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Synchronous circadian voltage rhythms with asynchronous calcium rhythms in the suprachiasmatic nucleus

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R.E., Y.O., S.H., and K.H. designed research; R.E., Y.O., and D.O. performed research; M.M. provided AVP-Cre mice and AAV-RGECO; R.E. analyzed data; R.E., S.H., and K.H. wrote the paper.

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Abstract
The suprachiasmatic nucleus (SCN), the master circadian clock, contains a network composed of multiple types of neurons which are considered to form a hierarchical and multi-oscillator system. The molecular clock machinery in SCN neurons drives membrane excitability and sends time cue signals to various brain regions and peripheral organs. However, how and at what time of the day these neurons transmit output signals remain largely unknown. Here, we successfully visualized circadian voltage rhythms optically for many days using a genetically encoded voltage sensor, ArcLightD. Unexpectedly, the voltage rhythms are synchronized across the entire SCN network of cultured slices, whereas simultaneously recorded Ca\(^{2+}\) rhythms are topologically specific to the dorsal and ventral regions. We further revealed that the temporal order of these two rhythms is cell-type specific; the Ca\(^{2+}\) rhythms phase-lead the voltage rhythms in AVP neurons, whereas they are nearly in-phase in VIP neurons. We confirmed that circadian firing rhythms are also synchronous and coupled with the voltage rhythms. These results indicate that SCN networks with asynchronous Ca\(^{2+}\) rhythms produce coherent voltage rhythms.
Significance Statement
The mammalian master circadian clock, the suprachiasmatic nucleus (SCN), is the network composed of various neuron types. The SCN network plays critical roles in expressing robust circadian rhythms in physiology and behavior, such as sleep-wake cycles. The molecular clock in individual SCN neurons controls membrane excitability, and sends output signals to various organs. However, how the SCN neurons transmit output signals remains unknown. Using a genetically encoded voltage sensor, we directly measured the circadian rhythms of membrane voltage in the SCN network. Remarkably, the circadian voltage rhythms are synchronous in the entire SCN network, whereas simultaneously recorded Ca\textsuperscript{2+} rhythms are asynchronous in the dorsal and ventral SCN regions. These results indicate that the SCN network produces coherent output signals.
Introduction
In mammals, daily rhythms in biochemistry, physiology, and behavior are coordinated by the master circadian clock located in the hypothalamic suprachiasmatic nucleus (SCN) in the brain (1). The SCN consists of approximately 20,000 neurons in rats and mice, each of which demonstrates self-sustained circadian oscillations (2, 3). In individual SCN neurons, cellular circadian rhythms are generated by an autoregulatory transcriptional and translational feedback loop (core loop) consisting of the clock genes Period (Per) 1, Per2, Cryptochrome (Cry) 1, Cry2, Bmal1 and Clock, and their protein products (4). These cellular clocks regulate membrane excitability and define firing patterns in the SCN neurons (5). In vivo and in vitro electrophysiological studies revealed that rhythms in resting membrane potentials and the frequency of action potential firings are high during the subjective day and low during the subjective night (5). Blockade of neuronal firings by tetrodotoxin (TTX) leads to the desynchronization of the SCN network in vitro (6, 7) and behavioral arrhythmicity in vivo (8). These studies indicate that output signals of neuronal firings in the SCN are crucial not only for intercellular communications within the SCN network but also for conveying time cues to synchronize rhythms in the peripheral tissues.

At the network level, bioluminescence and fluorescence imaging revealed the topologically specific patterns of rhythms in clock gene expressions, such as Per1 (6) and PER2 (9), and the intracellular Ca\(^{2+}\) concentration (7) in the dorsal to ventral SCN regions. In the SCN, it has been proposed that there are more than one regional oscillator wherein a group of oscillating neurons synchronize with each other and behave differently from other regional oscillators (10, 11). These groups include the oscillators in the dorsal SCN where arginine–vasopressin (AVP) neurons are abundant and those in the ventral SCN where vasoactive intestinal peptide (VIP) neurons predominate (10, 12–15).

To examine how and where different SCN neurons regulate membrane excitability and send output signals, we need to record neuronal activities on a large scale at a single cell resolution for several days. Conventional electrophysiology techniques, such as patch clamp recording, allow us to monitor only few neurons for few hours. Extracellular recordings, such as multi-unit activity (16–19) and multi-electrode array dish (MED) recordings (20) have limited spatial resolution. Recent advances in Ca\(^{2+}\) imaging methods enable us to optically record neuronal firing patterns in vitro and in vivo (21). However, sub-threshold membrane excitation or depolarizing state that does not change firing rate is also believed to contribute to information processing in the brain (22, 23). Indeed, in SCN neurons, daily silencing of neuronal firing was reported (24). Thus, there is a need to directly measure membrane potentials in master circadian clock neurons.

Over the last 40 years, since an organic voltage probe was first applied in the central nervous system (25), direct optical measurements of membrane potentials have long been one of the ultimate dreams in neuroscience. However, the voltage probe had drawbacks; its signal was very small compared with that of other probes, such as Ca\(^{2+}\) probes. Nevertheless, the recent improvement in
fluorescent protein-based voltage sensors enabled us to accurately record neuronal activities in real time (26, 27). Voltage sensors have the important advantage of being able to be specifically expressed in an individual cell type in the brain for several days. We recently developed high-resolution time-lapse imaging methods to visualize spatiotemporal dynamics of intracellular Ca$^{2+}$ in the SCN network for many days without noticeable photo-induced toxicity or bleaching. Thus, all necessary tools for direct monitoring of membrane potentials are now available.

In this study, we successfully measured the circadian rhythms of membrane voltage in the neurons of the cultured SCN using a genetically encoded voltage sensor, ArcLightD (27), and high-resolution confocal imaging microscopy (7, 28). Using RGECO (29), a red Ca$^{2+}$ indicator, and cell-type specifically expressed ArcLightD, we successfully measured the rhythmic change in membrane voltage and Ca$^{2+}$ rhythms simultaneously in the SCN network. Unexpectedly, circadian voltage rhythms are synchronous in the entire SCN network, whereas Ca$^{2+}$ rhythms are topologically specific to the dorsal and ventral regions in the same SCN slice. These findings give us an important insight as to how the SCN network encodes circadian time in vivo.
Results
Expression patterns of a genetically encoded voltage sensor, ArcLightD
To elucidate the spatiotemporal patterns of membrane potentials in SCN neurons, ArcLightD was transfected in cultured SCN slices of newborn mice [postnatal (P) day 4–6] using a recombinant adeno-associated virus (AAV) under a neuron-specific promoter, human synapsin I (hSynI). Figure 1A shows representative expression patterns of ArcLightD fluorescence in the SCN prepared from C57BL/6J mice. The expression pattern of ArcLightD in the SCN network was distinct from that of the Ca$^{2+}$ probes, such as Yellow Cameleon 3.60 (7, 28) and GCaMP6s (30). ArcLightD signals were detected in the plasma membrane of soma and fibers (i.e., axon and dendrites), but not in the cytosol or nucleus (Fig.1A bottom).

As ArcLightD is expressed throughout the plasma membrane including the axon and dendrites, it is difficult to identify the origin of the signals. To overcome this difficulty, the SCN slice from VIP- and AVP-Cre mice were transfected with AAV-encoding hSynI-Flex-ArcLightD in SCN slices. Cell-type specific expressions of ArcLightD allowed us to identify the VIP (Fig.1B) and AVP neurons (Fig.1C) separately and to estimate the distribution of fluorescence in dendrites and axons. The VIP neurons whose cell bodies were localized in the ventral SCN sent fibers to the dorsal SCN region (Fig.1B), whereas the AVP neurons with cell bodies located mostly in the dorsal SCN sent fibers to the entire SCN (Fig.1C). Cell-type specific expressions of ArcLightD in the VIP and AVP neurons were confirmed by immunohistochemical staining against VIP and AVP neuropeptides, respectively (Fig.S1).

Simultaneous recording of circadian rhythms in voltage and intracellular calcium
Using ArcLightD together with RGECO, we successfully detected the circadian rhythms in membrane potential (voltage rhythm) and intracellular Ca$^{2+}$ levels (Ca$^{2+}$ rhythm) simultaneously in the SCN (Fig.2). Remarkably, we found that the voltage rhythms were synchronous in the entire SCN network. For quantification of the rhythms at the SCN network, we created acrophase (peak phase) maps using pixel-based analysis program as described previously (7, 30, 31). The acrophase maps, expressed relative to the slice mean, revealed that the voltage rhythms were nearly in-phase in the entire SCN region (shown in yellow-green colors) (Fig.2B left). On the other hand, the Ca$^{2+}$ rhythms were topologically specific, with an advanced-phase in the dorsal region (shown in cold colors) and delayed-phase in the center and ventral regions (shown in warm colors) (Fig.2B right), as previously reported (7). We statistically compared the regional difference between the representative area (100 x 100 µm) each in the dorsal and ventral SCN. The mean acrophase of the voltage rhythm was not significantly different between them, indicating that they were synchronous (12.4 ± 0.5 h and 12.0 ± 0.5 h, n=6) (p = 0.61) (Fig.2C left), whereas the mean acrophase of the Ca$^{2+}$ rhythm was significantly different, indicating topological specificity (9.1 ± 0.3 h and 13.6 ± 0.4 h, n=6) (p < 0.001) (Fig.2C right). We compared the phase difference (Δ phase) between the voltage and Ca$^{2+}$ rhythms in these regions. The Ca$^{2+}$ rhythm was phase-advanced relative to the voltage...
rhythms in the dorsal region \((2.6 \pm 0.4 \text{ h}) (p < 0.001)\), whereas these two rhythms were nearly in-phase in the ventral region \((-0.3 \pm 0.3 \text{ h}) (p = 0.62)\) (Fig.2D, E).

**Signal origin of ArcLightD**

In order to validate the signal origin and dynamic range of ArcLightD fluorescence in our experimental conditions, we manipulated the membrane potentials of SCN neurons by changing extracellular concentrations of potassium ion \((0.5, 0.3, 5.4 \text{ and } 10 \text{ mM KCl})\), as reported previously (32). We recorded the resting membrane potentials and action potentials by whole-cell patch clamp recordings from single SCN neurons \((23 \text{ neurons in } 6 \text{ slices})\) and detected the fluorescence changes of ArcLightD in the SCN slices \((6 \text{ slices})\). We confirmed that ArcLightD reports changes in the membrane potentials with a dynamic range from \(-50 \text{ mV} \text{ to } -30 \text{ mV}\) (Fig.S3) under this condition. The mean membrane potentials were almost identical with or without firings (Fig.S3B). The estimated range of circadian oscillation was \(6.26 \pm 1.43 \text{ mV}\) in the dorsal region and \(6.35 \pm 1.36 \text{ mV}\) in the ventral, respectively. They were not statistically different \((p = 0.96)\) (Fig.S3D, E). We concluded that ArcLightD signals reflect the change in the resting membrane potential, not the firing level.

**Cell-type specific recording of the voltage rhythms**

**1. Voltage rhythms in VIP neurons**

Figure 3 shows cell-type specific monitoring of the voltage rhythms in the VIP neurons. The voltage rhythms were mostly detectable in the ventral region and absent in the dorsal region near the third ventricle (Fig.3B left). They were in phase throughout the SCN. The \(\text{Ca}^{2+}\) rhythms, monitored in entire SCN neurons by RGECO, were localized across the dorsal to the ventral SCN (Fig.3B right), as reported previously (7, 30). The voltage and \(\text{Ca}^{2+}\) rhythms in the ventral SCN were nearly in-phase \((-0.6 \pm 0.3 \text{ h}, n = 9 \text{ slices})\).

To examine the roles of SCN neural network in the circadian ArcLightD and RGECO rhythms, we tested the effects of blockers for voltage-dependent fast \(\text{Na}^{+}\) and \(\text{Ca}^{2+}\) channels. The amplitude of the circadian rhythms in terms of a peak-trough difference was compared before (pretreat) and after the drug application. To confirm the circadian rhythmicity, the fluorescence signals were fitted to a cosine curve using a least-square regression method, and the goodness of fit was statistically evaluated \((p < 0.001 \text{ by Percent Rhythm})\).

Each blocker differentially affected the amplitude of the two rhythms. A fast \(\text{Na}^{+}\) channel blocker, TTX \((1 \mu\text{M})\), reduced the amplitudes of the voltage \((30.8 \pm 13.7\% \text{ of pretreatment level}, p = 0.037)\) and \(\text{Ca}^{2+}\) rhythms \((32.1 \pm 12.4\% \text{ of pretreat}, p = 0.032)\) in the VIP neurons \((n = 3 \text{ slices})\). However, the two rhythms were still statistically significant even after TTX treatment (Fig.3C, 3F). These results indicate the presence of circadian voltage and \(\text{Ca}^{2+}\) rhythms independent of neuronal firings in VIP neurons.

\(\text{Ca}^{2+}\) channels are known to contribute to the membrane potential (32–35). Particularly, L-type \(\text{Ca}^{2+}\) channels are reported as the primal \(\text{Ca}^{2+}\) source in the SCN (34, 36). Indeed, we found that an L-type \(\text{Ca}^{2+}\) channel blocker, nimodipine \((3 \mu\text{M})\), significantly suppressed both the voltage \((40.9 \pm 10.2\% \text{ of pretreat, } p = 0.028)\) and \(\text{Ca}^{2+}\) rhythms \((41.2 \pm 2.2\% \text{ of pretreat, } p = 0.0014)\) \((n = \ldots)\).
3 slices) (Fig.3D, 3F). A blocker cocktail of TTX and nimodipine greatly suppressed the amplitude of the Ca²⁺ rhythms (6.1 ± 0.2% of pretreat, p < 0.001), whereas the effect was less profound in the voltage rhythms (45.0 ± 7.1% of pretreat, p = 0.016) (n = 3 slices) (Fig.3E, 3F). These results indicate that fast Na⁺ channels and L-type Ca²⁺ channels contribute additively to the Ca²⁺ rhythms, whereas the two channels contribute redundantly to the voltage rhythms. These findings suggest that the voltage rhythms are independent of the Ca²⁺ rhythms in the VIP neurons.

We then analyzed the effects of blockers on the phase relation in the VIP neurons. Phase differences between the Ca²⁺ and voltage rhythms were unchanged after TTX (0.8 ± 0.5 h, p = 0.24) (n = 3 slices) or nimodipine application (0.6 ± 1.1 h, p = 0.61) (n = 3 slices). However, they became more variable after co-application of TTX and nimodipine (1.0 ± 4.6 h, p = 0.85) (n = 3 slices) (Fig.3G).

2. Voltage rhythms in AVP neurons

We conducted the same series of experiments in the AVP neurons (Fig.4). As shown in Fig.1C, the cell bodies of the AVP neurons located in the dorsal SCN sent fibers to the entire SCN, including the ventral region. We found that the voltage rhythms of the AVP neurons were synchronous in large areas of the SCN. RGECO expressed in the entire SCN revealed that the Ca²⁺ rhythms were topologically specific in the dorsal to ventral SCN (Fig.4B right). The Ca²⁺ rhythms were phase-advanced relative to the voltage rhythms, and the phase difference (Δ phase) between the two rhythms was 3.1 ± 0.5 h in the AVP neurons (n = 9 slices).

Application of 1 μM TTX significantly reduced the amplitude of the Ca²⁺ rhythms (58.5 ± 10.9% of pretreat, p = 0.032), but did not change that of the voltage rhythms (119.8 ± 20.0%, p = 0.39) (n = 3 slices) (Fig.4C, 4G). Similarly, nimodipine (3 μM) reduced the amplitude of the Ca²⁺ rhythms (41.2 ± 22.2%, p < 0.001), but did not change that of the voltage rhythms (159.9 ± 42.8%, p = 0.26) (n = 3 slices) (Fig.4D). Co-application of TTX and nimodipine reduced the amplitude of both Ca²⁺ (17.5 ± 4.8%, p = 0.0034) and voltage rhythms (45.5 ± 9.0%, p = 0.026) (n = 3 slices) (Fig.4D). These results indicate that the Ca²⁺ rhythms were mediated by activation of fast Na⁺ and L-type Ca²⁺ channels in the AVP neurons, whereas the voltage rhythms were mediated by a TTX and nimodipine insensitive mechanism. Suppression of the voltage rhythms by co-application could be due to the reduced inputs from the damped VIP rhythms (Fig.3F). The phase difference between the Ca²⁺ and voltage rhythms were unchanged on the first cycle (4.2 ± 1.7 h, p = 0.13) but significantly changed on the second cycle (5.8 ± 1.1 h, p = 0.03) after TTX application (n = 3 slices). On the other hand, nimodipine (2.6 ± 2.4 h, p = 0.39) (n = 3 slices) or co-application (3.2 ± 4.6 h, p = 0.55) (n = 3 slices) did not change the phase difference (Fig.4G).

We confirmed these findings by double transfection of AAVs encoding hSynl-Flex-ArcLightD and hSynl-Flex-jRGECO (37) in the SCN from VIP-Cre and AVP-Cre mice. The circadian Ca²⁺ rhythms were in-phase with the voltage rhythms in the VIP neurons (−0.5 ± 0.3 h, n = 3 slices) (Fig.S4A, S4B), whereas
phase-advanced by 6.6 ± 1.4 h (n = 3 slices) relative to the voltage rhythms in the AVP neurons (Fig. S4C, D).

**Simultaneous recording of the voltage and firing rate rhythms**

To verify the synchronization of the voltage rhythms in the SCN, we performed simultaneous recordings of ArcLightD and neuronal firing using a MED with 8 x 8 planar electrodes (each, 20 x 20 μm) (Fig. 5). The cultured SCN slice transfected with AAV encoding hSynI-ArclightD was flipped over and placed on the MED probe (Fig. S2, Protocol 2). ArcLightD signals were measured by a high-sensitive CCD camera mounted on an upright microscope, and multi-unit spontaneous firings by the MED system.

The voltage and firing rhythms were analyzed in ROI (20 x 20 μm) on each MED electrode. A typical example is shown in Fig. 5D. The distribution of acrophase on pixel or ROI level was unimodal in respective voltage and firing rhythm, indicating synchronization of the circadian rhythms throughout the SCN (Fig. 5B, C). We analyzed the phase relation between the firing and voltage rhythms (Δ phase) in 39 electrodes (3 slices) and found that the firing rhythms were significantly phase-advanced relative to the voltage rhythms by 3.9 ± 0.3 h (Fig. 5E bottom). There was no significant regional difference in Δ phase between the dorsal (4.1 ± 0.5 h) and ventral (3.8 ± 0.4 h) SCN regions (p = 0.644) (Fig. 5F). Together, these results indicate that the voltage and firing rhythms are synchronous in the entire SCN network and were coupled to each other with a phase difference of approximately 4h.

**Voltage and the Ca²⁺ rhythms during development**

Previously, we observed that approximately 10% of MED electrodes demonstrated the firing rhythms anti-phasic to the majority rhythms (38, 39). However, the firing rhythms were synchronous in all MED electrodes in the present study. As the SCN slices were cultured for longer periods of time than we had done previously, we tested the possibility of developmental changes in the SCN network for synchronization.

To this end, we performed simultaneous recordings of the voltage and Ca²⁺ rhythms for over 10 days starting from an early developmental stage (Fig. 6). The SCN slice was prepared from mice at the P1 day 1. Entire SCN neurons were transfected with two AAVs, one encoding ArcLightD and the other RGECO, on P days 2 and 3, and confocal recordings were started 1 week later [corresponding (cP) day 10] (Fig. S2, Protocol 2). We found that the topological patterns of the voltage and Ca²⁺ rhythms in the SCN were similar in both the early and late recording stages (Fig. 6A) (n = 6 slices). However, judging from standard deviation (SD), the voltage rhythms were more synchronized at the late stage (sd = 3.0 ± 0.1 h) (cP days 21–22) than the early stage (sd = 4.0 ± 0.3 h) (cP days 10–11) (p = 0.038) (Fig. 6C). By contrast, the Ca²⁺ rhythms were not significantly different between the early (4.7 ± 0.1 h) and late stages (4.8 ± 0.03 h) (p = 0.87) (Fig. 6C). We calculated the phase difference (Δ phase) between the voltage and Ca²⁺ rhythms separately in the dorsal (3.2 ± 0.2 h vs 3.9 ± 0.5 h) and ventral SCN (−1.3 ± 0.6 h vs −0.9 ± 0.4 h) at both the early and late stage, and found no significant regional difference between the two stages (Fig. 6D).
These results indicate that the coherence of voltage rhythms, but not the Ca$^{2+}$ rhythms, are developmentally regulated in the SCN.
Discussion
In the present study, we found the cell-type specific couplings of circadian rhythms, the synchronous circadian voltage rhythms as well as the firing rhythms in the entire SCN, despite of topologically specific Ca\textsuperscript{2+} rhythms. We also found the differential effects of ion channel blockers on the amplitudes of voltage and Ca\textsuperscript{2+} rhythms in the AVP neurons but not in the VIP. These findings are explained by assuming the interaction between an intrinsic cellular oscillation and the inputs from the SCN neural network.

Cell-type specificity of the circadian functions
The circadian rhythms in clock gene expressions, such as Per1 and PER2, have been reported to have similar topological patterns as those in the Ca\textsuperscript{2+} rhythms (6, 7), thus the rhythms in Ca\textsuperscript{2+} and clock gene expression, as well as the rhythms in voltage and firing, behave similarly (Fig.6A). These results imply two functionally coupled oscillatory components in SCN neurons: one is composed of the voltage and neuronal firing rhythms, and the other is composed of Ca\textsuperscript{2+} and PER2 rhythms. These two oscillatory components might mutually interact directly or through the core molecular loop for circadian oscillation. The phase relation of the circadian PER2 and Ca\textsuperscript{2+} rhythms is not different between the VIP and AVP neurons. The same is true for the circadian voltage and firing rhythms. However, the phase relation between the former component and the latter was substantially different in the two neurons (Fig.2D, Fig.3G, Fig.4G). Since the voltage rhythms were synchronous throughout the entire SCN, the cell specific difference is ascribed to the oscillatory component of circadian Ca\textsuperscript{2+} rhythms. Interestingly, TTX changed the phase relation between the two components in the AVP neurons (Fig.4G), suggesting the SCN neural network is involved in the coupling between them. TTX did not affect the coupling in the VIP neurons (Fig.3G). As a result, the phase-relation of two oscillatory components was changed between the AVP and VIP neurons. The findings are consistent with our previous report that TTX desynchronize the circadian Ca\textsuperscript{2+} rhythms between the dorsal and ventral regions of the SCN (7).

Signal origin of the voltage rhythm
The voltage rhythms were synchronous throughout the entire SCN (Fig.1). The finding is unique in the face of regional differences in the circadian phase of other measures such as clock gene expression in the SCN (3). In this study, we used pixel-level analysis using time-series CCD images (7, 30, 40). Each pixel size is smaller than the average size of the cell body of a single SCN neuron. We are not able to exclude the possibility that some pixels represent the soma of a certain cell and others the processes of different cells. Such compartmentalization, however, cannot explain the synchrony of the voltage rhythms in the entire SCN (Fig.2B). We showed the synchronization of the voltage rhythms in the same group of SCN neurons (Fig.3B and Fig.4B), including the regions where the somas and processes exist intermixed (Fig.1B,C, Fig.S1).

The dendrite is known as the major target of synaptic inputs in a variety of neurons. The voltage changes in the dendrites propagate in the process and
soma within a few seconds based on the cable properties of the dendrite (41, 42). The synchrony of voltage rhythm throughout the SCN might be due to a rapid propagation of subcellular voltage change.

**Neural network and intracellular couplings of circadian rhythms**

Application of the channel blockers for either fast Na$^+$ or L-type Ca$^{2+}$ channels reduced the amplitude of both the Ca$^{2+}$ and voltage rhythms in the VIP neurons but did only diminish the amplitude of Ca$^{2+}$ rhythm in the AVP neurons. The amplitude of circadian voltage rhythm in the AVP neurons persisted without damping. The neuron-specific differences suggest an impairment of a specific pathway from the intrinsic circadian oscillation to both overt rhythms and/or desynchrony among cellular rhythms rather than a loss of cell-intrinsic rhythms in particular types of neurons. In addition, a significant change in the phase difference between the Ca$^{2+}$ and voltage rhythms indicates desynchrony between them and suggests that the two circadian rhythms are regulated by different mechanisms, or at least, through different pathways from the core molecular loop of circadian oscillation in the AVP neurons.

According to the theory of a multi-oscillator system (43), the phase of an intrinsic cellular circadian oscillation is affected, but not completely in phase with the system oscillation in which the cell involved. The circadian voltage and firing rhythms may represent the system oscillation in the SCN, while the circadian Ca$^{2+}$ and PER2 rhythms, the intrinsic cellular oscillation. Interruption of the inputs from the neural system by TTX may release the intrinsic oscillation from the impact of system oscillation to change the characteristics of cellular oscillation. The present findings are adequately explained by this theory.

**Possible mechanisms of intra and intercellular couplings**

Based on the present findings, we advanced a model wherein the cell-type specificity of circadian rhythms is explained by asymmetric impacts from the VIP to AVP neurons. In this model, the SCN neural network differentially influences onto the two oscillatory cell components in which the voltage and Ca$^{2+}$ rhythms are involved separately (Fig.7C). The notion is based on the differential effects of TTX and nimodipine on the amplitude of voltage rhythms in the two neuron groups (Fig.3F, Fig.4F). The neural network sensitive to TTX or nimodipine influences much greater on the Ca$^{2+}$ rhythms than on the voltage rhythms in the AVP neurons. In the SCN, neuronal firings and Ca$^{2+}$ conductance contribute the membrane potential (34, 36). TTX blocks fast Na$^+$ channels and neuronal firings, and it also suppresses the neuronal inputs from network (Fig.7C, 1). Nimodipine inhibits L-type Ca$^{2+}$ channels, which suppresses Ca$^{2+}$ and voltage rhythms in the AVP and VIP neurons (Fig.7C, 2). Inhibition of either fast Na$^+$ or L-type Ca$^{2+}$ channels enhances, probably by disinhibiting the coupling from the VIP to AVP neurons (Fig.7C, 3), the amplitude of voltage rhythm in AVP neurons. Alternatively, there might be an involvement of other ion channels such as calcium-activated BK potassium channel (5, 44, 45) which is more abundant in the AVP neurons than in the VIP. Suppression of rhythm amplitude may weaken the coupling between the two oscillatory components, thereby changing the phase relation of the rhythms. Another possibility is the change in threshold for
generating neuronal firings by modulating the ion channel activation or inactivation.

The firing rhythms phase-lead the voltage rhythms by nearly 4 h. This is not predictable from the experiments in molluscan clock neuron (46) or the Hodgkin-Huxley model in neurons. The maximum level of depolarization was associated with a decline of firing rate. Depolarization block is one of the possible explanations of this phase difference, which is, however, unlikely considering the estimated amplitude of voltage rhythm that was as small as 6.3 mV on average (Fig.S3). Unknown circadian mechanisms, such as activation of a calcium-activated BK-type potassium channel and a change in action potential threshold might account for this phenomenon.

**Developmental regulation of the voltage rhythms**

We found that the voltage rhythms became coherent during the course of recordings (Fig.6). This could be due to the maturation of the SCN network during the development. To support this idea, we recently found VIP and AVP signaling differentially integrated the SCN neural network during development (40). It is worth noting that the amplitude of the voltage and Ca\(^{2+}\) rhythms became larger during culturing. Rhythm amplitude could be reinforced by intercellular coupling during development. Coherent and robust voltage rhythms would lead to stable output signals, and ultimately sustain coherent and robust rhythms in animal behaviors. The function is of special importance to keep activity rest cyclicity under the perturbing influences such as transmeridian flights and extreme photoperiods.

**Physiological roles of differentially phased the voltage and Ca\(^{2+}\) rhythms**

The physiological roles of the synchronous voltage and asynchronous Ca\(^{2+}\) rhythms are unknown, but it is surmised that the SCN uses two rhythms differentially for the circadian clockwork: the voltage rhythms for the uniform sensitivity to environmental stimulation and the Ca\(^{2+}\) rhythms for the regional specific responses.

Previously, in vivo recording using stationary electrodes showed that the peak phase of the firing rhythms was similar in the dorsal and ventral SCN (16). Furthermore, a fixed phase relationship between Per1 and firing rate has been reported in the SCN slices however, the spatial pattern and regional specificity were not reported (47). On the other hand, differential responses in the firing and Per1 rhythms to a shifted light–dark cycle have been reported both in vivo and in vitro (18). The phase shift was large and persistent in the Per1 rhythm, while it was transient in the firing rhythm in vitro. The SCN network may keep the sensitivity of circadian clock through the circadian voltage rhythm and rapidly respond to environmental stimulation through the Ca\(^{2+}\) rhythms. Further studies will be required to clarify the physiological roles of the synchronous voltage rhythms in the SCN.
Materials and Methods

Animal care
C57BL/6J mice (Clea Japan), AVP-Cre mice (12), and Vip^tm1(cre)Zjh/J (Jackson laboratory) mice of C57BL/6J background were used for the experiments. Mice were born and bred in our animal quarters under controlled environmental conditions (temperature: 22 ± 2 °C, humidity: 60 ± 5%, 12-h light/12-h dark, with lights on from 0600–1800 hours). Light intensity was around 100 lx at the cage surface. The mice were fed commercial chow and tap water ad libitum. Experiments were conducted in compliance with the rules and regulations established by the Animal Care and Use Committee of Hokkaido University under the ethical permission of the Animal Research Committee of Hokkaido University (approval no. 15-0153).

SCN slice culture
Decapitation was performed in the middle of the light phase. The brains of neonate mice (1-d-old or 4 to 6-d-old, both male and female) were rapidly removed and dipped in ice-cold balanced salt solution comprising 87 mM NaCl, 2.5 mM KCl, 7 mM MgCl₂, 0.5 mM CaCl₂, 1.25 mM Na₂HPO₄, 25 mM NaHCO₃, 25 mM glucose, 10 mM HEPES, and 75 mM sucrose. A 200-μm coronal brain slice containing the mid rostrocaudal region of the SCN was carefully prepared using a vibratome (VT 1200; Leica). The bilateral SCNs were dissected from the slice using a surgical knife and explanted onto a culture membrane (Millicell CM; pore size, 0.4 μm; Millipore) in a 35 mm Petri dish containing 1.0 mL of DMEM (Invitrogen) and 5% FBS (Sigma-Aldrich). Before the recordings, the membrane containing the cultured SCN slice was cut out, flipped over, and transferred to glass base dishes (35 mm, No.1-S; AGC Techno Glass) or MED probes (20 × 20 μm) that were collagen-coated (Cellmatrix type 1-c; Nitta Gelatin) and supplemented with 180–250 μL DMEM containing 5% supplement solution. MED dishes were sealed with O₂-permeable filters (membrane kit, High Sens; YSI) using silicone grease compounds (SH111; Dow Corning Toray).

Adeno-associated virus-mediated gene transfer into SCN slices
Aliquots of the adeno-associated virus (AAV) (1 μL) harboring hSyn1-jRGECO1(37), hSyn1-ArcLightD, hSyn1-Flex-ArcLightD (produced by the University of Pennsylvania Gene Therapy Program Vector Core), and custom made Elongation Factor 1 (EF) α-RGECO were inoculated onto the surface of the SCN cultures on 4-6 days (Protocol 1) or 2 days of culture (Protocol 2) (Fig.S2). When multiple sensors were transfected in the SCN, AAVs were transfected in two subsequent days. Infected slices were further cultured for at least 14 d before confocal imaging (Protocol 1) or 7 days before CCD/MED imaging (Protocol 2) (Fig.S2). The titer of hSyn1-jRGECO1, hSyn1-ArcLightD, and hSyn1-Flex-ArcLightD vector was 1.1 × 10^{13}, 2.4 × 10^{13}, and 1.2 × 10^{13} genome copies/ml, respectively.

For making AAV encoding EF1α-RGECO, the AAV-2 ITR-containing plasmid pAAV-EF1α-DIO-hChR2(H134R)-EYFP (provided by Dr. K.Deisseroth, Stanford Univ) was modified to construct pAAV-EF1α-RGECO1 by replacing DIO-hChR2(H134R)-EYFP cDNA with RGECO1 cDNA fragment from the
plasmid CMV-RGECO1 (provided by Dr. T.Nagai, Osaka Univ). The AAV-RGECO, with a mutant form of the AAV-2 cap gene (provide by Dr. A.Srivastava, Univ of Florida) (48) was produced using a triple-transfection, helper-free method and purified as described previously (49). The titer of RGECO vector was $9.8 \times 10^{12}$ genome copies/ml.

**High-resolution confocal imaging of ArcLightD and RGECO**
Fluorescence images were captured at an exposure of 2–5s. Images of 100-μm depth in the z axis were obtained at 2-μm z-steps. The imaging system is composed of Nipkow spinning disk confocal (X-Light; Crest Optics), sCMOS CCD camera (2560 × 2160 pixels, 0.325-μm resolution, NEO; Andor Technology) or EM-CCD camera (1024 × 1024 pixels, -μm resolution, iXon3; Andor Technology), inverted microscope (Ti-E; Nikon), dry objectives (20 X, 0.75 NA, Plan Apo VC; Nikon), box incubator (TIXHB; Tokai-hit), and MetaMorph software (Molecular Devices). ArcLightD was excited by cyan color (475/28 nm) with LED light source (Spectra X Light Engine; Lumencor Inc) and the fluorescence was visualized with 495-nm dichroic mirror and 550/49-nm emission filters (Semlock). RGECO/jRGECO was excited by green color (542/27 nm) and the fluorescence was visualized with 593-nm dichroic mirror and 630/92-nm emission filters (Semlock). Expression patterns of ArcLightD fluorescence were visualized using confocal laser scanning microscopy (1024 × 1024 pixels) (A1R-FN1; Nikon). All experiments were performed at 36.5 °C and 5% CO₂.

**Simultaneous recording of the ArcLightD and spontaneous firing**
The SCN slice was cultured in 100% air at 36.5 °C on an MED probe with 64 electrodes of 20 × 20 μm arranged in an 8 × 8 grid with 100 μm distance. Before the recording, the MED dish was placed in a mini-incubator installed on the stage of a microscope (ECRIPSE 80i, Nikon). ArcLightD fluorescence and spontaneous firing were simultaneously recorded while culturing in culture media. Fluorescence was recorded with a cooled CCD camera at −80 °C (ImagEM, Hamamatsu Photonics) every 60 min with an exposure time of 2–3 s. ArcLightD was excited with a LED light (Light Engine; Lumencor Inc) at cyan color (475/28 nm), and the fluorescence was visualized with 495-nm dichroic mirrors and 520/35-nm emission filters (Semlock).

**Patch-clamp recordings**
Whole-cell patch-clamp recordings were made from cultured SCN slice neurons. Neurons were visualized with a 60× water-immersion objective lens (LUMPlanFLN, 1.0 NA; Olympus) using an upright microscope (BX50WI, Olympus) equipped with infrared/differential interference contrast systems and a EM-CCD camera (ImagEM, Hamamatsu Photonics) and a spinning disk confocal unit (CSU10; Yokogawa Electric). The whole-cell electrodes (resistance of 5-7 MΩ) were fabricated from borosilicate capillaries (GD-1.5; Narishige Scientific Instruments) and pulled on a micropipette puller (Sutter Instrument). Whole-cell current clamp recordings were made with an internal solution containing the followings: 140 mM K-gluconate, 4 mM KCl, 0.2 mM MgCl₂, 10 mM HEPES, 0.5 mM EGTA, and 2.5 mM NaCl. The extracellular solution contained 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, and 5 mM Glucose.
mM HEPES, 0.2 mM EGTA, 2 mM MgATP, 0.2 mM NaGTP, adjusted to pH 7.3 with KOH. Slices were continuously superfused with a physiological recording solution containing the following: 0.81 mM MgSO$_4$, 5.36 mM KCl, 0.44 mM KH$_2$PO$_4$, 1.26 mM CaCl$_2$, 136.9 mM NaCl, 0.34 mM Na$_2$HPO$_4$, 4.17 mM NaHCO$_3$, and 5.55 mM D-glucose, at flow rates of 2–3 mL/min. All experiments were performed at 32 °C. Responses were recorded using a MultiClamp700B amplifier, Digidata 1550A, and pClamp10.5 (Molecular Device), filtered at 10 kHz, and digitized at 10 kHz. For validation of ArcLightD signals, current-clamp recordings were obtained in solutions containing 0.5, 3, 5.36, and 10 mM KCl. Mean membrane potentials were calculated using 1-second data from individual cells.

**Immunohistochemistry**
The SCN slices expressing ArcLightD were incubated in DMEM containing 50 μg/ml colchicine for 24 hr and fixed with 4% paraformaldehyde in 0.1 M PBS for 60 min at room temperature. Nonspecific antibody binding was blocked by 60-min incubation with skim milk at room temperature. For labeling AVP and VIP, the SCN slices were stained using mouse anti-AVP monoclonal antibody (generous gift of Dr. H. Gainer, NIH/NIDDK) (1:1,000 dilution) and rabbit anti-VIP polyclonal antibody (1:10,000 dilution; Peptide Institute), respectively. Two days later, Alexa 594-conjugated goat IgG (1:200 dilution; Invitrogen) was used as the secondary antibodies for mouse IgG (AVP) and rabbit IgG (VIP), respectively. The slices were mounted on glass bottom dish with Prolong Gold-DAPI (Invitrogen). Fluorescence was visualized using Nipkow spinning disk confocal and EM-CCD camera (iXon3; Andor Technology) and MetaMorph software (Molecular Devices).

**Data analysis and statistics**
Statistical analyses were performed using Prism GraphPad (GraphPad Software). The group mean was presented as the mean ± sem. The t-test was used when two independent group means were compared, and Mann–Whitney U-test or Welch’s t-test was used when the variances of two group means were different. A paired t-test or one sample t-test was used when two dependent group means were compared. Peak phases of the rhythms were estimated by the midpoint of the rising and falling limbs of detrended circadian rhythm that intersected x-axis. For regional comparison of the rhythms, ROIs (100μm × 100 μm) in the upper 1/3 (near the third ventricle) and the bottom 2/3 (near optic chiasma) were selected as the dorsal and ventral regions.

For quantification of the rhythms at the SCN network, we used a custom-made program for creation of acrophase maps as described previously (7, 30, 31). Briefly, fluorescence images were smoothed with the median filter (one pixel) and converted to eight-bit intensity. Background signals were selected from a region where no cells were found (referred to as the background region). The mean +5 SDs of the signal intensity of the background region was set as the cutoff level of the signal and background. The time series of the images in each pixel, {Yj (ti); ti = 1,2,..,N (h)}, was fitted to cosine curve yj(t) = yj (t; Mj, Aj, Cj, Tj) = Mj + Aj * cosine (2pi (t - Cj)/Tj) using a least-square
regression method, where $j$, $y_j(t)$ is the signal intensity at time $t$ (h), $M_j$ is the mesor, $A_j$ is the amplitude, $C_j$ is the acrophase, and $T_j$ is the period of the images. The goodness of fitting was statistically evaluated by Percent Rhythm accounted for the fitted cosine wave (Pearson product-moment correlation analysis) at a significance level of $p < 0.001$. In all figures, acrophase maps were shown by pseudocolor, and pixels with unfitted rhythms and background level signals were shown by white color. The mean acrophase of the entire SCN regions is separately normalized to zero for voltage and Ca$^{2+}$ rhythms. To validate the rhythmicity in the ROIs, fluorescence signals in each ROIs were fitted to cosine curve using a least-square regression method, the goodness of fit was evaluated by cosine curve fitting and Percent Rhythm ($p < 0.001$).
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References


**Figure Legends**

**A** hSyn1-ArcLightD, C57BL/6J  
**B** hSyn1-Flex-ArcLightD, VIP-Cre  
**C** hSyn1-Flex-ArcLightD, AVP-Cre

![Images of expression patterns of voltage probe, ArcLightD, in the SCN network](image)

**Fig.1. Expression patterns of voltage probe, ArcLightD, in the SCN network**

Expression patterns of ArcLightD fluorescence in the entire SCN (top) and the dorsal/ventral regions (bottom) (red square ROIs in the top) of the cultured SCN slices from (A) C57BL/6J mice, (B) VIP-Cre mice, and (C) AVP-Cre mice, respectively. 3V, third ventricle; OC, optic chiasm.
Fig. 2. Simultaneous recordings of the voltage and Ca$^{2+}$ rhythms

(A) Expression patterns of ArcLightD (left) and RGECO (right) fluorescence at the peak circadian phase. Estimated border of the SCN is plotted with broken lines. (B) Acrophase maps of the voltage (left) and Ca$^{2+}$ rhythms (right). Mean acrophase of the entire SCN regions was separately normalized to zero for voltage and Ca$^{2+}$ rhythms. Color bars indicate the relative time of day (hours).

(C) The mean acrophase of the circadian voltage (left) and Ca$^{2+}$ rhythms (right) from two regions in the SCN (square ROIs in A). For a regional comparison of the rhythms, ROIs (100 μm × 100 μm) in upper 1/3 (near the third ventricle) and bottom 2/3 (near optic chiasma) were selected as the dorsal and ventral regions. Significant phase difference was detected between the dorsal (d) and ventral (v) regions in the Ca$^{2+}$ rhythms, but not in the voltage rhythms. (D) Raw (top) and 24h detrended traces (bottom) of the voltage (green) and Ca$^{2+}$ rhythms (red) in the dorsal (1) and ventral SCN (2) from square ROIs in panel (A). The circadian peak was indicated by arrowheads of each color. Detrended data were smoothed with a 3 h moving average method. (E) The phase difference (Δ Phase) between the voltage and Ca$^{2+}$ rhythms in the dorsal and ventral SCN (6 slices). All data are given as the mean ± sem. ***p < 0.001. Note that in all figures the vertical scale bar was inverted since ArcLightD fluorescence indicates that the fluorescence dims upon depolarization of the plasma membrane, reported as a characteristic feature of ArcLightD (27).
Fig. 3. Spatiotemporal profiles of the voltage and Ca$^{2+}$ rhythms in VIP neurons

(A) Expression patterns of ArcLightD in the VIP neurons (left) and RGECO in the entire in the same SCN (right). (B) Acrophase maps of the voltage rhythms (left) and the Ca$^{2+}$ rhythms (right). Mean acrophase of the entire SCN regions was separately normalized to zero for voltage and Ca$^{2+}$ rhythms. (C–E) Representative circadian voltage (green) and Ca$^{2+}$ rhythms (red). Top and bottom traces in each panel show raw and 24 h detrended data (smoothed with 3 h moving average). (c) Fast Na$^+$ channel blocker (1 μM TTX) (n = 3), (D) L-type Ca$^{2+}$ channel blocker (3 μM nimodipine) (n = 3). (E) Co-application of fast Na$^+$ channel blocker (1 μM TTX) and L-type Ca$^{2+}$ channel blocker (3 μM nimodipine) (n = 3). (F) Mean amplitudes of the Ca$^{2+}$ and voltage rhythms after respective channel blockers in terms of % of the pretreatment level. One sample t test was used to validate the blocker effects. (G) Differences in Δ Phase between the voltage and Ca$^{2+}$ rhythms after respective channel blockers (n = 3 in each condition). A paired t-test was used to validate the blocker effects. *p < 0.05, **p < 0.01, and ***p < 0.001. All data are given as the mean ± sem.
Fig. 4. Spatiotemporal profiles of the voltage and Ca\textsuperscript{2+} rhythms in AVP neurons

(A) Expression patterns of ArcLightD in the AVP neurons (left) and RGECO in the entire SCN (right). (B) Acrophase maps of the voltage rhythms (left) and the Ca\textsuperscript{2+} rhythms (right). Mean acrophase of the entire SCN regions was separately normalized to zero for voltage and Ca\textsuperscript{2+} rhythms. (C–E) Representative circadian voltage (green) and Ca\textsuperscript{2+} rhythms (red). (C) 1 μM TTX (n = 3), (d) 3 μM nimodipine (n = 3), (E) Co-application mixture of TTX (1 μM) and nimodipine (3 μM) (n = 3). (F) Mean amplitudes of the Ca\textsuperscript{2+} and voltage rhythms after respective channel blockers in terms of % of the pretreatment level. One sample t-test was used to validate the blocker effects. (G) Differences in Δ Phase between the voltage and Ca\textsuperscript{2+} rhythms after respective channel blockers (n = 3 in each condition). A paired t-test was used to validate the blocker effects. *p < 0.05, **p < 0.01, and ***p < 0.001. All data are given as the mean ± sem.
Fig. 5. Spatiotemporal profiles of the firing and voltage rhythms
(A) Images of ArcLightD fluorescence (left) and of MED in brightfield (right). (B) An acrophase map of the voltage rhythm (top) and phase distribution histogram (bottom). (C) Acrophase of the firing rhythm (top) mapped on each electrode covering the right SCN and the phase distribution histogram (bottom). (D) Representative circadian voltage (green) and firing rhythms (red). Data was detrended by 24h moving average subtraction and normalized relative to the peak amplitude. (E) Histograms of the acrophase of firing rhythm (top) and Δ phase between the two rhythms (bottom) examined in the ROIs covering each electrode (3 slices, 39 electrodes). Color columns represent three individual experiments. (F) The phase difference between the voltage and firing rhythms (Δ phase) in the dorsal and ventral regions. All data are given as the mean ± sem. n.s; statistically not significant.
Fig. 6. Long-term recording of the voltage and Ca\textsuperscript{2+} rhythms
(A) Acrophase maps of the voltage (top) and Ca\textsuperscript{2+} rhythms (bottom) at the early (cP days 10–11) (left) and the late stages (cP days 21–22) (right) of long-term recording. The mean acrophase of the entire SCN regions was separately normalized to zero for voltage and Ca\textsuperscript{2+} rhythms. (B) Time course of the voltage (green) and Ca\textsuperscript{2+} rhythms (red) in the dorsal (top) and ventral regions (bottom) during 13 days of recording. (C) Network synchronization of the voltage (left) and Ca\textsuperscript{2+} rhythms (right) at the early and late stages of the recordings. Variability of network synchrony is defined as acrophase standard deviation (SD). (D) Phase difference (Δ Phase) between voltage and Ca\textsuperscript{2+} rhythms in the dorsal and ventral SCN at the early (left) and late stages (right) of the recording (n = 5). cP day, corresponding postnatal day. *p < 0.05. Data are given as the mean ± sem.
Fig. 7. Summary schema of the spatiotemporal profiles and cell-type specificity of the circadian rhythms

(A) Spatial patterns of the circadian firing, voltage, Ca\textsuperscript{2+}, and PER2 rhythms. Acrophases are schematically shown in pseudocolor with the mean phase of the entire slice was set to 12h. (B) Estimated temporal orders of the circadian rhythms in the AVP (top) and VIP neurons (bottom). (C) Schematic drawing for the functional links between the two oscillating cell components and between the VIP and AVP neurons. Nerve terminals on the right represent inputs from the SCN networks. Double-headed arrows indicate the stable phase relation of the two functions suggesting strong coupling. Arrows indicate the direction of effect. +/− denotes enhancement or suppression effect, and numberings (①-③) show the effects of blockers (see discussion for the details).
Supplemental Figure Legends

**A**  **VIP-Cre**  
Flex-ArcLightD  Anti-VIP  Merge  

**B**  **AVP-Cre**  
Flex-ArcLightD  Anti-AVP  Merge  

Fig.S1. Comparison of Cre-dependent ArcLightD expressions and localization of VIP/AVP neuropeptides  
(A-B) Expression patterns of Flex-ArcLightD fluorescence of the cultured SCN slices prepared from (A) VIP-Cre mice and (B) AVP-Cre mice, respectively. Images of ArcLightD fluorescence (left), AVP and VIP immunopositive signals (middle), and the overlays (right) were displayed. Estimated border of the SCN is plotted with a white dotted line. 3V, third ventricle; OC, optic chiasm.
Fig. S2. Schema of experimental procedure

*Step 1*: The SCN slice was prepared from C57BL/6J or VIP/AVP-Cre newborn mice, and was explanted on a culture membrane. *Step 2*: Aliquots of the AAV (1 μL) harboring ArcLightD and RGECO were inoculated onto the surface of the SCN cultures. *Step 3*: The membrane with the cultured SCN slice was cut out, flipped over, and transferred to a glass bottom dish (for confocal) or multi-electrode array dish (MED) with 64 electrodes (for CCD image). Recording (confocal or CCD) was started from the corresponding postnatal (cP) days 25–27 (Protocol 1) or 10th (Protocol 2).
Fig.S3. ArcLightD reports change in membrane potentials

(A) ArcLightD fluorescence changes in the SCN slices under various extracellular KCl concentrations (0.5, 3.5, 5.4, 10 mM) (6 slices). (B) Mean membrane potentials (Vm) with (red) and without (blue) neuronal firings in various extracellular KCl concentrations (23 neurons in 6 slices). (C) Estimated ArcLightD fluorescence changes plotted against estimated Vm. (D) The voltage rhythms are shown in fluorescence (left ordinate) and in estimated Δ mV (right ordinate) in the dorsal and ventral SCN regions (100 µm x 100 µm). Each color trace represents different slices (n = 4). (E) Estimated amplitude expressed in ΔmV in the dorsal and ventral SCN regions. Note that all data in this figure were taken with same experimental settings (i.e., exposure time, excitation light intensity, EM gain) using EM-CCD camera (iXon, Andor). n.s; statistically not significant.
Fig. S4. The voltage and Ca\(^{2+}\) rhythms in VIP and AVP neurons
Using two Flex-AAVs, both ArcLightD and jRGECO are expressed only in the VIP (A,B) or AVP neurons (C,D). (a) Acrophase maps of the voltage (left) and Ca\(^{2+}\) rhythms (right) in the VIP neurons. The mean acrophase of the entire SCN regions is separately normalized to zero for voltage and Ca\(^{2+}\) rhythms. (B) Raw (top) and 24 h detrended traces (bottom) of the voltage (green) and Ca\(^{2+}\) rhythms (red). Triangles indicate the peak phase of each rhythm. (C) Acrophase maps of the voltage (left) and Ca\(^{2+}\) rhythms (right) in the AVP neurons. (D) Raw (top) and 24 h detrended traces of the voltage (green) and Ca\(^{2+}\) rhythms (red). Traces were taken from square ROIs in acrophase maps. (E) Phase difference (Δ phase) between voltage and Ca\(^{2+}\) rhythms in the AVP (n = 4) and VIP neurons (n = 3). 3V, third ventricle; OC, optic chiasm. Data are given as the mean ± sem.