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Authors

Vosko, Andrew
van Diepen, Hester C
Kuljis, Dika
[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 treatment 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 not reduced 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⁺; Irwin & Allen, 2007; Colwell, 2011) play a special role in mediating the effects of RHT activity. The influx of Ca²⁺ in retinally innervated SCN neurons triggers a number of signal transduction pathways that 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

Correspondence: Dr C. S. Colwell, as above.

E-mail: ccolwell@mednet.ucla.edu

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(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 $\mu\text{W}/\text{cm}^2$) 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: NaHCO_3 , 26; NaH_2PO_4 , 1.25; glucose, 10; NaCl, 125; KCl, 3; MgCl_2 , 5; CaCl_2 , 1) from *Per2::luciferase* (P2L) mice and VIP KO $\times$ 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% O_2 /5% CO_2) artificial cerebrospinal fluid (ACSF; in mM: NaHCO_3 , 26; NaH_2PO_4 , 1.25; glucose, 10; NaCl, 125; KCl, 3; MgCl_2 , 2; CaCl_2 , 2). Recording electrodes (4–8 M Ω) 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-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. 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 G Ω) 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 M Ω or holding currents larger than -30 pA (at $V_{\text{Hold}} = -70$ mV) were excluded from additional analysis. Junction potential between the pipette and extracellular 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 osmolality was adjusted to 290–310 mOsm.

Spontaneous firing rates (SFR) were recorded using current-clamp mode in 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 -aminobutyric 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 Ca^{2+} imaging

Brain slices containing the SCN were collected as described above from animals 6–10 weeks old, and loaded with the ratiometric Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} concentrations were examined using the mean and SD of signals 14 s before each peak in every cell. Cells with a baseline intracellular Ca^{2+} concentration $[\text{Ca}^{2+}]_i$ 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 $10\times$ concentrated DIG RNA Labeling Mix (Roche Applied Science, Indianapolis, IN, USA), 2 μL of $10\times$ 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 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, $5\times$ 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, $5\times$ 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 $5\times$ SSC, and then for 1 h in $0.2\times$ 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 MgCl_2]. 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 (unilateral) 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 Ca^{2+} 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 Sigmapstat software (version 3.5; Systat Software, San Jose, CA, USA). Values are shown as mean $\pm$ 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: $12 \pm 2\%$ change; WT: $16 \pm 6\%$; *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 = 23) 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

FIG. 2. Application of *N*-methyl-D-aspartate (NMDA) increased the firing rate of ventral suprachiasmatic nucleus (SCN) neurons during the night *in vitro*. (A) Representative examples illustrating the NMDA-induced increase in firing rate in a ventral SCN neuron from the vasoactive intestinal peptide (VIP) knockout (KO) and wild-type (WT) control mice. (B) Bar graphs showing the average firing rate for each group ($\pm$ SEM). *Indicates 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.

treatment conditions (WT: 4.11 ± 1.0 Hz vs. VIP KO: 1.44 ± 0.4 Hz, $P = 0.04$). Second, whereas all WT neurons (16/16) responded to NMDA treatment with increased firing rates, only 9 out of 13 mutant neurons did ($\chi^2 = 5.7$; $P = 0.008$). Two-way ANOVA (DF = 57) was also performed to test for genotype and treatment effects. Main effects of genotype ($F = 9.59$, $P = 0.003$) and NMDA treatment ($F = 8.17$, $P = 0.006$) were identified, but no interaction of genotype and treatment ($F = 1.20$, $P = 0.279$) was found. Thus, in the ventral SCN region, while the absolute levels of neural activity were reduced in VIP-deficient mice, NMDA still evoked a significant change in firing of these neurons although the excitatory effects were less uniform than in WT.

Ca²⁺ 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 $[Ca^{2+}]_i$ (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^{2+} transients in SCN cells in the night. RHT stimulation produced a reliable increase in $[Ca^{2+}]_i$ in a subset of SCN neurons in both ventral (WT: 40%; KO: 38%) and dorsal (WT: 20%; KO: 19%) cell populations (Fig. S4). In the ventral SCN region, the magnitude of the RHT-evoked Ca^{2+} 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^{2+} increases (Fig. 3B). The baseline Ca^{2+} 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^{2+} increases, but there were significant effects of region ($F = 50.860$; $P = 0.001$) and a significant interaction between genotype $\times$ region ($F = 9.473$; $P = 0.002$). Thus, as measured by Ca^{2+} 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

FIG. 3. Evoked Ca^{2+} transients in the suprachiasmatic nucleus (SCN) of vasoactive intestinal peptide (VIP) knockout (KO) and wild-type (WT) controls in response to retinohypothalamic tract (RHT) stimulation in a brain slice. (A) Examples of increases in $[Ca^{2+}]_i$ after electrical stimulation of the RHT (dotted line; 10 Hz for 1 s) in the ventral and dorsal region of the SCN for both WT and VIP KO mice. Each line represents the response of one cell to three repeated stimuli. (B) Average change in $[Ca^{2+}]_i$ expressed as a ratio (340 nm/380 nm) after RHT stimulation. Compared with WT controls, the VIP KO mice show higher responses in the ventral SCN ($P = 0.005$), but lower responses in the dorsal SCN ($P = 0.003$) after RHT stimulation. Error bars represent SEM. *Indicates significant difference ($P < 0.01$) compared with controls analysed by two-way ANOVA, followed by the Holm–Sidak method for multiple comparisons. (C) Average baseline $[Ca^{2+}]_i$ does not differ between the genotypes, or between ventral and dorsal 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,

FIG. 4. Examples of the temporal pattern of light-evoked changes in *Per1* mRNA. Suprachiasmatic nucleus (SCN) photomicrographs following digoxigenin (DIG) *in situ* hybridization (ISH) for *Per1* reveal a time course of induction in wild-type (WT) mice at circadian time (CT) 16. Message begins to appear about 30 min after the beginning of 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 $\times$ light treatment ($F = 4.109$,

FIG. 5. Quantification of the temporal patterns of light-evoked *Per1* expression in the core and shell suprachiasmatic nucleus (SCN). Cell counting of *Per1*+ cells following *in situ* hybridization (ISH) reveals that *Per1* is significantly induced by a circadian time (CT) 16 light pulse at all time points measured in wild-type (WT) mice, but only at 30 and 60 min post-pulse in vasoactive intestinal peptide (VIP) knockout (KO) mice. *Post hoc* analyses reveal significantly reduced *Per1*+ cell counts in the VIP KO compared with controls at 30, 60 and 90 min time points in the core SCN (denoted by *). In the shell, all *Per1*+ cell counts were significantly reduced. *Indicates significant difference ($P < 0.05$) compared with controls analysed by two-way ANOVA, followed by the Holm–Sidak method for multiple comparisons.

$P = 0.009$) 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 $\times$ 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

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.

brain slice preparation containing the SCN, application of VIP induces clock gene expression (Nielsen *et al.*, 2002), as well as phase shifts the circadian rhythm of vasopressin release (Watanabe *et al.*, 2000) and neural activity (Reed *et al.*, 2001). Behaviorally, the loss of VIP signaling alters the synchronization of the circadian system. This phenotype is best seen in the experiments in which VIP or VIPR2 KO animals are released into DD from an LD cycle. A normally entrained animal will start its activity from a phase predicted from the prior LD cycle. For example, WT mice begin their free-running rhythm within 30 min of the time of lights-off in the prior LD cycle. In contrast, the VIP KO animals start their activity about 8–10 h before the time of the prior lights-off (Colwell *et al.*, 2003; Aton *et al.*, 2005; Brown *et al.*, 2007; Ciarleglio *et al.*, 2009). Similarly, the VIPR2 KO mice also exhibit an extremely large advance in activity onset after release into DD (Harmar *et al.*, 2002; Hughes & Piggins, 2008). This altered synchronization in the VIP KO mice is also seen when the mice are held in a skeleton photoperiod consisting of one or two light exposures per 24-h cycle, and in the failure of these mice to respond to short light exposure with phase shift in their locomotor activity rhythms (Colwell *et al.*, 2003). The VIP KO mice fail to show behavioral and physiological adaptations to short or long photoperiods (Lucassen *et al.*, 2012). Thus, the data are consistent with the suggestion

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 retino-recipient 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-deficient 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

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; Shigeyoshi *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 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 (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 findings 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 neuropeptides 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 (Jeftinija *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; Berson *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; CREB, 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, γ -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; SDS, sodium dodecyl sulfate; SFR, spontaneous firing rates; SSC, standard sodium citrate; VIP, vasoactive intestinal peptide; WT, wild-type; ZT, zeitgeber time.

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