**VIP neurons of the SCN entrain circadian rhythms with distinct firing patterns**

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**Summary**

The mammalian suprachiasmatic nucleus (SCN) functions as a master circadian pacemaker, integrating environmental input to align physiological and behavioral rhythms to local time cues. Approximately 10% of SCN neurons express vasoactive intestinal polypeptide (VIP); however, it is unknown if VIP neurons entrain circadian rhythms through firing activity and VIP release. To identify physiologically relevant firing patterns, we optically tagged VIP neurons and characterized spontaneous firing over three days. VIP neurons had circadian rhythms in firing rate and exhibited two classes of instantaneous firing activity. We next tested whether physiologically relevant firing affected circadian rhythms through VIP release. We found that VIP neuron stimulation with high, but not low, frequencies shifted gene expression rhythms *in vitro* through VIP signaling. *In vivo*, high frequency VIP neuron activation rapidly entrained circadian locomotor rhythms. Thus, VIP neurons communicate daily cues through high frequency firing and VIP release to entrain molecular and behavioral circadian rhythms.

Highlights

* Mazuski et al. characterized two distinct classes (tonic and irregular) of firing activity from VIP SCN neurons.
* SCN neurons cluster into classes with distinct firing patterns consistent over multiple days
* Stimulation of VIP SCN neurons at high physiologically relevant frequencies phase shifts whole-SCN circadian rhythms in gene expression. These effects are blocked with VIP antagonists.
* High frequency firing of VIP SCN neurons entrains circadian rhythms in locomotor behavior in a frequency- and time-dependent manner solely through phase delays.

**Introduction**

Circadian rhythms are a ubiquitous adaptation to the 24-h daily cycle on Earth. Found in organisms as disparate as *Arabidopsis*, *Drosophila*, *Synechococcus*, and humans, these daily oscillations occur at molecular, cellular and systems levels, ultimately entraining an organism to environmental cycles(Dunlap, 1999). Dysregulation in the circadian system, also known as chronodisruption, is associated with a variety of physiological maladies in humans including diabetes, cancer, insomnia, and affective disorders(Van Someren, 2000, Musiek et al., 2013, McClung, 2011). Understanding how cellular circadian oscillators integrate environmental signals, communicate with each other, and entrain to their environment is key to preventing and treating chronodisruption.

The suprachiasmatic nucleus (SCN) is a master circadian pacemaker, which detects local light input through retinal release of glutamate and PACAP(Eastman et al., 1984, Ding et al., 1997, Harrington et al., 1999, Hannibal et al., 2000). Located in the ventral hypothalamus, the approximately 20,000 GABAergic neurons comprising the SCN are unique in that many express self-sustained and spontaneously synchronizing circadian rhythms in firing activity and gene expression(Cassone et al., 1993, Reppert and Weaver, 2002, Welsh et al., 1995). These rhythms are maintained through a near-24 h transcription-translation feedback loop of core clock genes including *Bmal1*, *Clock*, *Period1 and 2 (Per1 and 2)*,and *Cryptochrome1 and 2*(Ko and Takahashi, 2006, Okamura et al., 2002). Recently, researchers have shown that electrical stimulation of all SCN neurons was sufficient to phase shift and entrain circadian rhythms in gene expression and behavior(Jones et al., 2015). However, it is poorly understood how firing activity and neurotransmission from specific cell types within the SCN contribute to entrainment.

Although uniformly GABAergic (Moore and Speh, 1993), SCN neurons vary significantly in their neuropeptide content (Abrahamson and Moore, 2001). One anatomically and functionally distinct class of neurons expresses vasoactive intestinal polypeptide (VIP). Though neurons expressing VIP only make up approximately 10% of SCN neurons (Abrahamson and Moore, 2001), VIP neuron projections densely innervate the SCN and the majority of SCN neurons express its receptor VPAC2 (An et al., 2012). Functionally, genetic loss of VIP or VPAC2R weakens synchrony among SCN neurons and dramatically reduces circadian rhythms in the SCN and in behavior (Aton et al., 2005, Maywood et al., 2006, Brown et al., 2007). Additionally, exogenous application of VIP increases GABAergic neurotransmission (Reed et al., 2002), induces clock gene expression (Nielsen et al., 2002), and phase shifts daily rhythms in the SCN and locomotor activity (An et al., 2011, Piggins et al., 1995). With the development of Cre-lox technology allowing cell-type specific manipulation of SCN neurons, researchers found that VIP neurons have sparse GABAergic monosynaptic connections to approximately 50% of SCN neurons (Fan et al., 2015). In contrast, though VIP is the most potent neuropeptide in SCN function, little is known about how VIP is released to ultimately synchronize SCN neurons.

How VIP release occurs from neurons is not well understood. Within individual neurons, VIP is stored within dense core vesicles as opposed to clear synaptic vesicles that store small-molecular fast neurotransmitters like GABA (Castel et al., 1996). This has implications for the release of VIP. In contrast to synaptic vesicles, which are readily released through single action potentials, dense core vesicle contents such as neuropeptides are released through high calcium levels typically achieved through high frequency burst firing. However, neuropeptidergic dense core vesicle fusion has largely been studied in large neuromuscular synapses and hippocampal neurons where firing rates frequently exceed 50Hz (Xia et al., 2009, Pecot-Dechavassine and Brouard, 1997). In contrast, SCN neurons have relatively low sustained firing rates rarely exceeding 10-15Hz. Therefore it remains unclear what role, if any, firing activity in VIP neurons plays in neuropeptide release.

To address whether increases in VIP firing frequency are necessary for VIP-mediated entrainment, we take a multi-step approach: i) characterize the spontaneous firing frequencies and patterns of VIP neurons, and then ii) stimulate VIP neurons using different identified firing frequencies *in vivo* and *in vitro*.

**Results**

**Multi-day firing activity of VIP SCN neurons identified through optical tagging of VIP neurons on multielectrode arrays**

To identify physiologically relevant firing frequencies of VIP neurons, we identified and characterized the spontaneous firing activity of VIP neurons on multielectrode arrays (MEAs) using optical tagging. Overall, SCN neurons exhibit dynamic changes in spontaneous firing activity across the circadian day, which are heterogeneous on a single-neuron scale (e.g. individual neurons vary dramatically in frequency, firing pattern, phase, etc.) Therefore, we designed our experiment to i) characterize firing activity from single neurons across multiple days to capture the circadian variability within single-neurons, and ii) identify firing activity belonging to a specific subset (VIP) of SCN neurons.

s Extracellular MEA recording from SCN cell cultures allows us to follow single neuron firing activity for multiple days, and the novel use of optical tagging permits cell-specific identification of VIP SCN neurons. Importantly, previous studies have established that SCN cell cultures mimic the SCN *in vitro* and *in vivo* in many important aspects, including neuropeptide expression, release and function. Taken together, this suggests that our experimental setup provides a good starting point for identifying and characterizing the spontaneous firing frequencies of VIP that mediate VIP release.

Specifically, following multiday spontaneous activity recordings from SCN MEA neurons, we activated channelrhodpsin-2 (ChR2) expressed solely within VIP neurons (VIPCre crossed with floxed-ChR2) using blue light pulses (Figure 1a). To identify VIP neurons, we calculated the interspike interval histogram (ISIH) of each neuron that fired during the 1 h stimulation and found neurons whose firing matched the stimulation pattern (10.6 ± 1.7%, mean ± SEM, Figure 1b). To verify that VIP neurons selectively fired synchronously during the stimulation, we cross-correlated firing between each pair of active neurons. As predicted, we found that only VIP neurons increased firing synchronously in response to stimulation, while some non-VIP neurons decreased firing approximately 10-20 ms following stimulation, consistent with inhibitory neurotransmission from VIP SCN neurons (Figure 1c).

Based on our identification during stimulation, we classified the prior spontaneous activity firing as VIP or non-VIP (Supplementary Figure 1). Overall, we discriminated multiday electrical activity from 40 VIP neurons and 543 non-VIP neurons across 8 MEA preparations. Previously two reports measuring 1-30 min snapshots of VIP firing activity from slice recordings at two separate timepoints differed on whether VIP SCN neurons have circadian firing rhythms. The design of our study allows us to follow individual VIP neurons across multiple days and strongly confirms that a majority of VIP neurons have circadian firing activity (81.5 ± 4.0%).

Since our experimental design relied on direct ChR2-induced increases in firing within VIP neurons, we tested these assumptions. As utilized and quantified by previous researchers (Hermanstyne et al., 2016, Fan et al., 2015, Brancaccio et al., 2013), Cre-lox recombination selectively drives transgene expression within VIP neurons. Additionally, we verified that only ChR2-positive SCN neurons fired precisely in response to light pulses using whole-cell patch clamp recordings (Supplementary Figure 2). Finally, within our MEA preparation we validated that VIP neurons responded to light pulses within 5 ms (Supplementary Figure 3). In conclusion, optical tagging reliably identified the multiday firing activity of VIP SCN neurons.

**VIP neurons are a heterogeneous subclass of SCN neurons**

Most electrophysiological SCN studies have focused on broad changes in SCN neuronal firing rate, or the mean firing frequency (e.g. the number of spikes binned by time where time bins range from 10s to 10min). While mean firing rate quickly simplifies complex, dynamic SCN firing activity recordings, it can risk oversimplifying activity that occurs on the millisecond timescale also known as instantaneous firing frequency. Since we were interested in whether firing frequency of VIP neurons mediates neuropeptide release, we elected to analyze spike timing and frequency and characterized instantaneous firing of circadian SCN neurons (33 VIP and 268 non-VIP).

Based on the spike timing of spontaneous activity during an individual neuron’s daytime, we observed three distinct classes of spike timing within SCN neurons: tonic, irregular, and bursting (Figure 2a-b) similar to those previously described (Pennartz et al., 1998). Strikingly, when we looked at neuronal firing across the circadian day, individual SCN neurons did not change their spike timing (tonic, irregular, or bursting) with time of day, or across multiple days, including during periods of rapidly increasing or decreasing firing activity (Figure 2c) indicating that spike timing described separate electrophysiological classes of neurons. *Insert characterization of frequencies of these patterns – including the range (important to note that though e.g. 8Hz is the DIFF for an irregular neuron, that neuron fires at both higher and lower frequencies)*

To test whether expression of VIP influenced the electrophysiological class of the neuron, we used unbiased, hierarchical clustering based on the ISIH and the dominant instantaneous firing frequency (DIFF, the dominant spike timing frequency) to separate VIP and non-VIP neurons into their respective classes (Figure 2d). Surprisingly, this method revealed that VIP neurons are electrophysiolgocially heterogeneous, primarily firing with either tonic or irregular spike timing, and very seldom exhibiting >50Hz+ bursting firing activity.

To address whether our spike timing and frequency (DIFF) measurements were influenced by the artificial connectivity occurring during cell culture maturation, we preformed the same analysis on intracellular daytime adult VIP SCN slice recordings originally collected in Hermanstyne et al. (2016) (Supplementary Figure 4) and found comparable results. Taken together, these data indicate that despite dynamic fluctuations in overall firing activity, individual SCN neurons have distinct electrophysiological profiles that are stable across the circadian day. Surprisingly, though VIP SCN neurons are a relatively small subset of SCN neurons (10%) that all express and release VIP neuropeptide, they are still an electrophysiologically heterogeneous group that i) are predominately circadian ii) largely exhibit tonic or irregular spike timing with iii) peak frequencies occurring from 5-10Hz (Figure 2e-f).

**High frequency firing of VIP neurons phase shifts circadian gene expression rhythms *in vitro***

To test if VIP firing influences circadian entrainment, we stimulated VIP neurons with two firing frequencies. Based on our characterization of VIP neurons, we picked two stimulation paradigms that represent the high and low frequency ranges of VIP neurons: high instantaneous frequency (HIF, doublets of 20 Hz at 2 Hz) and low instantaneous frequency (LIF, 4 Hz pulses evenly spaced). These stimulation patterns had the same number of spikes per second (4 Hz), but differed in spike timing. Both HIF and LIF evoked firing in VIP neurons on MEAs, which reliably followed the stimulation frequency (Supplementary Figure 5). During the 1 h of stimulation we observed no evidence of depolarization block or habituation in stimulated VIP neurons.

We stimulated VIP neurons within intact SCN slices with HIF and LIF frequencies and found that only HIF frequencies shifted circadian gene expression. Briefly, we cultured SCN explants expressing ChR2 in VIP neurons and *Period2::Luciferase* (*PER2::LUC,* a knock-in fusion that reports PER2 protein abundance) or littermate controls lacking ChR2 expression. Following baseline bioluminescence recording of PER2 levels from the entire SCN, we stimulated all SCN for 1 h near the peak of PER2 expression (Circadian time, CT 9-12) with HIF or LIF patterns. We chose to stimulate at this time because it has been reported as the time when exogenous VIP application evoked large delays in PER2 rhythms(An et al., 2011). HIF stimulation significantly delayed the daily rhythms of SCN PER2 expression by over 1.5 h, whereas LIF stimulation did not (Figure 3). Additionally, three consecutive days of stimulation yielded similar results (Supplementary Figure 6). Thus, firing of VIP neurons can phase delay circadian gene expression but only if stimulated at sufficiently high frequencies.

To test whether the resulting phase delay was mediated by increases in VIP signaling, we stimulated SCN PER2 explants expressing ChR2 in VIP neurons with HIF frequencies in the presence of 10 um VPAC2R antagonist or vehicle control. We found that the presence of the antagonist reduced the resulting phase shift (Figure 3c). Thus, our data suggests that HIF firing of VIP neurons releases VIP, which phase delays circadian gene expression.

**Activation of VIP neurons induces cFOS expression throughout the SCN *in vivo***

Although VIP neuronal cell bodies localize to the ventral SCN, VPAC2 receptor is expressed in nearly all SCN neurons(An et al., 2012). Consistent with these anatomical data, we found that activation of VIP neurons *in vivo* increases cFOS protein throughout the SCN. Briefly, freely moving mice expressing ChR2 in VIP neurons (VIPChR2) and littermate controls received blue laser light via an implanted fiber optic cannula aimed at the SCN. After 1 h of 15Hz stimulation at CT 13, 88.9 ± 4.0% (mean ± SEM, n = 4 mice; Figure 4a) of VIP neurons expressed cFOS, a marker of neuronal activation. Furthermore, cFOS expression increased roughly 5-fold in the ventral SCN and almost 4-fold in the dorsal SCN (Figure 4b) compared to controls. We conclude that high frequency *in vivo* stimulation of VIP neurons increases activity both within VIP neurons and throughout the SCN.

**Activation of VIP neurons entrains locomotor activity *in vivo***

Next, we found that firing of VIP neurons underlies their role in circadian entrainment. Briefly, we monitored wheel-running activity before, during and after stimulation of SCN VIP neurons *in vivo* from mice enucleated to remove ambient light input and implanted with a fiber optic cannula. Before stimulation, all mice showed free-running circadian rhythms in locomotion (in hours, 23.4 ± 0.1, VIPChR2, 23.4 ± 0.2, Control, mean ± SEM, n = 7, 4). Mice then received daily HIF stimulation for 1 h for up to 30 days and only VIPChR2 mice entrained to the stimulation (Figure 5a). We calculated a phase response curve (Figure 5b) and found that surprisingly, only stimulation of VIP neurons between CT 10 – 18 lead to phase shifts and entrainment. Additionally, we observed that stimulation of VIP neurons acutely suppressed wheel running (Figure 5c) similar to light-induced masking.

Finally, we found that firing frequency plays a role in VIP-mediated entrainment *in vivo.* Mice were randomly assigned to receive either 1h of daily HIF or LIF stimulation for 4-10 days between CT 10-15. Then, following at least 4 days of free-running activity, the mice received the other (LIF or HIF, respectively) stimulation for 4-10 days at the same time of day. Overall, we found that either stimulation frequency entrained locomotor activity (Figure 6c), however HIF stimulation entrained more rapidly (Figure 6b). We conclude that firing of VIP neurons results in circadian entrainment and the frequency of firing determines the speed of entrainment.

**Discussion**

We consistently identified VIP SCN neurons that fired with either tonic or irregular patterns in extracellular and intracellular, slice and long-term multielectrode recordings, suggesting that spike timing characterizes functional classes of SCN neurons. These may correlate with the two populations of SCN VIP neurons that appear during development(Ban et al., 1997). We hypothesize that these firing patterns relate to different functional roles of SCN VIP neurons as previously suggested(Kawamoto et al., 2003). For example, these spontaneous firing patterns may identify VIP neurons that transduce direct vs. indirect retinal input(Fernandez et al., 2016) or that project primarily within the SCN to maintain synchrony among SCN neurons(Abrahamson and Moore, 2001) vs. outside the SCN to targets including the paraventricular nucleus of the hypothalamus and paraventricular nucleus of the thalamus(Abrahamson et al., 2001) to regulate daily rhythms in hormone release. Similar anatomical subgroups of brainstem serotonergic neurons have been associated, for example, with regulating distinct behaviors including respiration and aggression through distinct brain target areas(Niederkofler et al., 2016). Future studies should elucidate whether the physiological classes of VIP neurons map to specific anatomical groups within the SCN and targets outside the SCN.

VIP SCN neurons change firing frequency with time of day, and high and low frequency firing of VIP SCN neurons differentially affects circadian entrainment, suggesting that VIP neurons can modulate entrainment by increasing their firing frequency. In the unperturbed circadian system, this would occur during mid to peak firing activity of VIP neurons. We hypothesize that the firing frequency of VIP neurons may contribute to phase shifting and entrainment to new light schedules (e.g. in seasonal entrainment(Lucassen et al., 2012), and jet lag(An et al., 2013)).

Our data suggest that high frequency stimulation increases signaling from VIP neurons more than low frequency stimulation. Given the well-established links between high frequency firing, dense core vesicle fusion and neuropeptide release(Verhage et al., 1991, Iverfeldt et al., 1989), we hypothesize that high frequency firing results in more VIP release than low frequency firing. In the SCN, specifically, high frequency stimulation produces phase shifts consistent with the magnitude and timing of VIP-induced shifts *in vitro*(An et al., 2011) and *in vivo* (Pantazopoulos et al., 2010, Piggins et al., 1995). In addition, our stimulation may alter the release of GABA. However, since fast neurotransmission is more tightly coupled to spike count, which was similar between HIF and LIF stimulation, we believe this to be less likely.

Entrainment through stimulation of VIP neurons only partially mimics entrainment seen previously through stimulating all SCN neurons(Jones et al., 2015). Specifically, increases in VIP activity fail to advance phase or alter period of locomotor activity during late subjective night. This suggests that VIP activity likely plays a role in light-induced phase delays, but not in light-induced phase advances. Since our VIP stimulation bypasses any retinal input, we hypothesize that there is gating within the SCN mediating light responses. In addition to VIP firing frequency, daily rhythms in VIP abundance or release probability(Francl et al., 2010, Shinohara et al., 1999, Okamoto et al., 1991), VPAC2 receptor abundance(An et al., 2012), and responses in downstream SCN neurons(Enoki et al., 2017, Fan et al., 2015) likely contribute to shaping the phase response curve to firing of VIP neurons and, ultimately, circadian entrainment.

Intriguingly, locomotor activity decreases during stimulation of SCN VIP neurons. This is consistent with a report correlating SCN firing with locomotor inactivity(van Oosterhout et al., 2012) and provides the first causal demonstration that firing increases within VIP SCN neurons inhibit locomotor activity. This, combined with the broad projections of patterns of SCN VIP neurons within the hypothalamus, suggests a role for VIP neurons in regulating circadian behaviors including sleep timing(Aston-Jones et al., 2001) and daily hormonal levels(Fahrenkrug et al., 2012, Loh et al., 2007). How VIP SCN neurons couple anatomically and functionally to behavioral circuits is an open question.

In conclusion, we addressed outstanding questions about how firing activity of VIP neurons alters circadian activity and behavior. By altering the instantaneous firing frequency of SCN VIP neurons, we found that firing of VIP neurons had a larger effect on circadian entrainment when stimulated with short interspike intervals, regardless of the total number of action potentials. Strikingly, firing of VIP neurons shifted circadian phase only during the late subjective day and early subjective night. We conclude that VIP neurons can phase shift and ultimately entrain circadian behavior by firing during the day-to-night transition.

**Methods**

**Animals.** VIPCre knock-in mice (VIPtm1(cre)Zjh, Jackson Laboratories), floxed ChR2 (Ai32, Gt(ROSA)26Sortm32(CAG-COP4\*H134R/EYFP)Hze, Jackson Laboratories) and *Per2::Luc(Yoo et al., 2004)* knock-in mice (founders generously provided by Dr. Joseph Takahashi, UTSW) were housed in a 12h:12h light:dark cycle in the temperature and humidity controlled Danforth Animal Facility at Washington University in St. Louis. All animals were congenic on a C57BL/6JN background. Combinations of these genotypes were used for all experiments, with VIPChR2 animals being heterozygous for both VIPCre and floxed-ChR2 and controls being littermate animals heterozygous for only VIPCre or floxed-ChR2. Mice were genotyped by PCR before and by presence (ChR2-positive) or absence of eYFP fluorescence microscopy following each experiment. All procedures were approved by the Animal Care and Use Committee of Washington University and followed National Institutes of Health guidelines.

**Multielectrode array cell electrophysiology.** Homozygous VIPCre-J mice were crossed with homozygous floxed-ChR2 mice to generate heterozygous litters expressing ChR2 solely in VIP+ neurons (VIPChR2). Extracellular recordings were made from multielectrode arrays (Multichannel Systems, Reutlingen, Germany) plated with SCN cells as previously described (Aton et al., 2005, Webb et al., 2012, Freeman et al., 2013). Briefly, following decapitation, the brains were rapidly removed from postnatal day 4-5 (P4-P5) VIPChR2 pups. We dissected the bilateral SCN from 250-um thick coronal brain slices, papain-dissociated and dispersed the cells at high density onto sixty, 30µm-diameter electrodes (200µm spacing) pre-treated with poly-D-lysine/laminin. Cultures were maintained in Air-DMEM (Dulbecco’s Modified Eagles Medium, DMEM, supplemented with 10% fetal bovine serum for the first week of recording) for 3 weeks prior to recording.

Multielectrode arrays were covered with a fluorinated ethylene-polypropylene membrane before transfer to a recording incubator maintained at 36oC. We waited 24h before the start of digitization to ensure culture health and stability. For extracellular recordings, spikes that exceeded a manually set threshold (~3-4 standard deviations from noise level) were digitized (1 ms before and after crossing the threshold; MC-Rack software, Multichannel Systems) at 20,000 Hz sampling. Subsequently, the culture was stimulated with 15ms pulses of light from a high-power 470nm LED (Cree XLamp XP-E2 Blue High Power LED, LEDsupply) at frequencies between 2-20Hz for 1h. Culture light intesntiy was verified to fall between 5 – 10 mW.

**Whole-cell patch clamp electrophysiology**. Whole cell patch clamp recordings from SCN neurons were obtained using the procedures described in Hermanstyne et al. (Hermanstyne et al., 2016). Specifically, SCN slices were prepared from 3 month old adult VIPChR2 heterozygous mice. After anesthesia with 1.25% Avertin, brains were removed into a cold cutting solution (in mM 240 sucrose, 2.5 KCL, 1.25 NaH2Po4, 25 NaHCo3, 0.5 CaCl2 and 7 MgCl2, saturated with 95% O2/5%Co2). 300um coronal slices were cut on a Leica VT1000 S vibrating blade microtome and incubated in oxygenated artificial cerebrospinal fluid (in mM 125 NaCl, 2.5 KCL, 1.25 NaH2PO4, 25 NaHCO3, 2 CaCl2, 1 MgCl2, 25 dextrose, saturated with 95% O2/5% CO2) for at least 1 h. Using glass pipettes (4-7 MΩ) containing an intracellular solution (in mM 120 KMeSO4, 20 KCl, 10 HEPES, 0.2 EGTA, 8 NaCl 4 Mg-ATP 0.3 Tris-GTP, and 14 phosphocreatine), a “loose patch” cell-attached recording was obtained. A gigaOhm seal (>2 GΩ) was formed and spontaneous firing was recorded for approximately 1 min. We then evoked firing in ChR2-positive neurons with 15ms pulses from a 465 nm laser (DPPS MDL-III-447 100mW, 5% stability, Information Unlimited) positioned over the slice controlled by a TTL input from a Grass stimulator (S88, Grass Instrument Company, Quincy, MA) at the desired frequency (2 to 15Hz). Electrophysiological data were compiled and analyzed using ClampFit, Mini Analysis, and Prism 7.0 (Graphpad Software, La Jolla California).

**Isolation of individual neuronal firing patterns.** Software was created for semi-automated spike sorting to allow discrimination of neuronal activity from MEA recordings across multiple days. Briefly, time-stamped spikes from a given electrode were separated into 24-h epochs, subsampled by taking a random 10% of the total spikes from each epoch, and then sorted based on principal component analysis (PCA) and fitting a Gaussian mixture model (GMM) to the principal components that contained >10% of the explained variance using a Bayesian information criterion (BIC) cutoff. The noise was identified as the cluster with the average spike shape with the lowest magnitude. The data was split into each remaining cluster. PCA and GMM clustering and splitting was then applied recursively until each cluster could not be split based on the BIC cutoff. We used the Mahalanobis distance to keep only spikes sufficiently close to the center of the distribution. The PCA components and GMM parameters used to sort the subsampled day of spikes were saved and used to sort all spikes on that electrode from that day. The spike trains identified on each electrode were then combined across days by correlating spike shapes (Pearson r >0.95) to recover the activity of a single neuron throughout the multiday recording. If neuronal firing could not be connected across multiple days, it was excluded from subsequent analysis. Firing activity during optogenetic stimulation was similarly sorted and matched to the third day of spontaneous firing to identify VIP+ neurons. This algorithm was constructed in the Python language using packages scipy (Walt et al., 2011), and scikit-learn (Pedregosa et al., 2011) for sorting, and neuroshare for reading raw MEA data files. All scripts used in spike sorting are publicly available at: http://github.com/JohnAbel/spikesort. This method produced similar numbers of circadian neurons and spike times to manual sorting (Freeman et al., 2013) in approximately 90% less time. To calculate circadian rhythmicity, we binned the average firing rate of each neuron over 10min. Using the MetaCycle package (Wu et al., 2016) (<https://cran.r-project.org/web/packages/MetaCycle/index.html> in R), we calculated circadian rhythmicity using JTK cycle and Lomb-Scargle (range 20 to 28h). If a neuron was deemed rhythmic with p < 0.05 after controlling for multiple comparisons on both methods, we considered that neuron circadian.

**Classification of individual neuronal firing patterns.** Neuronal firing patterns from the three days of spontaneous activity were identified in Python using scipy hierarchical clustering (Johnson, 1967) of interspike interval histogram (ISIH) with a bin size of 0.01 s and the dominant instantaneous firing rate (DIFR, the peak of the ISIH), using dynamic time warping (Salvador and Chan, 2007) as the correlation metric, and complete linkage. Varying ISIH bin size (0.005 s to 0.05 s) did not alter results significantly. The threshold for dendrogram cluster identification was set to 70% of the maximum distance between data points (default scikit-learn setting). Following clustering, DFIR was identified with a bin size of 0.0001 s to construct summary statistics with high temporal resolution. In-slice recording and whole-cell patch clamp recording data were analyzed in an identical fashion, except ISIH bin size was changed to 0.05 s for patch clamp recording due to the short 60 s recording interval.

**Recording and analysis of real-time clock gene expression.** To record circadian PER2 protein expression, we crossed heterozygous VIPChR2 and *PER2::Luc* mice. Control mice were littermate animals lacking either VIPCre or floxed-ChR2. Adult (> P15) mice of both sexes were sacrificed with CO2 and 300µm coronal brain slices were sectioned. The bilateral SCN was dissected out and cultured on Millicell-CM inserts (Millipore, Billerica, MA) in pre-warmed culture medium (AirDMEM supplemented with 10mM HEPES and 100uM beetle luciferin, Promega, Madison, WI). The sealed 35-mm Petri dishes (BD Biosciences, San Jose, CA) were transferred to a light-tight incubator kept at 36oC. As described previously (Freeman et al., 2013, Aton et al., 2005), bioluminescence from the *Per2*-luciferase reporter was counted with a photomultiplier tube (PMT; HC135-11 MOD, Hamamatsu Corp., Shizuoka, Japan) in 6 min bins for at least three days prior to optogenetic stimulation. PMT recordings were paused during 1 h of optogenetic stimulation between CT9-12 for either one day or three consecutive days. Using a custom-made LED array (Cree XLamp XP-E2 Blue Color High powered LEDs, LEDsupply, Randolph, VT) that delivered light flashes (15 ms, 5 mW, 470 nm) per dish, SCN were stimulated for 1 h at either high (20 Hz pulses at 2 Hz) or low instantaneous frequencies (4 Hz). The LED array was powered by a supply (LEDD1B high-powered LED driver, Thorlabs, Newton, NJ) under TTL control from a stimulator (S88, Grass Instrument Company, Quincy, MA). Care was taken to minimize any mechanical disturbance to the SCN, and during stimulation, the temperature underneath the LED array remained at 36oC. After stimulation, the dishes were repositioned underneath their PMT and recording continued for at least four days.

For pharmacology experiments we stimulated VIPChR2 PER2 slices in the presence or absence of 10um VPAC2R antagonist in AirDMEM([D-p-Cl-Phe6,Leu17]-VIP, Tocris Bioscience, Bristol, UK). Following 1h of stimulation with HIF frequency, all slices were transferred briefly into a fresh, prewarmed dish of AirDMEM and then transferred back into their original dishes and placed back underneath their PMT channels.

All data were analyzed blinded to genotype. SCN traces that did not retain rhythmicity due to media evaporation or fungal infection were excluded (N=4), and all other data were analyzed in a stereotyped, reproducible manner. In addition, due to inherent variability in period and amplitude within our PMT systems, all comparable experiments were performed in the same incubator. Each experiment (HIF stimulation, LIF stimulation and antagonist treatment) represents at least 3 separate runs with control and experimental conditions run in parallel. Raw counts from the PMT were detrended using a running 24-hour smooth, discarding the first and last 12 h of the recording as previously described (Herzog et al., 2015). For single-pulse experiments, the data from the day of stimulation also was excluded from analyses. Circadian period was calculated from a linear fit to times of the daily acrophase of PER2 expression (baseline= second through fourth days of recording; after-stimulation= fifth through seventh days; Clocklab, Actimetrics). The difference between the baseline-extrapolated and observed acrophases in the three days following stimulation was reported as the phase shift. For PMT traces stimulated for three consecutive days, we used the rising phase as a stable phase marker. Given the natural spread of PER2, we normalized the data based on the rising phase on the day before stimulation.

***In vivo* stimulation of VIP+ neurons.** To stimulate VIP+ neurons *in vivo*, mice underwent stereotactic surgery to implant a fiberoptic cannula capable of delivery light to the bilateral SCN. Specifically, anesthetized mice (2% Isofluorane) were placed into a stereotaxic device and implanted with a sterilized fiber-optic cannula (5.8 mm in length, 200uM diameter core, 0.39 NA; Thorlabs, Newton, NJ). The cannula was implanted at +0.4mm anterior, +0.0 mm lateral and -5.5 mm ventral to Bregma. Mice received analgesic treatment during recovery. Following recovery, mice were enucleated as previously described (Aton et al., 2004, Hermanstyne et al., 2016) so that mice did not respond to ambient light. Additionally, mice were tethered to a flexible fiberoptic cable (Thorlabs, Newton, NJ) attached to a laser (465 nm, 100 mW, 5% stability, DPPS MDL-III-447, Information Unlimited) and allowed to freely roam with *ad lib* access to food, water and an open-faced wheel in a custom-built cage. Wheel revolutions were counted with a reed switch (Clocklab, Actimetrics). After free-running locomotor behavior returned to pre-enucleation levels, mice were stimulated for up to 40 days with different frequency patterns (HIF or LIF). Mice that received both stimulation patterns had the order randomized and separated by at least four days without stimulation.

**Immunohistochemistry.** To test for neuronal activation, we measured cFOS protein induction in mice implanted with a fiberoptic aimed at the SCN after 1 h of stimulation (15Hz, 15ms pulses) during early subjective night (CT 13). Immediately following stimulation, mice were anesthetized with 1.25% Avertin (2,2,2-tribromoethanol and tert-amyl alcohol in 0.9% NaCl; 0.025 ml/g body weight) and transcardially perfused with phosphate-buffered saline (PBS) and 4% paraformaldehyde (PFA). The brain was rapidly dissected and transferred to 30% sucrose following 24 h in 4% PFA. Frozen coronal sections cut at 40 um were collected in 3 separate wells. cFOS immunofluorescence or avidin-biotin immunohistochemistry was performed using two using a rabbit anti-cFOS antibody (Santa Cruz Biotechnology, Santa Cruz, CA). For immunofluorescence, free-floating sections were washed for 1 h in PBS, incubated overnight at 4oC in anti-rabbit cFOS antibody (1:1000 in PBSGT). Slices were washed again and incubated for 2 h at room temperature in donkey anti-rabbit Cy3 secondary antibody (1:500 in PBSGT). Sections were washed again in PBS for 30min, mounted, and cover-slipped with DABCO (1,4-Diazobicyclo[2,2,2]-octane) mounting medium. Sections were imaged in 4-um z-stacks on a Nikon A1 Confocal microscope. Two independent investigators quantified the fraction of ChR2-eYFP positive neurons that also expressed nuclear cFOS and results differed by less than 10% per brain. For DAB immunohistochemistry, free-floating sections were incubated for 72 h in rabbit c-Fos antibody (1:2500). Subsequently, sections were processed with the avidin-biotin method for immunohistochemistry. Tissues were reacted in diaminobenzidine with 0.01% H2O2, mounted, dehydrated and cover-slipped. Sections were imaged using the Alafi Nanozoomer at Washington University in St. Louis Medical School. Tissues were always processed together and mid-SCN sections were selected from all animals for quantification. An investigator blinded to the genotype of the mouse quantified the number of cFOS positive cells within the SCN using ImageJ software. The SCN was located and boundaries were drawn to demarcate the ventral and dorsal SCN in each animal (250 um x150 um, ventral SCN per side, 150 um x 150 um, dorsal SCN per side).

**Locomotor analysis.** We identified the daily onset of locomotor activity from wheel running data in 15-min bins as the zero-crossing of the continuous wavelet transform using the Mexican Hat wavelet as described for temperature rhythms (Leise et al., 2013). We calculated the phase response curve by identifying the change in activity onsets evoked by optogenetic stimulation compared to the same mouse under free running conditions. Calculating phase change in comparison to the same mouse under free-running conditions (rather than comparison with a separate control group) allowed inclusion of mice with visibly different periods. To measure the time to entrainment, we constructed Rayleigh plots of activity onset for days 2-10 of optogenetic stimulation and calculated the Kuramoto parameter (i.e. synchronization index)(Kuramoto, 2012). Finally, we measured optogenetically induced activity suppression by comparing wheel revolutions during the stimulated hours between CT 12 – 16 and equivalent time bins from free-running days with no stimulation.

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