Published in final edited form as:

Eur J Neurosci. 2010 November ; 32(9): 1497–1506. doi:10.1111/j.1460-9568.2010.07411.x.

Neuropeptide mediated calcium signaling in the suprachiasmatic nucleus network

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Abstract

Neuroactive peptides and the intracellular calcium concentration ($[Ca^{2+}]_i$) play important roles in light-induced modulation of gene expression in the suprachiasmatic nucleus (SCN) neurons that ultimately control behavioral rhythms. Vasoactive intestinal peptide (VIP) and arginine vasopressin (AVP) are expressed rhythmically within populations of SCN neurons. Pituitary adenylate cyclase-activating peptide (PACAP) is released from retinohypothalamic tract (RHT) terminals synapsing on SCN neurons. Nociceptin/orphanin FQ (OFQ) receptors are functionally expressed in the SCN. We examined the role of several neuropeptides on Ca^{2+} signaling, simultaneously imaging multiple neurons within the SCN neural network. VIP reduced the $[Ca^{2+}]_i$ in populations of SCN neurons during the day, but had little effect at night. Stimulation of the RHT at frequencies that simulate light input signaling evoked transient $[Ca^{2+}]_i$ elevations that were not altered by VIP. AVP elevated the $[Ca^{2+}]_i$ during both the day and night, PACAP produced variable responses, and OFQ induced a reduction in the $[Ca^{2+}]_i$ similar to VIP. During the day, VIP lowered the $[Ca^{2+}]_i$ to near nighttime levels while AVP elevated $[Ca^{2+}]_i$ during both the day and night, suggesting that the VIP effects on $[Ca^{2+}]_i$ were dependent, and the AVP effects independent of the action potential firing activity state of the neuron. We hypothesize that VIP and AVP regulate, at least in part, Ca^{2+} homeostasis in SCN neurons and may be a major point of regulation for SCN neuronal synchronization.

Keywords

Circadian Rhythm; Peptides; Vasoactive intestinal peptide; Vasopressin; Retinohypothalamic tract

Introduction

Several neuropeptides including VIP, AVP, PACAP, and OFQ have been shown to play important roles in the regulation of circadian clock function and entrainment (Piggins *et al.*, 1996; Maywood *et al.*, 2007; Miyakawa *et al.*, 2007; Morin, 2007; Butler & Silver, 2009; Welsh *et al.*, 2010). Calcium is important in the signal transduction pathways mediating photic entrainment and an essential component of the feedback loops that generate circadian rhythms (Albrecht *et al.*, 1997; Shearman *et al.*, 1997; Shigeyoshi *et al.*, 1997; Morris *et al.*, 1998; Lundkvist & Block, 2005; Lundkvist *et al.*, 2005). Yet currently little is known of the role that Ca^{2+} plays in the neuropeptide regulation of the circadian clock.

The effect of neuropeptides on neuronal $[Ca^{2+}]_i$, typically involve G-protein coupled receptors resulting in alterations of the membrane potential or second messenger-mediated release from intracellular stores (Maywood *et al.*, 2006; Pakhotin *et al.*, 2006; O'Neill *et al.*, 2008). Disruption of VIP signaling alters the action potential firing of SCN neurons, clock gene expression, behavioral circadian rhythms, and can be rescued by application of VIP or the VPAC2 receptor agonist RO 25-1553 (Aton *et al.*, 2005; Maywood *et al.*, 2006; Pakhotin *et al.*, 2006; Brown *et al.*, 2007). The role of Ca^{2+} in VIP-mediated signaling in SCN neurons is currently unknown. Vasopressin is rhythmically released in the SCN with a peak during the day (Yamase *et al.*, 1991; Tominaga *et al.*, 1992; Nakamura *et al.*, 2001), which can be phase shifted by VIP (Watanabe *et al.*, 2000), and may play a role in the generation of circadian rhythms (Gouzenes *et al.*, 1999; Kuhlman *et al.*, 2003; Li *et al.*, 2009). Vasopressin agonists increase $[Ca^{2+}]_i$ in neurons of the supraoptic nucleus, an effect that was attenuated by IP_3 -receptor blockers and PLC and PKA inhibitors, suggesting that both phospholipase C and adenylate cyclase pathways are involved (Sabatier *et al.*, 2004). PACAP is localized with glutamate from RHT terminals (Hannibal *et al.*, 2000; Hannibal *et al.*, 2002). PACAP application produces phase delays early in the night and phase advances late in the night similar to a light pulse (Harrington *et al.*, 1999; Piggins *et al.*, 2001). PACAP potentiates the increased $[Ca^{2+}]_i$ produced by AMPA, but not NMDA receptor activation in populations of SCN neurons (Kopp *et al.*, 2001; Dziema & Obrietan, 2002). Activation of OFQ receptors in the SCN reduces action potential firing, phase shifts the circadian clock, and down regulates mPER2 (Anton *et al.*, 1996; Allen *et al.*, 1999; Neal *et al.*, 1999a; Teshima *et al.*, 2005; Miyakawa *et al.*, 2007). OFQ decreases calcium and increases a potassium conductance in multiple brain regions including the SCN (Allen *et al.*, 1999; Meis, 2003; Gompf *et al.*, 2005).

To clarify the role that Ca^{2+} plays in the neuropeptide regulation within the SCN network, we recorded Ca^{2+} from multiple neurons simultaneously examining the actions of VIP, AVP, PACAP, and OFQ on Ca^{2+} signaling.

Methods

Tissue preparation

Hypothalamic slices were prepared, imaged and test agents applied using previously published methods (Irwin & Allen, 2009). Briefly, male Sprague-Dawley rats (Charles River, Wilmington MA) were housed for at least 1 week on a 12:12-hr light:dark schedule. During the light phase, 4 to 6 week old rats were anesthetized with isoflurane (Novaplus, UK), their brains removed, and coronal hypothalamic slices (250 μ m) containing the SCN were cut with a vibrating blade microtome (Leica VT1000S, Nussloch, Germany), while the tissue was surrounded by ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 120, KCl 2.5, NaH_2PO_4 1.2, $MgCl_2$ 5, $CaCl_2$ 0.5, glucose 10, $NaHCO_3$ 26, adjusted to 300 mOsm with sucrose and saturated with 5% CO_2 and 95% O_2 . The Institutional Animal Care and Use Committee of OHSU approved, in advance, all procedures involving animals.

Calcium Imaging

Multiple SCN neurons maintained in hypothalamic slices were loaded with the Ca^{2+} -sensitive probe fura-2 AM for the simultaneous recording of Ca^{2+} responses using previously published methods (Irwin & Allen, 2009). Briefly, freshly prepared SCN slices were treated with fura-2 AM (50 μ M) for 1 – 2 minutes (Invitrogen, CA; from stock 3.3 mM in DMSO) followed by incubation with fura-2 AM (10 – 25 μ M) in ACSF for 1–2 hours (Irwin & Allen, 2009). The slices were washed for at least 30 min prior to recording to allow for fura-2-AM deesterification and maintained in a recording chamber (35°C) with a continuous laminar flow (1 – 2 ml/min) of an ACSF solution consisting of (in mM): NaCl

120, KCl 2.5, NaH₂PO₄ 1.2, MgCl₂ 1.2, CaCl₂ 2.4, glucose 10, HEPES 10, NaHCO₃ 26; adjusted to 300 mOsm and bubbled with 5% CO₂ and 95% O₂ (Fig. 1). Calcium measurements were obtained by recording a pair of images at excitation wavelengths of 340 and 380 nm supplied via a monochromator (Polychrome IV; Till Photonics, Martinsried, Germany) passing through a UG11 optical filter to restrict harmonic wavelengths above 400 nm, reflected via a 400 nm DCLP dichroic mirror, and through a Leica 40x/0.80 UV water immersion objective. Emitted light passed through a 510 ± 40 nm filter (Chroma Technology Corp, Rockingham, VT) and was recorded with a cooled charge-coupled device camera (CCD, ORCA-ER 12 bit level, Hamamatsu, Japan) with acquisition time and binning adjusted to minimize photobleaching and maximize recording speed via the digital imaging software Metafluor (Molecular Devices Corporation, Sunnyvale, CA). Optical data was converted to relative fluorescence intensity units for each cell. Data was presented as the emission following excitation at 340 and 380 nm and background subtraction at each wavelength as previously described (Irwin & Allen, 2009). Neurons were identified by an increase in the Ca²⁺ ratio in response to NMDA (200 μM for 5 sec). Cells that did not respond to NMDA or were dim were excluded. The Ca²⁺ response (an increase, decrease or no effect) to VIP, AVP or OFQ was defined by a ratio change ≥2 standard deviations from the mean of 6 – 11 baseline Ca²⁺ ratios recorded prior to the peptide application. The magnitude of the Ca²⁺ response to peptides having prolonged changes in Ca²⁺ was determined from the mean of six Ca²⁺ ratios at the apparent maximal response for each experiment. GABA, VIP(6-28) and PACAP produced transient neuronal Ca²⁺ responses, and were coded as an increase, decrease or no response by visual inspection. The magnitude of the transient Ca²⁺ response was determined as the maximum (or minimum for Ca²⁺ decreases) response within the first 30 sec of application. The SCN neurons were localized to the dorsomedial (DM) and ventrolateral (VL) regions of the SCN as previously described (Irwin & Allen, 2009).

Chemicals

VIP, VIP(6-28), [Arg8]-Vasopressin, PACAP(1-38), GABA, and NMDA were obtained from Tocris Bioscience (Ellisville, MO). Fura-2 AM was purchased from Invitrogen (Carlsbad, California). Other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Data and statistical analysis

Igor Pro (Version 6.0.4, Wavemetrics, Lake Oswego, OR, USA), Statview 5.0.1 (SAS Institute, Cary, NC, USA) and Excel 11.5.1 (Microsoft, Redmond, WA) were used for curve fitting and data analysis. Unless otherwise stated data are presented as the mean ± S.E.M. and t-tests (two tailed). A *p* level of ≤0.05 was used to determine statistical significance.

Results

VIP reduces Ca²⁺ in SCN neurons during the day but not the night

The role of VIP in regulating Ca²⁺ was examined by simultaneously imaging the mean Ca²⁺ ratio of multiple neurons before and following VIP application during the day and night and at several locations within the SCN (Fig. 2). During the day VIP (250 nM) reduced the Ca²⁺ ratio [VIP(Ca²⁺)] in 27% of neurons (ZT5-8 hours, *n* = 96), while VIP (1 μM) reduced the Ca²⁺ ratio in 54% of neurons (ZT3-9 hours, *n* = 152 neurons). The proportion of neurons responding to 1 μM VIP was significantly greater than at 250 nM (*z* = 4.2, *p* < 0.0001). Regression analysis of the baseline Ca²⁺ ratio in VIP(Ca²⁺) neurons during the day plotted versus the change in the Ca²⁺ ratio induced by VIP (1 μM), results in a slope of -0.50 ± 0.07 (*R*² = 0.41, *n* = 82, *p* < 0.0001) (Fig. 2C), suggesting that neurons with higher Ca²⁺ ratios and presumably faster action potentials firing frequencies, have a greater VIP-induced reduction of Ca²⁺ than quiescent neurons with low Ca²⁺ baseline ratios (Irwin & Allen,

2007;2009). In contrast, but consistent with this hypothesis, at night, VIP (1 μM) reduced the Ca^{2+} ratio in only 7% of SCN neurons (ZT: 15–18 hours, $n = 121$ neurons) (Fig. 2A&B). The few neurons observed at night with a VIP(Ca^-) response had a higher mean baseline Ca^{2+} ratio (1.30 ± 0.06 , $n = 8$, $\Delta = -0.12 \pm 0.04$ ratio units) that differed significantly from the mean baseline Ca^{2+} ratio in neurons that did not respond to VIP [VIP($\text{Ca}0$)] (1.16 ± 0.03 , $n = 105$, $\Delta 0.001 \pm 0.005$; $t_{12} = 2.12$, $p = 0.028$ one-tail with unequal variances). The Ca^{2+} ratio in 64% of neurons was not altered by treatment with VIP (250 nM) during the day. Similarly, VIP (1 μM) did not change the Ca^{2+} ratio of 39% of SCN neurons recorded during the day nor 87% during the night. VIP elevated the Ca^{2+} ratio in a small subset of neurons during both the day (9% at 250 nM; 7% at 1 μM) and night (7% at 1 μM).

In SCN neurons, the mean baseline Ca^{2+} ratio is higher during the day than the night (Colwell, 2000; Ikeda *et al.*, 2003; Irwin & Allen, 2007; 2009). During the day, VIP (1 μM) lowered Ca^{2+} to near baseline nighttime levels (Fig. 2A). The reduction of the Ca^{2+} ratio in VIP(Ca^-) neurons during the day was larger at 1 μM than at 250 nM (0.35 ± 0.029 vs. 0.25 ± 0.038 ratio units respectively, $t_{106} = 1.75$, $p = 0.042$ one-tail). These data demonstrate that while VIP can lower $[\text{Ca}^{2+}]_i$ during both the day and night a much greater proportion of VIP(Ca^-) responding neurons were observed during the day when the mean baseline $[\text{Ca}^{2+}]_i$ is higher.

Localization of VIP-induced Ca^{2+} response

We examined whether the VIP induced Ca^{2+} changes differed regionally within the SCN during the day and night. The location of each VIP(Ca^-) and VIP($\text{Ca}0$) neuron was superimposed over a representative drawing of the SCN (Fig. 2D). VIP(Ca^-) responding neurons demonstrated no clear regional pattern.

VIP modulation of RHT signaling in the SCN network

Since VIP reduced the daytime baseline $[\text{Ca}^{2+}]_i$, it may also play an important role in modulating light-responsive retinal ganglion cell signaling via the RHT to the SCN. Electrical stimulation of the RHT evokes divergent Ca^{2+} responses in SCN neurons that were dependent on the action potential firing frequency and modulation by endogenous GABA_A activity (Irwin & Allen, 2007; 2009). Stimulation of the RHT via a bipolar electrode (100 pulses at 20 Hz) placed in the optic chiasm induced transient Ca^{2+} elevations, reductions or no responses in the postsynaptic SCN neurons (Fig. 3A). Application of VIP (1 μM) did not alter the magnitude of the Ca^{2+} elevations induced by RHT stimulation during the day or the night.

During the day when $[\text{Ca}^{2+}]_i$ is generally high and neurons are rapidly firing, a RHT-induced reduction of $[\text{Ca}^{2+}]_i$ reflects an activation of GABA_A ergic neurons and subsequent release of GABA; while at night when baseline $[\text{Ca}^{2+}]_i$ is low, a RHT-induced reduction of the $[\text{Ca}^{2+}]_i$ occurs in only a small number of neurons, suggesting that $[\text{Ca}^{2+}]_i$ cannot be reduced further (Irwin & Allen, 2009). Therefore, since VIP reduced the baseline Ca^{2+} ratios during the day to near nighttime levels, the daytime RHT-evoked Ca^{2+} reductions were also reduced (Fig. 3B); at night the baseline Ca^{2+} ratios were generally low and VIP had little effect on RHT-evoked Ca^{2+} reductions. These data suggest that, while VIP induces a reduction of $[\text{Ca}^{2+}]_i$, it does not impair the ability of RHT input to elevate $[\text{Ca}^{2+}]_i$ in SCN neurons.

The role of GABA_A receptor activation in the VIP-induced reduction of Ca^{2+}

VIP increases the frequency of GABA-mediated synaptic currents (Itri & Colwell, 2003; Itri *et al.*, 2004). Additionally, GABA_A receptor activation can either raise or lower $[\text{Ca}^{2+}]_i$ in SCN neurons (Choi *et al.*, 2008; Irwin & Allen, 2009). We therefore examined the

relationship between VIP- and GABA-induced Ca^{2+} responses in 122 SCN neurons. No association was observed between VIP responsive (VIP(Ca⁺), $n = 71$) and non-responsive (VIP(Ca⁰), $n = 51$) neurons, in relation to GABA- (200 μM , 10 sec) -evoked Ca^{2+} ratio increases (GABA(Ca⁺), $n = 47$), decreases (GABA(Ca⁻), $n = 45$) or no responses (GABA(Ca⁰), $n = 30$; $\chi^2 = 0.23$, $p = 0.89$). Since GABA_A antagonists can alter baseline $[\text{Ca}^{2+}]_i$ (Irwin & Allen, 2009) and VIP(Ca⁻) responses are dependent on baseline $[\text{Ca}^{2+}]_i$ (Fig. 2C), experiments with GABA_A antagonists were not performed. These data suggest that the VIP-induced reduction of $[\text{Ca}^{2+}]_i$ is not mediated via GABA activity.

A VIP antagonist, VIP(6-28) modifies $[\text{Ca}^{2+}]_i$ in SCN neurons

Since VIP reduced $[\text{Ca}^{2+}]_i$ in SCN neurons during the day, we tested the hypothesis that endogenous VIP facilitates the $[\text{Ca}^{2+}]_i$ reduction observed during the night using the VIP antagonist VIP(6-28). Application of VIP(6-28) (2 – 4 μM , ZT = 14 – 18 hrs, $n = 53$) induced a variety of Ca^{2+} responses in SCN neurons including transient and sustained elevations (41.5%), reductions (18.9%), and no responses (39.6%) (Fig. 4). VIP(6-28) elevated the Ca^{2+} ratio in the majority (~69%, $n = 32$) of responding neurons. Further, VIP(6-28) evoked larger Ca^{2+} elevations than reductions (Fig. 4B). During the day (ZT = 4–6 hrs, $n = 33$), the pattern of VIP(6-28) induced Ca^{2+} responses was similar to that observed during the night (39.4% elevations, 12.1% reductions, 48.5% no response; $\chi^2 = 0.96$, $p = 0.62$). The magnitude of the VIP(6-28) evoked Ca^{2+} excursions were similar during the day and night (elevation day 0.31 ± 0.05 , $n = 13$, night 0.30 ± 0.06 , $n = 22$, $t_{33} = 0.11$, $p = 0.91$; reduction day 0.11 ± 0.07 , $n = 4$, night 0.15 ± 0.02 , $n = 10$; $t_{12} = 0.74$, $p = 0.47$) (Fig. 4B). These data suggest that VIP may have a role in lowering $[\text{Ca}^{2+}]_i$ during the night.

AVP induces elevations of Ca^{2+} during the day and night in SCN neurons

The effect of AVP on $[\text{Ca}^{2+}]_i$ of SCN neurons was evaluated with applications of AVP (1 μM) during the day and night (Fig. 5). During the day, AVP elevated the Ca^{2+} ratio [AVP(Ca⁺)] in 74%, generated no response [AVP(Ca⁰)] in 22% and lowered the Ca^{2+} ratio [AVP(Ca⁻)] in 4% of SCN neurons ($n = 72$). Similarly, during the night the $[\text{Ca}^{2+}]_i$ was increased in 75% and generated no response in 25% of SCN neurons ($n = 73$). While the baseline Ca^{2+} ratios of AVP(Ca⁺) neurons differed between the day (1.41 ± 0.05 , $n = 53$) and night (1.11 ± 0.42 , $n = 55$, $t_{104} = 4.78$, $p < 0.0001$), the magnitudes of the Ca^{2+} elevations were similar (day 0.28 ± 0.03 , $n = 53$; night 0.31 ± 0.04 , $n = 55$, $t_{106} = 0.54$, $p = 0.59$). Further, blocking action potential firing with tetrodotoxin (TTX; 0.5 μM) did not block the AVP-induced Ca^{2+} elevation (Fig. 5B). The AVP-induced change in $[\text{Ca}^{2+}]_i$ was slow to develop with a mean time to peak ($\pm$ SEM) of 27.0 ± 1.2 seconds.

While AVP induced $[\text{Ca}^{2+}]_i$ elevations during both day and night, VIP-induced $[\text{Ca}^{2+}]_i$ reductions almost exclusively during the day. There was however an association between responses to AVP and VIP ($\chi^2 = 17.5$, $n = 66$, $p < 0.0001$) during the day in SCN neurons. In 41% of neurons where AVP induced a Ca^{2+} ratio elevation ($n = 51$), VIP induced a Ca^{2+} ratio reduction ($n = 21$). And 87% of neurons that did not respond to AVP during the day ($n = 15$) also did not respond to VIP ($n = 13$). The baseline Ca^{2+} ratio of AVP(Ca⁺) responding neurons showed little correlation to the magnitude of the AVP-induced change in the Ca^{2+} ratio during the day ($R^2 = 0.03$, $n = 53$, $p = 0.24$) or night ($R^2 = 0.09$, $n = 55$, $p = 0.03$). These data suggest that the Ca^{2+} response to AVP was independent of the firing activity state of the SCN neuron, and that AVP(Ca⁺) sensitive neurons were more likely to be located in the dorsomedial region (Fig. 5C).

The modulation of SCN neuronal $[\text{Ca}^{2+}]_i$ by PACAP

PACAP is thought to be co-released with glutamate from RHT terminals (Hannibal *et al.*, 2000; Hannibal & Fahrenkrug, 2004). PACAP(1-38) applied to SCN neurons during the

day, activated a variety of transient changes in $[Ca^{2+}]_i$ without obvious alteration of RHT stimulation-induced transients (Fig. 6). PACAP (250 nM) elevated the Ca^{2+} ratio in 45.7%, reduced the Ca^{2+} ratio in 10.6% and had no change of the Ca^{2+} ratio in 43.6% of 94 neurons. PACAP (500 nM) induced similar ($\chi^2 = 2.12$, $p = 0.35$) changes, with an elevation of Ca^{2+} in 35.6%, a reduction in 16.9% and no-response in 47.5% of 59 neurons. Note that PACAP induced a response in only about 50% of neurons, and that reapplication often did not evoke subsequent Ca^{2+} transients (data not shown). Consistent with previous studies (Kopp *et al.*, 2001; Dziema & Obrietan, 2002; Michel *et al.*, 2006), these data suggest that PACAP acts to modulate the response of SCN neurons to input pathways.

The modulation of SCN neuronal $[Ca^{2+}]_i$ by OFQ

Orphanin-FQ (OFQ) (1 μ M) produced the most dramatic and prolonged effect on the Ca^{2+} ratio of SCN neurons during the day, reducing the Ca^{2+} ratio in 83% of the neurons tested ($n = 29$; Fig. 7A). In these neurons, the magnitude of the OFQ-induced reduction (-0.45 ± 0.08 ratio units) correlated linearly with the baseline Ca^{2+} ratio (1.63 ± 0.09 ratio units, $n = 24$, slope = -0.83 , $R^2 = 0.82$, $p < 0.0001$) (Fig. 7B). Non-responding neurons already had lower baseline ratios (0.98 ± 0.13 , $n = 5$). Unlike VIP, the OFQ effect was extremely long lasting and did not wash out during the remainder of the recording session. Five minutes after OFQ application, the Ca^{2+} ratio remained low and even continued to slightly decline (-0.03 ± 0.01 ratio units, $t = 2.84$, $p = 0.0094$). These data demonstrated that OFQ induces a robust and prolonged reduction of $[Ca^{2+}]_i$ and suggests, that like VIP, this reduction is dependent on the action potential firing activity state of the SCN neuron.

Discussion

Neuroactive peptides are released from distinct neuronal populations and act on selective receptors to activate a variety of intracellular signaling pathways that ultimately may alter gene transcription (Maywood *et al.*, 2006; Maywood *et al.*, 2007; Pfeffer *et al.*, 2009). Changes of $[Ca^{2+}]_i$ are linked to alterations of gene expression by neurotransmission and is one signal that may mediate the actions of some neuropeptides. Neuropeptides may change the $[Ca^{2+}]_i$ by multiple mechanisms, including opening Ca^{2+} permeable channels, membrane hyperpolarization and depolarization with activation of voltage-gated Ca^{2+} channels, and sequestration or release of Ca^{2+} from intracellular stores. Neuroactive peptides present in the SCN were found to have distinct effects on $[Ca^{2+}]_i$. VIP and OFQ produced a greater reduction of $[Ca^{2+}]_i$ in neurons with an elevated baseline $[Ca^{2+}]_i$, such as occurs during the day and is consistent with a reduction of the action potential firing frequency. While VIP appears to reduce the activity of SCN neurons, the Ca^{2+} transient increases induced by RHT stimulations were not altered, suggesting that an SCN neuron “quieted” by VIP can still respond to photic input as occurs following light applied during the dark period. Conversely, AVP slowly elevated the $[Ca^{2+}]_i$ without an apparent dependence on the firing activity state of the neuron. PACAP application resulted in transient and variable effects on $[Ca^{2+}]_i$, conceivably this peptide may be acting as a modulator of glutamate co-released from RHT terminals in the SCN. Calcium perturbations resulting from voltage-(firing activity) dependent processes and from intracellular stores can produce both transient or prolonged changes of $[Ca^{2+}]_i$. However, in general voltage-dependent processes are more rapid and Ca^{2+} release from intracellular stores more gradual, yet both appear to make contributions to the overall circadian oscillation of $[Ca^{2+}]_i$ (Colwell, 2000; Ikeda, 2004; Irwin & Allen, 2007; 2009). We hypothesize that induction of Ca^{2+} -mediated transcriptional and translational changes to the clock are dependent on the activity state of the neuron and its ability to rapidly alter $[Ca^{2+}]_i$ and are facilitated by neuropeptide modulation of both external and internal sources of Ca^{2+} .

VIP has significant effects on the circadian clock and the activity of SCN neurons (Cutler *et al.*, 2003; Pakhotin *et al.*, 2006). VIP acting on the VPAC2 receptor synchronizes the circadian clocks of individual SCN neurons (Cutler *et al.*, 2003; Piggins & Cutler, 2003; Aton *et al.*, 2005). In smooth muscle, VIP has been thought to reduce Ca^{2+} via membrane hyperpolarization, sequestration into intracellular stores, and activation of G-proteins (Ohta *et al.*, 1991; Kawasaki *et al.*, 1997). VIP reduced the $[\text{Ca}^{2+}]_i$ in populations of SCN neurons during the day but has little effect at night. The day-night difference may reflect the action potential firing activity of SCN neurons at different parts of the circadian cycle. The magnitude of the Ca^{2+} change was dependent on the baseline $[\text{Ca}^{2+}]_i$, such that VIP (and OFQ) induced large reductions in Ca^{2+} in neurons with high baseline $[\text{Ca}^{2+}]_i$, as occurs in fast firing neurons, and had little or no effect on neurons with low baseline $[\text{Ca}^{2+}]_i$, associated with low firing rates. Blocking action potentials with TTX similarly reduces $[\text{Ca}^{2+}]_i$ in more neurons during the day than the night (Colwell, 2000; Irwin & Allen, 2009). Our findings are consistent with the observation that VIP reduces the action potential firing of SCN neurons by inhibiting voltage-dependent Na^+ and K^+ channels (Pakhotin *et al.*, 2006). Since the firing activity of an SCN neuron contributes to the overall $[\text{Ca}^{2+}]_i$ (Irwin & Allen, 2007; 2009), these data suggest that VIP alters the physiological action potential firing activity of the neuron to reduce the $[\text{Ca}^{2+}]_i$.

A key question is whether VIP is contributing to the lower $[\text{Ca}^{2+}]_i$ and action potential firing rates observed during the night. VIP is released rhythmically in the SCN with higher levels at night than the day (Morin *et al.*, 1993; Shinohara & Inouye, 1995; Nakamura *et al.*, 2001; Aton & Herzog, 2005), and stimulated release of VIP in rat hypothalamus occurs only during the night (Nicholson *et al.*, 1983). We found that the VIP antagonist VIP(6-28) induced transient elevations of $[\text{Ca}^{2+}]_i$ in a proportion of SCN neurons during the night, suggesting a potential role for VIP activity in regulation of the SCN firing activity. However, while VIP(6-28) is frequently used as a VIP antagonist, it is only moderately effective even at large concentrations, may not be a competitive antagonist and may interact with other receptors (Markos *et al.*, 2002). While these data suggest that VIP may have a role in lowering the $[\text{Ca}^{2+}]_i$ of SCN neurons during the night, highly competitive antagonists are needed to confirm this mechanism.

While VIP expression is localized heavily in the ventral part of the SCN (Belenky *et al.*, 2008), we did not observe regional differences in the VIP-induced reductions in $[\text{Ca}^{2+}]_i$. This is consistent with the wide distribution of VPAC2 expression, including about half of AVP-positive neurons (Kallo *et al.*, 2004b) in the SCN, and consistent with a role of VIP in SCN synchronization. The SCN neurons with higher levels of $[\text{Ca}^{2+}]_i$ that did not respond to VIP, may represent neurons without VIP receptors, different VPAC2 receptor signaling pathways, quiescent neurons with high $[\text{Ca}^{2+}]_i$ due release from intracellular stores, or other mechanisms (Kallo *et al.*, 2004b; Irwin & Allen, 2007; 2009).

Since VIP reduces the excitability of SCN neurons, and VIP release, VIP mRNA, and VPAC2 expression are higher during the night (Albers *et al.*, 1990; Rea, 1990; Morin *et al.*, 1993; Shinohara *et al.*, 1994; Shinohara *et al.*, 1999), an important question was whether light-induced glutamate release from RGC terminals in the SCN would be less likely to trigger the post-synaptic action potentials required to phase shift the clock. Stimulating the RHT, evokes divergent Ca^{2+} responses in neurons within the SCN (Irwin & Allen, 2009). VIP did not alter the magnitude of RHT-evoked Ca^{2+} elevations during either the day or night. These data suggest a model in which VIP reduces the firing of populations of SCN neurons, without altering the ability of the glutamatergic RHT input to elicit action potentials and elevate $[\text{Ca}^{2+}]_i$. This may be important in the ability for light to induce phase changes during the night.

GABA- and VIP- expressing neurons in the SCN network are known to frequently interconnect and colocalize (Francois-Bellan *et al.*, 1990; Francois-Bellan & Bosler, 1992; Buijs *et al.*, 1995; Castel & Morris, 2000). VIP regulates GABA release in the SCN and modulates SCN GABAergic signaling to subparaventricular zone neurons (Itri & Colwell, 2003; Itri *et al.*, 2004; Hermes *et al.*, 2009). Conceivably VIP might reduce the $[Ca^{2+}]_i$ by evoking GABA release and lowering excitability in SCN neurons. However, GABA may lower or elevate $[Ca^{2+}]_i$ in SCN neurons (Choi *et al.*, 2008; Irwin & Allen, 2009). We found no association between the VIP and GABA activity, and observed that VIP-induced reductions of Ca^{2+} occurred in many neurons where GABA-induced an elevation (Fig. 3A), suggesting that GABA does not mediate the VIP-induced reductions of $[Ca^{2+}]_i$.

V_{1a} and V_{1b} -type AVP receptors are expressed in the SCN and couple to G_q -type G proteins, which activates phospholipase C activity and subsequently increases inositol-1,4,5 trisphosphate-induced Ca^{2+} release from intracellular stores and diacylglycerol-mediated activation of protein kinase C (Majewski & Iannazzo, 1998; Birnbaumer, 2000). While the rhythmic release of AVP by SCN neurons peaks during the day (Yamase *et al.*, 1991; Tominaga *et al.*, 1992), the expression of V_{1a} -type receptor mRNA, but not the V_{1b} -type, peaks during the night (Young *et al.*, 1993; Kalamatianos *et al.*, 2004). We found that AVP can induce elevations of $[Ca^{2+}]_i$ in the majority of SCN neurons during both the day and night. We observed a lag of approximately 30 sec from the onset of AVP application to the maximal response, suggesting that the increased Ca^{2+} was not a result of the rapid opening of voltage dependent Ca^{2+} channels, but rather required second messenger-mediated process likely involving at least in part Ca^{2+} release from intracellular stores. During the night AVP evoked similar elevations of Ca^{2+} in the presence of TTX, further suggesting a mechanism of Ca^{2+} release from intracellular stores. However, the closing of G protein-coupled inwardly-rectifying potassium channels inducing membrane depolarization may also make a contribution to the rise in $[Ca^{2+}]_i$ (Zhang *et al.*, 2006). In the supraoptic nucleus $[Ca^{2+}]_i$ is elevated by a V_{1a} -type AVP receptor agonist and attenuated by blocking IP_3 receptors. Similarly, Ca^{2+} increases induced by V_{1a} - and V_{2-} type, but not V_{1b} -type, AVP receptor agonists were attenuated by PLC and PKA inhibitors, suggesting that both phospholipase C and adenylate cyclase pathways are involved (Gouzenes *et al.*, 1999; Sabatier *et al.*, 2004). This supports our observations that AVP elevates $[Ca^{2+}]_i$ in SCN neurons during both the day and night. Further, the regional location of AVP-sensitive SCN neurons appearing to overlap regions with known AVP immunoreactivity (Yamase *et al.*, 1991; Moore *et al.*, 2002) and the slightly higher dorsomedial expression of V_{1a} receptor mRNA (Young *et al.*, 1993; Kalamatianos *et al.*, 2004) suggest that AVP may be autoregulating its own release.

In addition to VIP and AVP, which appear to play key roles in generating and maintaining circadian timing, other neuropeptides modify the activity of the SCN (Teshima *et al.*, 2005; Miyakawa *et al.*, 2007). While the application of PACAP and VIP produced different types of Ca^{2+} responses, both peptides have been reported to have activity at the VPAC2 receptor while PACAP is more selective for the PAC1 receptor, and both receptors are expressed in the SCN (Ajpru *et al.*, 2002; Kallo *et al.*, 2004a; Yang *et al.*, 2010). PACAP's effect on $[Ca^{2+}]_i$ was variable with the Ca^{2+} elevations tending to be transient and located more ventrolaterally, consistent with regions receiving greater RHT and PACAP associated input (Hannibal & Fahrenkrug, 2004). The different responses observed using PACAP may be a consequence of PACAP modulating the activity of neurotransmission pathways (Kopp *et al.*, 1999; Kopp *et al.*, 2001; Dziema & Obrietan, 2002; Michel *et al.*, 2006). We found little or no change was observed in the Ca^{2+} transients induced by RHT stimulation before and after application of PACAP. Previously, we have shown that RHT stimulation induces glutamate receptor-mediated excitatory postsynaptic potentials (EPSPs), but does not produce a change in somatic $[Ca^{2+}]_i$ without subsequently triggering an action potential and opening of

voltage dependent Ca^{2+} channels (Irwin & Allen, 2007). This suggests that if endogenous PACAP is also released with RHT stimulation, it does alter somatic $[\text{Ca}^{2+}]_i$, and is consistent with the relatively small, variable and often transient effects on $[\text{Ca}^{2+}]_i$ associated with pharmacologic applications of PACAP.

While SCN neurons have little or no OFQ mRNA expression (Neal *et al.*, 1999b), the OFQ receptor mRNA is expressed along with high levels of radioligand binding (Anton *et al.*, 1996; Neal *et al.*, 1999a). Further, OFQ can functionally alter circadian rhythms (Teshima *et al.*, 2005; Leggett *et al.*, 2007). However, the anatomy of OFQ input to the SCN remains unclear. Several brain regions expressing OFQ mRNA also have neurons that project to the SCN including the raphe, the periaqueductal gray, and the lateral geniculate (Boom *et al.*, 1999; Neal *et al.*, 1999b; Hay-Schmidt *et al.*, 2003; Horowitz *et al.*, 2004). We found OFQ application induced a sustained reduction of Ca^{2+} that was strongly dependent ($R^2 = 0.82$) on the baseline $[\text{Ca}^{2+}]_i$ and consistent with prior observations (Allen *et al.*, 1999; Ikeda *et al.*, 2003). Like VIP, OFQ appeared to quiet SCN neurons during the day with a reduction of the $[\text{Ca}^{2+}]_i$ to levels and variances more typical of neurons during the night (Irwin & Allen, 2009), and this is consistent with a reduction in baseline action potential firing by OFQ in SCN neurons (Allen *et al.*, 1999; Teshima *et al.*, 2005). While OFQ is known to reduce, it does not eliminate presynaptic glutamate release from RHT terminals (Gompf *et al.*, 2005), and RHT stimulation can still trigger postsynaptic action potential firing consistent with the RHT-induced Ca^{2+} transients observed in a number of neurons. Thus these data demonstrate that OFQ, like VIP lowers the $[\text{Ca}^{2+}]_i$ in populations of SCN neurons consistent with a reduction in the state of action potential firing activity.

The neuropeptides expressed in the SCN demonstrated unique effects on the $[\text{Ca}^{2+}]_i$. VIP lowers $[\text{Ca}^{2+}]_i$ in SCN neurons in an activity state-dependent manner. Conversely, AVP elevated $[\text{Ca}^{2+}]_i$ independent of the activity state of the neuron. For a threshold change in $[\text{Ca}^{2+}]_i$ to subsequently evoke phase changes to the clock, the initial Ca^{2+} concentration, amplitude and duration are likely critical factors. We hypothesize that VIP facilitates photic entrainment of SCN neurons by lowering the action potential firing frequency and $[\text{Ca}^{2+}]_i$, which may facilitate the RHT input to induce a threshold change in $[\text{Ca}^{2+}]_i$. Conceivably AVP, which appears to raise $[\text{Ca}^{2+}]_i$ may impede this process. While the contribution, significance and proportions of Ca^{2+} originating from extracellular and intracellular sources to the $[\text{Ca}^{2+}]_i$ remain unclear, these data suggest that the activity state of the neuron, autoregulated at least in part by neuropeptides, may be a major point of regulation for SCN synchronization and modulation of light-input induced changes to the clock.

Acknowledgments

Support contributed by Grant MH 70922 (CNA).

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Figure 1. Measurement of intracellular Ca^{2+} simultaneously in multiple SCN neurons

A. Schematic illustration of the recording setup showing a coronal hypothalamic slice containing the SCN and third ventricle (3V) with a bipolar stimulating electrode (SE) used to stimulate the RHT positioned in the optic chiasm (OC) and a perfusion pipette (P) positioned to flow solutions over the SCN. B. High power (40X objective) fluorescent image of fura-2 loaded SCN neurons. C. Example showing NMDA (200 μM , 5 sec) inducing a transient increase in the Ca^{2+} ratio (340nm/380nm) in SCN neurons during the day (ZT = 6 hours). D. Example showing changes in the Ca^{2+} ratio of a SCN neuron with responses to NMDA (200 μM , 5 sec), GABA (200 μM , 10 sec), RHT stimulation (arrows; 100 pulses, 200 μs at 20 Hz) and VIP (1 μM) application.

Figure 2. VIP induces a reduction of baseline $[\text{Ca}^{2+}]_i$ during the day but not the night

A. Comparison of the mean Ca^{2+} ratio of SCN neurons at baseline and following application of VIP (250 nM) during the day ($n = 96$). VIP (250 nM) induced a significant reduction from baseline in SCN neurons showing a VIP(Ca-) response ($n = 26$, $t = 7.24$, *** $p < 0.0001$), while most neurons had little or no response (VIP(Ca0), $n = 61$, $t = 1.15$, $p = 0.25$), and only 9 neurons had a Ca^{2+} increase (data not shown). B. Comparison of the mean Ca^{2+} ratio of SCN neurons at baseline and following application of VIP (1 μM) during the day ($n = 152$) and night ($n = 121$). During the day, VIP (1 μM) significantly reduced the baseline Ca^{2+} ratio (mean $\pm$ SEM) in VIP(Ca-) neurons ($n = 82$, $t = 11.9$, *** $p < 0.0001$), but not in the VIP(Ca0) group ($n = 59$, $t = 1.71$, $p = 0.093$), while only 11 neurons showed a Ca^{2+} increase (data not shown). During the night, VIP reduced the Ca^{2+} ratio in 7% of neurons. The data shown is the mean $\pm$ SEM of all nighttime neurons ($n = 121$ neurons, $t = 0.114$, $p = 0.91$). Note the baseline Ca^{2+} ratios were higher during the day than the night, and that VIP reduced the Ca^{2+} ratios of VIP(Ca-) responding neurons to near nighttime levels. C. Plot of the baseline Ca^{2+} ratio versus the VIP (1 μM)-induced change in the Ca^{2+} ratio in VIP(Ca-) neurons during the day. Note that larger reductions in Ca^{2+} occur in neurons with higher baseline Ca^{2+} ratios ($R^2 = 0.41$). D. Regional location of VIP-treated neurons during the day and night. The position for each VIP(Ca-) and VIP(Ca0) neuron was superimposed on a representative drawing of the SCN with the third ventricle (3V) on the left and optic chiasm (OC) at the bottom. Neither VIP(Ca-) nor VIP(Ca0) neurons demonstrated a clear regional expression pattern.

Figure 3. VIP modulation of RHT signaling in the SCN network

A. The postsynaptic Ca^{2+} ratio in two SCN neurons with similar reductions in response to VIP (1 μM), but opposing responses to both GABA (200 μM , 10 sec) and stimulation of the RHT (arrows, 100 pulses, 200 μs at 20 Hz). B. VIP (1 μM) had little or no effect on RHT-evoked elevations of Ca^{2+} (top) during the day ($n = 36$ neurons, $t = 0.43$, $p = 0.67$) and night ($n = 22$, $t = 0.39$, $p = 0.70$), but attenuated RHT-evoked Ca^{2+} reductions during the day ($n = 10$, $t = 2.82$, $**p < 0.020$), but not the night ($n = 3$, $t = 1.06$, $p = 0.40$). Data is the mean $\pm$ SEM of maximum (Ca^{2+} elevations) and minimum (Ca^{2+} reductions) responses to RHT-evoked changes in the Ca^{2+} ratio before and during VIP (1 μM) application. Note that since VIP reduced the baseline Ca^{2+} , the magnitudes of RHT-evoked Ca^{2+} reductions were also reduced, while at night the baseline Ca^{2+} ratio was low with few RHT-evoked Ca^{2+} transient reductions.

Figure 4. The VIP antagonist VIP(6-28) modified $[Ca^{2+}]_i$ in SCN neurons at night

A. Example showing three SCN neurons during the night with different responses to application of VIP(6-28) (2 μ M), a transient rise, a prolonged elevation and a reduction in the Ca^{2+} ratio. B. Pie chart of VIP(6-28) data showing the proportion of neurons with elevations ($\uparrow$, $n = 22$) reductions ($\downarrow$, $n = 10$) and no responses ($\emptyset$, $n = 21$) in Ca^{2+} . The bars represent the mean $\pm$ SEM of the maximal (black) and minimum (gray) responses (0-30 sec) of neurons showing VIP(6-28) (2-4 μ M) evoked Ca^{2+} changes.

Figure 5. AVP elevates the $[Ca^{2+}]_i$ of SCN neurons during the day and night

A. Example showing SCN neurons responding to AVP during the day and night. Tracings show treatments with NMDA (200 μ M), GABA (200 μ M), VIP (1 μ M) and AVP (1 μ M). Note that VIP lowered the Ca^{2+} ratio during the day but not at night, while AVP elevated the Ca^{2+} ratio [AVP(Ca+)] during both the day and night. B. Bar graph of AVP(Ca+) responding neurons showing the mean $\pm$ SEM Ca^{2+} ratio before and during AVP application during the day ($n = 53$, $t = 8.25$, *** $p < 0.001$) and night ($n = 55$, $t = 8.44$, *** $p < 0.001$), and during night with TTX (ANOVA, $F_{3,54} = 28.6$, $p < 0.001$, Bonferroni adjusted $p = 0.48$ [night-Baseline] and $p = 0.09$ [night-AVP]). C. Regional location of AVP-treated neurons (day and night combined). The position for each AVP(Ca+) and non-responding [AVP(Ca0)] neuron was superimposed on a representative drawing of the SCN with the third ventricle (3V) on the left and optic chiasm (OC) at the bottom. AVP(Ca+)-responding neurons appeared to be located primarily in the dorsomedial region.

Figure 6. The Effects of PACAP on $[Ca^{2+}]_i$ in SCN neurons

A. Examples of the variety of Ca^{2+} responses observed with PACAP (1–38) application onto SCN neurons during the day. i & ii) PACAP (250 nM) evoked transient Ca^{2+} elevations without altering the RHT stimulation-induced transients (arrows, 100 pulses at 20 Hz). iii) PACAP (500 nM) evoked a Ca^{2+} reduction in one neuron and an elevation in another. B. Regional localization of SCN Ca^{2+} responses to PACAP. The position of each neuron where PACAP elevated [PACAP(Ca+)], reduced [PACAP(Ca-)] or produced no response [PACAP(Ca0)] was superimposed on a representative drawing of the SCN with the third ventricle (3V) on the left and optic chiasm (OC) at the bottom.

Figure 7. The Effects of orphanin-FQ (OFQ) on $[Ca^{2+}]_i$ in SCN neurons

A. Example showing the effect of OFQ (1 μ M) on two SCN neurons during the day with different responses to GABA (200 μ M) and RHT stimulation (arrows, 100 pulses at 20 Hz). B. The magnitude of the OFQ reduction of Ca^{2+} was linearly correlated with the baseline Ca^{2+} ratio (filled circles, $n = 24$, slope = -0.83 , $R^2 = 0.82$). Non-responding neurons already had low baseline ratios (open circles, $n = 5$). Note that the effect of OFQ on the $[Ca^{2+}]_i$ was long-lasting.