, wheel-running activity was measured for ≥1 wk in LD to ensure that animals were entrained and running on wheels. Once a clear pattern of wheel-running activity was established in LD, the lights were discontinued, and the animals were maintained in the same light-tight chamber in DD for ≥4 days before death. Only animals maintaining a coherent wheel-running pattern in DD were used in electrophysiological experiments. The locomotor activity rhythms of mice were analyzed by generating actograms (El Temps, Barcelona, Spain) to determine the circadian phase, with the onset of activity defined as circadian time 12 (CT 12). Animals were removed during the appropriate phase and killed in complete darkness using IR viewers 1 h prior to

recording. Data for sIPSC frequency were acquired during the subjective day (CT 4–8), early subjective night (CT 13–15), and late subjective night (CT 20–22).

Statistics

Between-group differences were first evaluated using an ANOVA to determine if there were any significant differences among means of all groups. Post hoc pairwise comparisons were performed using *t*-test or Mann-Whitney rank sum tests when appropriate. Values were considered significant if $P < 0.05$. All tests were performed using SigmaStat or SigmaPlot (SPSS, Chicago, IL). In the text, values are shown as means $\pm$ SE. Each group of data are collected from ≥ 3 animals, with *n* representing the number of cells recorded.

Results

Characterization of GABA-mediated sIPSCs recorded in the SCN

We used the whole cell voltage-clamp technique to record sIPSCs from neurons in the SCN. sIPSCs were widespread in the SCN and could be detected from every neuron recorded ($n = 552$; Fig. 1). Each of these cells was determined to be within the SCN by directly visualizing the cell's location with IR-DIC videomicroscopy before any data were collected. In most cases, the IR-DIC video images were sufficient to identify a cell as being in either ventral or dorsal regions of the SCN (see Itri and Colwell 2003). Across all phases and regions and in the absence of drug treatments, the mean frequency and mean amplitude of GABA-mediated sIPSCs recorded in the SCN at a holding potential of -70 mV was 7.9 ± 0.4 (SE) events/s and 15.5 ± 0.8 pA, respectively. The time to rise and time to decay (latency of the inward current peak from the baseline) were 2.5 ± 0.1 and 15.4 ± 0.6 ms, respectively. In the whole cell voltage-clamp configuration, sIPSCs exhibited a reversal potential between -40 and -50 mV. The chloride equilibrium potential (E_{Cl}) set by the concentration of chloride in the bathing media and internal solution (see METHODS) was calculated to be -45.6 mV at 25°C , which is consistent with the reversal potential measured during whole cell recording. The reversal potential for sIPSCs stabilized within 3 min of entering the whole cell configuration and remained constant for the duration of the experiment. Subsequently, GABA-mediated sIPSCs appear as inward currents because the reversal potential for chloride was more positive than the holding potential. All sIPSCs were completely abolished with the GABA_A antagonist bicuculline ($25 \mu\text{M}$, 8/8 neurons tested), indicating that they were mediated by GABA_A receptors. In contrast, the sIPSCs were unaffected by the AMPA/KA glutamate receptor (GluR) antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; $20 \mu\text{M}$; 8/8 neurons tested; data not shown).

IPSC frequency in the dorsal SCN exhibited a TTX-sensitive daily rhythm

The next experiment was designed to determine whether sIPSCs recorded in SCN neurons show a daily rhythm in frequency. These experiments were performed with brain slices taken from animals at different phases during the LD cycle. Other than the time that the animals were killed, all conditions between the groups remained constant. The data for this experiment were collected between ZT 4–12 and ZT 13–22 and pooled into 1-h time bins. In the dorsal SCN (dSCN), the sIPSC frequency exhibited a daily rhythm that peaked during the late day (ZT 11–12) and remained significantly increased during the early night (ZT 13–15; Fig. 2). The frequency of GABA sIPSCs was significantly increased during ZT 11–12 (11.9 ± 1.5 Hz; $n = 14$; $P < 0.05$), ZT 13–14 (12.3 ± 1.9 ; $n = 11$; $P < 0.05$), and ZT 14–15 (11.9 ± 1.6 ; $n = 17$; $P < 0.05$) compared with all 1-h bins from ZT 4–11 and ZT 18–22. There were no significant differences in the amplitude, rise, or decay times of sIPSCs between any of the 1-h time bins.

When separated into three phases (day ZT 4–8, early night ZT 13–15, and late night ZT 20–22), the sIPSC frequency was significantly greater during early night (11.9 ± 1.2 Hz; $n = 28$) compared with day (7.7 ± 0.8 Hz; $n = 51$; $P < 0.01$) and late night (7.2 ± 1.0 Hz; $n = 14$; $P <$

0.01). These data confirm results reported in a previous study (Itri and Colwell 2003) focusing primarily on VIP-sensitive neurons. There was no significant daily rhythm in sIPSC frequency observed in the ventral SCN (vSCN) between the day (3.9 ± 0.6 Hz; $n = 21$), early night (4.0 ± 1.0 Hz; $n = 29$), or late night (3.0 ± 1.8 Hz; $n = 10$). A significant proportion of sIPSCs recorded in the dSCN were actively driven by action potential firing because the frequency of currents was significantly reduced by the application of the sodium (Na^+) channel blocker TTX ($1 \mu\text{M}$). Furthermore, action potential firing is required for expression of the daily rhythm in sIPSC frequency, because in the presence of TTX, the frequency of mIPSCs was not significantly different in the dSCN between day (4.0 ± 0.8 Hz; $n = 10$), early night (3.3 ± 0.7 Hz; $n = 13$), or late night (2.8 ± 0.8 Hz; $n = 7$). The amplitudes of GABA-mediated currents were not significantly affected by TTX (data not shown).

Rhythmicity of GABA-mediated IPSCs is dependent on VIP and the VPAC₂ receptor

Previous studies have shown that VIP is expressed in the SCN (Moore et al. 2002) and is a potent regulator of sIPSC frequency (Itri and Colwell 2003). The following experiments were designed to determine if the rhythm in sIPSC frequency is dependent on the neuropeptide VIP. Pretreatment with the VPAC₁/VPAC₂ receptor antagonist {[Tyr1,DPhe2]GHRF(1-29); 100 nM} significantly reduced the peak in GABA sIPSCs during the early night (7.9 ± 2.0 Hz; $n = 10$) compared with control (11.9 ± 1.2 Hz; $n = 28$) in dSCN neurons. Pretreatment with a VPAC₂ receptor-specific antagonist (PG 99-465; Moreno et al. 2000) also reduced the peak in sIPSC frequency observed during early night (200 nM; 7.9 ± 2.3 Hz; $n = 8$) compared with control (11.9 ± 1.2 Hz; $n = 28$; Fig. 3). Pretreatment with either antagonist during the day had no significant effect on the frequency of GABA-mediated sIPSCs (VPAC₁/VPAC₂ antagonist: 7.1 ± 2.3 Hz; $n = 7$; VPAC₂ receptor-specific antagonist: 7.6 ± 2.3 Hz; $n = 13$) compared with control (7.7 ± 0.8 Hz; $n = 51$) in dSCN neurons. Additionally, pretreatment with PG 99-465 during late night had no significant effect on the frequency of GABA-mediated sIPSCs (VPAC₂ receptor-specific antagonist: 6.8 ± 1.3 Hz; $n = 9$; data not shown in figure) compared with control (7.1 ± 1.0 Hz; $n = 14$) in dSCN neurons.

Next, we determined whether the frequency of sIPSCs recorded in dSCN neurons varied with the phase of the daily cycle in VIP/PHI-deficient mice (Colwell et al. 2003). The data were collected at various phases of the animal's daily cycle corresponding to ZT 4–8, ZT 13–15, and ZT 20–22 as in the experiments referenced above. In contrast to wild-type animals, the sIPSC frequency in VIP/PHI-deficient animals remained relatively constant throughout the day (7.4 ± 1.9 Hz; $n = 6$), early night (7.7 ± 1.2 Hz; $n = 13$), and late night (7.6 ± 1.0 Hz; $n = 6$). Together, data from the receptor antagonists and VIP/PHI-deficient mice strongly indicate that VIP, acting through the VPAC₂ receptor, is responsible for the rise in sIPSC frequency observed in the early night.

We also characterized the sensitivity of SCN neurons to exogenous VIP during various phases of the animal's daily cycle. In control animals, the magnitude of VIP-mediated enhancement of sIPSC frequency was phase-dependent (Fig. 4). During the day, application of VIP (100 nM) increased the frequency of sIPSCs by $40 \pm 7\%$ ($n = 7$). In contrast, the same VIP treatment increased the frequency of sIPSCs $16 \pm 5\%$ ($n = 6$) during the early night and $15 \pm 3\%$ ($n = 6$) during the late night. The effect of VIP on sIPSC frequency was significantly greater ($P < 0.05$) during the day than either early or late night. Similar experiments performed in VIP/PHI-deficient animals revealed that, in the absence of endogenous VIP, there was no longer a phase-dependent effect of exogenous VIP on GABA-mediated sIPSCs (Fig. 4). Before VIP treatment, the frequency of sIPSCs in VIP/PHI-deficient animals during the day (7.4 ± 1.9 , $n = 6$) was indistinguishable from the frequency measured during the early night (7.2 ± 1.6 , $n = 6$) and the late night (7.6 ± 1.0 , $n = 6$). Application of VIP (100 nM) increased the frequency of sIPSCs by $49 \pm 10\%$ (11.0 ± 1.2 , $n = 6$) during the day, $51 \pm 9\%$ (10.9 ± 2.3 , $n = 6$) during the early

night, and $51 \pm 5\%$ (11.5 ± 1.8 , $n = 6$) during the late night. These data show that VIP/PHI-deficient mice are sensitive to VIP treatment in a manner similar to wild-type animals treated during the day when VIP levels are lowest. The effect of VIP was long-lasting and did not wash out after 30 min of drug-free ACSF ($n = 10$).

Rhythmicity in GABA-mediated sIPSCs is dependent on the cAMP/PKA pathway

Previous studies have found that VIP acting through VPAC₂ receptors is positively coupled to the cAMP/PKA secondary messenger system (Harmar et al. 1998; Nowak and Kuba 2002). We used agonists and antagonists of PKA to determine if the cAMP/PKA system was necessary for the observed peak in GABA-mediated IPSCs during the early night (Fig. 5). Indeed, pretreatment of dSCN neurons with the PKA inhibitor H-89 (20 μ M) completely blocked the increase in GABA sIPSCs during the early night (7.3 ± 1.7 Hz; $n = 7$) compared with control (11.9 ± 1.2 Hz; $n = 28$). The frequency of GABA sIPSCs did not change significantly in response to H-89 (20 μ M) during the day (7.3 ± 1.8 Hz; $n = 7$). Furthermore, pretreatment of the slice with forskolin (5 μ M), a potent activator of PKA, enhanced the frequency of sIPSCs both during the day (13.0 ± 2.2 Hz; $n = 7$) and the early night (13.9 ± 2.7 Hz; $n = 7$) compared with control. Neither PKA modulator significantly affected the amplitude, rise time, or decay time of GABA sIPSCs (data not shown). Overall, the rhythm in sIPSCs was abolished by both an inhibitor and activator of PKA, strongly implicating the cAMP/PKA system in the expression of this daily rhythm.

Circadian rhythm in GABA IPSCs in animals housed under constant conditions

Temporal profiles of VIP release examined in organotypic SCN slice cultures over a 2-day period revealed a circadian rhythm in VIP release from the ventrolateral region (Shinohara et al. 1994). Since the daily rhythm in GABAergic sIPSCs is driven by VIP in LD, it is possible that rhythmic VIP release in DD continues to drive a circadian rhythm in GABAergic sIPSCs in the dSCN. For these experiments, mice were placed in DD, and wheel-running activity rhythms were recorded. The data for sIPSC frequency were collected between CT 4–8 (subjective day), CT 13–15 (early subjective night), and CT 20–22 (late subjective night). Although a similar trend in sIPSC frequency existed in animals housed in DD, the clear peak previously observed in LD during the day (ZT 4–8) was no longer present in DD (Fig. 6). Unlike under LD conditions, the GABA frequency measured in the dSCN during subjective day (9.5 ± 1.2 Hz; $n = 23$) was not significantly different from the early subjective night (10.3 ± 1.4 Hz; $n = 25$). However, the frequency of sIPSCs was significantly lower during the late subjective night (6.3 ± 1.0 Hz; $n = 21$; $P < 0.05$) compared with subjective day and early night, indicating that a rhythm in GABA sIPSCs remained under DD conditions. Pretreatment of dSCN neurons with a VPAC₂ receptor-specific antagonist (PG-465 200 nM) blocked the rhythm in GABA currents by reducing the frequency of GABAergic sIPSCs during the subjective day (7.4 ± 2.0 Hz; $n = 9$) and the early subjective night (6.9 ± 0.8 Hz; $n = 8$) compared with the late subjective night (6.7 ± 1.1 Hz; $n = 8$; Fig. 6). As observed under LD conditions, VIP/PHI-deficient mice housed in DD did not exhibit a rhythm in sIPSC frequency between subjective day (8.2 ± 1.8 Hz; $n = 9$), early subjective night (7.7 ± 1.9 Hz; $n = 10$), or late subjective night (7.3 ± 1.0 Hz; $n = 8$; data not shown in figure). These data confirm that the rhythm in GABA-mediated sIPSCs is circadian and that, as observed in LD, this rhythm is dependent on VIP.

Sensitivity to VIP was also altered in animals housed in DD. As described above, in mice held in LD, the magnitude of VIP-mediated enhancement of GABA frequency in the dSCN was phase-dependent, with peak responses recorded during the day. In the absence of light, however, there was no longer a phase-dependent effect of VIP on GABA-mediated sIPSCs. Application of VIP (100 nM) increased the frequency of sIPSCs by $23 \pm 5\%$ ($n = 6$) during

subjective day, $23 \pm 7\%$ ($n = 6$) during the early subjective night, and $25 \pm 3\%$ ($n = 6$) during the late subjective night.

Discussion

In this study, we used whole cell patch-clamp techniques to characterize GABA_A-mediated sIPSCs from neurons in both the dorsal and ventral subdivisions of the SCN. The sIPSC frequency was consistently higher in the dorsal subdivision compared with the ventral subdivision, although every neuron exhibited sIPSCs. The sIPSC frequency measured in the dSCN varied significantly with the daily cycle and exhibited a peak during ZT 11–15, spanning across late day and early night. In contrast, sIPSC amplitude, rise time, or decay time did not vary between SCN subdivisions or with the daily cycle. The rhythm in sIPSCs was dependent on synaptic activity because it was not seen in the presence of a blocker of voltage-dependent sodium channels (TTX). We used various manipulations of the VIP signaling system and its associated secondary messenger ensemble cAMP/PKA to conclusively show that the rhythm in GABAergic activity was dependent on endogenous VIP release. To determine if the day-night difference in sIPSC frequency was circadian, we examined GABA-mediated sIPSCs in mice housed in DD. Under these conditions, we were unable to detect a significant peak in sIPSC frequency during the early subjective night. However, the frequency of GABA sIPSCs during the late subjective night (CT 20–22) was significantly reduced compared with the other times (CT 4–8 and CT 13–15), indicating that a rhythm in GABA sIPSCs persisted under DD conditions. Additionally, day-night differences in GABA sIPSCs were absent in VIP/PHI-deficient mice housed under both LD and DD cycles.

SCN neurons are well known to exhibit a daily rhythm in electrical activity and membrane conductance with peak electrical activity occurring during the subjective day (Schaap et al. 2003). The neurotransmitter GABA is frequently thought of as an inhibitory neurotransmitter in the adult mammalian nervous system, activating chloride-permeable GABA_A ion channels that serve to block action potential generation by either hyper-polarizing the membrane or acting as a transmembrane shunt to prevent rapid depolarization. Our observation that all SCN neurons in this study were under tonic GABAergic influence would suggest that this transmitter plays a significant role in regulating spontaneous firing and excitability during all phases of the circadian cycle. Recently, several groups have suggested that GABA may have an excitatory role in the adult SCN (de Jeu and Pennartz 2002; Wagner et al. 1997, 2001). One possible explanation is that the chloride equilibrium potential shifts with the 24-h cycle and with development in SCN neurons (Shimura et al. 2002). This issue is as yet unsettled, with one group reporting that GABA is excitatory only during the subjective day (Wagner et al. 1997), another concluding that GABA is excitatory only during the subjective night (de Jeu and Pennartz 2002), and yet a third group stating that GABA is inhibitory regardless of the phase (Gribkoff et al. 1999, 2003). In any case, the daily rhythm in GABAergic tone observed in the dSCN undoubtedly impacts both spontaneous electrical activity as well as how these cells respond to excitatory synaptic inputs.

Although the preparation used in this study does not allow us to identify the source of GABAergic input while recording, previous anatomical and electrophysiological studies have described various sources of GABAergic inputs onto SCN neurons. Several electrophysiological studies have observed that SCN neurons receive a tonic level of GABA_A-mediated postsynaptic currents, while focal stimulation in the vicinity of recorded neurons reveal that nearly all SCN neurons receive local or extranuclear GABAergic inputs (de Jeu and Pennartz 2002; Jiang et al. 1997; Kim and Dudek 1992; Strecker et al. 1997). Other proposed sources of GABAergic input include retinal ganglion cells directly innervating the SCN (Jiao and Rusak 2003), the contralateral SCN (Buijs et al. 1994), arcuate nucleus, supraoptic nucleus, and intergeniculate leaflet (Morin and Blanchard 2001; Saeb-Parsy et al.

2000). The observed peak in GABA currents during the early night is TTX-dependent, indicating that neuronal activity is driving the circadian rhythm in inhibitory transmission in the dSCN. The fact that the day-night difference persists in an acute slice preparation suggests that GABAergic input to the dSCN is mediated either by active GABAergic neurons within the SCN or at least within the brain slice preparation.

It has been proposed that electrical signals generated by core SCN neurons (vSCN), which are adjusted to the LD cycle by retinal inputs, are transmitted to the shell region of the SCN (dSCN) through monosynaptic connections (van Esseveldt et al. 2000). Output from the shell region is synchronized by virtue of direct connectivity to the core region and modified by cortical inputs. However, if sIPSCs recorded from dSCN neurons are driven by neuronal activity within the SCN, a seemingly contradictory situation arises in that the highest level of activity-driven GABAergic transmission in the dSCN occurs during the early night, a time period when the spontaneous firing rate of SCN neurons is significantly reduced (Schaap et al. 2003). However, recent work suggests that the SCN is a heterogeneous cell population (Karatsoreos et al. 2004) and that some cells exhibit peak activity during the night. A recent study using time-lapse imaging of a green fluorescent protein (GFP) reporter of the clock gene *Period 1* (*Per1*) found a positive linear correlation between neuronal spike frequency and *Per1* transcription, indicating that *Per1* rhythms are representative of physiological activity (Quintero et al. 2003). Importantly, it was also noted that a small proportion of the sampled neurons cycled in antiphase to the principal phase peak, raising the possibility that a subgroup of SCN neurons acts as inhibitory interneurons within the SCN. Thus we speculate that the dSCN neurons are receiving rhythmic inhibitory input from a distinct population of SCN interneurons that are electrically silent during the day, but highly active during the early night.

We have previously demonstrated that VIP is a potent regulator of sIPSC frequency in SCN neurons (Itri and Colwell 2003), and in this study, sought to determine the role of the endogenous peptide in driving a circadian rhythm in inhibitory synaptic transmission. An important difference between this study and the previous study (Itri and Colwell 2003) describing the effects of VIP on GABA-mediated sIPSCs is that, previously, VIP was bath-applied to the slice to demonstrate that VIP is capable of enhancing GABA release in the SCN. In this study, we manipulated the endogenous VIP signal to determine if we could prevent the peak in GABA-mediated sIPSC frequency, providing a physiologically relevant measure of VIPs effect in the SCN. We found that pretreatment of SCN slices with a VPAC₁/VPAC₂ antagonist prevented the increase of IPSC frequency during the early night in the dSCN, as did pretreatment with the VPAC₂-specific receptor antagonist PG 99-465. Our interpretation is that VIP release is initiated in anticipation of the transition from light to dark (ZT 11-12) and continues to be released during the early night (ZT 13-15), signaling the onset of darkness by presynaptically enhancing GABA release onto dSCN neurons. This is supported by the observation that VIP content in the SCN decreases monotonically in animals maintained in illumination and that light pulses given during the night are more effective at suppressing VIP levels than during the day (Shinohara et al. 1999). By blocking the receptors for VIP (VPAC₂ receptors), we effectively prevent the endogenous VIP signal from reaching GABAergic terminals synapsing on dSCN neurons. Since the activated VPAC₂ receptor utilizes the cAMP/PKA second messenger system to enhance GABA release (Itri and Colwell 2003), we concluded that blocking these intermediates would also prevent the peak in GABA sIPSCs observed during the early night. Pretreatment of SCN slices during the early night with the PKA inhibitor H-89 completely blocked the nocturnal increase in sIPSC frequency. Similar experiments performed in transgenic VIP/PHI-deficient animals housed under an LD cycle revealed that, in the absence of endogenous VIP, the daily rhythm in inhibitory synaptic transmission was nonexistent. Thus using a variety of manipulations, our data strongly suggest that the peak in GABA frequency during the early night in an LD cycle is dependent on the VIP signaling system and associated cAMP/PKA second messenger pathway.

Temporal profiles of VIP release reveal a circadian rhythm that is maintained in organotypic SCN slice culture, suggesting that rhythmic VIP release may drive a circadian rhythm in GABAergic sIPSCs in the dSCN under constant conditions (Shinohara et al. 1994). We examined sIPSCs in control mice housed in DD, and under these conditions, we were unable to detect a significant peak in sIPSC frequency during the early subjective night. The frequency of GABA sIPSCs between subjective day (CT 4–8) and early subjective night (CT 13–15) were nearly indistinguishable. However, we found that the frequency of GABA sIPSCs was significantly reduced during the late subjective night (CT 20–22) compared with the rest of the cycle in DD, indicating that the rhythm in GABA sIPSCs was sustained in DD but different from what was predicted from the data observed in a LD cycle. The correlation between rhythmic VIP release and rhythmic sIPSC frequency suggests that changes in VIP release underlie the differences in sIPSC frequency observed during the late subjective night compared with subjective day and early subjective night. Based on these observations, we believe that VIP plays a critical role in driving the daily rhythm in GABA frequency under both LD and DD conditions.

It has been suggested that GABA can modulate light- and *N*-methyl-D-aspartate (NMDA)-induced phase-shifts by direct regulation of excitability in SCN neurons (Gillespie et al. 1997; Mintz et al. 2002). By modulating the frequency of GABA release in the SCN, VIP may be an important mediator in the neuronal pathway responsible for light-induced phase-shifting in mammals. Indeed, behavioral experiments performed in heterozygous and homozygous VIP/PHI-deficient mice indicate that both maintenance of a daily rhythm in wheel-running activity and phase-shifting of this daily rhythm are significantly affected in animals with a reduction or absence of endogenous VIP (Colwell et al. 2003). VIP has a fundamental functional role in biological clock function (Hannibal and Fahrenkrug 2003; Harmar 2003), which may manifest through regulation of GABA release in the dorsal cell population. It has also been shown that daily treatments with GABA are sufficient to synchronize electrical activity in SCN cell populations in culture (Liu and Reppert 2000; Shirakawa et al. 2000). In fact, it has been surmised that circadian fluctuations in GABA release within the SCN may be important for synchronizing clock cells with widely different phases and period lengths (Liu and Reppert 2000). In this study, we have identified a circadian rhythm in GABAergic tone in the dorsal SCN that persists in DD and is driven by the endogenous VIP/VPAC₂ signaling system.

Acknowledgements

We thank Drs. Robberecht for kindly supplying the VPAC₂ receptor antagonist PG 99-465 and N. Wayne for comments on the manuscript.

Grants: This study was supported by National Institutes of Health Grants HL-64582, NS-043169, DA-05010, and MH-68087.

References

- Abrahamson EE, Moore RY. Suprachiasmatic nucleus in the mouse: retinal innervation, intrinsic organization and efferent projections. *Brain Res* 2001;916:172–191. [PubMed: 11597605]
- Allada R, Emery P, Takahashi JS, Rosbash M. Stopping time: the genetics of fly and mouse circadian clocks. *Annu Rev Neurosci* 2001;24:1091–1119. [PubMed: 11520929]
- Buijs RM, Hou YX, Shinn S, Renaud LP. Ultrastructural evidence for intra- and extranuclear projections of GABAergic neurons of the suprachiasmatic nucleus. *J Comp Neurol* 1994;340:381–391. [PubMed: 8188857]
- Colwell CS. NMDA-evoked calcium transients and currents in the suprachiasmatic nucleus: gating by the circadian system. *Eur J Neurosci* 2001;13:1420–1428. [PubMed: 11298803]

- Colwell CS, Michel S, Itri J, Rodriguez W, Tam J, Lelievre V, Hu Z, Liu X, Waschek JA. Disrupted circadian rhythms in VIP and PHI deficient mice. *Am J Physiol Regul Integr Comp Physiol* 2003;285:R939–R949. [PubMed: 12855416]
- de Jeu M, Pennartz C. Circadian modulation of GABA function in the rat suprachiasmatic nucleus: excitatory effects during the night phase. *J Neurophysiol* 2002;87:834–844. [PubMed: 11826050]
- Francois-Bellan AM, Segu L, Hery M. Regulation by estradiol of GABA_A and GABA_B binding sites in the diencephalon of the rat: an autoradiographic study. *Brain Res* 1989;503:144–147. [PubMed: 2558776]
- Gillespie CF, Mintz EM, Marvel CL, Huhman KL, Albers HE. GABA(A) and GABA(B) agonists and antagonists alter the phase-shifting effects of light when microinjected into the suprachiasmatic region. *Brain Res* 1997;759:181–189. [PubMed: 9221935]
- Gribkoff VK, Pieschl RL, Dudek FE. GABA receptor-mediated inhibition of neuronal activity in rat SCN in vitro: pharmacology and influence of circadian phase. *J Neurophysiol* 2003;90:1438–1448. [PubMed: 12750413]
- Gribkoff VK, Pieschl RL, Wisialowski TA, Park WK, Strecker GJ, de Jeu MT, Pennartz CM, Dudek FE. A reexamination of the role of GABA in the mammalian suprachiasmatic nucleus. *J Biol Rhythms* 1999;14:126–130. [PubMed: 10194649]
- Hamada T, LeSauter J, Venuti JM, Silver R. Expression of period genes: rhythmic and nonrhythmic compartments of the suprachiasmatic nucleus pacemaker. *J Neurosci* 2001;21:7742–7750. [PubMed: 11567064]
- Hannibal J, Fahrenkrug J. Circadian rhythm regulation: a central role for the neuropeptide vasoactive intestinal polypeptide. *Am J Physiol Regul Integr Comp Physiol* 2003;285:R935–R936. [PubMed: 14557231]
- Harmar AJ. An essential role for peptidergic signalling in the control of circadian rhythms in the suprachiasmatic nuclei. *J Neuroendocrinol* 2003;4:335–338. [PubMed: 12622830]
- Harmar AJ, Arimura A, Gozes I, Journot L, Laburthe M, Pisegna JR, Rawlings SR, Robberecht P, Said SI, Sreedharan SP, Wank SA, Waschek JA. International Union of Pharmacology. XVIII. Nomenclature of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. *Pharmacol Rev* 1998;50:265–270. [PubMed: 9647867]
- Herzog ED, Schwartz WJ. A neural clockwork for encoding circadian time. *J Appl Physiol* 2002;92:401–408. [PubMed: 11744683]
- Itri J, Colwell CS. Regulation of inhibitory synaptic transmission by vasoactive intestinal peptide (VIP) in the mouse suprachiasmatic nucleus. *J Neurophysiol* 2003;90:1589–1597. [PubMed: 12966176]
- Jiang ZG, Yang Y, Liu ZP, Allen CN. Membrane properties and synaptic inputs of suprachiasmatic nucleus neurons in rat brain slices. *J Physiol* 1997;499:141–159. [PubMed: 9061646]
- Jiao YY, Rusak B. Electrophysiology of optic nerve input to suprachiasmatic nucleus neurons in rats and degus. *Brain Res* 2003;960:142–151. [PubMed: 12505666]
- Karatsoreos IN, Yan L, LeSauter J, Silver R. Phenotype matters: identification of light-responsive cells in the mouse suprachiasmatic nucleus. *J Neurosci* 2004;24:68–75. [PubMed: 14715939]
- Kim YI, Dudek FE. Intracellular electrophysiological study of suprachiasmatic nucleus neurons in rodents: inhibitory synaptic mechanisms. *J Physiol* 1992;458:247–260. [PubMed: 1302267]
- Kuhlman SJ, Silver R, Le Sauter J, Bult-Ito A, McMahon DG. Phase resetting light pulses induce Per1 and persistent spike activity in a subpopulation of biological clock neurons. *J Neurosci* 2003;23:1441–1450. [PubMed: 12598633]
- Lee HS, Nelms JL, Nguyen M, Silver R, Lehman MN. The eye is necessary for a circadian rhythm in the suprachiasmatic nucleus. *Nat Neurosci* 2003;6:111–112. [PubMed: 12536213]
- Liou SY, Albers HE. Single unit response of neurons within the hamster suprachiasmatic nucleus to GABA and low chloride perfusate during the day and night. *Brain Res Bull* 1990;25:93–98. [PubMed: 2207720]
- Liu C, Reppert SM. GABA synchronizes clock cells within the suprachiasmatic circadian clock. *Neuron* 2000;25:123–128. [PubMed: 10707977]
- Michel S, Itri J, Colwell CS. Excitatory mechanisms in the suprachiasmatic nucleus: the role of AMPA/KA glutamate receptors. *J Neurophysiol* 2002;88:817–828. [PubMed: 12163533]

- Mintz EM, Jasnow AM, Gillespie CF, Huhman KL, Albers HE. GABA interacts with photic signaling in the suprachiasmatic nucleus to regulate circadian phase shifts. *Neuroscience* 2002;109:773–778. [PubMed: 11927159]
- Moore RY, Speh JC. GABA is the principal neurotransmitter of the circadian system. *Neurosci Lett* 1993;150:112–116. [PubMed: 8097023]
- Moore RY, Speh JC, Leak RK. Suprachiasmatic nucleus organization. *Cell Tissue Res* 2002;309:89–98. [PubMed: 12111539]
- Moreno D, Gourlet P, De Neef P, Cnudde J, Waelbroeck M, Robberecht P. Development of selective agonists and antagonists for the human vasoactive intestinal polypeptide VPAC(2) receptor. *Peptides* 2000;21:1543–1549. [PubMed: 11068102]
- Morin LP, Blanchard JH. Neuromodulator content of hamster intergeniculate leaflet neurons and their projection to the suprachiasmatic nucleus or visual midbrain. *J Comp Neurol* 2001;437:79–90. [PubMed: 11477598]
- Nowak JZ, Kuba K. Pituitary adenylate cyclase-activating polypeptide and vasoactive intestinal peptide-stimulated cyclic AMP synthesis in rat cerebral cortical slices: interaction with noradrenaline, adrenaline, and forskolin. *J Mol Neurosci* 2002;18:47–52. [PubMed: 11931349]
- Quintero JE, Kuhlman SJ, McMahon DG. The biological clock nucleus: a multiphasic oscillator network regulated by light. *J Neurosci* 2003;23:8070–8076. [PubMed: 12954869]
- Reppert SM, Weaver DR. Molecular analysis of mammalian circadian rhythms. *Annu Rev Physiol* 2001;63:647–676. [PubMed: 11181971]
- Saeb-Parsy K, Lombardelli S, Khan FZ, McDowall K, Au-Yong IT, Dyball RE. Neural connections of hypothalamic neuroendocrine nuclei in the rat. *J Neuroendocrinol* 2000;12:635–648. [PubMed: 10849208]
- Schaap J, Bos NP, de Jeu MT, Geurtsen AM, Meijer JH, Pennartz CM. Neurons of the rat suprachiasmatic nucleus show a circadian rhythm in membrane properties that is lost during prolonged whole-cell recording. *Brain Res* 1999;815:154–166. [PubMed: 9974136]
- Schaap J, Pennartz CM, Meijer JH. Electrophysiology of the circadian pacemaker in mammals. *Chronobiol Int* 2003;20:171–188. [PubMed: 12723879]
- Shimura M, Akaike N, Harata N. Circadian rhythm in intracellular Cl^- activity of acutely dissociated neurons of suprachiasmatic nucleus. *Am J Physiol Cell Physiol* 2002;282:C366–C373. [PubMed: 11788348]
- Shinohara K, Honma S, Katsuno Y, Abe H, Honma K. Circadian rhythms in the release of vasoactive intestinal polypeptide and arginine-vasopressin in organotypic slice culture of rat suprachiasmatic nucleus. *Neurosci Lett* 1994;170:183–186. [PubMed: 8041503]
- Shinohara K, Tominaga K, Inouye ST. Phase dependent response of vasoactive intestinal polypeptide to light and darkness in the suprachiasmatic nucleus. *Neurosci Res* 1999;33:105–110. [PubMed: 10211775]
- Shirakawa T, Honma S, Katsuno Y, Oguchi H, Honma KI. Synchronization of circadian firing rhythms in cultured rat suprachiasmatic neurons. *Eur J Neurosci* 2000;12:2833–2838. [PubMed: 10971625]
- Strecker GJ, Wuarin PJ, Dudek FE. GABA_A -mediated local synaptic pathways connect neurons in the rat suprachiasmatic nucleus. *J Neurophysiol* 1997;78:2217–2220. [PubMed: 9325388]
- Tominaga K, Shibata S, Hamada T, Watanabe S. GABA_A receptor agonist muscimol can reset the phase of neural activity rhythm in the rat suprachiasmatic nucleus in vitro. *Neurosci Lett* 1994;166:81–84. [PubMed: 8190363]
- van Esseveldt KE, Lehman MN, Boer GJ. The suprachiasmatic nucleus and the circadian time-keeping system revisited. *Brain Res Rev* 2000;33:34–77. [PubMed: 10967353]
- Wagner S, Castel M, Gainer H, Yarom Y. GABA in the mammalian suprachiasmatic nucleus and its role in diurnal rhythmicity. *Nature* 1997;387:598–603. [PubMed: 9177347]
- Wagner S, Sagiv N, Yarom Y. GABA-induced current and circadian regulation of chloride in neurones of the rat suprachiasmatic nucleus. *J Physiol* 2001;537:853–869. [PubMed: 11744760]
- Yan L, Silver R. Differential induction and localization of mPer1 and mPer2 during advancing and delaying phase shifts. *Eur J Neurosci* 2002;16:1531–1540. [PubMed: 12405967]

Fig. 1.

GABAergic spontaneous inhibitory postsynaptic currents (sIPSCs) recorded from neurons located in the dorsal suprachiasmatic nucleus (dSCN). *A*: sIPSCs recorded from a dSCN neuron during the early night (ZT 13–15, *top*) are reduced in frequency but not in amplitude when treated with TTX (*bottom*). Frequency was determined by counting the number of events during a 1- to 2-min time bin and comparing the frequency before and after treatments. *B*: amplitude, rise time, and decay time measurements were recorded for each event. There were no significant differences in the amplitude, rise, or decay times when comparing sIPSCs before (*left*) and after (*right*) TTX application.

Fig. 2. GABAergic sIPSC frequency peaks between ZT 11 and ZT 15 in mice held under 12-h light:dark (LD) conditions. Each data point represents the average frequency of GABAergic sIPSCs during a 1-h time bin $\pm$ SE ($n = 8-17/\text{bin}$).

Fig. 3.

Vasoactive intestinal peptide (VIP) and the VPAC₂ receptor are necessary for the peak in GABA sIPSCs observed during the early night. Pretreatment of dSCN neurons with a VPAC₂ receptor-specific antagonist (PG-465; 200 nM; $n = 23$) blocked the peak in GABA sIPSCs during the early night (ZT 13–15) while having no effect during the day (ZT 4–8). The frequency of GABA sIPSCs in dSCN neurons of VIP/PHI-deficient mice (VIP KO) did not exhibit a daily rhythm that peaked during the early night.

Fig. 4.

The phase-dependent effect of VIP on the frequency of GABAergic sIPSCs in control animals was absent in VIP/PHI-deficient animals. The effect of VIP (100 nM) on sIPSC frequency of dSCN neurons was significantly greater ($P < 0.05$) during the day (ZT 4–8) than during either the early night (ZT 13–15) or the late night (ZT 20–22). In VIP/PHI-deficient animals, VIP significantly increased the frequency of GABA IPSCs by the same magnitude during all phases.

Fig. 5.

The cAMP/protein kinase A (PKA) signaling system is necessary for the peak in GABA sIPSCs observed during the early night. Pretreatment of dSCN neurons with a PKA inhibitor (H-89; 20 μ M, $n = 14$) blocked the peak in GABA sIPSCs during the early night (ZT 13–15) while having no effect during the day (ZT 4–8). Pretreatment of dSCN neurons with a potent activator of PKA (forskolin; 5 μ M) increased the frequency of GABA sIPSCs during both day (13.0 Hz, $n = 7$) and early night (13.9 Hz, $n = 7$), effectively abolishing any significant difference between these phases.

Fig. 6.

Circadian modulation of GABA-mediated IPSC frequency. GABA frequency measured in dSCN neurons during the subjective day (CT 4–8; 9.5 Hz; $n = 23$) was not significantly different from the early subjective night (CT 13–15; 10.3 Hz; $n = 25$). However, frequency of GABA sIPSCs was significantly lower during the late subjective night (CT 20–22; 6.3 Hz; $n = 21$) compared with subjective day ($P < 0.05$) and early subjective night ($P < 0.05$), indicating that a rhythm in GABA currents persists in DD. Pretreatment of dSCN neurons with a VPAC₂ receptor-specific antagonist (PG-465; 200 nM) blocked the rhythm in GABA currents by reducing the frequency of GABAergic sIPSCs during subjective day (7.4 Hz; $n = 9$) and early subjective night (6.9 Hz; $n = 8$) compared with late subjective night (6.7 Hz; $n = 8$).