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Authors
Vosko, A
van Diepen, HC
Kuljis, D
et al.

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Role of vasoactive intestinal peptide in the light input to the circadian system

Andrew Vosko,1 Hester C. van Diepen,2 Dika Kuljis,3 Andrew M. Chiu,4 Djai Heyer,2 Huub Terra,2 Ellen Carpenter,3 Stephan Michel,2 Johanna H. Meijer2 and Christopher S. Colwell3

1Department of Structural Medicine, Rocky Vista University, Parker, CO, USA
2Laboratory of Neurophysiology, Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, the Netherlands
3Department of Psychiatry & Biobehavioral Sciences, University of California – Los Angeles, Los Angeles, CA 90024, USA
4Medical Scientist Training Program, Northwestern University, Evanston, IL, USA

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Abstract

The neuropeptide vasoactive intestinal peptide (VIP) is expressed at high levels in a subset of neurons in the ventral region of the suprachiasmatic nucleus (SCN). While VIP is known to be important for the synchronization of the SCN network, the role of VIP in photic regulation of the circadian system has received less attention. In the present study, we found that the light-evoked increase in electrical activity in vivo was unaltered by the loss of VIP. In the absence of VIP, the ventral SCN still exhibited N-methyl-D-aspartate-evoked responses in a brain slice preparation, although the absolute levels of neural activity before and after light exposure were significantly reduced. Next, we used calcium imaging techniques to determine if the loss of VIP altered the calcium influx due to retinohypothalamic tract stimulation. The magnitude of the evoked calcium influx was unchanged in the ventral SCN, but did decline in the dorsal SCN regions. We examined the time course of the photic induction of Period1 in the SCN using in situ hybridization in VIP-mutant mice. We found that the initial induction of Period1 was not reduced by the loss of this signaling peptide. However, the sustained increase in Period1 expression (after 30 min) was significantly reduced. Similar results were found by measuring the light induction of cFOS in the SCN. These findings suggest that VIP is critical for longer-term changes within the SCN circuit, but does not play a role in the acute light response.

Introduction

Daily biological rhythms are intrinsically generated, synchronized, and regulated by networks of circadian oscillators. These oscillations are generated by robust negative feedback mechanisms that occur at the molecular, cytoplasmic and membrane levels within single cells (Mohawk et al., 2012; O’Neill et al., 2013). In mammals, the suprachiasmatic nucleus (SCN) of the hypothalamus contains the master oscillatory neurons necessary for coordinating these rhythms throughout the body (Dibner et al., 2010; Welsh et al., 2010). These SCN pacemaker neurons receive and integrate timing cues such as daylight and food availability to adjust their rhythmic output (Challet et al., 2003). The SCN is divided into core and shell regions (Abrahamson & Moore, 2001): the dorsally situated shell region contains the majority of the endogenously oscillating neurons in the nucleus; and the ventral core region receives afferent sensory signals (Yan et al., 2007).

Light is the most powerful environmental cue to which circadian rhythms synchronize (Czeisler et al., 1981; Khalsa et al., 2003). Photic cues are detected by specialized intrinsically photosensitive retinal ganglion cells (ipRGCs) containing the photopigment melanopsin (Lucas et al., 2012). The axons of ipRGCs form the retinohypothalamic tract (RHT), which terminates on the ventral aspect of the SCN (Morin & Allen, 2006). RHT terminals release glutamate and, under certain conditions, the neuropeptide PACAP (Hannibal et al., 2008), with the result of photic stimulation being the increase in firing rate of SCN neurons (Shibata et al., 1984; Meijer et al., 1998). In SCN neurons, N-methyl-D-aspartate (NMDA) receptor activation and increases in intracellular calcium (Ca2+) promote the transcription of a set of genes through cyclic AMP-responsive element (CRE)-binding protein (CREB) phosphorylation, including the clock gene Per1 (Travnickova-Bendova et al., 2002; Yan & Silver, 2004) and the immediate-early gene cFOS (Kornhauser et al., 1996). These molecular light-evoked changes in gene expression are thought to be necessary to cause light-induced phase shifts in physiology and behavior (Albrecht, 2012).

Although the detailed mechanisms by which the SCN circuit operates remain unknown, a major role for vasoactive intestinal peptide (VIP) in photic resetting is indicated. Behaviorally, VIP-deficient mice show clear deficits in their circadian light response...
(Colwell et al., 2003), and our hypothesis was that the light-evoked changes in SCN physiology would also be compromised. Therefore, in the present study, we first examined the SCN response to light in vivo using multi-unit activity (MUA) recordings of freely-moving VIP knockout (KO) mice and littermate wild-type (WT) controls. We then measured NMDA-evoked responses in ventral SCN neurons in a brain slice preparation of both genotypes. Next, fura-2-acetoxymethyl ester (Fura-2-AM) calcium imaging techniques were used to measure the calcium transients evoked by electrical stimulation of the RHT in vitro. Digoxigenin (DIG) in situ hybridization (ISH) was used to follow the light induction of Per1 message in the SCN of both genotypes. Light induction of cFOS in the SCN was examined with immunohistochemical (IHC) techniques.

Materials and methods

Animals

Experimental protocols used in this study were approved by the University of California, Los Angeles or Leiden University Animal Research Committee. Recommendations for animal use and welfare, as dictated by the UCLA Division of Laboratory Animals and the guidelines from the National Institutes of Health, were followed. Adult male (1.5–5 months) WT C57Bl/6 mice and mice lacking the gene encoding for the neuropeptides VIP (VIP KO; Colwell et al., 2003) were obtained from a breeding facility at the University of California, Los Angeles, or from the breeding facility at Leiden University. Mice were group-housed until they were used for experiments. A total of 102 C57 mice were used, with half being the VIP KO.

In vivo SCN recordings

Mice were implanted with a tripolar stainless-steel micro-electrode (Plastics One, Roanoke, VA, USA) using a stereotaxic instrument (Stoelting, Wood Dale, IL, USA), as previously described (Lucassen et al., 2012; van Diepen et al., 2013). Two polyimide-insulated and twisted electrodes were aimed at the SCN, and a third uncoated electrode was placed in the cortex as a reference. Mice were anesthetized using a mix of ketamine (100 mg/kg), xylazine (20 mg/kg) and atropine (1 mg/kg). The electrodes were implanted under a 5° angle at the same rostrocaudal level as bregma, 0.61 mm lateral to midline and 5.38 mm ventral to the dura mater. The electrode was fixed to the skull using three screws and dental cement. After 1 week of recovery, animals were placed in a custom-designed recording chamber to measure SCN electrical activity and behavioral activity using passive infrared sensors simultaneously. Animals were connected to the recording system using a counterbalanced swivel system in which they were able to freely move. The electrical signal was amplified and bandwidth filtered (0.5–5 kHz). Window discriminators were used to convert action potentials into digital pulses that were counted in 2-s epochs. Physiological responses were used to confirm electrode placement within the light-responsive part of the SCN.

Animals were recorded over at least 2 days in continuous darkness. After 2 days of continuous darkness, animals were exposed to 5 min of light (fluorescent light source; 150 μW/cm²) at circadian time (CT) 14–16 (2–7 pulses). The animals received multiple 5-min pulses within the same day at this phase. The CT of the light response was calculated per day on the basis of the onset of behavioral activity recorded by a passive infrared sensor in the recording chamber. For quantification of the response, the increases in SCN electrical activity were compared with baseline levels. Baseline levels were defined as 50 s before lights on, and the level of sustained light-induced increase was defined as the average firing rate during lights on (> 5 s after light onset). At the end of each recording, animals were killed, and brain tissue was collected for histological verification of the electrode location. After brain fixation in a 4% paraformaldehyde solution containing ferrocyanide, brains were sectioned coronally and stained with Cresyl violet. The position of the electrode was determined by microscopic inspection (Fig. S1). Electrodes outside the SCN were excluded from analysis.

Whole-cell patch-clamp electrophysiology

Mid-SCN coronal slices were collected with a vibratome in slice solution (in mM: NaHCO3, 26; NaH2PO4, 1.25; glucose, 10; NaCl, 125; KCl, 3; MgCl2, 5; CaCl2, 1) from Per2::Luciferase (P2L) mice and VIP KO × P2L littermates between 1.5 and 3 months old. Slices were attached to the stage of a fixed-stage upright DIC microscope (Olympus, Tokyo, Japan), and superfused continuously with aerated (95% O2/5% CO2) artificial cerebrospinal fluid (ACSF; in mM: NaHCO3, 26; NaH2PO4, 1.25; glucose, 10; NaCl, 125; KCl, 3; MgCl2, 5; CaCl2, 2). Recording electrodes (4–8 MΩ) were pulled from glass capillaries (WPI, Sarasota, FL, USA) on a multistage puller (Sutter P97, Novato, CA, USA), and recording electrodes were filled with standard internal solution (in mM): K-glucuronate, 112.5; EGTA, 1; Hepes, 10; MgATP, 5; GTP, 1; leupeptin, 0.1; phosphocreatine, 10; NaCl, 4; KCl, 17.5; CaCl2, 0.5; and MgCl2, 1. Recordings were obtained using the AXOPATCH 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) and monitored on-line with pCLAMP (Ver. 10; Molecular Devices). Each cell was visualized to be within the SCN by means of DIC microscopy, ventral cells were adjacent to or in close proximity with the optic chiasm. After forming a high-resistance seal (> 1 GΩ) in voltage-clamp configuration, a second pulse of negative pressure was used to break the membrane. Most cells had a capacitance between 6 and 15 pF, and cells with access resistance higher than 60 MΩ or holding currents larger than −30 pA (at Vm = −70 mV) were excluded from additional analysis. Junction potential between the pipette and extra-cellular solution was canceled by voltage offset of the amplifier before establishing a seal. The pH of all solutions was adjusted to 7.25–7.3, and osmolarity was adjusted to 290–310 mOsm.

Spontaneous firing rates (SFR) were recorded using current-clamp mode within whole-cell patch-clamp configuration. After entering whole-cell mode in voltage-clamp and switching to current-clamp mode, neuronal firing was allowed 3–5 min to stabilize. Baseline SFR was then calculated using the number of action potentials detected over the subsequent 60 s. NMDA (25 μM) was then applied for 2–3 min, and SFR was calculated using the number of action potentials detected during the subsequent minute of NMDA treatment. Following NMDA treatment, ACSF was used to wash slices and determine whether cells were able to re-hyperpolarize following pharmacological excitation to ensure only healthy cells were included in the analysis. All recordings were made in the presence of the γ-aminoobutyric acid (GABA)A receptor blocker gabazine (10 μM; Tocris; Minneapolis, MN, USA). Chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA), unless otherwise noted.

In vitro Ca2+ imaging

Brain slices containing the SCN were collected as described above from animals 6–10 weeks old, and loaded with the ratiometric Ca2+ indicator dye Fura-2-AM (Teflabs, Austin, TX, USA), as previously
described (Michel et al., 2013). The slices were prepared at zeitgeber time (ZT) 11 and the recordings made between ZT 15 and 17. A monochromator (Polychrome V, TILL Photonics, Gräfeling, Germany) was used to deliver paired 50-ms light pulses of two excitation wavelengths (340 and 380 nm). Emitted light (505 nm) was detected by a cooled CCD camera (Sensicam; TILL Photonics), and images were acquired at 2-s intervals (0.5 Hz). Single-wavelength images were background subtracted, and ratio images (340/380) generated. Cells were defined as ventral or dorsal based on the proximity to the optic chiasm or third ventricle, respectively (Fig. S2), and the mean ratio values for these region-of-interest-defined cells were used to calculate intracellular Ca²⁺ concentration. Experiments were conducted using imaging software TILLvisION (TILL Photonics).

For electrical stimulation of the RHT, a concentric bipolar electrode (125 μm/Rnd/25 μm Pt-Ir, purchased from FHC, Bowdoin, ME, USA) connected to a Grass S88 Stimulator (Warwick, RI, USA) was placed in the center of the optic chiasm of a coronal hypothalamic slice. The stimulation strength was adjusted to elicit clear Ca²⁺ transient with a fast recovery to baseline levels. The cells were stimulated at 10 Hz for 1 s with a pulse duration of 200 μs. This train of stimuli was repeated three times with a 60-s pause between stimulations.

Data were collected and analysed using Tillvision, Igor Pro (Wavemetrics, Portland, OR, USA), Excel (Microsoft, Redmond, WA, USA) and SPSS 20 (IBM, Armonk, NY, USA). First, baseline Ca²⁺ concentrations were examined by using the mean and SD of signals 14 s before each peak in every cell. Cells with a baseline intracellular Ca²⁺ concentration [Ca²⁺]ᵢ larger than 600 nM were excluded from the data set. Values that were >2 SDs from the mean were identified as outliers by the SPSS software and excluded from the analysis.

DIG ISH
A plasmid (pCRII; Invitrogen, Carlsbad, CA, USA) containing the cDNA for Per1 (340–761 nucleotides, accession number AF022992) was generously provided by Dr D. Weaver (University of Massachusetts), and insert identity was confirmed by sequencing using the M13R primer. To generate antisense and sense templates for hybridization, plasmids were linearized overnight, phenol–chloroform extracted, ethanol precipitated and re-suspended in diethyl pyrocarbonate-treated water.

DIG-labeled riboprobes were generated from 1 μg of template cDNA in a reaction mixture containing 2 μL of 10 × concentrated DIG RNA Labeling Mix (Roche Applied Science, Indianapolis, IN, USA), 2 μL of 10 × concentrated Transcription Buffer (Roche Applied Science), 40 U RNase Block (Stratagene, La Jolla, CA, USA) and 2 μL of the appropriate RNA transcriptase (SP6 or T7; Roche Applied Science) for 2 h at 37 °C. The in vitro transcription reaction was terminated by the addition of 2 μL of 0.2 M EDTA, and precipitated with 2.5 μL of 4 μ M LiCl and 100% ethanol overnight at −20 °C. The precipitate was extracted with 70% ethanol and reconstituted in 100 μL of sterile water. Probe yield estimates were determined by comparison to known concentrations of untranscribed, linearized plasmid in gel electrophoresis, and also from serial dilutions of cross-linked riboprobe spotted to nitrocellulose membranes, bound to alkaline phosphatase-conjugated anti-DIG antibody (Roche Applied Science) and visualized with 4-nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Roche Applied Science).

Prior to brain collection, wheel-running activity was measured as described previously (Loh et al., 2013). Male VIP-deficient mice were housed in cages containing running wheels (Mini Mitter, Bend, OR, USA) from 6 to 7 weeks old, and their wheel-running activity was recorded as revolutions (rev) per 3-min intervals. Animals were exposed to a 12 : 12 h light–dark cycle (LD; light intensity 350 lux) for 10 days, and then released to 24 h of constant darkness (DD) to assess their free-running activity pattern for 7–10 days. Animals were exposed to light (10 min, light intensity ≈ 50 lux) at CT 16 based on wheel-running activity records (CT 12 was defined as activity onset), and killed after either 30, 60, 90 or 120 min under anesthesia (n = 3–5 in each condition). Control animals were killed at the same time without a light pulse. Brains were removed, flash-frozen, sectioned at 20 μm and slide mounted, then stored at −80 °C until used for ISH or immunofluorescence.

On the first day of hybridization, slides were warmed to room temperature and fixed in 4% paraformaldehyde. Following brief washes in phosphate-buffered saline (PBS), slides were placed in prehybridization buffer [50% formamide, 5 × standard sodium citrate (SSC), 1% sodium dodecyl sulfate (SDS), 0.2% Tween-20, 0.1% heparin and 50 ng/mL Torula RNA] at 60 °C for 1–2 h. Sections were then hybridized overnight at 60 °C in hybridization buffer (50% formamide, 5 × SSC, 1% SDS, 0.2% Tween-20, 0.1% heparin and 50 ng/mL Torula RNA) and ≈50–100 pg/μL of riboprobe in sealed slide mailers. Following hybridization, slides were washed briefly in 5 × SSC, and then for 1 h in 0.2 × SSC at 60 °C to remove unbound probe. Slides were then briefly washed with maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl) and blocked in 20% heat-treated sheep serum in maleic acid buffer. After blocking, slides were incubated with 1 : 500 anti-DIG antibody conjugated to alkaline phosphatase (Roche Applied Science) in a humid chamber at 4 °C overnight. After antibody incubation, slides were washed in maleic acid buffer and then in Tris buffer [0.1 M Tris (pH 9.5), 0.1 M NaCl and 5 mM MgCl₂]. For revelation, slides were incubated in a color reaction solution (Tris buffer, 0.3375% NBT, 0.35% BCIP, 1 mM levamisole) at room temperature overnight in a humid chamber. After revelation, slides were washed with PBS and color was preserved via a final incubation in 4% paraformaldehyde containing EDTA. Slides were then cover-slipped and imaged on a Zeiss microscope using Axiovision software (Carl Zeiss, Thornwood, NY, USA) for analysis. Sense probe hybridization showed no positive staining.

For quantification, SCN sections were imaged and SCN borders were determined by 4’,6-diamidino-2-phenylindole, dihydrochloride (DAPI) and arginine vasopressin (AVP) distribution dorsal to the optic chiasm in the anterior hypothalamus. For each SCN, a template was created to define core and shell subregions delineated by AVP expression (Fig. S3). This delineation was confirmed by additionally double-immunostaining against androgen receptor (AR) to confirm that a lack of AVP signal coincided with the SCN core. Templates from adjacent sections were superimposed onto both Per1+ and c-FOS+ sections, and the resulting mid-SCN image (uni-lateral) for each animal was counted by an experimenter blind to condition.

IHC
Brains were processed as described above, and alternate sections were used to delineate SCN subregions chemo-architecturally. Sections were fixed in 4% paraformaldehyde at room temperature, washed with PBS, and incubated in blocking solution (3% normal goat serum, 0.1% Triton X-100 in PBS). After blocking, sections were incubated with a guinea pig polyclonal antibody raised against AVP (1 : 1000; Bachem, Torrance, CA, USA); a rabbit polyclonal
antibody raised against FOS (1 : 30 000; Merck Millipore, Darmstadt, Germany); or a rabbit polyclonal antibody raised against AR (1 : 150; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in blocking solution at 4 °C for 4 days. Sections were then washed and incubated with Alexa Fluor® 568-conjugated goat anti-guinea pig IgG antisera (Molecular Probes, Eugene, OR, USA), diluted to 1 : 300 with blocking solution at room temperature. If tissue was processed for double-immunostaining, primary antibody incubation was carried out with both the primary AVP antibody and a rabbit polyclonal antibody raised against AR (Santa Cruz Biotechnology) diluted at 1 : 150. Also, during the secondary antibody incubation, Alexa Fluor® 488-conjugated secondary antisera (Molecular Probes), diluted at 1 : 200, was additionally used. After incubation with secondary antibody, sections were again washed with PBS, cover-slipped with Vectashield Mounting Media containing DAPI (Vector Laboratories, Burlingame, CA, USA), and stored in the dark at 4 °C until imaged.

**Statistical measurements**

The data sets were analysed for equal variance and normal distribution to help select the appropriate statistical test. Significance for electrophysiological recordings and Ca²⁺ imaging was assessed using a Student’s t-test as well as two-way analysis of variance (ANOVA), with genotype and light or NMDA exposure as factors. Paired Student’s t-tests were used to detect significant changes due to pharmacological treatment, and unpaired Student’s t-tests were used to assess differences in spontaneous electrical activity due to genotype. Effects were reported to be significant if \( P < 0.05 \). For ISH, the data sets were analysed by two-way ANOVA, with genotype and light exposure as factors. If significant group differences were detected (\( P < 0.05 \)) by ANOVA, then the Holm–Sidak method for pair-wise multiple comparisons was used. For all tests, values were considered significantly different if \( P < 0.05 \). All tests were performed using Sigmastat software (version 3.5; Systat Software, San Jose, CA, USA). Values are shown as mean ± SEM.

**Results**

**In vivo electrophysiology finds no deficits in light-evoked responses in SCN of VIP mutants**

Using in vivo extracellular recording techniques, SCN electrical activity was measured in both VIP KO (\( n = 7 \)) and WT controls (\( n = 5 \)). Physiological responses were used to confirm that the electrode was placed in a light-response region of the SCN, and histological analysis confirmed that each of these recordings was made in the SCN. Successful recordings showed high SCN electrical activity during the day and low electrical activity during the night. Light exposure induced an increase in the SCN electrical discharge pattern with a transient overshoot at lights on and a sustained elevation in SCN electrical activity throughout light exposure (Fig. 1A). No differences in light-response characteristics were detected between VIP KO and WT mice (KO: 12 ± 2% change; WT: 16 ± 6%; \( t \)-test: \( P > 0.05 \); Fig. 1B). Both genotypes showed a significant increase in SCN electrical activity upon light exposure during the night (CT 14–16; Fig. 1C). Two-way ANOVA (DF = 25) was also performed to test for genotype and treatment effects. Main effect of light treatment (\( F = 15.610, P = 0.003 \)) was identified, but no effect of genotype (\( F = 0.299, P = 0.596 \)) or an interaction (\( F = 0.173, P = 0.687 \)) was found. These results demonstrate that photic information is reaching the retino-recipient cells in the SCN in the VIP KO mice.

**Fig. 1. Light-evoked changes in multi-unit activity (MUA) recorded from the suprachiasmatic nucleus (SCN) of freely moving mice. (A, B) Representative examples of MUA rhythms recorded from the SCN of a wild-type (WT) and vasoactive intestinal peptide (VIP) knockout (KO) mouse during the night. Light pulses are indicated above the graphs. SCN firing rate is increased in both VIP KO and WT mice, with a response latency of 0.04 s. Bin size is 1 s. (C) Bar graphs showing the mean and SEM of the MUA before and during light exposure. *Indicates significant difference (\( P < 0.05 \)) compared with controls analysed by two-way ANOVA, followed by the Holm–Sidak method for multiple comparisons. There were no differences between the two genotypes.**

**In vitro electrophysiology demonstrates reduced activity but robust NMDA-evoked changes in ventral SCN neurons**

A variety of evidence suggests that the effects of light on the mammalian circadian system are mediated by glutamatergic mechanisms, and that the NMDA receptor plays an important role in this regulation. Using the whole-cell patch-clamp recording technique in current-clamp mode, we measured the SFR in ventral SCN neurons in a brain slice preparation in response to bath application of NMDA (25 μM, 3 min). Each of these cells was determined to be within the ventral region of the SCN by directly visualizing the location of the cell as being immediately adjacent to or in close proximity with the optic chiasm using infrared DIC video microscopy. In WT mice, ventral SCN neurons responded to bath application of NMDA with an increase in SFR (Fig. 2; \( P < 0.001 \)). The VIP KO also showed a significant response to NMDA treatment (\( P = 0.02 \)), but there were differences between the genotypes. First, the absolute SFR was reduced in mutant SCN compared with WT in both baseline (WT: 1.76 ± 0.4 Hz vs. VIP KO: 0.48 ± 0.2 Hz, \( P = 0.03 \)) and NMDA
compared with controls analysed by two-way ANOVA, followed by the Holm Sidak method for multiple comparisons. #Indicates a significant difference (P < 0.05) compared with controls analysed by two-way ANOVA, followed by the Holm-Sidak method for multiple comparisons. #Indicates a significant difference between the two genotypes.

**Ca**

 imaging indicates RHT stimulation to be normal in the ventral SCN cell population but reveals deficits in dorsal SCN in VIP KO mice

Release of glutamate from RHT terminals in the SCN after electrical stimulation will lead to an increase in [Ca

]

 (Irwin & Allen, 2007) and ultimately phase shifts of the circadian system. Optical imaging techniques and the Fura-2-AM indicator dye were used to measure RHT-stimulated Ca

 increases in SCN cells in the night. RHT stimulation produced a reliable increase in [Ca

] in a subset of SCN neurons in both ventral (WT: 40%; KO: 38%) and dorsal (WT: 20%; KO: 19%) cell populations (Fig. 3A). In the ventral SCN region, the magnitude of the RHT-evoked Ca

 transient was enhanced in the VIP-deficient mice (Fig. 3A), while in the dorsal SCN there was a 30% reduction in the amplitude of RHT-evoked Ca

 increases (Fig. 3B). The baseline Ca

 levels measured during the night were low and did not vary between the genotypes (Fig. 3C). Two-way ANOVA (DF = 580) indicated no main effect of genotype (F = 0.04; P = 0.841) on the magnitude of the Ca

 increases, but there were significant effects of region (F = 50.860; P = 0.001) and a significant interaction between genotype \texttimes\ region (F = 9.473; P = 0.002). Thus, as measured by Ca

 transients, the ventral SCN cell population appears to be receiving the signal from the RHT, but the dorsal SCN cells exhibit a weakened response in the absence of VIP.

The loss of VIP alters the temporal and spatial distribution of light-evoked increases in Period1 expression in the SCN

To establish how light-induced Per1 expression varied between the genotypes, ISH using DIG probe for Per1 was performed on SCN tissue at 30, 60, 120 min after light exposure at CT 16 (50 lux, 10 min). SCN photomicrographs illustrate that WT and VIP-deficient mice differ in the spatio-temporal expression profiles of Per1 (Figs 4 and 5). As has been previously reported (Yan & Silver,
In the VIP KO mice, the two waves of Per1 induction follow a different pattern. Although there was an immediate response to the light pulse, with well-defined expression in a subpopulation of cells in the ventro-middle region of the nucleus about 45–60 min after the initial pulse. Message expression spreads across the nucleus and disappears by 4 h after the initial pulse. This pattern is disrupted in the vasoactive intestinal peptide (VIP) knockout (KO) mice in which the initial induction of the Per1 in response to light is strong but the signal is not sustained or communicated to the shell SCN region. 3rd V; 3rd ventricle; OC, optic chiasm; PVN, paraventricular nucleus; SCN, suprachiasmatic nucleus.

2004) in WT SCN, Per1 is photically induced in two separate waves: one in the core; followed by one in the shell. The majority of immediate light-induced Per1 expression occurs during the first wave in the SCN core. This is followed by a second wave in the shell, characterized by a steady increase of Per1 from 30 to 90 min. In VIP KO mice, the two waves of Per1 induction follow a different pattern. Although there was an immediate response to the light pulse with a sizable induction of Per1 seen 30 min after the initial light exposure in the SCN core, this first wave of Per1 induction decreased faster in the VIP KO, at least 1 h earlier when compared with WT. Next, the second wave of Per1 induction in the SCN shell was markedly attenuated in VIP KO mice. In fact, Per1 levels were similar to untreated controls, although there was more animal to animal variation in mutants. Two-way ANOVA analysis (DF = 38) of the Per1 expression in the core revealed effects of genotype (F = 50.216, P = 0.001), light treatment (F = 9.272, P = 0.001) and interaction of genotype × light treatment (F = 4.109, P = 0.009) on light-induced Per1 expression levels in the mouse SCN.

We also examined the light induction of FOS in the core and shell SCN (Fig. 6). Compared with untreated controls, WT SCN exhibited a significant FOS induction when measured 60 and 120 min after light exposure (50 lux, 10 min) in both the core and shell regions. In contrast, the VIP KO mice only exhibited a significant FOS induction when measured 60 min after light exposure in the core SCN. By 120 min, the FOS counts were back at baseline in the core and never increased in the shell region. Two-way ANOVA analysis (DF = 22) of FOS expression in the core revealed effects of genotype (F = 24.851, P = 0.001), light treatment (F = 21.776, P = 0.001) and interaction of genotype × light treatment (F = 4.668, P = 0.02) on light-induced FOS expression levels in the mouse SCN. Thus, in the VIP KO mice, the light induction of cFOS and Per1 are attenuated in duration and most significantly impacted in the shell region of the SCN.

Discussion

Previous studies have provided evidence that VIP signaling is important for synchronization of the circadian oscillator to the environment. Behavioral pharmacology studies have shown that the application of VIP alone (Piggins et al., 1995) or in combination with other peptides (Albers et al., 1991) can mimic the phase-shifting effects of light. In a
that VIP is required for normal light-induced synchronization of the circadian system, but does not tell us where in the circadian circuit the deficits lie.

To examine this issue, we first implanted electrodes into the SCN of VIP KO mice and litter-mate WT controls, and recorded MUA in freely moving mice. In response to retinal illumination, light-responsive SCN neurons show an increase in electrical impulse frequency (Meijer et al., 1998; Nakamura et al., 2004; Drouyer et al., 2007; Brown et al., 2011; van Diepen et al., 2013). We found that the VIP KO mice exhibited robust light responses that could not be distinguished from those recorded from the SCN of WT mice (Fig. 1). All of the cells were light responsive, but the nature of the extracellular recording makes it difficult to determine the region of the SCN from which the neurons were sampled. Prior work has established that the RHT mostly terminates on the ventral aspect of the SCN (Ibata et al., 1989; Morin & Allen, 2006; Kiss et al., 2008), and we assume that this is where the greatest percentage of light-responsive units are located. Given the severe behavioral deficits observed in the photic response of the VIP KO mice (Colwell et al., 2003), these results were unexpected. These results indicate that the retinal illumination is reaching the SCN of the mutant mice without difficulty.

A variety of evidence indicates that NMDA receptors play a critical role in transducing the glutamatergic RHT signal to an electrical response in the SCN (Colwell et al., 1990; Ding et al., 1994; Colwell, 2001; Pennartz et al., 2001; Wang et al., 2008). Therefore, we next directly measured NMDA-evoked changes in firing rate in SCN neurons in a brain slice preparation. One caveat here is that in order to isolate the NMDA currents, we blocked GABA-mediated synaptic transmission, which could influence the findings. Focusing on neurons in the retinorecipient ventral region of the SCN, we found that VIP KO and WT mice exhibited clear NMDA-evoked changes in firing rate (Fig. 2). This observation fits with the extracellular recording described above. The single cell resolution of the whole-cell patch-clamp recordings offers a more nuanced view of the impact of the loss of VIP. The absolute level of SCN activity was significantly reduced in the absence of VIP, and a smaller percentage of neurons exhibited a significant NMDA-evoked change in firing. These genotypic changes would be very difficult to detect with the extracellular technique used above. So, the patch-clamp recording indicates that, while the KO mice exhibit a clear NMDA response, the absolute firing rate with and without NMDA treatments was reduced in the mutant ventral SCN neurons.

After NMDA receptor activation, the next step in the signaling cascade in SCN neurons appears to be an increase in intracellular Ca\(^{2+}\) mediated by both glutamate receptor activation as well as depolarization-driven activation of voltage-sensitive Ca\(^{2+}\) channels (Kim et al., 2005; Irwin & Allen, 2007; Colwell, 2011). Optical imaging techniques and the Fura-2-AM indicator dye were used to measure RHT-stimulated Ca\(^{2+}\) transients in SCN cells in the night. While the percentage of cells responding to RHT did not vary between the genotypes (Fig. S4), we saw a striking difference in the magnitude of responses between the ventral and dorsal cell populations. In the ventral SCN region, the RHT-evoked Ca\(^{2+}\) transient was actually enhanced in the VIP-deprived mice compared with WT (Fig. 3A). This fits with our in vivo recordings, as shown in Fig. 1. In contrast, in the dorsal SCN region, there was a 30% reduction in the amplitude of RHT-evoked Ca\(^{2+}\) increases (Fig. 3B). So the physiological measures indicate that the loss of VIP does not alter the transmission of light information to the SCN circuit, but raise the possibility that the VIP prevents the spread of photic information from the ventral neurons to the rest of the SCN circuit. This

Fig. 6. Quantification of the temporal patterns of light-evoked c-FOS expression in the core and shell of suprachiasmatic nucleus (SCN). Cell counting of c-FOS+ cells in the SCN core following immunofluorescence reveals that c-FOS is significantly induced by a circadian time (CT) 16 light pulse at both 60 and 120 min following light exposure in wild-type (WT) mice, but only at 60 min post-pulse in vasoactive intestinal peptide (VIP) knockout (KO) mice. In the SCN shell, c-FOS is significantly induced at both 60 and 120 min following light exposure in the WT mice. There was no significant c-FOS induction in the SCN shell of the mutant mice. Post hoc analyses reveal significantly reduced c-FOS+ cell counts in VIP KO mice compared with controls at both the 60 and 120 min time points. *Indicates significant difference (P < 0.01) compared with controls analysed by two-way ANOVA, followed by the Holm–Sidak method for multiple comparisons.
hypothesis fits with recent observations suggesting that VIP and its receptor (VIPR2) are necessary for circuit-level integration with the SCN (Brancaccio et al., 2013; Pauls et al., 2014).

Finally, we turned to anatomical techniques to further test this model. The increase in Ca$^{2+}$ activates a number of signaling pathways that converge to alter transcriptional and/or translational regulators, including CREB. Phosphorylated CREB is translocated into the nucleus where it can bind to CREs in the promoter regions of c-Fos and Period1 (Per1), and drives transcription of these genes over the course of hours (Kornhauser et al., 1996; Shearman et al., 1997; Shigeysoshi et al., 1997; Gau et al., 2002; Travnickova-Bendova et al., 2002). We have previously found that the photic induction of Per1 in the SCN was reduced in the VIP KO (Dragich et al., 2010) at 60 min following the light exposure, but did not examine the spatio-temporal patterns in the mutants. So, in our final set of experiments, we examined the light-evoked changes in Per1 and FOS in the SCN of the VIP KO mice. For each SCN, a template was created to define core and shell regions delineated by expression of AVP (shell) and AR (core; Fig. S3). As has been previously reported (Hamada et al., 2004; Yan & Silver, 2004; Koch et al., 2009) in WT SCN, Per1 is pholtically induced in two separate waves: one in the core followed by one in the shell. The majority of immediate light-induced Per1 expression occurs during the first wave in the SCN core. This is followed by a second wave in the shell, characterized by a steady increase of Per1 from 30 to 90 min (Figs 4 and 5). The light induction of Per1 expression in the VIP KO showed a strikingly different pattern. As we have seen with the physiology, the immediate response to light exposure was a sizable induction of Per1 in the core SCN of the VIP KO mice. However, this increase declined faster and a second wave of Per1 induction in the SCN shell was markedly attenuated in VIP KO mice. As far as we know, this pattern of gene expression has only been reported once before. Mice heterozygous for a mutation in the NaV1.1 channel (Scn1a +/-) also show normal light induction of c-Fos and Per1 mRNA in ventral SCN but impaired gene expression responses in dorsal SCN (Han et al., 2012). We do not know the impact of the reduction in the Scn1a gene on SCN electrical activity, but a reasonable speculation is that the excitability is reduced in both the Scn1a +/- and the VIP –/– mice.

Together these finding raise questions about mechanisms and functional significance. The ventral SCN receives most of the retinal input (Ibata et al., 1989; Morin & Allen, 2006; Kiss et al., 2008), and shows more robust light-induced changes in electrical activity (Shibata et al., 1984; Meijer et al., 1998) and gene expression (Antle et al., 2009). Many of the neurons that receive retinal input within the core SCN express the neuromodulators VIP and gastrin-releasing peptide, as well as the neurotransmitter GABA. In contrast, neurons of the dorsal shell appear to generate the most robust circadian oscillations, at least at the level of gene expression (Yan & Okamura, 2002; Hamada et al., 2004; Nakamura et al., 2005). The neurons in the shell express AVP, prokineticin 2 as well as GABA. Therefore, VIP is well positioned to mediate core to shell communication. While the VIP receptors (VIPR2) are expressed throughout the SCN, the expression is more abundant in the dorsal region (An et al., 2012), especially in the central SCN where we do our physiological recordings.

Both light exposure as well as treatment with gastrin-releasing peptide or VIP can also cause persistent increases in neural activity within the SCN at night (Kuhlman et al., 2003; Gamble et al., 2007, Gamble et al., 2011; LeSauter et al., 2011; Kudo et al., 2013). Thus, peptide transmitters can drive long-lasting changes in the excitability within the SCN network. We speculate that VIP and gastrin-releasing peptide may work together functionally to regulate the excitability of the SCN circuit. VIP is expressed with a subset of GABAergic interneurons found throughout the CNS, and has been shown to alter the excitability of several neural populations (Jeltinjia et al., 1982; Pawelzik et al., 1992; Lee & Cox, 2006; Hermes et al., 2009). There is also evidence that SCN neurons from VIPR2–/– mice may be chronically hyperpolarized (Pakhotin et al., 2006), consistent with the findings in the present study. These findings suggest that VIP-induced changes in electrical activity may be critical for the light-induced changes in gene expression especially in the dorsal SCN. During the night, SCN neurons are normally silent, but do respond to photic stimulation transduced by ipRGCs that generate action potentials up to 20 Hz (Meijer et al., 1998; Benson et al., 2002; Warren et al., 2003; Tu et al., 2005; Irwin & Allen, 2007). This light-induced increase in neural activity drives synaptic communication with the rest of the cells in the circuit. Prior work in both mollusks and mammals suggests that the electrical activity of circadian pacemaker neurons determines how these cells respond to photic stimulation (McMahon & Block, 1987; Irwin & Allen, 2007; Colwell, 2011). This regulation can explain why the loss of VIP or its receptor has such a dramatic effect on photic entrainment of the circadian system and impairs the ability of the SCN to encode seasonal information.

Supporting Information

Additional supporting information can be found in the online version of this article:

Fig. S1. An example of a coronal slice of the mouse brain with the SCN right above the optic chiasm at the base of the hypothalamus (left panel).

Fig. S2. Image of Fura-2-AM-labeled SCN slice.

Fig. S3. Different SCN markers were used to create core and shell templates to overlay on alternately stained Per1-expressing SCN photomicrographs.

Fig. S4. Distribution of Ca$^{2+}$ responses as a result of RHT stimulation.

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Abbreviations

ACSF, artificial cerebrospinal fluid; AR, androgen receptor; AVP, arginine vasopressin; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; CCREB, cyclic AMP-responsive element (CRE)-binding protein; CT, circadian time; DAPI, 4',6-diamidino-2-phenylindole, dihydrochloride; DD, constant dark; DIG, digoxigenin; Fura-2-AM, fura-2-acetoxymethyl ester; GABA, -aminobutyric acid; IHC, immunohistochemistry; ipRGCs, intrinsically photosensitive retinal ganglion cells; ISH, in situ hybridization; KO, knockout; LD, light-dark; MUA, multi-unit activity; NBT, 4-nitro blue tetrazolium; NMDA, N-methyl-D-aspartate; PBS, phosphate-buffered saline; RHT, retinohypothalamic tract; SCN, suprachiasmatic nucleus; SD, sodium dodecyl sulfate; SFR, spontaneous firing rates; SSC, standard sodium citrate; VIP, vasoactive intestinal peptide; WT, wild-type; ZT, zeitgeber time.

References


