Suprachiasmatic VIP neurons are required for normal circadian rhythmicity and comprised of molecularly distinct subpopulations

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The hypothalamic suprachiasmatic (SCN) clock contains several neurochemically defined cell groups that contribute to the genesis of circadian rhythms. Using cell-specific and genetically targeted approaches we have confirmed an indispensable role for vasoactive intestinal polypeptide-expressing SCN (SCN\text{VIP}) neurons, including their molecular clock, in generating the mammalian locomotor activity (LMA) circadian rhythm. Optogenetic-assisted circuit mapping revealed functional, di-synaptic connectivity between SCN\text{VIP} neurons and dorsomedial hypothalamic neurons, providing a circuit substrate by which SCN\text{VIP} neurons may regulate LMA rhythms. In vivo photometry revealed that while SCN\text{VIP} neurons are acutely responsive to light, their activity is otherwise behavioral state invariant. Single-nuclei RNA-sequencing revealed that SCN\text{VIP} neurons comprise two transcriptionally distinct subtypes, including putative pacemaker and non-pacemaker populations. Altogether, our work establishes necessity of SCN\text{VIP} neurons for the LMA circadian rhythm, elucidates organization of circadian outflow from and modulatory input to SCN\text{VIP} cells, and demonstrates a subpopulation-level molecular heterogeneity that suggests distinct functions for specific SCN\text{VIP} subtypes.
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current understanding holds that circadian rhythms are generated within individual cells of the suprachiasmatic nucleus (SCN) and that cell–cell interactions within the SCN network are required to sustain them. One of the emergent properties of these cell–cell circuit interactions is the circadian period, a fundamental property of the SCN circadian clock. While the SCN contains a variety of neuropeptides, as well as the fast transmitter GABA, that might variably contribute to SCN network function and hence the ensemble period, previous work has suggested an especially important role for SCNVIP cells as “master pacemakers” of circadian rhythms. In general support of this view, developmental disruption of vasoactive intestinal polypeptide (VIP−/− mice) or VIP signaling through its cognate receptor, VPAC2 (Vipr2−/− mice), severely compromises both synchrony among SCN neurons and behavioral rhythms. However, more recent studies have suggested an alternate view; that, in combination with a variety of neuropeptides, including neuropeptide S (NMS) within the SCN, are hierarchically downstream to SCNVIP neurons with respect to circadian pacemaking. This hypothesis is surprising given that: (1) despite alterations in circadian period, circadian rhythms are preserved following disruption of the molecular clock in SCNAVIP cells, (2) the effects of selective disruption of the molecular clock in SCNVIP neurons have not been determined, and (3) the effect of selective, non-germline ablation of SCNVIP neurons has also not been tested.

Therefore, to more clearly and definitively delineate the role of SCNVIP neurons in the regulation of circadian rhythms, in particular the question of necessity, here we selectively disrupted the molecular clock in SCNVIP cells in mice and recorded circadian rhythms of locomotor activity (LMA), body temperature (Tb), sleep-wake, and wheel running activity. We also used a genetically targeted ablation approach to selectively eliminate SCNVIP cells in adult mice and compared this with genetically targeted lesions of SCNAVIP neurons and SCN GABA-ergic neurons, which enabled us to evaluate the effect of non-germline disruption of SCNVIP neurons on circadian rhythms. On the basis of the outcomes of these physiological experiments, which identified necessity of SCNVIP neurons in the regulation of the LMA circadian rhythm, we then extended this experimental work to include a comprehensive interrogation of the cellular and synaptic properties of SCNVIP neurons. To do so, we first employed fiber photometry to evaluate the state-dependent activity dynamics of SCNVIP neurons in vivo. We then used channelrhodopsin (ChR2)-assisted circuit mapping (CRACM) to inform a more detailed understanding of the synaptic output pathways by which SCNVIP cells may produce organismal-level rhythmicity. We similarly employed modified rafibers tracing to define synaptic inputs that might modulate SCNVIP activity in vivo. Finally, we used high-throughput single-nuclei sequencing (sNuc-seq) of SCNVIP cells to determine whether SCNVIP neurons comprise distinct subtypes and to define their molecular identities.

Results

Selective disruption of the molecular clock in SCNVIP cells. Expression of the Bmal1 transcription factor within the cellular SCN is both necessary and sufficient for coherent circadian rhythmicity. Whether or not a functional cellular clock in SCNVIP neurons, which comprise ~10% of the cellular SCN population, is necessary for establishing and maintaining circadian rhythms remains unclear. To explore this question, we first generated VIP-IRES-Cre mice using previously described recombinering techniques (Methods). As shown in Fig. 1a, our reporter cross (VIP-IRES-Cre::R26-loxSTOPlox-L10-GFP) indicated that expression of the Cre transgene in our mice was restricted to the ventrolateral (core) SCN. To generate mice lacking a functional cellular clock in SCNVIP neurons, we crossed our VIP-IRES-Cre mice with mice harboring floxed Bmal1 alleles (Bmal1fl/fl)15. While mice homozygous for the VIP-Cre allele showed the expected complete deletion of Bmal1 in SCNVIP neurons, mice that were heterozygous for the VIP-Cre allele continued to, unexpectedly, express Bmal1 in ~70% of VIP-Cre neurons (Fig. S1). We therefore measured the diurnal and circadian rhythms of LMA and Tb in mice with: (1) complete deletion of Bmal1 in SCNVIP neurons, and hence loss of the molecular clock in these neurons (VIPcre/cre::Bmal1fl/fl); (2) partial (VIPcre/cre::Bmal1fl/+ and VIPwt/cre::Bmal1fl/fl) and (3) no deletion of Bmal1 in SCNVIP cells (VIPcre/cre) (Fig. 1B1–E2). Under a 12:12 light-dark (LD) cycle, Bmal1-deleted mice exhibited a consistent and significantly advanced phase of entrainment (~3 h advance) in LMA and Tb (Fig. 1b) as compared to mice with partial (Fig. 1C1–D2) or no deletion (Fig. 1e) of Bmal1 in VIP neurons, indicating altered synchronization to the external LD cycle. When the VIPcre/cre::Bmal1fl/fl mice were released into constant darkness (DD), their LMA rhythms slowly, but also inexorably, became arrhythmic (Fig. 1B1 and 1B2; 1G1), demonstrating that the molecular clock in SCNVIP cells is necessary for sustaining coherent circadian rhythms of LMA. Despite clear visible effects on the amplitude and architecture of the Tb waveform in DD (Fig. S2), our periodogram analysis revealed that the Tb rhythm (Fig. 1B1–2), unlike the LMA rhythm (Fig. 1B1-2), retained a circadian harmonic in the Bmal1-deleted mice, with only two of the seven mice exhibiting a period outside of the circadian range (18.5 and 27.7 h, Fig. 1G1). We finally noted that non-crossed VIPcre/cre mice (i.e., parent strain in homozygous condition) under 12:12 LD conditions displayed a more variable, although not statistically advanced or delayed, phase angle of entrainment in LMA and Tb (Fig. 1F1-1) as compared with mice heterozygous for the cre allele or wild-type littermate controls. VIPcre/cre mice also displayed a range of free running LMA and Tb periods in DD (Fig. 1E1-2, 1E1–2; 1G1–1) that were comparable to those expressed by VIPcre/cre::Bmal1fl/fl and VIPwt/cre::Bmal1fl/fl mice. We interpret the more variable phase angle of entrainment as evidence of a possible hypomorphic allele in the VIPcre/cre condition, although we did not observe a reduction in VIP mRNA, and hence putative production of VIP, in VIPcre/cre homozygous state as compared with mice heterozygous for the cre allele or wild-type littermate controls (Fig. S3). As a genetic comparator, we deleted Bmal1 in AVP neurons by crossing AVP-ires-Cre mice14 with Bmal1fl/fl mice (AVPcre/cre, Bmal1fl/fl) and analyzed their LMA and Tb rhythms under LD and DD (Fig. S4). We again found that complete deletion of Bmal1 required that the AVP-ires-Cre mice be in the homozygous state (i.e., AVPcre/cre); unfortunately, this also produced profound diabetes insipidus in these mice, likely secondary to disruption of AVP function (allele itself or Bmal1) in the supraoptic hypothalamus. Regardless, we found that deletion of Bmal1 from AVP neurons did not produce arrhythmia in LMA or Tb under DD conditions, but did result in a lengthening of the circadian period of both rhythms (Fig. S4) in the AVPcre/cre::Bmal1fl/fl mice, a finding that recapitulates that previously reported by Mieda and colleagues. Taken together, these findings clearly demonstrate that a functional molecular clock in SCNVIP, but not SCNAVIP, neurons is necessary for circadian rhythms of LMA, and that the absence of this clock in SCNVIP neurons also affects the expression, including phasing and amplitude, of the circadian rhythm of Tb.

SCNVIP neurons and the circadian rhythm of sleep-wake. The SCN plays an important role in regulating the timing and architecture of the sleep-wake cycle. Additional data suggest a
possible role for the SCN in regulating the amount of sleep and wake, although this remains a point of controversy. A decrease in rapid-eye movement (REM) sleep has also been reported in \textit{VIP}^{-/-} mice, although whether or not these effects on REM sleep in the \textit{VIP}^{-/-} mouse link to \textit{SCN}^{VIP} or extra-

\textit{SCN}^{VIP} regulation of REM sleep remains unclear. Regardless, the cellular and synaptic bases by which the SCN clock, including possibly \textit{SCN}^{VIP} neurons, might regulate the sleep-wake cycle remains to be determined. Given our foregoing findings, we asked whether \textit{SCN}^{VIP} neurons, or the molecular clock thereof, might

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**Fig. 1 Disruption of the molecular clock in \textit{SCN}^{VIP} neurons results in eventual loss of the LMA circadian rhythm.** a Representative microphotographs at three rostro-caudal levels of the ventrolateral SCN from a VIP-\textit{ires-Cre:lox-L10-GFP} reporter mouse reveals eutopic expression of GFP (scale bar, 300 µm; \(n=2\)). See also Fig. S1. b-e Representative actograms showing locomotor activity (LMA) and body temperature (Tb) circadian rhythms (gray scale: darker represents higher temperature) and associated periodograms during at least 10 days in light/dark (LD), followed by at least three weeks in constant dark (DD), followed by at least 7 days of LD from two \textit{VIP^{cre/cre::Bmal1fl/fl}} mice (\textit{b1}, \textit{b1'}), two \textit{VIP^{cre/wt::Bmal1fl/fl}} mice (\textit{c1}, \textit{c1'}), two \textit{VIP^{wt/wt::Bmal1fl/wt}} mice (\textit{d1}, \textit{d1'}), and two \textit{VIP^{cre/wt::Bmal1fl/wt}} mice (\textit{e1}, \textit{e1'}). Note a profound disruption of LMA rhythms in \textit{VIP^{cre/cre::Bmal1fl/fl}} mice (\textit{b1}-2), whereas these rhythms remain unaltered in \textit{VIP^{cre/wt::Bmal1fl/fl}} mice (\textit{c1'}), \textit{VIP^{wt/wt::Bmal1fl/wt}} mice (\textit{d1'}), and \textit{VIP^{cre/wt::Bmal1fl/wt}} mice (\textit{e1'}). See also Figure S2. Phase angle of entrainment for LMA (\textit{f1}) and Tb (\textit{f1'}) in DD across all mice. Period length of LMA (\textit{g1}) and Tb (\textit{g1'}) in DD across all mice. Note that \textit{VIP^{cre/cre::Bmal1fl/fl}} mice (\textit{n=6}) display significantly advanced phase angles of entrainment in DD for both LMA and Tb rhythms compared to \textit{VIP^{cre/wt::Bmal1fl/fl}} and \textit{VIP^{wt/wt::Bmal1fl/wt}} mice (\textit{n=8}), and \textit{VIP^{cre/wt::Bmal1fl/wt}} mice (\textit{n=5}). (For LMA: 1-way ANOVA, \(F_{2,36}=27.57, P<0.0001\), Sidak's post hoc: **\(P<0.0001\) and \(P<0.0001\); For Tb: 1-way ANOVA, \(F_{2,17}=32.48, P<0.0001\), Sidak's post hoc: **\(P<0.0001\) and \(P=0.0002\)). In DD, \textit{VIP^{cre/cre::Bmal1fl/fl}} mice display period lengths outside of the circadian range for LMA rhythms compared to \textit{VIP^{cre/wt::Bmal1fl/fl}}, \textit{VIP^{wt/wt::Bmal1fl/wt}}, and \textit{VIP^{cre/wt::Bmal1fl/wt}} mice, and \textit{VIP^{cre/cre}} mice (note \textit{VIP^{cre/wt::Bmal1fl/fl}} and \textit{VIP^{wt/wt::Bmal1fl/wt}} mice are included together as \textit{VIP^{cre/wt::Bmal1fl/fl}} in \textit{f-g}). Means ± s.e.m. III, third ventricle; OC, optic chiasm; PV, paraventricular nucleus of the hypothalamus; SPZ, subparaventricular zone; SON, supraoptic nucleus. See also Figs. S3 and S4.
contribute to the regulation of the sleep-wake cycle. We did so by recording the electroencephalogram (EEG) to monitor wake, REM and non-REM (NREM) sleep, in the same cohorts of mice as used for the Tb and LMA recordings, while mice were under DD. We found that NREM sleep was increased at the beginning of the subjective day in homozygous Bmal1-deleted mice as compared to mice with partial Bmal1 deletions. And while we did not observe an effect of genotype on the quantity of each vigilance state, as measured over the 24 h period (Fig.2a), we did find that Bmal1-deleted mice exhibited decreased NREM sleep during the subjective light phase, leading to an apparent redistribution of sleep-wake activity (as demonstrated by a subjective dark to subjective light ratio (SD/SL) that is closer to 1 (Fig.2B2), suggesting a potential role for SCN VIP neurons in regulating the amplitude of the sleep-wake cycle. In order to test if disruption of the molecular clock in SCN VIP neurons affected sleep quality, we examined the vigilance state episodes during the SD and SL periods, as well as performed an analysis of the cortical EEG spectral power. Aside from sporadic individual differences in wakefulness episode duration, Bmal1-deleted mice did not demonstrate differences in sleep fragmentation or consolidation as compared to mice with partial Bmal1 deletions (Fig. S5).

Selective ablation of SCN VIP cells. Nearly all in vivo work to date on SCN VIP neurons, including the foregoing experiments in this study, have been performed in developmental mutant models, which have included transgenic crosses. While these models have provided important insights into the neurobiology of the SCN pacemaker, they also have important limitations, namely that developmental disruption of transcription factors, receptors, and neuropeptides, can complicate interpretation secondary to unintended or otherwise unrecognized effects on intercellular

Fig. 2 Disruption of the molecular clock in SCN VIP neurons reduces the amplitude of the sleep-wake circadian rhythm. a Hourly amounts (mean ± s.e.m.) of wakefulness (a1), NREM sleep (a2) and REM sleep (a3) in VIPcre/cre::Bmal1fl/fl (blue, n = 8) and VIPcre/wt::Bmal1fl/fl (red, n = 8) mice during the 4th and 5th day in constant darkness. b Upper panels: Amounts (mean ± s.e.m.) of the vigilance stages during the 4th and 5th subjective dark (SD4, SD5) and subjective light (SL4, SL5) periods. Lower panels: 24 h amounts (mean ± s.e.m.) of the vigilance stages during the 4th and 5th day in constant darkness (DD4, DD5; lower left panels) and subjective dark to subjective light (D/L) ratio for each vigilance stage during the 4th and 5th day in constant darkness (lower right panels). n = 8 mice per genotype, *p < 0.05, **p < 0.01, two-way ANOVA followed by a post hoc Bonferroni test. See also Figs. S5 and S6. Note: VIPcre/wt::Bmal1fl/fl and VIPwt/wt::Bmal1fl/wt mice are included together under VIPcre/wt::Bmal1fl/fl in all panels.
signaling and overall network function. We therefore pursued a genetically targeted approach for chronically disrupting SCN VIP neuron function in adult mice. To do this, we placed bilateral injections of a viral vector expressing a Cre-dependent dipheria toxin (DIO-DTA-AAV) into the SCN of VIP-IRES-Cre mice (Fig. 3). We also placed bilateral injections of this same toxin into the SCN of AVP-ires-Cre and Vgat-ires-Cre mice to serve as genetic comparators. While the DIO-DTA-AAV has been previously validated, and exhibits exquisite selectivity in producing cell death, we confirmed histologically that bilateral injections of this vector into the SCN of VIP-IRES-Cre (VIPDTA) and AVP-IRES-Cre (AVPDTA) mice resulted in cell-specific ablation, with no indication of "off target" cell death (Fig. 3a–i). As shown in Fig. 3j–n, VIPDTA mice with complete lesions (n = 6; >95% of VIP cell loss bilaterally) demonstrated a phase angle of entrainment of LMA and Tb in LD that was significantly advanced as compared with AVPDTA mice, and more strikingly, both the Tb and LMA rhythms of these mice became severely fragmented in DD, with all mice exhibiting periods outside of the circadian range (12.33–30.5 h, shortest and longest tau; also see DD Tb waveform in Fig S3). By comparison, AVPDTA mice with complete lesions (n = 6) exhibited high-amplitude rhythms of LMA and Tb in DD. Of note, AVPDTA mice did not show the diabetic insipidus phenotype observed in the AVPcre/cre::Bmalfl/fl mice, reinforcing the extra-SCN etiological basis of this phenotype. Finally, bilateral injections of DIO-DTA-AAV into the SCN of Vgat-IRES-Cre mice approximated a near complete cellular lesion of the SCN (due to the near ubiquitous expression of Vgat in the cellular SCN) and produced a more dramatic phenotype than seen in the VIPDTA mice, including complete arrhythmicity.
of LMA and Tb in LD as well as in DD (Fig. 3). The apparent absence of light-masking by the LD cycle in the Vgat<sup>DTA</sup> mice may reflect the intensity of the light in our LD cycle, but may also link to some DTA-induced cell loss in the dorsally adjacent subparaventricular zone (SPZ), which is largely GABAergic (Vgat+) and is a region previously shown to play a role in LD masking<sup>24,25</sup>.

**Selective ablation of SCN<i>VIP</i> cells and sleep-wake.** We next performed EEG analysis on the VIP<sup>DTA</sup> mice to determine the effects of SCN<i>VIP</i> cell loss upon on the circadian rhythms of sleep-wake (Fig. 4). Given the absence of LMA and Tb phenotypes in the AVP<sup>DTA</sup> mice, and because they received the same vector injections, we used these mice as comparators. As compared to AVP<sup>DTA</sup> mice, VIP<sup>DTA</sup> mice exhibited a reduced circadian amplitude of the sleep-wake rhythm in DD. Selective ablation of SCN<i>VIP</i> neurons did not produce significant changes in vigilance stage episode number or duration (Fig. S7). However, wakefulness and NREM sleep cortical EEG power circadian variations appeared phase-reversed across all frequency bands and at all vigilance stages (Fig. S8), resulting in significant differences in power variation at multiple time periods between AVP<sup>DTA</sup> and VIP<sup>DTA</sup> mice. These findings, in particular the observed reduction in circadian amplitude, are largely consistent to those in the VIP<sup>cre/cre::Bmal<sup>fl/fl</sup></sup> mice, reinforcing the interesting possibility that SCN<i>VIP</i> neurons may exert an influence on the expression of the sleep-wake rhythm, presumably by influencing the level of arousal or sleep.

**SCN<i>VIP</i> and wheel running behaviors.** We next evaluated the effects of (1) disruption of the molecular clock in SCN<i>VIP</i> neurons, and (2) genetically driven ablation of SCN<i>VIP</i> neurons on wheel running behavior in LD and DD. To do so, we generated new cohorts of mice and evaluated wheel running rhythms in LD, DD, and in a 1:1:1:1 skeleton photoperiod (SKP; Fig. 5a–e). In contrast to the arrhythmic LMA phenotype observed in the VIP<sup>cre/cre::Bmal<sup>fl/fl</sup></sup> mice under DD (see Fig. 1b1-b2), wheel-running behavior in DD was strikingly normal in VIP<sup>cre/cre::Bmal<sup>fl/fl</sup></sup> mice (Fig. 5d) as compared with VIP<sup>cre/wt</sup> and VIP<sup>cre/cre</sup> mice (Fig. 5a–b). Interestingly, however, we did observe mildly abnormal wheel running behavior in three (of seven) of our VIP<sup>cre/cre</sup> mice, taking the form of an immediate advance (~2–4 h) of wheel running activity on transition from LD to DD and a lower amplitude/more fragmented circadian rhythm of wheel running activity in DD (Fig. 5c), which we view as additional evidence for a hypomorphic allele in the homozygous state. This phenotype was not observed in the remainder of our VIP<sup>cre/cre</sup> or VIP<sup>cre/wt</sup> controls, nor was it observed in any of the VIP<sup>cre/cre::Bmal<sup>fl/fl</sup></sup> mice (Fig. 5d1–2), the latter suggesting that disruption of the molecular clock in SCN<i>VIP</i> cells somehow compensated for the putative hypomorphic phenotype observed in some of the VIP<sup>cre/cre</sup> mice. Compared to VIP<sup>cre/wt</sup> controls, which showed the expected unitary bout of wheel running activity under the 1:11:1:11 SKP, all of the VIP<sup>cre/cre::Bmal<sup>fl/fl</sup></sup> and VIP<sup>cre/cre</sup> mice showed splitting (i.e., 2 daily bouts; Fig. 5b–d2) of their rhythms, which is consistent with that previously reported in VIP knockout (VIP<sup>−/−</sup>) and VIP receptor knockout (Vipr2<sup>−/−</sup>) mice<sup>6</sup>, and continues to point to a potential hypomorphic condition in the VIP<sup>cre/cre</sup> mouse. On the other hand, and similar to our telemetry findings, wheel running rhythms in VIP<sup>DTA</sup> mice during DD were severely disrupted. Despite appearance of arrhythmicity in several mice, all but one VIP<sup>DTA</sup> mouse expressed a detectable circadian component in their wheel running rhythms, although the rhythms were low in amplitude and their period generally shorter than that of the other groups (Fig. 5 e1–f). Hence the genetically driven loss of SCN<i>VIP</i> neurons resulted in a wheel running rhythm in DD that was similar, though not identical to that previously reported in the congenital VIP and VPAC2 KO models. Taken together, these findings suggest the interesting possibility that non-photic sensory flow during wheel running may provide an adequate forcing signal to ‘clockless’ VIP neurons (i.e., VIP<sup>cre/cre::Bmal<sup>fl/fl</sup></sup> mice), resulting in the appearance of a consolidated wheel running rhythm. But in the absence of VIP neurons (i.e., VIP<sup>DTA</sup> mice), and hence synaptic release of VIP, GABA or other diffusible factors used by SCN<i>VIP</i> neurons, the SCN is compromised in its ability to convey these non-photic inputs to downstream intra- and extra-SCN effector circuits and hence produce a high-amplitude rhythm of wheel running activity. These results may also reflect a differential role for VIP in circadian function across development, as acute ablation in adulthood produces similar yet distinct phenotypes from genetic germ-line deletions. It is also worth mentioning that while wheel running behavior ostensibly provides another readout of SCN phase and period, at least one prior study has raised the concern that wheel running activity may not track with other physiological readouts of SCN phase, especially when animals have altered
circadian phenotypes\textsuperscript{26}. Our finding of similar, but also somewhat divergent not identical, effects of SCNVIP manipulation on wheel running versus cage LMA is consistent with this prior suggestion. We opted not to perform a similar experiment with AVPcre/cre::Bmal1\textsuperscript{fl/fl} mice due to concerns that they were too sickly for the running wheel.

**Activation of SCNVIP neurons does not drive sleep or arousal.**

Given the significantly blunted circadian amplitude of the sleep-wake cycle in both VIP\textsuperscript{cre/cre::Bmal1\textsuperscript{fl/fl}} and VIP\textsuperscript{DTA} mice, we asked whether acute and selective activation of SCNVIP neurons might increase sleep or wake (Fig. 6). To do this, we placed bilateral injections of Cre-dependent hM3Dq into the SCN of heterozygous VIP-ires-Cre mice (n = 4) as previously described\textsuperscript{27}, and administered the chemogenetic ligand clozapine-N-oxide (CNO; 0.3 mg/kg) at ZT4 (during the middle of the day when mice are typically less active) or ZT13 (1 h after lights off, when mice are typically most active). Strikingly, administration of CNO, which produced robust expression of c-Fos in mCherry\textsuperscript{+} (hM3Dq+) neurons, did not affect sleep-wake amounts (Fig. 6a), the number and length of vigilance state episodes (Fig. 6b), or their distribution (Fig. 6c) at ZT13. Similarly, no changes in the cortical EEG were observed in response to CNO administration (Fig. 6d). Finally, circadian sleep-wake distribution was not affected by activation of SCNVIP neurons in DD condition (Fig. 6f) and the same results were obtained when CNO was injected at ZT4. These findings suggest that SCNVIP neurons likely do not directly play a role in the modulation of behavioral state, i.e., not wake- or sleep-promoting per se. Rather, and instead, these findings inform the more parsimonious explanation that the blunted circadian amplitude of the sleep-wake rhythms observed in the VIP\textsuperscript{cre/cre::Bmal1\textsuperscript{fl/fl}} mice and VIP\textsuperscript{DTA} mice were secondary to changes (i.e., arrhythmicity and fragmentation) of the LMA rhythm.

**Light but not behavioral state alters SCNVIP cell activity.**

To help reconcile the observed reduction in the amplitude of the sleep-wake cycle in the VIP\textsuperscript{cre/cre::Bmal1\textsuperscript{fl/fl}} mice and VIP\textsuperscript{DTA} mice with the contrasting absence of effect of acute activation of
SCNVIP neurons on sleep or wake, we next examined the state-dependent population Ca\(^{2+}\) activity dynamics of SCNVIP neurons using in vivo fiber photometry (Fig. 7). To do so, we placed unilateral injections of DIO-GCaMP6s-AAV10 into the SCN of VIPcre/wt mice and implanted a photometry fiber immediately dorsal to the SCNVIP cell bodies (Fig. 7a–c). Four–six weeks after injections of the viral vector, mice were habituated to the recording conditions before being placed in DD at ZT12. After the mice were released into DD, on the next day, simultaneous sleep–wake and photometry recordings were carried out at CT 2–5 and CT 12–15 with a 20-min light pulse applied at CT14 (i.e., 26 h after onset of DD, Fig. 7d). SCNVIP neurons were immediately activated by the light pulse (Fig. 7e–g), indicating that light stimulation at the retina results in robust excitation of the SCNVIP cell population. As an important control, VIP-containing neurons in the rostrally adjacent ventromedial preoptic nucleus (VMPO) were not activated by the light pulse (Fig. S9 A–B), indicating that neuronal activation by light is selective to SCNVIP neurons and not simply an artifact of external light pollution. Following the initial peak induced by the light pulse, Ca\(^{2+}\) activity declined to baseline levels. Interestingly, further Ca\(^{2+}\) peaks were observed throughout the remainder of the light pulse when the mice were awake, but not when they were in NREM or REM sleep (Fig. 7e). These peaks in Ca\(^{2+}\) activity were only observed during the 20-min light pulse and did not occur during waking episodes either prior to, or immediately after completion of, the light pulse (Fig. 7h), nor were they present during wake in the absence of light—hence this was not a movement artifact. Apart from the activity observed during the light pulse, the activity profile of SCNVIP neurons did not fluctuate significantly over sleep–wake states or at arousal state transitions, and this was independent of the time of day (Fig. S9C–I). In sum our findings show that the SCNVIP population is highly responsive to light input in vivo, which is congruent with a recent report\(^{28}\), but also that their activity is behavioral state independent. The latter observation is consistent with our chemogenetic findings which suggest that SCNVIP neurons may not contribute directly to the regulation of sleep–wake architecture, timing or amounts.

Mapping functional synaptic outflow of SCNVIP cells. While the foregoing work points to an indispensable role for SCNVIP neurons in producing coherent circadian rhythms of LMA, the post-synaptic targets by which SCNVIP neurons drive the LMA rhythm (or nearly any output rhythm) remain incompletely understood. To initially map efferent projections of SCN VIP neurons may not contribute directly to the regulation of sleep–wake architecture, timing or amounts.

**Fig. 5** Circadian rhythms of wheel running behavior remain intact following loss of the molecular clock in SCNVIP neurons, whereas their ablation disrupts this rhythm. a–f Wheel running behavior and associated periodograms from a control VIPcre/wt mouse (a), two VIPcre/wt mice (b, c), two VIPcre/cre-Bmal1fl/fl mouse (d), two VIPcre/wt mice that received bilateral injections of the DTA vector into the SCN (e) and a VIPcre/cre mouse that received bilateral injections of the DTA vector into the SCN (f) under a 12:12 light-dark (LD, 10 days) cycle, in constant darkness (DD, 22 days), and under a 1:11 light photoperiod. (Fig. S9 A–B). SCNVIP neurons were immediately activated by the light pulse (Fig. 7e–g), indicating that light stimulation at the retina results in robust excitation of the SCNVIP cell population. As an important control, VIP-containing neurons in the rostrally adjacent ventromedial preoptic nucleus (VMPO) were not activated by the light pulse (Fig. S9 A–B), indicating that neuronal activation by light is selective to SCNVIP neurons and not simply an artifact of external light pollution. Following the initial peak induced by the light pulse, Ca\(^{2+}\) activity declined to baseline levels. Interestingly, further Ca\(^{2+}\) peaks were observed throughout the remainder of the light pulse when the mice were awake, but not when they were in NREM or REM sleep (Fig. 7e). These peaks in Ca\(^{2+}\) activity were only observed during the 20-min light pulse and did not occur during waking episodes either prior to, or immediately after completion of, the light pulse (Fig. 7h), nor were they present during wake in the absence of light—hence this was not a movement artifact. Apart from the activity observed during the light pulse, the activity profile of SCNVIP neurons did not fluctuate significantly over sleep–wake states or at arousal state transitions, and this was independent of the time of day (Fig. S9C–I). In sum our findings show that the SCNVIP population is highly responsive to light input in vivo, which is congruent with a recent report\(^{28}\), but also that their activity is behavioral state independent. The latter observation is consistent with our chemogenetic findings which suggest that SCNVIP neurons may not contribute directly to the regulation of sleep–wake architecture, timing or amounts.

Mapping functional synaptic outflow of SCNVIP cells. While the foregoing work points to an indispensable role for SCNVIP neurons in producing coherent circadian rhythms of LMA, the post-synaptic targets by which SCNVIP neurons drive the LMA rhythm (or nearly any output rhythm) remain incompletely understood. To initially map efferent projections of SCNVIP neurons we placed unilateral injections of a genetically encoded anterograde tracer into the SCN of VIPcre/wt mice. As shown in Fig. S10, intra-hypothalamic and preoptic regions were primary targets of SCNVIP neurons. The presumptive terminal fields within the dorsomedial hypothalamus (DMH) were of particular interest given previous lesion studies that identified an important role for the DMH in the expression of the LMA circadian rhythm\(^{29}\). The density of SCNVIP terminal fields within the DMH was nevertheless rather modest, suggesting that a portion of SCNVIP inputs to the DMH may be indirect (polysynaptic). To this end, prior lesion studies have suggested an important role for...
a polysynaptic pathway, involving the SPZ, in regulating the circadian rhythm of LMA. We therefore asked whether SCNVIP neurons are functionally, synaptically coupled with DMH neurons via the SPZ. Indeed, a SCN → SPZ → DMH circuit has been previously proposed, but never functionally confirmed.

To assess functional polysynaptic connectivity, we first tested whether SCNVIP neurons inhibit SPZ neurons that project to the DMH (SCNVIP → SPZ → DMH) by performing Channelrhodopsin (Chr2)-assisted circuit mapping (CRACM) in brain slices, as previously described. We expressed ChR2-mCherry in SCNVIP neurons by injecting AAV-DIO-ChR2-mCherry into the SCN. This allowed us to manipulate SCNVIP activity and observe its effects on SPZ and DMH activity.
the SCN of VIPcre/wt mice. In the same mice, we placed injections of green-fluorescent CTB (F-CTB) into the ipsilateral DMH. Five to six weeks later, we recorded from SPZ neurons that were retrogradely labeled from the DMH while photostimulating fibers and terminals from SCNVIP neurons in SPZ (Fig. 8a). Of the 20 recorded SPZ-DMH projecting neurons, we labeled 11 of these neurons with biocytin and confirmed that they were located in the SPZ (Fig. 8b–c). Histological assessment confirmed that expression of ChR2-mCherry and F-CTB were restricted to the 30 μm in diameter. These results also re-emphasize the functional and anatomical importance of the cellular SPZ as a postsynaptic target of, and relay for, SCN neurons and their synaptic outflow33–35.

**SCN VIP** neurons comprise two molecularly distinct subtypes. Previous studies in rodent models have raised the possibility that SCN VIP neurons are a heterogenous population containing anatomically and physiologically distinct subtypes40,41. However, the molecular identity of these subtypes and the full extent of their differences remain unclear. We therefore used sNuc-Seq42, a systematic means of classifying cell types based on the nuclear mRNA profiles of single cells, to characterize the molecular diversity of SCN VIP neurons. First, to label VIP neuron nuclei for sampling, we crossed VIP-ires-Cre to a transgenic reporter mouse line that Cre-dependently expresses a nuclear-localized mCherry (H2b-TRAP)43. We then performed sNuc-Seq on mCherry+ cell nuclei sorted from juvenile and adult mice VIP-ires-Cre::H2b-TRAP SCN tissue that had been dissected one hour after light cycle onset (Fig. 10a). We sequenced each of 192 single-nuclei samples to a depth of 3.0 M ± 1.4 M reads per sample, aligned the reads to the mouse genome (mean and S.D. reads aligned per sample, 79% ± 22%), and quantified expression values based on the number of reads aligning to each gene (see “Methods”). After filtering out the 7% of samples in which fewer than 500 genes were detected, we used the remaining 178 single-nuclei transcriptomes, averaging (±S.D.) 3694 ± 1425 genes each, for unsupervised clustering and differential expression analysis.

Our initial results showed three clusters of VIP neurons (Fig. S11A). Each cluster contained cells from each age group (Fig. S11A), indicating that the transcriptional identities of these groups are stable across late development and thereby justifying our pooling of the data for analysis. However, only two of the three initial clusters were confirmed as SCN neurons based on their expression of the regional marker genes, Lhx1 and Vipr244,45 (Fig. S11B) and so were the focus of our subsequent analysis.

Re-clustering only the neurons that contained SCN marker genes revealed two subtypes of SCN VIP neurons with distinct transcriptional profiles (Fig. 10b, c). We annotated these two subtypes as Gpr5 VIP neurons and Nms VIP neurons based on their enriched expression of the neuropeptide-encoding genes. These subtypes are consistent with the two VIP neuron subtypes, similarly called Vip/Gpr neurons and Vip/Nms neurons, identified by a study published during the revision of this manuscript (Fig. S11C). Contrasting their transcriptional profiles revealed gene sets which are consistent with distinct functional roles. For instance, consistent with a central role in circadian clock function, Nms VIP neurons were enriched with transcripts encoding components and regulators of the circadian clock, including: Per2; salt inducible kinase 1 (Sik1)46, Prok247, and Prokr248. On the other hand, Gpr5 VIP neurons had higher expression of GABA receptor genes (GabrA2, Gabrg1, GabrrB1).
and androgen receptors (Ars) and therefore GrpVIP SCN neurons may be an important cellular point of entry to the SCN clock for non-photic inhibitory inputs, including androgens that can potently alter the circadian period49,50.

Technical consideration: a hypomorphic allele? While our physiological and behavioral recordings in the VIPcre/cre mouse are potentially consistent with a hypomorphic allele, the expression of VIP transcript (per RNAscope in situ hybridization) and AAV10-EF1α-DIO-GCaMP6s in SCNVIP EMG electrode EEG electrode 465 nm LED (30 μW) FILTERS/DICHROICS AMPLIFIER/AD CONVERSION Connector Fiber ferrule Photoreceptor AMPLIFIER Ca2+ signal EEG/EMG signal

**Fig. 7** In vivo fiber photometry reveals SCNVIP neurons are activated by light stimulation of the retina. **a** Experimental schema showing viral vector targeting and headset equipment for simultaneous in vivo fiber photometry and EEG/EMG recordings from mice expressing GCaMP6s in SCNVIP neurons. **b** Representative photomicrograph of GCaMP6s expression in the SCN and location of the photometry optical fiber. Scale bar: 200 μm. OC; optic chiasm, III; 3rd ventricle. **c** Schematic of SCN area showing fiber placement from experimental mice over the SCN. **d** Behavioral experimental paradigm showing times of photometry/EEG recording related to the time of day and the light pulse stimulus. **e** Representative example showing GCaMP6s signal (upper panel), EEG frequency spectrogram (2nd panel from top), raw EEG (3rd panel from top) and raw EMG (bottom panel) before, during and after the 20-min light pulse at ZT14. Red dotted rectangle indicates time displayed in (f, g). **f** Mean GCaMP6s activity over all mice at the initiation of the light pulse. Black dotted line indicates onset of the light pulse. Shaded area indicates s.e.m. Paired t-test (two-tailed) * <0.0206. n = 4. **g** Heatmap depicting GCaMP6s activity at the light pulse for each mouse. White dotted line indicates onset of the light pulse. **h** Mean (+ s.e.m.) GCaMP6s activity during wake and NREM sleep before, during and after the light pulse. RM two-way ANOVA (Interaction: Light Pulse x Arousal State F (2, 6) = 62.82, p < 0.0001; n = 4 followed by Sidak’s post hoc tests (* p < 0.05, ** p < 0.01, **** p < 0.0001)). See also Fig. S9.
number of VIP+ cell bodies (per GFP+ cells in the genetic cross) within the SCN did not appear to be appreciably altered in the homozygous state. It is nevertheless possible, however, that less VIP was being produced in the SCN of the homozygous VIPcre/cre::Vgatfl/fl mice. Rather remarkably, we found that disruption of GABAergic signaling by VIP neurons in the VIPcre/cre mouse (putative hypomorphic) reduced the variability in the LD phase angle of entrainment for Tb, but not for LMA (Fig. S12). While a finding that could have explanatory power for the previously reported “desynchronizing” effects of SCN GABA as well as implications for the molecular basis of entrainment, future intersectional studies will be needed to better understand the functional relationship between GABA and VIP synaptic release by SCN VIP and their individual roles in SCN circuit function.

Discussion

Altogether considerable evidence has accumulated pointing to an important role for VIP, and its cognate receptor, VPAC2, in SCN clock function, yet more recent studies have suggested that other, non-VIP SCN cell groups may play a more dominant role in circadian pacemaking. Here we show that SCN VIP neurons, and their internal molecular clock, are in fact necessary for establishing and maintaining coherent circadian rhythms of LMA, and for the normal expression of the circadian rhythms of Tb, sleep-wake and wheel running behavior. While our findings do not exclude the possibility that SCN VIP neurons may also directly

Fig. 8 SCN VIP neurons inhibit SPZ neurons that project to the DMH (SCN VIP → SPZ → DMH). a Schematic of the experimental design used to map SCN VIP → SPZ → DMH connectivity in brain slices. AAV-DIO-ChR2-mCherry was injected into the SCN of Vipcre/wt mice and in the same animals, green-fluorescent CTB (F-CTB; Alexa Fluor-488 conjugated-CTB) was injected into the ipsilateral DMH, to retrogradely label SPZ neurons that project to the DMH. Recordings were made from SPZ neurons labeled with F-CTB in green while photostimulating SCN VIP axon terminals in the SPZ. b Examples of SPZ neurons retrogradely labeled (upper panels) from the DMH and imaged during whole-cell recordings under fluorescent and IR-DIC visualization (lower left) or after post hoc labeling of biocytin with streptavidin-conjugated AlexaFluor-405 (blue; lower right). Scale bar: 20 µm. c Map of 11 recorded SPZ DMH projecting neurons identified after post hoc labeling of biocytin (scale bar: 500 µm). d Photomicrograph, illustrating ChR2-mCherry expression in SCN VIP neurons (marked by arrows) at low (left; scale bar: 100 µm) and higher (right; scale bar: 20 µm) magnifications. e Heatmap of the distribution of F-CTB in the DMH in five recorded mice (left) and an example of green F-CTB in the DMH (right; scale bar: 200 µm). f, g Photostimulation of SCN VIP input inhibited action potential firing of SPZ DMH projecting neurons (f cell-attached recording, blue bar represents a 10-s train stimulation at 10 Hz; 10 ms light pulses) and in the same neuron in whole-cell configuration, elicited photo-evoked IPSCs (g blue bar represents a 10-ms light pulse; \( V_h = 0 \) mV). Individual photo-evoked IPSCs are shown in gray and the average trace in black. OC: optic tract; III third ventricle.
contribute to the regulation of the sleep-wake cycle, both the absence of an effect of acute chemogenetic activation of SCN\textsuperscript{VIP} neurons on sleep-wake, and our finding that the activity dynamics of SCN\textsuperscript{VIP} neurons are behavioral state invariant, instead support the null hypothesis. Regardless, our in vitro CRACM experiments establish the presence of a functional SCN\textsuperscript{VIP} → SPZ → DMH synaptic pathway by which these neurons may regulate circadian rhythms of LMA. We also found that SCN\textsuperscript{VIP} neurons are post-synaptic targets of key structures mediating non-photic and photic modulation of the SCN clock and hence likely comprise a cellular point of integration of these inputs. Finally, our sNuc-seq results reveal that a subset of
Fig. 9 Presynaptic inputs to SCNVIP neurons arise from areas involved in photic and nonphotic sensory regulation of the SCN clock. a Schematic of experimental procedure. AAV8-DIO-TVA-mCherry and AAV8-DIO-RG were co-injected into the SCN of VIP-IRESCre mice at Day 1. 28 days later, the pseudotyped modified rabies virus (EnvA-ΔG-eGFP) was injected at the same site in the SCN and the mice perfused 11 days later. b Photomicrograph of the SCN illustrating AAV8-DIO-TVA-mCherry expressing neurons (magenta) and EnvA-ΔG-eGFP expressing neurons (green). Co-expression of AAV8-DIO-TVA-mCherry and EnvA-ΔG-eGFP represents the starter population. Scale bar: 200 μm. (C1-3) High magnification image of box in B (C1), red channel only (C2) and green channel only (C3). Filled arrows point towards starter population neurons expressing both AAV8-DIO-TVA-mCherry and EnvA-ΔG-eGFP. Unfilled arrows point towards neurons expressing EnvA-ΔG-eGFP only (retrogradely labeled neurons). Scale bar: 100 μm. d-h Photomicrographs showing SCNVIP brain input populations in: LS, BNST and SHy (d); VMPO, MPO, AVPe (e); PV (f); Arc, VMH, DMH (g); IGL and PH (h). Scale bar: 200 μm. i Photomicrograph of retina showing EnvA-ΔG-eGFP expressing neurons (green, filled arrows), counter-stained with DAPI. Scale bar: 200 μm. Also see Fig S10. For abbreviations and quantitative proportion of inputs from regions throughout the brain, see Supplementary Information.

Fig. 10 sNuc-seq analysis reveals two transcriptionally distinct populations of SCNVIP neurons. a Schematic of method for labeling, isolating, and profiling SCNVIP neuron transcriptomes for sNuc-Seq; images are from samples used in this study. b t-Distributed Stochastic Neighbor Embedding (tSNE) plot of 144 VIP SCN neurons (dots) profiled by sNuc-Seq and clustered according to their expression of 689 high-dispersion genes; colors indicate cluster identity. c Heatmap showing single-cell (column) expression of top cluster marker genes (rows), with cells (columns) binned by cluster identity. d Violin plots of single-cell expression values of Vip and selected cluster marker genes. See also Fig. S11.

SCNVIP neurons, NmsVIP neurons, express core components and regulators of the circadian clock, including pacemaker potential, and that another subset of SCNVIP neurons, GrpVIP, more uniquely expresses high levels of GABA and Ars and hence may function as cellular effectors for non-photic inputs, including gonadal hormones.

In the present study, we found that developmental disruption of the molecular clock within SCNVIP neurons produced, in DD, arrhythmicity in LMA and altered the expression of the Tb and sleep-wake rhythms. Selective, genetically driven ablation of SCNVIP neurons in adult mice recapitulated the arrhythmic LMA phenotype but also profoundly altered the expression of Tb and wheel-running activity rhythms. With respect to other established pacemaker cell populations, seminal work by Lee and colleagues found that a subset of SCN neurons expressing NMS (~45–50% of total SCN neuronal population) plays an essential role in the
generation of daily rhythms in behavior. These authors specifically showed that disruption of the molecular clock in NMS neurons produces arrhythmic wheel running activity and, moreover, that NMS neurons control in vivo circadian rhythms through intercellular synaptic transmission. Our data suggest the possibility that the arrhythmic phenotype observed in the NMS mice experiments may link to disruption of the Nms

The fact that non-VIP NMS, including Grp

Our transcriptional data confirm that Ars are localized to the Grp

While SCNVIP neurons comprise only ~10% of the total cellular SCN, our findings indicate an indispensable role for this cell population in the regulation of the LMA rhythm as well as for the normal expression of the Tb, sleep-wake and wheel-running rhythms. Specifically, we found that other neuropeptide systems in the SCN network, in either the acute or developmental framework, were unable to compensate for the loss of SCNVIP neurons, or their cellular clocks, to maintain coherent LMA rhythms or high-amplitude Tb and sleep-wake rhythms. Given differential disruptive effects of our SCNVIP cell manipulations on LMA, Tb, sleep-wake, and wheel running rhythms, our findings also lend support to a model in which neurochemically defined SCN cell groups utilize differential synaptic outflow pathways and downstream circuit targets to impart circadian timing information on a wide range of physiological and behavioral processes. This model derives additional support from a recent paper by Paul and colleagues who showed that selective manipulation of SCNVIP cells can acutely modulate cardiovascular function, likely via direct synaptic inputs to pre-autonomic neurons within the dorsal cap of the PVH (a region we also found to be a dense post-synaptic target of SCNVIP cells—see Fig. S10). Our results further align with findings in aging humans, wherein the LMA, rhythm is disrupted in association with loss of SCNVIP neurons and also with recent findings linking a polysynaptic SCNVIP—VMH circuit with rhythms of aggression.

The present study also provides a template for future experimental interrogation of the cellular SCN, including intersectional approaches. As examples, such approaches would help address clear knowledge gaps that exist with respect to (1) molecular heterogeneity within other canonical SCN cell populations (e.g., AVP, NMS), (2) how temporal information (phase) is synaptically communicated to downstream effector networks, (3) the behavioral state activity dynamics of different SCN cell populations, and (4) how the various SCN cell populations, including functional subpopulations, are regulated synaptically by sensory flow to modulate fundamental properties of the circadian clock such as phase, amplitude and period. Hence, for example, future CRACM work using other Cre-driver mouse lines, including the
development of newer lines informed by cell-type specific transcriptions, will facilitate the development of a comprehensive map of SCN synaptic outflow. In turn this will enable a more detailed understanding of the circuit and synaptic bases by which various SCN cell populations impart temporal organization on a broad range of physiological and behavioral outputs.

Methods

Generation and validation of VIP-IRESCre mice. VIP-IRESCre mice were generated using standard recombineering techniques. In a brief, a selection cassette containing an optimized internal ribosomal entry sequence linked to Cre recombinase and a Frt-flanked kanamycin resistance gene was targeted 3 bp downstream of the stop codon of the VIP gene in a bacterial artificial chromosome (RP24-216N8; Children’s Hospital Oakland Research Institute). A targeting plasmid containing the Cre-reconstituting selection cassette and ~4 kb genomic sequence upstream and downstream of the VIP stop codon was isolated and used for embryonic stem cell targeting by the Beth Israel Deaconess Transgenic Core. Correctly targeted clones were identified by long range PCR. Chimeric animals generated from blastocyst implantation were then bred for germline transmission of the VIP-IRESCre allele. EIIa-deleter mice were then used to remove the neomycin selection cassette. Mice were subsequently intercrossed to generate heterozygous Cre reporter strains and bred to the B6a129S7-SDSPTG-lt10-GFP reporter line. Results from the reporter cross indicated that expression of the transgene was restricted to the ventrolateral (core) SCN (Fig. 1a). In the VIP-IRESCre-lt10-GFP cross we also confirmed the presence of a previously described population of VIP neurons in the ventromedial SCN — in proximity of the preoptic and SCN VIP populations, we confirmed the volumes of our AAV injections targeting the SCN to avoid transient misexpression of the VIP transgene. We subsequently confirmed that the preoptic VIP population is transcriptionally unique from the SCN VIP cell population and, per fiber photometry, exhibits markedly different population cell activity dynamics across the 24 h day and does not respond to an administration of a light pulse (see Fig. S9).

Stereotoxic brain injections. For selective ablation of SCN VIP and SCN AVP neurons, VIP-IRESCre and AVP-IRESCre mice received bilateral injections of AAV16-hSyn-mCherry-DIO-DTA (DTA-AAV; 30-40 nl per side, acquired from PMF27, was injected into the bilaterally into the SCN in male VIP-IRESCre mice. For ChR2-assisted circuit mapping (CRACM) experiments, we injected VIP-IRESCre mice (n = 5) unilaterally with 30 nl of AAV-DIO-Chr2-mCherry (UNC vector core) and contralateral injection of 10 nl of Fluorescent-conjugated Cholera Toxin Subunit B (F-CtB; green Alexa Fluor 488 conjugated CtB; Invitrogen) into the ipsilateral DMH of the same mice, to retrogradely label SPZ neurons projecting to the DMH.

For photobiomodulatory experiments, AAV10-EF1a-DIO-M3Dq-mCherry (30-40 nl per side; acquired from PMF27), was injected into the bilaterally into the SCN in male VIP-IRESCre mice. For monosynaptic retrograde modified rabies tracing we first made injections of a mixture of 60 nl AAV8- EF1a-DIO-TVA (UNC vector core) and 60 nl AAV8-CAG-DIO-RG (GVVC-AAV-99, Stanford vector core) into the left SCN. Four weeks after the initial surgeries, 200 nl of EnVag-AG-eGFP (Salk Institute) was injected into the same place.

In brief, mice were anesthetized with ketamine/xylazine mixture (100 and 10 mg/kg, respectively, IP) and administered slow release meloxicam subcutaneously (4 mg/kg) for pain relief. Fur was removed from the top of the skull with clippers and the area sterilized with iodopovidone, followed by isopropyl alcohol. The muscle was secured in a stereotaxic frame and an incision was made down the midline of the skull and the skin was retracted to expose bregma. A small burr hole was drilled through the skull above the SCN and a fine glass pipette containing the viral vector was slowly lowered down to the SCN (AP: −0.4 from Bregma, DV: −5.0, ML: 0.3, using coordinates derived from the atlas of Paxinos and Franklin) using a Kopf high precision micro-manipulator. Viral vector was dispelled slowly at a rate of <1 nl/second using a pico-spritzer air puff system. Once the required volume of virus had been dispelled, the pipette was left in position for 3 min before being slowly raised out of the brain. For CRACM experiments, a second injection of F-CtB was made into the DMH (AP: −1.6 mm, DV: −3.5 mm, ML: −2.0 mm) during the photostimulation. The skin was sutured back together and 0.5 ml sterile saline administered subcutaneously before the mouse was allowed to resume consciousness on a heating pad.

Biotlemetry implantations and recordings. Mice were anesthetized with ketamine/xylazine [100 and 10 mg/kg, respectively, intraperitoneal (IP)]. An incision was made in the IP cavity and biotlemetry transmitters (ETA-F10, Data Sciences International, St. Paul, MN) were implanted. Incisions were sutured and treated with topical antibiotics. After at least 5 days in LD conditions for at least 7 days. LMA and Tb data were first analyzed and plotted using Clocklab (Coulbourn Instruments, Natick, MA). For each mouse, we obtained LMA and Tb rhythm period (Chi-squared periodogram) and cosinor amplitude for the last 7 days in LD and the last 7 days in DD. The phase angle of entrainment (Ψ) was calculated as the difference (in hours) between the light-dark transition (ZT 12) and the acrophase of the Tb and LMA rhythms were calculated from a least squared fit during the last 7 days of LD. Chi-square periodograms were generated from the last 7 days of wheel-running data in constant darkness. The largest peak in the periodogram in the range of 12–36 h was selected as the circadian period. The amplitude of this peak was used in subsequent analysis as a proxy for the degree of circadian rhythmicity in individual animals.

Running wheels. Mice were housed in individual cages equipped with running wheels with free access to food and water. The cages were placed in an environmental chamber (Percival Scientific, Perry, IA) maintained at 20 – 21 °C on a 12:12 h light-dark (LD) cycle. The environmental chamber light intensity was approximately 300 lux during L and 0 lux during D. Wheel rotations were monitored through magnetic induction, acquired continuously and stored in 5-min bins with Vitalview software (Mini-Mitter Co.). The mice were maintained in the LD cycle for at least ten days then placed into constant darkness for 21 days. Finally, a skeleton photoperiod consisting of one hour of light and 11 h of darkness was applied. Time series data were imported into Clocklab (Actimetrics) for analysis of behavioral and circadian rhythmicity. Zeitgeber time (ZT) 12 was defined as the time of wheel running data in constant darkness. The largest peak in the periodogram in the range of 12–36 h was selected as the circadian period. The amplitude of this peak was used in subsequent analysis as a proxy for the degree of circadian rhythmicity in individual animals.

Sleep–wake monitoring and analysis. Mice were anesthetized with ketamine/xylazine [100 and 10 mg/kg, respectively, IP] and then placed in a stereotaxic apparatus. Mice were implanted with four EEG screw electrodes (two frontal and two parietal electrodes; Pineapple Technology Inc. #8403) and two flexible electronic recording (EMG) wire electrodes (Plastics One #E363/76/SPC) previously sol- dered to a 6-pin connector (Heilind Electronics, Inc. #853-43-006-10-001000) and the assembly was secured with dental cement. The frontal electrodes were positioned 1 mm frontal and 1 mm lateral from bregma whereas the parietal electrodes were positioned 1 mm lateral from bregma and midway between bregma and lambda.

Two weeks after EEG/EMG implantation, the mice were housed individually in transparent barrels in an insulated sound-proofed recording chamber maintained at an ambient temperature of 22 ± 1 °C and on a 12 h, 12 h day/night cycle (lights-on at 6 A.M., Zeitgeber time: ZT0) with food and water available ad libitum. Mice were habituated to the wheel conditions for 5–7 days before polysomnographic recordings. Cortical EEG (ipsilateral frontoparietal leads) and EMG signals were amplified and digitalized with a resolution of 500 Hz using VitalRecordor software (Kissel, Japan). Sleep was recorded in baseline LD conditions followed by 5 days in constant dark.

Using SleepPhen for Animal (Kissei, Japan), and with assistance of spectral analysis using fast Fourier transform (FFT), polysomnographic records were visually scored in 10 s epochs for wakefulness, NREM sleep, and REM sleep. The percentage of time spent in each condition was summarized for each group and each condition. To study sleep–wake fragmentation we analyzed the distribution of each vigilance stage occurrence as a function of episode length in discrete 5 min bins. EEG power spectra were computed into eight episode lengths (<30, 40–70, 80–150, 160–310, 320–630, 640–1270, 1280–2550, and >2550) min. For each vigilance stage, the number of episodes and the percentage of the vigilance stage occurring in each episode length bin were used to produce a time-weighted frequency histogram.

Recordings were scored again in 5 s epochs to allow for performance of an EEG power spectrum analysis. On the basis of visual and spectral analysis, epochs containing artifacts occurring during active wakefulness (with large movements) or containing two vigilance states were visually identified and omitted from the spectral analysis. Recordings containing wake artifact for more than 20% of the analysis period were removed from the spectral analysis. EEG power spectra were computed for consecutive 5 s epochs within the frequency range of 0.5–120 Hz using a FFT. The data were collapsed into 0.5 Hz bins and summed in delta, θ, 5–5 Hz, theta, δ: 5–5 Hz; sigma, σ: 9–15 Hz; beta, β: 15–30 Hz, low gamma, γ: 30–60 Hz and high gamma, high gamma, γ: 60–120 Hz. For chemogenetics experiments (ChR2-activated circuit mapping), all EEG power spectra were averaged during the 10 min immediately after the final CNO injection, starting 10 min after the injection to capture the complete range of time in which CNO affects vigilance state as previously described. The data were...
standardized by expressing each frequency bin as a percentage relative to the same bin under baseline conditions from the same mouse and from the same time of the day (same Zeitgeber time). To analyze the FFT across the whole width of the
"Bmal1+/-mice, VIP+/−/Bmal1+/-mice, AVPΔDTA mice and VIPΔDTA mice, the FFT
was analyzed in 3 h periods throughout the 24 h circadian period. The data were
standardized by expressing each frequency band as a percentage relative to the 24 h
frequency band power.

In vivo fiber photometry. A total of 12 heterozygous VIP-ires-Cre mice were used for
photometry experiments. Only mice bearing accurate targeting of the
photometer fiber and injection sites are included in the results, hence the final cohort
size of 4.

Surgery: Two weeks after the viral vector injection of AAV10-DIO-EEFluoraGCaMP6s,
the mouse was implanted with a custom-made photofiber and EEG/EMG headset.
The photofiber was manufactured in-house and comprised a fiber optic cable (400 µm outer diameter, ThorLabs FP400URT)
inserted into an aluminum ferrule (400 µm internal diameter, ThorLabs SFL400-10),
epoxyed into place (PF3 353ND, Precision Fiber Products), cleaved to size
and polished. The EEG headset was also manufactured in-house (as described above),
with the addition of an optical window. The optical window (25 µm thick) was
removed from the assembly to allow photometry fiber implantation. The mouse was prepared
for stereotaxic surgery as described above (Stereotaxic brain injections). Once secured
in the stereotaxic frame, the EEG headset was implanted as described above (Sleep-
wake monitoring and analysis). The burr hole over the SCN was reopened and the
photofiber was implanted immediately dorsally to the SCN (0.5 mm posterior to
the injection site). The whole assembly was affixed to the skull using a mixture of
cyanoacrylate glue and dental cement. An additional layer of dental cement was
then applied to insulate the headset and provide structural stability. The mouse was
then allowed to recover for 2 weeks prior to recordings.

Photometry behavioral recordings: Recordings were carried out in a soundproofed
and light-tight chamber, maintained at a constant temperature and humidity.
Prior to the experimental recordings, mice were kept on a 12:12 light-
dark schedule. Clear, plexiglass barrels were used as the animal’s home cage and
food and water were available ad libitum. Mice were allowed to habituate to the
recording chambers, EEG/EMG cable and photometry patch cable for at least
3 days prior to behavioral recordings before being placed in DD at lights off on
the last day of habituation. Simultaneous EEG/EMG and in vivo fiber photometry
recordings were then made at CT2-5 in the first cycle after the onset of DD and at
CT 12–16 (24 h after the onset of DD). A 20-min light pulse was administered at CT 14
after the onset of DD.

Simultaneous EEG/EMG and photometry recording setup and analyses: For
EEG/EMG recordings, the EEG/EMG headset was connected, via a low weight
custom-made cable, to a freely moving electrical commutator. The signal from the
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Basic procedures were performed with P4506S1 containing (mouse: build: mm10, GRCm38) by HISAT2 v2.1.0 (using “-k 2”)73. Aligned reads were converted to bam using SAMtools v1.0.474. Duplicate and low-quality reads were removed with Picard 2.1.07 (http://broadinstitute.github.io/picard). Reads were assigned to features (genes) using the feature Counts from the Subread package v1.6.0 (using recommended setting and a custom meta-annotation that merges overlapping isoforms)75. Expression values were calculated by counting the reads assigned to each gene.

VIP RNAseq™ in situ hybridization. RNAseq™ in situ hybridization was performed to label VIP mRNA in mouse brain tissue. For this, 30 μm SCN brain sections were cut on a freezing microtome and mounted on Superfrost Plus slides in RNase free conditions. RNAseq™ hybridization was performed on these sections using the RNAseq™ Multiplex Fluorescent Reagent Kit V2 (Catalog # 323100, Advanced Cell Diagnostics). The sections were then pretreated with hydroxyperoxide for 20 min in room temperature and then blocked with ultrapure water to a size-corrected concentration of 2 nM. The diluted library was averaged during the 10 seconds preceding the light pulse or the state transition upon GCaMP6s activity, the mean GCaMP6s signal of top cluster markers observed in the Allen Mouse Brain Atlas77 (Lhx1; Vipr2; Reln; Ecel1). The SCN clusters were re-clustered by excluding the non-SCN cluster and then repeating the workflow described above, beginning with the variable gene selection (689 variable genes used for PCA, 12 PCs used for clustering).

Plots were generated with Seurat v2.3.4 and heatmap v1.0.12 packages for R.
Harmar, A. J. et al. The VPAC(2) receptor is essential for circadian function in Syrian hamsters. J. Comp. Physiol. A Neuroethol. Sens Neural Behav. Physiol. 191, 23–30 (2005).

Kramer, A. et al. Regulation of daily locomotor activity and sleep by a hypothalamic enhancer of neuronal activity. Science 294, 2111–2135 (2001).

Hannibal, J., Hsiung, M. H. & Fahrenkrug, J. Temporal phasing of locomotor activity, heart rate rhythmicity, and core body temperature is disrupted in VIP receptor 2-deficient mice. Am. J. Physiol. Regul. Integr. Comp. Physiol. 300, R519–R530 (2011).

Pedersen, N. F. et al. Supramammillary glutamate neurons are a key node of the arousal system. Nat. Commun. 8, 1405 (2017).

Jones, J. R., Simon, T., Lones, L. & Herzog, E. D. SCN VIP neurons are essential for normal light-mediated resetting of the circadian system. J. Neurosci. 38, 7986–7995 (2018).

Chou, T. C. et al. Critical role of dorsomedial hypothalamic nuclei in a wide range of behavioral circadian rhythms. J. Neurosci. 23, 10691–10702 (2003).

Lu, J. et al. Contrasting effects of lesions of the paraventricular nucleus and subparaventricular zone on sleep-wake cycle and temperature regulation. J. Neurosci. 21, 4864–4874 (2001).

Venners, A. et al. Newly identified sleep-wake and circadian circuits as potential therapeutic targets. Sleep https://doi.org/10.1093/sleep/zsz023 (2019).

Anaclet, C. et al. The GABAergic parafacial zone is a mediatory slow wave sleep-promoting center. Nat. Neurosci. 17, 1217–1224 (2014).

Todd, W. D. et al. A hypothalamic circuit for the circadian control of aggression. Nat. Neurosci. 21, 717–724 (2018).

Vujovic, N., Gooley, J. J., Jhou, T. C. & Saper, C. B. Projections from the subparaventricular zone define four channels of output from the circadian timing system. J. Comp. Neurol. 523, 2714–2737 (2015).

Fuller, P. M., Gooley, J. J. & Saper, C. B. Neurobiology of the sleep-wake cycle: sleep architecture, circadian regulation, and regulatory feedback. J. Biol. Rhythms 21, 482–493 (2006).

Pickard, G. E. The apparent connections of the suprachiasmatic nucleus of the golden hamster with emphasis on the retinohypothalamic projection. J. Comp. Neurol. 211, 65–83 (1982).

Krou, K. E., Kawano, J., Mettenleiter, T. C. & Loewy, A. D. CNS inputs to the suprachiasmatic nucleus of the rat. Neuroscience 110, 73–92 (2002).

Tanaka, M., Ichitani, Y., Okamura, H., Tanaka, Y. & Ibata, Y. The direct retinal projection to VIP neuronal elements in the rat SCN. Brain Res. Bull. 31, 637–640 (1993).

Moga, M. M. & Moore, R. Y. Organization of neural inputs to the suprachiasmatic nucleus in the rat. J. Comp. Neurol. 389, 508–534 (1997).

Mazuki, C. et al. Entrainment of circadian rhythms depends on firing rates and neuropeptide release of VIP SCN neurons. Neuron 99, 555–563 e553 (2018).

Kawamoto, K. et al. Two types of VIP neuronal components in rat suprachiasmatic nucleus. J. Neurosci. Res. 74, 852–857 (2003).

Habib, N. et al. DivSeq: Single-nucleus RNA-Seq reveals dynamics of rare cell types within heterogeneous tissues in vivo. Cell Rep. 18, 1048–1061 (2017).

Bedont, J. et al. An LH1X-regulated transcriptional network controls sleep/wake coupling and thermal resistance of the central circadian clocks. Curr. Biol. 27, 128–136 (2017).

Kalamatianos, T., Kallo, L., Jiggins, H. D. & Coen, C. W. Expression of VIP and/or PACAP receptor mRNA in peptide synthesizing cells within the suprachiasmatic nucleus of the rat and in its efferent target sites. J. Comp. Neurol. 475, 19–35 (2004).

Jagannath, A. et al. The CRTC1-SIK1 pathway regulates entrainment of the circadian clock. Cell 154, 1100–1111 (2013).

Chen, M. Y. et al. Prokineticin 2 transmits the behavioural circadian rhythm of the suprachiasmatic nucleus. Nature 417, 405–410 (2002).

Prosser, H. M. et al. Prokineticin receptor 2 (Prokr2) is essential for the regulation of circadian behavior by the suprachiasmatic nuclei. Proc. Natl Acad. Sci. USA 104, 648–653 (2007).

Karatsoreos, I. N., Wang, A., Sasian, J. & Silver, R. A role for androgens in regulating circadian behavior and the suprachiasmatic nucleus. Endocrinology 148, 5487–5495 (2007).

Karatsoreos, I. N., Butler, M. P., Lesauter, J. & Silver, R. Androgens mediate structure and function of the suprachiasmatic nucleus brain clock. Endocrinology 152, 1970–1978 (2011).

Cheng, A. H., Pung, S. W. & Cheng, H. M. Limitations of the Avp-IRE52-Cre (JAX #023350) and Vip-IRE52-Cre (TAX #010908) models for chronobiological investigations. J. Biol. Rhythms 34, 634–644 (2019).
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Competing interests

The authors declare no competing interests.

Additional information

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