Presynaptic regulation of inhibitory synaptic transmission by vasoactive intestinal peptide (VIP) in the mouse suprachiasmatic nucleus

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Running title: VIP modulates IPSCs in the SCN
Pages: 18
Figures: 7
Title: 134 characters
Abstract: 229 words

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Abstract

Circadian rhythmicity in mammals is generated by a pair of nuclei in the anterior hypothalamus known as the suprachiasmatic nuclei (SCN), whose neurons express a variety of neuropeptides that are thought to play a vital role in the circadian timing system. To evaluate the influence of VIP on inhibitory synaptic transmission between SCN neurons, we used whole-cell patch-clamp recording in an acute brain slice preparation of mouse SCN. Bath-applied VIP caused a significant increase in the frequency of spontaneous inhibitory postsynaptic currents (sIPSC) in a reversible and dose-dependent manner with no effect on the mean amplitude or kinetic parameters. The effect of VIP was widespread throughout the SCN and observed in both ventrolateral (VL) and dorsomedial (DM) regions. In the presence of tetrodotoxin, VIP increased the frequency of miniature IPSCs without affecting the mean magnitude or kinetic parameters. The magnitude of the enhancement by VIP was significantly larger during subjective day than during the subjective night. Pre-treatment with the VPAC receptor antagonist [Ac-Tyr^1, D-Phe^2]-GHRF 1-29 or the selective $VPAC_2$ receptor antagonist PG 99-465 completely blocked the VIP-induced enhancement. The effect of VIP appears to be mediated by a cAMP/PKA-dependent mechanism as forskolin mimics while the PKA antagonist H-89 blocks the observed enhancement of GABA currents. Our data suggest that VIP activates presynaptic $VPAC_2$ receptors to regulate inhibitory synaptic transmission within the SCN and that this effect varies with the circadian cycle.

Key words: circadian rhythms, GABA, $VPAC_2$, presynaptic modulation, cAMP/PKA

Introduction

Daily rhythms in our behavior, most notably the sleep-wake cycle, are primarily regulated by a timekeeping structure in the hypothalamus known as the suprachiasmatic nucleus (SCN) (Gillette 1997). When SCN neurons are removed from an organism and maintained in a brain slice preparation, they continue to generate 24-hour rhythms in electrical activity, peptide secretion, and gene expression (Prosser 1998; Earnest and Sladek 1986; Dunlap 1999). Previous studies suggest that the basic mechanism responsible for the generation of these rhythms is intrinsic to individual cells in the SCN (Welsh et al. 1995). Morphological studies based on peptide phenotypes and neuronal projections delineate two discrete subregions within the SCN, the ventrolateral (or core) region and the dorsomedial (or shell) region (Moore et al. 2002). Each subregion is functionally distinct and plays an important role in the translation of both photic and non-photic stimuli into a coherent circadian rhythm that is entrained to the subjective LD cycle and capable of adapting to changes in the environment (Gillette 1997). Of recent interest is the physiological significance of various neuropeptides expressed in SCN neurons and co-released at active synapses with neurotransmitters such as glutamate and GABA (Peytevin et al. 2000; Moore et al. 2002). These neuropeptides are regulated in a circadian manner and are likely to transmit information that is distinct from that of classical neurotransmitters (Inouye 1996).

VIP-expressing neurons found in the core region of the SCN are thought to mediate entrainment to the LD cycle by receiving photic information from the retina via the RHT and GHT (van Esseveldt et al. 2000). This is further supported by the observation that VIP protein and mRNA cycle diurnally in the adult rat in a light-dark (LD) cycle, but remain constant in prolonged darkness (DD; Takahashi 1989; Shinohara 1993, 1999a). Using extracellular recording in an acute brain slice preparation, Reed and coworkers (2001) demonstrated that VIP treatment during the early subjective night evoked a small phase delay and during the late subjective night evoked a large phase delay. Functionally, the microinjection of VIP into the SCN region of Syrian hamsters during the early or late subjective night produced similar phase-shifts in locomotor activity rhythms (Albers et al. 1991; Piggins 1995). Short light pulses administered during the

early or late subjective night also phase-shift rodent behavioral rhythms in a manner similar to VIP application, raising the possibility that VIP is an intermediate in the process of entraining the circadian system to light pulses. Additionally, administration of VIP induces mPer expression in SCN neurons presumably through activation of $VPAC_2$ receptors, which are widely distributed throughout the SCN (Nielsen et al. 2002; Shinohara et al. 1999a; Cagampang et al. 1998). Mice overexpressing $VPAC_2$ receptors exhibit a shorter free-running period (Shen et al. 2000), while mice deficient in this receptor exhibit a profound disruption in both wheel-running activity and rhythmic gene expression (Harmar et al. 2002). Given this background, it would be surprising if VIP did not play an important modulatory role in regulating cell-to-cell communication within the SCN.

Although clearly functionally important, the effects of VIP on SCN neurons are largely unknown. Since VIP is co-expressed with GABA (Moore et al. 2002), one hypothesis is that VIP modulates inhibitory synaptic transmission within the SCN. VIP is a potent stimulator of adenylyl cyclase (AC) and is expected to stimulate cAMP and protein kinase A (PKA) via the $VPAC_2$ receptor (e.g. Harmer et al. 1998; Nowak and Kuba 2002). Furthermore, VIP has been shown to modulate GABAergic synaptic transmission in the hippocampus (Wang et al. 1997). Therefore, it is likely that VIP acts through the cAMP/PKA cascade to regulate GABA release in neurons of the SCN. The $VPAC_2$ receptor is abundantly expressed throughout the SCN (Cagampang et al. 1998), suggesting that the effects of VIP may not be restricted to the shell region. In the present study, we utilized whole-cell patch electrophysiological techniques to record GABA-mediated currents in SCN cells in a brain slice preparation. As a first step, spontaneous inhibitory postsynaptic currents (sIPSC) were recorded from SCN slices from animals maintained in a LD cycle. Comparisons were made between ventrolateral (VL) and dorsomedial (DM) regions of the SCN as well as between day and night. Next, the effects of VIP on sIPSCs recorded in the VL and DM SCN at different phases were determined. Finally, the possibility that VIP's actions are mediated by a $VPAC_2$ receptor and cAMP/PKA dependent pathway was evaluated.

Materials and Methods

Animals and brain slice preparation

The UCLA Animal Research Committee approved the experimental protocols used in this study. Brain slices were prepared using standard techniques from C57 BL/6 mice between 3 and 8 weeks of age. Mice were killed by decapitation, brains dissected and placed in cold oxygenated ACSF containing (in mM) NaCl 130, $NaHCO_3$ 26, KCl 3, $MgCl_2$ 5, NaH_2PO_4 1.25, $CaCl_2$ 1.0, glucose 10 (pH 7.2-7.4). After cutting slices (Microslicer, DSK Model 1500E) from areas to be analyzed, transverse sections (350μm) were placed in ACSF (25-27^oC) for at least 1 hour (in this solution $CaCl_2$ is increased to 2mM, $MgCl_2$ is decreased to 2mM). Slices were constantly oxygenated with 95%O_2-5% CO_2 (pH 7.2-7.4, osmolality 290-300mOsm).

Whole-cell patch clamp electrophysiology

Methods are similar to those described previously (Colwell 2001; Michel et al. 2002). Slices were placed in a chamber (PH-1, Warner Instruments) attached to the stage of a fixed-stage upright microscope. The slice was held down with thin nylon threads glued to a platinum wire and submerged in continuously flowing, oxygenated ACSF (25^oC) at 2-4ml/min. Electrodes were pulled on a multistage puller (Sutter P-97; 1.5 mm o.d. borosilicate capillary glass) and resistance in the bath was typically 3-6MΩ. The standard solution in the patch pipette contained (in mM): K-gluconate, 112.5; EGTA, 1; Hepes, 10; MgATP, 5; GTP, 1; leupeptin, 0.1; phosphocreatine, 10; NaCl, 4; KCl, 17.5; $CaCl_2$, 0.5; and $MgCl_2$, 1. 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 Instr.). To minimize changes in off-set potentials with changing ionic conditions, the ground path used a KCl agar bridge to an Ag/AgCl ground well. Cells were approached with slight positive pressure (2-3cm H_2O) and offset potentials were corrected. The pipette was lowered to the vicinity of the membrane keeping positive pressure. After forming a high-resistance seal (2-10GΩ) by applying negative pressure, a second pulse of negative pressure was used to rupture the membrane.

While entering the whole-cell mode, a repetitive test pulse of 10mV was delivered in a passive potential range (≈-60 to -70 mV). Once the whole cell configuration was established, whole-cell capacitance was calculated from voltage transients produced by a 20 mV voltage step lasting 40 msec according to standard methods (Colwell 2001; Michel et al. 2002). Whole-cell capacitance and electrode resistance were neutralized. The series and input resistances were monitored throughout the experiment by checking the response to small pulses in the passive potential range. Data were not collected if series resistance was greater than 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. When necessary, various specific blocking agents were used to isolate currents under investigation. 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 sec and were typically maximal by 3-5 min.

The current required to maintain the cell's membrane potential at –70 mV was monitored throughout the experiment. Spontaneous currents were recorded with pClamp in the gap-free mode and analyzed using Minianalysis software (Ver. 5.2.1, Synaptosoft). Events were detected using the following criteria: threshold 5 pA, period to find local maximum 5 msec, time before peak for baseline 6 msec, period to search decay time 50 msec, fraction of peak to find decay time 0.38, period to average baseline 2.5 msec, area threshold 15, and detect complex peak enabled. Events were excluded if the decay time was less than 6 msec (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 1-2 min time bin and reporting this number as events/sec or Hz. 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. Using these criteria in untreated cells, baseline IPSC frequency typically did not vary significantly over the 12 min time span.

Cell identification

After electrophysiological analysis, whole slices (350 μm) containing biocytin-filled cells were fixed by overnight immersion in paraformaldehyde (4%) in phosphate buffered saline (PBS). The slices were then washed with Tris-buffered saline for 1hr and processed histochemically for biocytin staining. The purpose of staining recorded neurons is to identify the type of cell. Neurons were initially designated as either VL or DM based on visualization with IR-DIC optics. This characterization was then verified by filling the cell with biocytin and counterstaining the slice with a nissl stain to confirm the location of the neuron within the SCN.

Lighting conditions

Animals were maintained on a daily light-dark cycle consisting of 12 h of light followed by 12 h of dark. In order to evaluate diurnal variations in IPSCs and the VIP-mediated enhancement of these GABA-mediated currents, animals were sacrificed ~1 h before the beginning of the phase of interest. Zeitgeber time (ZT) is used to describe the projected time of the oscillator

within the SCN based on the previous light cycle, with lights-on defined at ZT 0. Animals were sacrificed between ZT 3-4 in the LD cycle for recording during subjective day (ZT 4-8), while animals were sacrificed 30 min before lights-off (ZT 12) for recording during early subjective night (ZT 13-15). The remainder of animals was sacrificed in the dark using IR viewers at ZT 19 for recording during late subjective night (ZT 20-22).

Statistics

Between group differences were evaluated using t-tests 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, USA). In the text, values are shown as mean ± SEM. Each group of data is collected from at least 3 animals, with n representing the number of cells recorded.

Results

Spontaneous Inhibitory Post-Synaptic Currents (sIPSCs) Recorded in VL and DM SCN.

In this study, we used the whole-cell voltage-clamp technique to record spontaneous inhibitory postsynaptic currents (sIPSCs) from neurons in the SCN (**Fig. 1**). IPSCs were widespread in the SCN and could be detected from every neuron recorded (n=187). Overall, the mean frequency and the mean amplitude of sIPSCs recorded at a holding potential of –70 mV were 5.9 ± 0.6 events/s (mean ± SEM, n=107) and –14.3 ± 0.8 pA, respectively. The time to rise and the time to decay (latency of the inward current peak from the baseline) were 2.4 ± 0.1 ms and 14.2 ± 0.5 ms, respectively. The IPSCs exhibited a reversal potential between –40 and –50 mV (n= 8), a range consistent with the calculated chloride equilibrium potential (E_{Cl}) of –45.6 mV at 25^{o} C. The reversal potential for IPSCs, and presumably E_{Cl}, stabilized within 3 min of entering the whole-cell configuration and remained constant for the duration of the experiment (n=8). Because the reversal potential for chloride was more positive that the holding potential used to measure GABA currents, all recordings show IPSCs as inward currents. All sIPSCs were completely abolished with $GABA_A$ antagonist bicuculline (25 µM, 8 of 8 neurons tested), indicating that they are mediated by $GABA_A$ receptors. In contrast, the

sIPSCs were unaffected by the AMPA/KA GluR antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 μM, 8 of 8 neurons tested).

Neurons were initially designated as either ventrolateral (VL) or dorsomedial (DM) based on visualization with IR-DIC optics and landmarks such as the third ventricle and optic chiasm. This localization was then verified by filling the cell with biocytin and counterstaining the slice with a Nissl stain to confirm that the neuron was in the VL (6 of 6 neurons examined) or DM (6 of 6 neurons examined) region. Overall, the frequency of sIPSCs in the VL region (3.9 ± 0.6 Hz, n=44) differed significantly from IPSCs measured in the DM region (8.1 ± 1.0 Hz, n=41, $p < 0.01$). No significant differences were detected in the amplitude, rise time or decay time of sIPSCs between the VL or DM SCN regions. Thus, sIPSCs are general feature of cells within the SCN but are less frequent in the retinal recipient VL region.

Spontaneous IPSCs frequency varied between day and night in the SCN

The next experiment was designed to determine whether sIPSCs recorded in SCN neurons varied with the phase of the daily cycle. The data was collected at various phases of the animals daily cycle corresponding to day (ZT 4-8), early night (ZT 13-15) and late night (ZT 20-22). The sIPSC frequency in the DM SCN exhibited a daily rhythm that peaked during the early night (**Fig. 2**). The IPSC frequency was significantly greater in the early evening (12.0 ± 1.6 Hz, n=16) compared to day (7.9 ± 1.1 Hz, n=28, $p < 0.05$) and late night (7.7 ± 1.9 Hz, n=6, $p < 0.05$). This diurnal rhythm was restricted to the cells in the DM region as there was no significant temporal variation in the IPSC frequency measured from the VL region (**Fig. 2**). Similarly, no significant differences were detected in the amplitude, rise time or decay time of sIPSCs between the three phases of the daily cycle. These data indicate that the frequency of inhibitory synaptic transmission varies with a daily cycle in SCN neurons in the DM cell population.

Application of the neuropeptide VIP enhanced inhibitory synaptic transmission.

The application of VIP was found to increase the sIPSC frequency but not other properties of sIPSCs recorded in SCN neurons (**Fig. 3**). For example, during the day, VIP (100nM) increased the sIPSC frequency by 41 ± 6% above baseline (n=12, $p<0.01$).

This effect of VIP was widespread in the SCN and, overall, about two-thirds of SCN neurons tested (37/58 cells) responded to VIP with an increase in sIPSC frequency. We compared the effect of VIP on sIPSCs between the DM and VL regions of the SCN, but found that there were no significant differences between these regions during day, early night or late night. Therefore, the data from both regions were pooled for the remainder of the experiments evaluating the effect of VIP on sIPSCs. The effect of VIP on SCN neurons was concentration dependent with a maximal effect observed at or above 100 nM and a half-maximal response at 42 nM (**Fig. 4**). The magnitude of VIP-mediated enhancement of sIPSC frequency was phase-dependent (**Fig. 5**). During the day, application of VIP (100nM) increased the frequency of sIPSCs by 36 ± 2% (n=14). In contrast, the same VIP treatment increased the frequency of sIPSCs 16 ± 2% during the early subjective night (n=12) and by 13 ± 2% (n=11) during the late subjective night. The effect of VIP on sIPSC frequency was significantly greater ($p < 0.01$) in the day than in either the early or late night. The percentage of cells responding to 100 nM VIP did not change from day to night (60-65%).

VIP enhanced IPSC frequency through a presynaptic mechanism.

To determine if a presynaptic mechanism was responsible for VIP's ability to enhance sIPSCs throughout the SCN, we performed similar whole-cell patch-clamp recordings of SCN neurons in the presence of the Na^+ channel blocker tetrodotoxin (TTX). In the presence of TTX (0.5 μM), VIP administered during the subjective day significantly increased the frequency but not amplitude of miniature IPSCs (mIPSCs) 21 ± 3% above baseline (n=13; **Fig. 5**). Additionally, VIP administered during the early subjective night increased the frequency but not amplitude of mIPSCs 10 ± 3% above baseline (n=12), reflecting the same phase-dependent relationship noted above in the absence of TTX ($p < 0.01$). Cumulative distributions of sIPSC and mIPSC amplitudes and inter-event intervals also confirm that VIP enhances GABA release by a presynaptic mechanism (**Fig. 6**). The inter-event interval curve is shifted to the left with no change in the cumulative amplitude curve for 4/5 SCN neurons in TTX and 4/6 SCN neurons in the absence of TTX, indicating that the time interval between each event is shorter after VIP administration. The remaining 3 neurons showed a shift to the left in both the inter-event

interval and cumulative amplitude plots, indicating that the amplitude of IPSCs were smaller after VIP administration and suggesting that a subset of VIP-responsive neurons display both presynaptic and postsynaptic changes when treated with VIP.

VIP enhanced IPSC frequency through mechanisms dependent upon $VPAC_2$ receptors and adenylyl cyclase signaling.

The next set of experiments was designed to characterize the receptor and associated signaling pathway mediating VIP's enhancement of inhibitory synaptic transmission. First, we found that enhancement of GABA release was selective for VIP in that the related peptide PHI (100 nM: 0.2 ± 2%, n=6) had no significant effect on sIPSC frequency. Treatment with the peptide PACAP (100 nM), however, resulted in a 15 ± 3% increase in sIPSC frequency (6/10 cells) during the day. We expected PACAP to have a similar effect on GABAergic sIPSCs because it has been previously reported that PACAP binds and activates the $VPAC_2$ receptor at approximately the same concentration as VIP (Laburthe and Couvineau 2002). Next, we found that treatment with the VPAC receptor antagonist [Tyr1,DPhe2]GHRF(1-29) (100 nM) by itself reduced sIPSC frequency (11% decrease, n=9) as well as completely prevented any stimulatory effect of VIP (50 nM) on sIPSC frequency during the day (0 ± 3%, n=8; **Fig. 7**). Similarly, in the presence of the selective $VPAC_2$ antagonist PG 99-465, VIP (50 nM) had no significant effect on IPSC frequency (3 ± 3%, n=6). Since the $VPAC_2$ receptor can be positively coupled to the cAMP/PKA cascade, we sought to determine the involvement of this signaling pathway. Pretreatment of the slices with H-89 (20 μM), an inhibitor of PKA, completely prevented the stimulatory effect of VIP (50 nM) on sIPSC frequency during the day (-1 ± 3% change; n=6; **Fig. 7**). Conversely, bath application of forskolin (10 μM), a potent activator of PKA, significantly enhanced the sIPSC frequency during the subjective day (77 ± 4%; n=6). To determine if VIP and forskolin were both acting through the cAMP/PKA pathway to enhance inhibitory currents, hypothalamic slices were pretreated with forskolin before VIP (100 nM) treatment. If both drugs were working through the cAMP/PKA pathway, then pretreatment with a saturating dose of forskolin would prevent any additional effect with VIP treatment. As expected, VIP administered after forskolin pretreatment resulted in no further increase in the frequency

of GABA currents (0 ± 3%, n = 6). The inactive analog of forskolin, 1,9 dideoxy-forskolin, did not significantly alter the frequency of GABA currents in SCN neurons (0 ± 3%, n = 6). All experiments were performed in the presence of TTX (0.5 μM).

Discussion

In the present study, whole-cell patch clamp techniques were used to measure sIPSCs from SCN neurons. While these currents were widespread in the SCN and the sIPSC frequency was consistently higher in the DM subdivision when compared to the VL subdivision of the SCN. The sIPSC frequency also varied with the daily cycle and exhibited a peak in the early night (ZT 13-15). In contrast, the sIPSC amplitude, rise time or decay time did not vary between the SCN regions or with the daily cycle. Application of the neuropeptide VIP increased the sIPSC frequency but not other properties of the sIPSCs recorded in the SCN. This enhancement was seen in about two-thirds of the SCN neurons examined and was similar in both DM and VL SCN regions. The magnitude of the VIP enhancement exhibited a daily rhythm with significantly larger effects observed during the subjective day. This effect of VIP on IPSC frequency, as well as the daily variation in the magnitude of the response, was observed in the presence of TTX. Furthermore, VIP's actions were blocked by antagonists for VPAC receptors as well as the PKA antagonist H-89. Forskolin, an AC activator, mimicked the stimulatory action of VIP and pretreatment with forskolin precluded further stimulation by VIP. These results indicate that VIP enhances IPSC frequency through presynaptic activation of $VPAC_2$ receptors and positive coupling to AC cascade. Overall, these observations also raise the possibility that VIP may play an important role in regulating inhibitory synaptic transmission throughout the SCN.

Spontaneous IPSCs are widespread within the SCN

Anatomical studies support the subdivision of the SCN into at least two subdivisions (Abrahamson and Moore 2001). The core subdivision (VL) of the SCN receives photic input from the retina and intergeniculate leaflet as well as non-photic input from the

raphe. Neurons in this region express GABA as well as several peptides including VIP and gastrin releasing peptide. The shell subdivision (DM) division does not appear to receive direct retinal innervation, but rather receives input from core SCN neurons as well as other brain regions (Moga and Moore 1997). These neurons express GABA as well as several peptides including, most notably, arginine vasopressin (AVP). The efferent projects of the two subdivisions are also distinct and generally include projections to the hypothalamus, thalamus and cortical regions (Leak and Moore 2001). We were able to record inhibitory currents in every neuron in the SCN (n=187). Although peptide expression was not characterized in all of these cells, video microscopy was used to visually place neurons within these two general regions of the SCN. We observed a significant regional difference in that the DM subdivision received a higher frequency of inhibitory currents regardless of the phase. There were no other significant differences between the currents recorded from the two regions.

The slice preparation used in the present study does not allow us to define the source of GABA input onto the cells from which we are recording. It is widely accepted that most SCN neurons are GABAergic and are likely to use this transmitter to communicate with other neurons within and beyond the SCN (Castel and Morris 2000; Abrahamson and Moore 2001). Electrophysiological analysis indicates that SCN neurons receive a high frequency of $GABA_A$ mediated postsynaptic currents (Kim and Dudek 1992; Jiang et al. 1997; De Jeu and Pennartz 2002) that, at least partly, originate within the SCN itself (Strecker et al. 1997). Other sources of GABAergic input include the arcuate nucleus, supraoptic nucleus, and the intergeniculate leaflet (e.g. Saeb-Parsy et al. 2000, Morin and Blanchard 2001). Based in part on the observation that photic stimulation inhibits electrical discharge of some SCN neurons, the hypothesis has been raised that GABA may even being released directly from retinal ganglion cells innervating the SCN (Jiao and Rusak 2003). Certainly our data is consistent with earlier work suggesting that SCN neurons are under tonic GABAergic control. Moreover, our data demonstrates that the frequency of this GABAergic release onto SCN neurons varies with a diurnal rhythm that peaks during the early night. While not the focus of the current study, the presence of a rhythm in IPSC frequency may have important implications for SCN function.

Effects of VIP on IPSCs in the SCN.

While many core SCN neurons express VIP, the physiological roles of this peptide in the SCN are not well defined. Previous studies have shown that VIP and VIP mRNA levels are regulated by lighting conditions, suggesting that VIP release is dependent on photic input and peaks during the night (Shinohara et al. 1999ab). Physiologically, extracellular recordings of the firing rate of SCN neurons in a brain slice indicate that VIP administration can cause phase shifts of the circadian rhythm of electrical activity (Reed et al. 2001). Since VIP is co-expressed with GABA, we hypothesized that VIP modulates inhibitory synaptic transmission within the SCN. Furthermore, a recent study by Reed and coworkers (2002) showed that VIP elicited predominantly suppressions in SCN cellular activity, some of which were modulated by bicuculline, implicating the involvement of GABAergic mechanisms. Our present results demonstrate that VIP can profoundly enhance GABAergic IPSCs throughout the SCN during both day and night. In addition, we observed a significantly greater effect of VIP during the day compared to early and late night. We do not know if this rhythm is self-sustained nor did we identify the mechanisms responsible for the rhythm. For example, it is possible that we observed a reduced effect of VIP during the night because endogenous levels of VIP were already increased, occluding further enhancement of GABAergic IPSCs with exogenous VIP application during this phase. It may also be possible that VIP receptors ($VPAC_2$) are less sensitive to VIP during the night.

Mechanisms of VIP modulation

While the mechanisms underlying the diurnal modulation of VIP-response are not yet understood, our results have clarified the mechanisms through which VIP is acting to regulate inhibitory synaptic transmission within the SCN. First, VIP enhanced mIPSC frequency in the presence of TTX. Each miniature synaptic current is thought to result from the spontaneous fusion of an individual synaptic vesicle with the pre-synaptic membrane subsequently resulting in quantal release of transmitter molecules. Changes in the frequency at which this process occurs are normally associated with alterations in the presynaptic release process while changes in the amplitude of the currents reflect

postsynaptic changes in receptor sensitivity or ionic driving force. So the finding that VIP changed the mIPSC frequency certainly indicates a presynaptic mechanism. However, there were a few cells that also exhibited a change in IPSC amplitude. This observation, coupled with the previous work demonstrating that VIP can regulate potassium channels in cells in other brain regions, suggest that VIP may have multiple actions on SCN neurons (Haug and Storm 2000). Second, the effects of VIP are likely to be mediated by VPAC receptors. Both VPAC and selective $VPAC_2$ antagonists were found to prevent VIP enhancement of IPSC frequency. Previous studies have established that the $VPAC_2$ subtype of receptor is expressed in the SCN (Cagampang et al. 1998; Shinohara et al. 1999) and mediates the VIP-induced suppression of the frequency of action potentials recorded in SCN neurons (Cutler et al. 2003). In other preparations, this receptor is positively coupled to AC and is expected to act via stimulation of cAMP and PKA (Harmar et al. 1998). Furthermore, in other systems, there is evidence that GABA currents are positively regulated by this second messenger cascade (e.g. Poisbeau et al. 1999; Shindou et. al. 2002). In the present study, we demonstrate the forskolin mimics while H-89 prevents the sIPSC enhancement by VIP. In summary, our data suggests that VIP acts through $VPAC_2$ receptors to stimulate the cAMP/PKA cascade and presynaptically enhance GABA release in the SCN.

Functional importance

Our data indicate that VIP can broadly regulate GABA-mediated synaptic input to SCN neurons. Through this mechanism, VIP could function to modulate the photic input to the SCN as well as coupling between neurons in the SCN. Previous studies have shown that injection of GABA agonists into the SCN can modulate light and NMDA induced phase shifts (Gillespie et al. 1997; Mintz et al. 2002) as well as *fos*-induction (Gillespie et al. 1999). These data suggest that GABAergic tone of the SCN can modulate light induced phase shifts perhaps by direct regulation of the amplitude of glutamate-evoked responses in retino-recipient SCN neurons. Second, most SCN neurons are GABAergic and are likely to use this transmitter to communicate with other neurons within the SCN. Previous studies suggest that GABAergic mechanisms appear to have

the capability to synchronize SCN cell populations in culture conditions (Liu and Reppert 2000; Shirakawa et al. 2000). Therefore, by modulating the frequency of GABA release, VIP could function to alter the degree of synchronization between SCN cell populations. This suggestion is supported by the finding that VIP, but not glutamate, can cause phase shifts of the rhythms in vasopressin release from cultured SCN neurons (Watanabe et al. 2000). Of course, it may be that VIP has multiple actions within the SCN cell populations and our data suggest that neuropeptides in the VIP family play a central role in the mammalian circadian timing system.

Acknowledgements:

Supported by NIH #HL64582, #NS043169 and NRSA # MH68087. We would like to thank Dr. S. Michel for his technical assistance as well as comments on a draft of this manuscript.

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Figure Legends

Figure 1. GABAergic IPSCs recorded from neurons in the mouse SCN. A. (top) Spontaneous IPSCs recorded from a dorsal SCN neuron during the day (top) are completely and reversibly blocked by 25 μM bicuculline (bottom). Frequency was determined by counting the number of events during a 1-2 min bin and comparing the frequency before and after drug treatments. B. Amplitude, rise time and decay time measurements were recorded for each event.

Figure 2. Frequency of GABAergic sIPSCs in dorsomedial and ventrolateral divisions of the mouse SCN during three phases, day (ZT 4-8), early night (ZT 13-15) and late night (ZT 20-22). Data are represented as mean frequency ± SEM. The sIPSC frequency was significantly greater in the DM division during early evening (12.0 Hz, n=16) compared to day (7.9 Hz, n=28) and late night (7.7 Hz, n=6). There were no significant differences detected in the VL division between day (3.7 Hz, n=15), early night (4 Hz, n=22), and late night (1.5 Hz, n=6).

Figure 3. VIP significantly increases the frequency of GABAergic sIPSCs in the mouse SCN. Trace is representative of a DM neuron recorded during the day, demonstrating a 49% increase in sIPSC frequency with VIP treatment (100 nM). IPSC amplitude, rise time and decay time were unaffected by VIP in this particular example.

Figure 4. Dose-response curve for the effects of VIP on GABAergic IPSCs. EC_{50} was calculated to be 42 nM. Data represented as normalized mean ± SEM. Curve fit to data using a Hill equation, n=6 at each data point.

Figure 5. Phase-dependent effect of VIP on the frequency of GABAergic IPSCs in the presence and absence of TTX. In TTX, magnitude of change is smaller but still significant between day and early night.

Figure 6. VIP enhances the frequency, but not amplitude, of GABAergic sIPSCs in SCN neurons. **A.** Graph demonstrating a leftward shift in the inter-event interval curve of a DM neuron recorded during the day. **B.** Cumulative amplitude curve for the same neuron is unchanged, indicating that VIP enhances the frequency of sIPSCs without altering the amplitude.

Figure 7. VIP enhances GABA release via the $VPAC_2$ receptor and cAMP/PKA-dependent pathway. VIP does not increase GABAergic IPSCs in the presence of VPAC receptor ([Tyr1,DPhe2]GHRF(1-29) 100 nM, n=8), $VPAC_2$ receptor (PG-465 200 nM, n=6), or PKA (H-89 20 μM, n=8) antagonists. Forskolin (10 μM, n=7), a potent activator of PKA, enhanced GABA release whereas the inactive analog, 1,9 dideoxyforskolin (10 μM, n=6), had no effect. In an occlusion experiment, pretreatment of the slice with forskolin prevented bath-applied VIP (100 nM, n=8) from having any additional effect, suggesting that both drugs are working through the same mechanism. Data represented as means ± SEM.

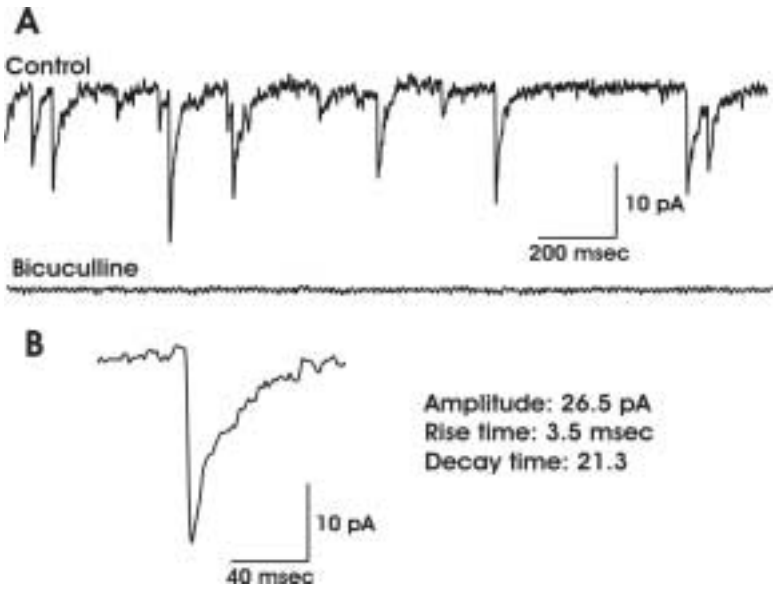
A
Control
10 pA
200 msec
Bicuculline
B
Amplitude: 26.5 pA
Rise time: 3.5 msec
Decay time: 21.3
10 pA
40 msec

Figure 1.

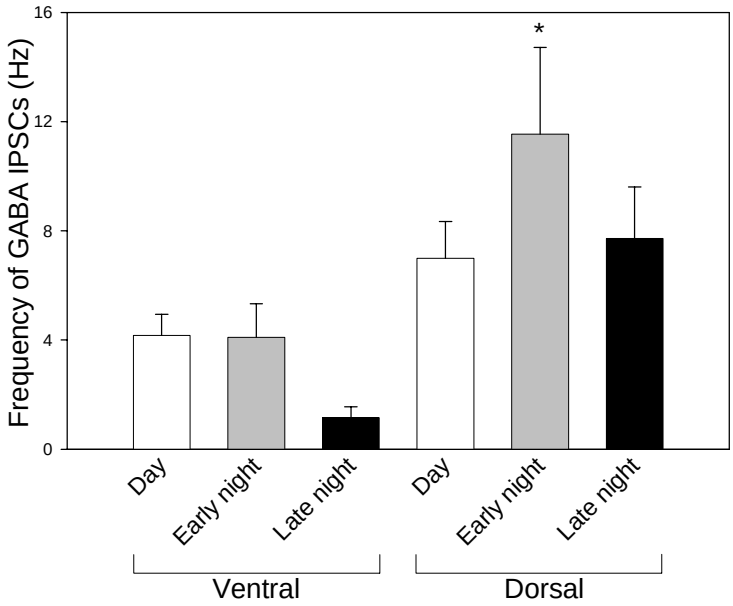
Frequency of GABA IPSCs (Hz)
16
12
8
4
0
*
Day
Early night
Late night
Day
Early night
Late night
Ventral
Dorsal

Figure 2.

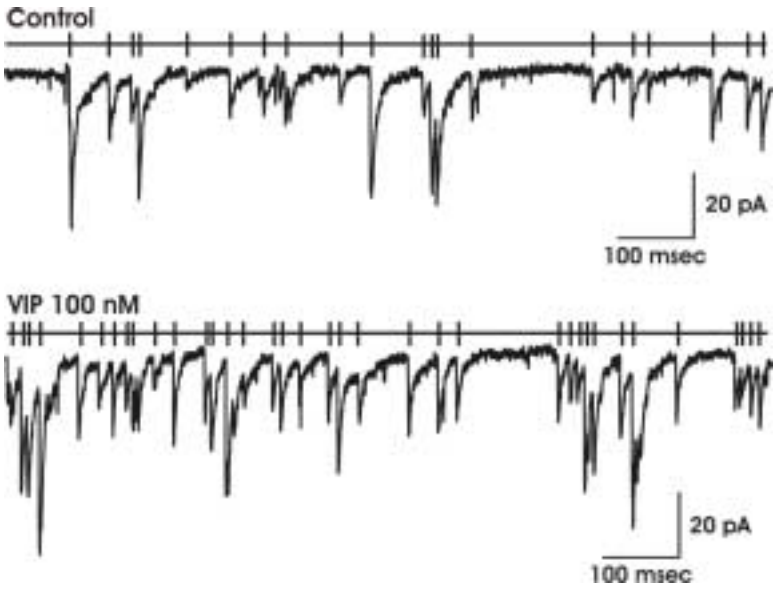

Figure 3.

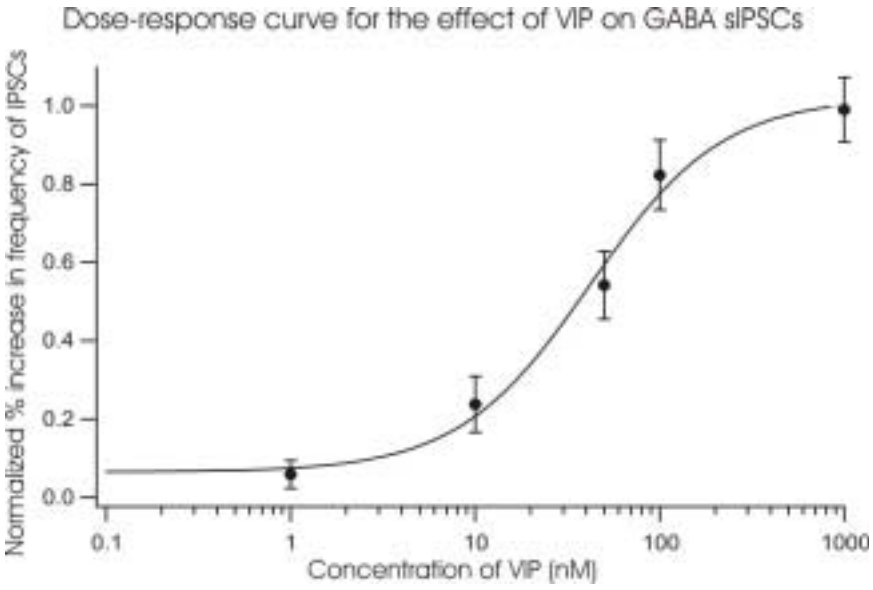
Dose-response curve for the effect of VIP on GABA sIPSCs
Normalized % increase in frequency of IPSCs
1.0
0.8
0.6
0.4
0.2
0.0
0.1
1
10
100
1000
Concentration of VIP [nM]

Figure 4.

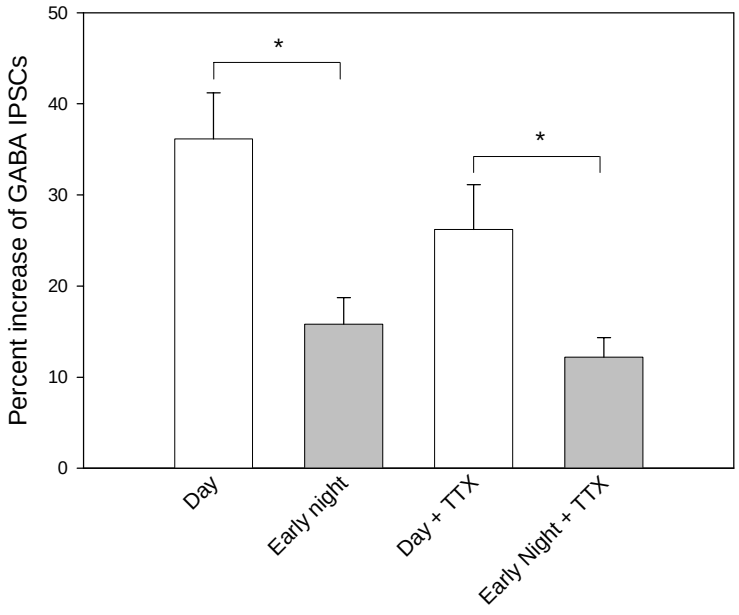
Percent increase of GABA IPSCs
50
40
30
20
10
0
*
*
Day
Early night
Day + TTX
Early Night + TTX

Figure 5.

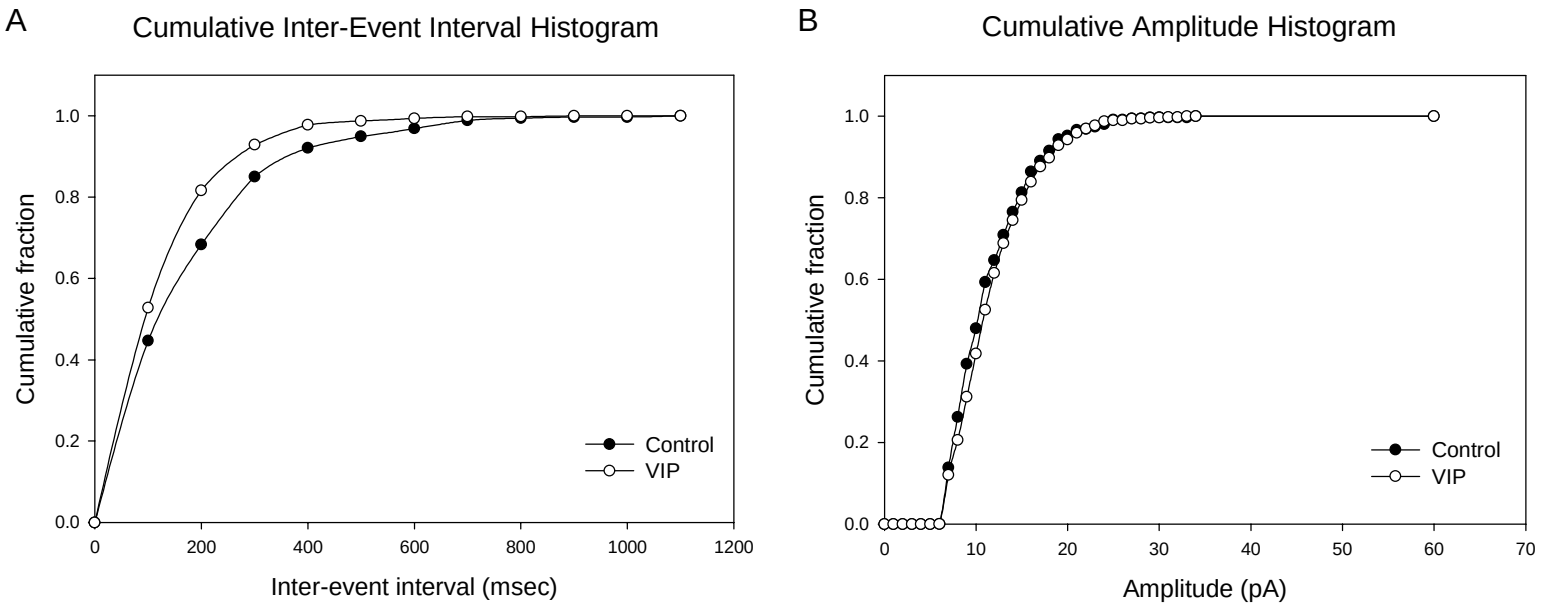
A
Cumulative Inter-Event Interval Histogram
Cumulative fraction
1.0
0.8
0.6
0.4
0.2
0.0
0
200
400
600
800
1000
1200
Inter-event interval (msec)
Control
VIP
B
Cumulative Amplitude Histogram
Cumulative fraction
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
70
Amplitude (pA)
Control
VIP

Figure 6.

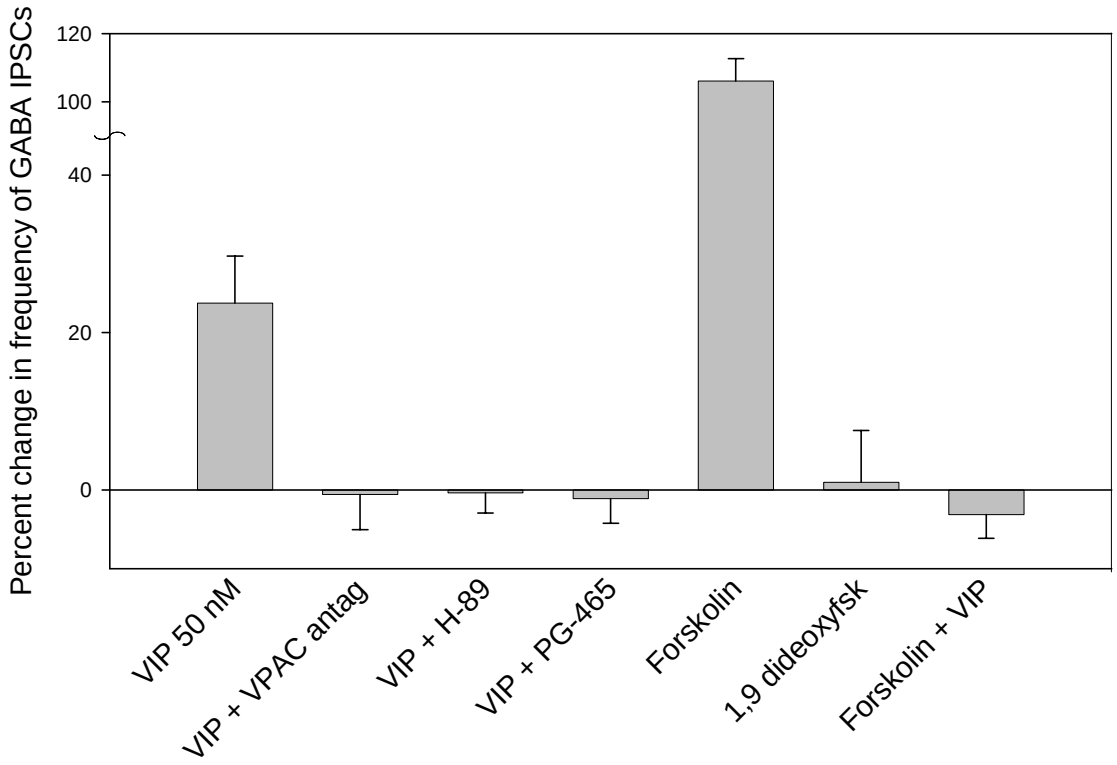
Percent change in frequency of GABA IPSCs
120
100
40
20
0
VIP 50 nM
VIP + VPAC antag
VIP + H-89
VIP + PG-465
Forskolin
1,9 dideoxyfsk
Forskolin + VIP

Figure 7.