



HOKKAIDO UNIVERSITY

Title	Topological specificity and hierarchical network of the circadian calcium rhythm in the suprachiasmatic nucleus.
Author(s)	Enoki, Ryosuke; Kuroda, Shigeru; Ono, Daisuke et al.
Citation	Proceedings of the National Academy of Sciences of the United States of America, 109(52), 21498-21503 https://doi.org/10.1073/pnas.1214415110
Issue Date	2012-12-26
Doc URL	https://hdl.handle.net/2115/52918
Type	journal article
File Information	PNAS109-52_21498-21503.pdf



Classification: Biological Sciences, Neuroscience

Topological specificity and hierarchical network of the circadian calcium rhythm in the suprachiasmatic nucleus

Ryosuke Enoki^{1,2,3}, Shigeru Kuroda^{4,7}, Daisuke Ono¹, Mazahir T. Hasan^{5,6},
Sato Honma^{1,3*}, Tetsuo Ueda⁴, and Ken-ichi Honma^{1,3}

¹*Photonic Bioimaging Section, Research Center for Cooperative Projects;* ²*Department of Physiology;*

³*Department of Chronomedicine, Hokkaido University Graduate School of Medicine, N15 W7, Kita-ku,*

Sapporo 060-8638, Japan. ⁴*Laboratory of Cellular Informatics, Research Institute for Electronic Science,*

Hokkaido University, N20 W10, Kita-ku, Sapporo 001-0020, Japan. ⁵*Department of Molecular Neurobiology,*

Max Planck Institute for Medical Research, Jahnstrasse 29, D-69120, Heidelberg, Germany. ⁶*Charité –*

Universitätsmedizin, NeuroCure Cluster of Excellence, Berlin, D-10117, Germany.

Present Address

⁷*The School of Systems Information Science, Future University Hakodate, Hakodate 041-8655, Japan.*

Corresponding Author

Sato Honma, M.D., Ph.D.

Hokkaido University Graduate School of Medicine, N15 W7, Kita-ku, Sapporo 060-8638, Japan

E-mail: sathonma@med.hokudai.ac.jp, Tel.: +81-11-706-4778, Fax: +81-11-706-4737

Author contributions

R.E., D.O., S.H., and K.H. designed research; R.E. performed research and analyzed data;

S.K. and T.U. developed analytical software; M.T.H. developed the viral transduction method;

R.E., S.H., and K.H. wrote the paper.

Abstract

The circadian pacemaker in the hypothalamic suprachiasmatic nucleus (SCN) is a hierarchical multi-oscillator system in which neuronal networks play crucial roles in expressing coherent rhythms in physiology and behavior. However, our understanding of the neuronal network is still incomplete. Intracellular calcium mediates the input signals, such as phase resetting stimuli, to the core molecular loop involving clock genes for circadian rhythm generation and the output signals from the loop to various cellular functions, including changes in neurotransmitter release. Using a new large-scale calcium imaging method with genetically-encoded calcium sensors, we visualized intracellular calcium from the entire surface of SCN slice in culture including the regions where autonomous clock gene expression was undetectable. We found circadian calcium rhythms at a single-cell level in the SCN, which were topologically specific with a larger amplitude and more delayed phase in the ventral region than the dorsal. The robustness of the rhythm was reduced but persisted even after blocking the neuronal firing with tetrodotoxin (TTX). Notably, TTX dissociated the circadian calcium rhythms between the dorsal and ventral SCN. In contrast, a blocker of gap junctions, carbenoxolone, had only a minor effect on the calcium rhythms at both the single-cell and network levels. These results reveal the topological specificity of the circadian calcium rhythm in the SCN and the presence of coupled regional pacemakers in the dorsal and ventral regions. Neuronal firings are not necessary for the persistence of the calcium rhythms but indispensable for the hierarchical organization of rhythmicity in the SCN.

\body

Introduction

In mammals, the master circadian clock is located in the suprachiasmatic nucleus (SCN) of the hypothalamus and regulates various physiological functions and behaviors (1). The SCN comprises ~20,000 neurons and generates coherent circadian rhythms via oscillatory coupling (2). Recent advances in molecular and cellular biology have proposed the molecular machinery for intracellular circadian rhythm generation, which is transcriptional/translational auto-feedback loops involving several clock genes and their protein products (3). The SCN is a hierarchical multi-oscillator system in which neuronal networks play a critical role in producing coherent circadian rhythms (2). Without neuronal networks as in dispersed SCN cell culture, the phase and period of the cellular circadian rhythm become more variable (4). The coupled SCN networks are resistant to genetic mutations (5) and temperature resetting (6). Whereas the oscillatory coupling in the SCN is altered by light input such as an abrupt shift of a light-dark cycle (7) and seasonal changes in day length (8, 9). These studies indicate that neuronal couplings and regional interactions play important roles in the hierarchy of the SCN circadian pacemaker.

Heterogeneous distribution of the circadian phase in SCN slices was shown by in situ hybridization and bioluminescence studies examining clock gene expression (e.g., *Per1* or *Per2*) (10-14). Disruption of intercellular coupling by inhibiting neuronal firing with tetrodotoxin (TTX) or mechanical isolation of SCN subregions severely reduced clock gene expression and mutual synchronization (10, 11). In addition, SCN neurons are connected through gap junctions (15, 16) which were reported to synchronize neuronal firings under circadian regulation (17). Therefore, gap junctions may also play some roles in the expression of circadian rhythm at both single cell and network levels. Previous studies have reported specific patterns in clock gene expression rhythm in the SCN (10, 11), and there are SCN subregions lacking autonomous clock gene rhythm (18), particularly in the regions where neurons receive major inputs from the retina. Thus, our understanding of the hierarchy of SCN circadian pacemaker is still far from complete.

The cytosolic calcium concentration in the SCN is reported to fluctuate spontaneously in a circadian fashion and to change rapidly in response to the photic signals from the retina (19-21). The intracellular calcium is regulated by the input and/or output signals to/from the molecular feedback loop. To visualize the intracellular calcium throughout the SCN, we recently developed a

large-scale time-lapse calcium imaging method using a genetically-encoded FRET-based calcium sensor (i.e., yellow cameleon 3.60, YC 3.60) and Nipkow spinning disk confocal system (22). In the present study, by taking advantage of this new technology, we analyzed the topological and temporal specificities as well as hierarchical structures of the circadian calcium rhythm in the SCN.

Results

Detection of the circadian calcium rhythm in the SCN network

By using recombinant adeno-associated virus and the neuron specific promoter (i.e., human synapsin), YC 3.60 was expressed in the entire surface of cultured SCN slice (Fig. S1) including the subregion where PER2::LUC expression was not detected (Fig. S2A-C). With specific labeling for neurons and glial cells (i.e., Nissl and GFAP), we confirmed that YC 3.60 expression was neuron specific (Fig. S3), and absent in glial cells (Fig. S4). The transduction efficiency of YC 3.60 was $98.2 \pm 0.4 \%$ and $97.7 \pm 0.3 \%$ in the dorsal and ventral regions of SCN slice, respectively (Fig. S3C).

We successfully monitored the spatio-temporal dynamics of the intracellular calcium (Venus/CFP ratio) at 1-h intervals in all regions of the SCN for several days at single cell resolution (Fig. 1 and Video S1), and demonstrated that the SCN at the slice as well as single cell levels exhibit robust circadian rhythms. Excitation/emission of FRET acceptor (Venus) alone did not show any circadian periodicity. The results indicate that circadian variation in FRET signals of YC 3.60 reflects primarily the circadian rhythms in intracellular calcium concentration (Fig. S1D). The circadian calcium rhythms in the dorsal region showed an advanced phase relative to the ventral one (Fig. 1B and C, a-c). In contrast, no circadian calcium rhythm was detected in the subparaventricular zone (SPZ), indicating that a robust circadian calcium rhythm is a characteristic feature of the SCN (Fig. 1B and C, d). Simultaneous recording of circadian calcium and PER2::LUC rhythms in the same SCN slices revealed that the former phase advanced by 5.67 ± 0.29 h as compared with the later (n=3, Fig. S2D). To quantify and visualize the spatio-temporal profiles of the circadian calcium rhythm throughout the SCN, we developed a custom-made software program for analyzing essential parameters of the circadian rhythm (see Fig. S5 and SI text). Briefly, in each pixel, the Venus/CFP signals were fitted to a cosine curve. And, the acrophase (peak phase), period, amplitude (peak-trough difference), and trough level (the lowest value) of the best fitted curve were obtained. Spatial profiles of the 4 parameters were generated and presented as pseudo-color maps; typical examples are presented in Fig. 1D. An acrophase map, constructed by normalizing phases to the mean of acrophase in pixel of entire SCN slice, revealed regional heterogeneity in the circadian calcium rhythm. In particular, the calcium rhythm was phase advanced in the dorsal region near the third ventricle as compared to the ventral region

(Fig. 1D, 1, and E). Similar results were obtained in all 5 slices examined, confirming that the topological pattern of the acrophase was stereotyped (Fig. S6). The other parameters (period, amplitude, and trough level) are also presented (Fig. 1D, 2-4). Since these parameters in each pixel contained some degree of variance due to estimation error of fitting, we compared the parameters at region levels. Variance in the circadian period could well be within the range of fitting error, when the cellular rhythms are mutually synchronized. For quantitative analysis and regional comparisons, the SCN was divided into 2 regions, the dorsal and ventral, based on the neuropeptide distributions of arginine vasopressin (AVP) and vasoactive intestinal polypeptide (VIP), both of which are marker peptides of the dorsal and ventral SCN subregions, respectively (Fig. 1E). The standard deviation (SD) of the phase distribution was used as an index of network synchronization and compared between the dorsal and ventral regions (Fig. 2A). The SD of the ventral region (2.0 ± 0.3 h) was significantly smaller than those of the dorsal region (3.2 ± 0.6 h) and of the whole SCN (3.2 ± 0.7 h). The period thus calculated in the ventral region (23.8 ± 0.4 h) was not different either from that in the dorsal region (23.5 ± 0.4 h) or in the whole SCN (23.6 ± 0.4 h) (Fig. 2B). The amplitude of the calcium rhythm was significantly higher in the ventral region (0.38 ± 0.04) than that in the dorsal region (0.29 ± 0.05) (Fig. 2C). The trough level of calcium rhythm was not different regionally (1.84 ± 0.05 and 1.83 ± 0.05 in the dorsal and ventral regions, respectively) (Fig. 2D). The non-rhythmic calcium level in the SPZ was 1.93 ± 0.01 , which was slightly higher than the trough level in the SCN regions. Based on the calculation of the intracellular calcium concentrations (see SI text), the amplitude and trough level were estimated to be 30.4 ± 1.6 nM and 84.9 ± 3.8 nM in the dorsal region and 39.7 ± 1.6 and 84.1 ± 4.2 nM in the ventral region, respectively.

TTX disrupts synchronization of the circadian calcium rhythm

We applied 1 μ M TTX to block sodium-dependent action potentials in SCN neurons and found that the calcium rhythm was TTX sensitive in terms of the amplitude and coupling. However, cellular rhythms were still robust even on the 3rd day of treatment (Fig. 3). Following TTX application, the amplitude of the calcium rhythm as well as the trough level in both the dorsal and ventral regions decreased, as compared with the pretreat levels (Fig. 3C and 4A and B; Tables S1 and S2) ($n = 4$). The calcium level in the SPZ was also slightly decreased following TTX application (1.84 ± 0.01 at

day 1, 1.80 ± 0.01 at day 3). Importantly, TTX application led to desynchronization of the SCN network (Fig. 3C and Fig. 4C). The SD of acrophases at pixel level in the whole SCN increased significantly (3.2 ± 0.3 h in the pretreat, 4.2 ± 0.2 h in TTX at day 1, 4.7 ± 0.2 h in TTX at day 3) (Fig. 4C1, Tables S1 and S2). Desynchronization significantly proceeded in the ventral region but not in the dorsal after TTX application (Fig. 4C2 and 4C3; Tables S1 and S2), indicating the heterogeneity of the TTX-sensitive mechanisms within SCN subregions. Rayleigh test indicated that TTX application reduced the average length of mean vector (r) significantly in the dorsal (Fig. S7B) as well as in the ventral regions (Fig. S7C), confirming that TTX desynchronized the SCN.

Notably, TTX enlarged the phase difference of the circadian calcium rhythms between the dorsal and ventral regions (Fig. 5). The distribution of acrophase in the entire SCN became flattened following TTX application (Fig. 5B). We generated histograms of acrophase in the dorsal (green) and ventral regions (red) separately in all 4 slices examined (Fig. 5C). Histogram shows a Gaussian-like distribution and the mean phase of respective distribution was calculated. The difference of two mean phases was enlarged by TTX application, which were 3.5 ± 0.2 h in the pretreat, 4.5 ± 0.2 h in TTX at day 1, and 4.4 ± 0.2 h in TTX at day 3. The intervals of two phases became significantly larger after the TTX application ($p < 0.01$). The dissociation was also seen in the raster plots across the dorsal to ventral regions (arrowhead in Fig. 3C). Furthermore, Rayleigh test showed that the phase angle difference between the two regions became larger following TTX application in all 4 slices (Fig. S7A). Medium change containing vehicle or extended long-term culturing did not affect any parameter of calcium rhythm (Fig. S8), indicating that the dissociation did not occur by vehicle treatment or in the course of culturing.

Gap junctions do not contribute to the circadian calcium rhythm and network synchronization

We next investigated whether gap junctions contributed to circadian calcium rhythm generation and synchronization in the SCN network. After application of a widely used specific gap-junction blocker, carbenoxolone (CBX), the circadian parameters of calcium rhythm were analyzed ($n = 3$) (Fig. 6). CBX application did not cause any notable change in the rhythm parameters. Individual neurons were able to sustain a robust circadian calcium rhythm with only a slight reduction in amplitude in the dorsal region on day 3 of the treatment (Fig. 6B-C and Fig. 7A-B, Tables S3 and

S4). The calcium levels in the SPZ did not change following CBX application (1.92 ± 0.01 at day 1, 1.91 ± 0.01 at day 3). Similarly, the SD of acrophases did not show any change in the whole SCN as well as the dorsal and ventral regions (Fig. 7C, Tables S3 and S4).

Discussion

Topological characterization of the SCN network

Topological specificity of the SCN network has been characterized by the patterns of clock gene expression using in situ hybridization and bioluminescence imaging (2, 10-14). However, the autonomous expression of a clock gene is either absent or undetectable in certain regions, particularly those where neurons receive major light information from the retina (18). And the reporter of clock gene (i.e., GFP) and its protein product (PER1) do not co-localize in certain neurons (18). These findings indicate the limitation of SCN network analysis only by clock gene expressions. In contrast, the calcium probes used in this study are widely expressed in the entire surface of the SCN slice and allowed the topological patterns of the calcium rhythm as well as regional interactions to be characterized. We found that the circadian calcium rhythm in the ventral region had a larger amplitude than the dorsal. In addition, the circadian phase in the ventral region was more delayed and less variable than in the dorsal region. TTX experiments revealed that the circadian rhythms in the dorsal and ventral regions were coupled by neuronal firing-mediated mechanisms.

Our rhythm analysis at pixel level indicated that the dorsal and ventral regions of the SCN showed substantial difference in circadian calcium rhythms. Previously, the differential induction as well as localization of *Per1* and *Per2* was shown in the core and shell regions of the SCN (23). Recent reports indicate that the circadian peaks of *Per1* or *Per2* appear first in the dorsal region and flow progressively towards the ventral region like a “wave” (10-14). However, the temporal order of the circadian peak detected in different SCN areas does not necessarily mean that the circadian signals flow and transfer along this direction via neuronal couplings, because neuronal interactions via synaptic (e.g., GABA) or diffusible factors (e.g., AVP, VIP or GRP) are more rapid processes compared to the spread of “wave” in the SCN. Instead, the appearance of the circadian peak at different times of the day may result from differences in the intrinsic circadian period among subregions under mutually synchronized condition. The circadian period was reported to be shorter in the dorsal region than that in the ventral one (24-26). These results are well consistent with the present findings.

In the present study, we observed the topological specificity of circadian calcium rhythm. Previous studies using biolistic particle delivery of YC 2.1 with a gene gun (27, 28) showed that

only 60-65 % of YC 2.1-expressing neurons exhibited circadian calcium rhythm. In contrast, virtually all neurons in the surface of the SCN slice showed circadian calcium rhythm in the present study. Our success to find the topological specificity is presumably due to high transduction efficiency and less invasive delivery of transgenes in SCN slices.

The amplitude of the calcium rhythm is larger in the ventral than in the dorsal SCN. In contrast, *Per1* and *Per2* expressions have been reported to show a larger amplitude in the dorsal than in the ventral SCN (10, 11). The amplitude of bioluminescence signals (e.g., *Per1* and *Per2*) is generally regarded to reflect the oscillator amplitude. By contrast, the amplitude of calcium rhythm involves in both the circadian rhythms of the input and output signals. Therefore the difference in amplitude could reflect the different roles in circadian oscillation between clock gene and calcium. Alternatively, the calcium rhythms of high amplitude were detected in the region where autonomous clock gene expression is not evident, which could explain the discrepancy between clock genes and cellular calcium. In addition, the results suggest that clock mechanisms other than those involving PER1 or PER2 exist in the region where clock gene expression is undetectable, since the calcium rhythms in this region continued even after the shut down of neural inputs by TTX from other areas where autonomous clock gene expression exists.

Neuronal firings reinforce circadian calcium rhythm and network synchronization.

Previously, Ikeda et al., (27) demonstrated that the circadian calcium rhythm was insensitive to TTX at single-cell levels. They also found that the calcium rhythms preceded the neuronal firing rhythms by approximately 4 h, indicating the independence of the calcium rhythms from the neural firing rhythms. The findings were further elaborated by showing 3 types of neurons responding differently to TTX (no change, gain and loss of the calcium rhythms) (28). These studies employed a biolistic particle delivery method of YC2.1 to neurons. On the other hand, other studies reported that TTX blocked neuronal firing and eliminated the day-night difference in the intracellular calcium concentration using a synthetic calcium dye, fura-2 (20-21, 29). These results are different from our present findings that the calcium rhythms persisted with reduced amplitude and circadian phases were dissociated between the dorsal and ventral SCN after TTX treatment. The discrepancy may be caused by the differences in experimental methods. First, the dynamic range of intracellular calcium estimation was wider in YC3.60 than in YC2.1 (30), which allowed us to

estimate more precise evaluation of intracellular calcium. Actually, the cellular calcium concentration was estimated as 80-120 nM in the present study that was similar to the estimation by fura-2 (20-21, 29), while lower than those in the studies of Ikeda et al., (120-440 nM) (27). Second, the number of analyzed neurons in the SCN was different among studies. rAAV in the present study labeled the entire surface of SCN slices, while biolistic delivery labeled randomly a relatively small number of SCN neurons (27, 28). The former method enabled us to analyze calcium rhythms more systematically in the SCN.

The persistence of circadian rhythm with reduced amplitude by TTX is consistent with the idea of reinforcement of circadian rhythms by mutual synchronization through neuronal networks (2). By TTX application, the neuronal connection was shut down and the reinforcing effect of networks did not work, resulting in the reduction of circadian amplitude in constituent neurons. The mechanism of reinforcement is not well understood but could well be calcium influx through voltage-gated calcium channels that are opened by action potentials or synaptic/paracrine input from other neurons (20, 21). Calcium rhythms phase lead the PER2 by ~5 hours, suggesting that calcium is involved in the input to the molecular feedback loop. Theoretically, intracellular calcium is also involved in the output from the loop to the mechanisms of, for instance, neurotransmitter release (e.g., GABA) (31, 32) or paracrine signaling (e.g., AVP, VIP, or GRP) (33). TTX shut down the input signals from other neurons and reduced the amplitude of intracellular calcium rhythm by ca. 30 %, which may confirm the contribution of calcium in the input pathway to the rhythmicity, but also indicate the involvement of calcium in the output pathway. In this respect, it is interesting to note that amplitude reduction by TTX was more pronounced in the ventral SCN than in the dorsal. The ventral SCN receives major input signals from outside the SCN. Therefore, the contribution of calcium rhythm in the input pathway is more important in the ventral SCN than in the dorsal, which could reflect the differential response of amplitude to TTX application. In the present study, the trough level was also suppressed by TTX, indicating that the TTX-sensitive and non-rhythmic components present in the intracellular calcium concentration. The TTX-insensitive component could arise from calcium released from ryanodine-sensitive internal store (27).

In addition to the reduction of reinforcement through shut down of neuronal networks, TTX affected the hierarchical structure of SCN circadian system. Previously, two distinct regional pacemakers were found in the SCN by demonstrating that the AVP and VIP release showed

independent circadian rhythms in organotypic slice cultures (34). Dissociation of the regional pacemakers in the dorsal and ventral SCN was also reported to occur after an abrupt shift of a light-dark cycle (7), photoperiodic change (35, 36), and temperature change (6). In the present study, TTX enlarged the phase difference between the population rhythms in the dorsal and ventral SCN. The finding indicates that TTX sensitive mechanism is involved in the coupling of the regional pacemakers. Furthermore, TTX affected differentially the synchrony of population rhythms constituting the regional pacemakers. Desynchrony was more pronounced in the ventral pacemaker than in the dorsal one. The finding suggests differential network structures in the regional pacemakers, which could relate to the fact that neurons in the dorsal region are more tightly packed than those in the ventral (37). Previously, a loss of synchronization among cellular circadian rhythms was reported in *Per1* bioluminescence by TTX application for 7 days (10). However, the regional specificity was not examined in this study.

Minor role of gap junctions

The present study showed that gap junctions have only a minor effect on the circadian calcium rhythm both at the single-cell and network levels. Previously, the gap junction antagonists, octanol and halothane, were shown to reduce the amplitude of the circadian rhythm of AVP and VIP release in the SCN (16). However, these drugs are known to have poor selectivity and considerable side effects. In the present study, a most widely used gap-junction blocker of high selectivity, CBX, slightly suppressed the amplitude of the circadian calcium rhythm only in the dorsal region, while other parameters remained unchanged. Mice lacking Connexin36, a structural component of gap junction in the SCN, displayed altered circadian behaviors (17). Whereas, a few “miniature” gap junctions mediating weak electrotonic coupling were recently demonstrated between limited numbers of neuron pairs, suggesting only minor roles of gap junctions in the SCN network (38). The electrical synapses via gap junctions may contribute to SCN coupling under a certain condition, but our data showed that chemical interactions (e.g., synaptic or paracrine) are more important than electrotonic signal transduction.

Possible role of the circadian calcium rhythm

We report here the topological characterization of the circadian calcium rhythm in the SCN and the oscillatory coupling between the dorsal and ventral SCN. However, very little is known about the physiological importance of circadian calcium rhythm in the SCN. Calcium influx has been reported to be essential for generation of the circadian rhythm in the SCN (39). In addition, calcium-activated potassium channels are highly expressed in the SCN and regulate behavioral rhythms as well as neuronal firing (40). High intracellular calcium level in the subjective day is hypothesized to prevent light induced phase shifts of the SCN cellular rhythms since the already high calcium level does not allow further increase (41). This may be the cellular/network mechanism of the “gating” in which light input to the circadian clock is shut down depending on the circadian phase. Applying the same logic to the interaction of dorsal-ventral SCN, the dorsal pacemaker may be highly sensitive to the phase shifting signals from the ventral when the circadian calcium rhythm reaches the trough level.

Materials and Methods

The experiments in this study were ethically approved by Animal Research Committee of Hokkaido University (approval number 0800277) and performed following the Guide for the Care and Use of Laboratory Animals at Hokkaido University. Neonate mice were decapitated, and SCN slices were cultured as described previously with minor modifications (42). More information of materials and methods is available in SI Text.

Acknowledgments

This work was supported by MEXT/JSPS KAKENHI (Grant Number 21890003 & 23790268 to R.E., 21390064 & 24390055 to S.H, and 20249010 to K.H.); and by the Research Foundation for Opto-Science and Technology to R.E.; and by Fritz Thyssen Stiftung and the Max Planck Society to M.T.H. This work was also supported by the Cooperative Research Project for Advanced Photonic Bioimaging and by the Project for Developing Innovation Systems of MEXT. We thank Takeharu Nagai (Osaka Univ) for valuable advice on calcium imaging, Harold Gainer (NIH/NINDS) for providing anti-mouse AVP monoclonal antibody, and Joseph S. Takahashi (Southwestern) for providing us with the PER2::LUC mice.

References

1. Moore RY & Eichler VB (1972) Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. *Brain Res* 42(1):201-206.
2. Welsh DK, Takahashi JS, & Kay SA (2010) Suprachiasmatic nucleus: cell autonomy and network properties. *Annu Rev Physiol* 72:551-577.
3. Reppert SM & Weaver DR (2002) Coordination of circadian timing in mammals. *Nature* 418(6901):935-941.
4. Webb AB, Angelo N, Huettner JE, & Herzog ED (2009) Intrinsic, nondeterministic circadian rhythm generation in identified mammalian neurons. *Proc Natl Acad Sci U S A* 106(38):16493-16498.
5. Liu AC et al., (2007) Intercellular coupling confers robustness against mutations in the SCN circadian clock network. *Cell* 129(3):605-616.
6. Buhr ED, Yoo SH, & Takahashi JS (2010) Temperature as a universal resetting cue for mammalian circadian oscillators. *Science* 330(6002):379-385.
7. Nagano M et al., (2003) An abrupt shift in the day/night cycle causes desynchrony in the mammalian circadian center. *J Neurosci* 23(14):6141-6151.
8. Hazlerigg DG, Ebling FJ, & Johnston JD (2005) Photoperiod differentially regulates gene expression rhythms in the rostral and caudal SCN. *Curr Biol* 15(12):R449-450.
9. Inagaki N et al., (2007) Separate oscillating cell groups in mouse suprachiasmatic nucleus couple photoperiodically to the onset and end of daily activity. *Proc Natl Acad Sci U S A* 104(18):7664-7669.
10. Yamaguchi S et al., (2003) Synchronization of cellular clocks in the suprachiasmatic nucleus. *Science* 302(5649):1408-1412.
11. Maywood ES et al., (2006) Synchronization and maintenance of timekeeping in suprachiasmatic circadian clock cells by neuropeptidergic signaling. *Curr Biol* 16(6):599-605.
12. Evans JA, Leise TL, Castanon-Cervantes O, & Davidson AJ (2011) Intrinsic regulation of spatiotemporal organization within the suprachiasmatic nucleus. *PLoS One* 6(1):e15869.
13. Fukuda H, Tokuda I, Hashimoto S, & Hayasaka N (2011) Quantitative analysis of phase wave of gene expression in the mammalian central circadian clock network. *PLoS One* 6(8):e23568.

14. Foley NC et al., (2011) Characterization of orderly spatiotemporal patterns of clock gene activation in mammalian suprachiasmatic nucleus. *Eur J Neurosci* 33(10):1851-1865.
15. Colwell CS (2000) Rhythmic coupling among cells in the suprachiasmatic nucleus. *J Neurobiol* 43(4):379-388.
16. Shinohara K, Funabashi T, Mitushima D, & Kimura F (2000) Effects of gap junction blocker on vasopressin and vasoactive intestinal polypeptide rhythms in the rat suprachiasmatic nucleus in vitro. *Neurosci Res* 38(1):43-47.
17. Long MA, Jutras MJ, Connors BW, & Burwell RD (2005) Electrical synapses coordinate activity in the suprachiasmatic nucleus. *Nat Neurosci* 8(1):61-66.
18. LeSauter J et al., (2003) A short half-life GFP mouse model for analysis of suprachiasmatic nucleus organization. *Brain Res* 964(2):279-287.
19. Honma S & Honma K (2003) The biological clock: Ca²⁺ links the pendulum to the hands. *Trends Neurosci* 26(12):650-653.
20. Irwin RP & Allen CN (2007) Calcium response to retinohypothalamic tract synaptic transmission in suprachiasmatic nucleus neurons. *J Neurosci* 27(43):11748-11757.
21. Irwin RP & Allen CN (2010) Neuropeptide-mediated calcium signaling in the suprachiasmatic nucleus network. *Eur J Neurosci* 32(9):1497-1506.
22. Enoki R et al., (2012) Single-cell resolution fluorescence imaging of circadian rhythms detected with a Nipkow spinning disk confocal system. *J Neurosci Methods* 207(1):72-9.
23. Yan L & Silver R (2002) Differential induction and localization of mPer1 and mPer2 during advancing and delaying phase shifts. *Eur J Neurosci* 16(8):1531-1540.
24. Nakamura W, Honma S, Shirakawa T, & Honma K (2001) Regional pacemakers composed of multiple oscillator neurons in the rat suprachiasmatic nucleus. *Eur J Neurosci* 14(4):666-674.
25. Noguchi T & Watanabe K (2008) Regional differences in circadian period within the suprachiasmatic nucleus. *Brain Research* 1239:119-126.
26. Noguchi T, Watanabe K, Ogura A, & Yamaoka S (2004) The clock in the dorsal suprachiasmatic nucleus runs faster than that in the ventral. *European Journal of Neuroscience* 20(11):3199-3202.

27. Ikeda M et al., (2003) Circadian dynamics of cytosolic and nuclear Ca^{2+} in single suprachiasmatic nucleus neurons. *Neuron* 38(2):253-263.
28. Hong JH, Jeong B, Min CH, & Lee KJ (2012) Circadian waves of cytosolic calcium concentration and long-range network connections in rat suprachiasmatic nucleus. *European Journal of Neuroscience* 35(9):1417-1425.
29. Colwell CS (2000) Circadian modulation of calcium levels in cells in the suprachiasmatic nucleus. *Eur J Neurosci* 12(2):571-576.
30. Nagai T et al., (2004) Expanded dynamic range of fluorescent indicators for Ca^{2+} by circularly permuted yellow fluorescent proteins. *Proc Natl Acad Sci U S A* 101(29):10554-10559.
31. Albus H et al., (2005) A GABAergic mechanism is necessary for coupling dissociable ventral and dorsal regional oscillators within the circadian clock. *Current Biology* 15(10):886-893.
32. Han S, et al. (2012) Na(V)1.1 channels are critical for intercellular communication in the suprachiasmatic nucleus and for normal circadian rhythms. *P Natl Acad Sci USA* 109(6):E368-E377.
33. Maywood ES, Chesham JE, O'Brien JA, & Hastings MH (2011) A diversity of paracrine signals sustains molecular circadian cycling in suprachiasmatic nucleus circuits. *P Natl Acad Sci USA* 108(34):14306-14311.
34. Shinohara K et al., (1995) Two distinct oscillators in the rat suprachiasmatic nucleus in vitro. *Proc Natl Acad Sci U S A* 92(16):7396-7400.
35. de la Iglesia HO, Cambras T, Schwartz WJ, & Diez-Noguera A (2004) Forced desynchronization of dual circadian oscillators within the rat suprachiasmatic nucleus. *Current Biology* 14(9):796-800.
36. VanderLeest HT, et al. (2007) Seasonal encoding by the circadian pacemaker of the SCN. *Current Biology* 17(5):468-473.
37. Moore RY & Leak RK (2001) in *Handbook of Behavioral Neurobiology*, eds Takahashi JS, Turek FW, & Moore RY (Kluwer Academic / Plenum Publishers, New York), pp 141-179.
38. Rash JE, et al. (2007) Connexin36 vs. connexin32, "miniature" neuronal gap junctions, and limited electrotonic coupling in rodent suprachiasmatic nucleus. *Neuroscience* 149(2):350-371.

39. Lundkvist GB et al., (2005) A calcium flux is required for circadian rhythm generation in mammalian pacemaker neurons. *J Neurosci* 25(33):7682-7686.
40. Meredith AL et al., (2006) BK calcium-activated potassium channels regulate circadian behavioral rhythms and pacemaker output. *Nature Neuroscience* 9(9):1193-1193.
41. Colwell CS (2011) Linking neural activity and molecular oscillations in the SCN. *Nat Rev Neurosci* 12(10):553-569.
42. Nishide SY, Honma S, & Honma K (2008) The circadian pacemaker in the cultured suprachiasmatic nucleus from pup mice is highly sensitive to external perturbation. *Eur J Neurosci* 27(10):2686-2690.

Figure Legends

Fig. 1. Topological characterization of the circadian calcium rhythm.

(A) Pseudocolor image of YC 3.60 signals in the SCN slice. 3V: third ventricle, OC: optic chiasm. The image was taken near the peak phase of the rhythm. (B) Hourly montage of circadian signals in 4 representative areas. Scale bar is 10 μm . Left images show the locations of individual neurons. (C) Left: signal intensity of individual neurons over time (72 h). A horizontal bar indicates the time when the montage of images was collected. Right: raster plots of signal intensity across the dorsal tip to the end of ventral region as indicated by a red line in A. (D) Mapping of rhythm parameters. 1) Acrophase map: peak phase time is depicted and normalized relative to the mean phase of the whole slice. 2) Period map. 3) Amplitude map. 4) Trough map. (E) Bihourly images of the acrophase distribution. Black pixels indicate those with acrophase in this bin. Number on the upper margin of the top panel indicates the difference from the mean acrophase of the whole slice (hours). Estimated borders of the SCN and the dorsal/ventral regions are shown as broken lines. Scale bars are 100 μm (except B).

Fig. 2. Statistical comparison of circadian rhythm parameters in different SCN regions.

(A) Standard deviation (SD) of phase distribution in the whole, dorsal and ventral SCN. Period (B), amplitude (C), and trough level (D) in the dorsal and ventral SCN are demonstrated as the mean $\pm$ SD. The estimated calcium concentration is shown on the right of graph in C and D. * $p < 0.05$, ** $p < 0.01$. $n = 5$ slices.

Fig. 3. Tetrodotoxin affects the calcium rhythm and disrupts synchronization.

(A) YC 3.60 signals in a representative SCN slice at the peak phase of calcium rhythm on the pretreat day. (B) Image of the SCN immunolabeled with AVP and VIP antibodies. (C) Top: changes in the signal intensity of YC 3.60 over time (142 h) in 3 individual neurons, the location of which is indicated in A with the same color. Bottom: raster plots of calcium rhythm from the dorsal tip to the end of ventral region as indicated by a red line in A. The arrowhead is the approximate boundary between the two regions. (D) Mapping of the rhythm parameters before and during TTX application. Scale bars are 100 μm .

Fig. 4. Statistical comparison of tetrodotoxin effects.

Effect of TTX on the amplitude (A), trough (B), and (C) distribution of phase expressed in SD are analyzed on the pretreat, 1st day and 3rd day of TTX treatment. Data are expressed as the mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 4$ slices. See also the legend of Fig.2 for the details.

Fig. 5. Enlargement of phase difference between the dorsal and ventral regions by tetrodotoxin.

(A) Bihourly images of the acrophase distribution before (pretreat) and during TTX application. The estimated borders of the SCN and the dorsal/ventral regions are shown as broken lines. Number on the upper margin of the top panel indicates the difference from the mean acrophase (hours). Data was obtained from the same SCN as displayed in Fig. 3. Scale bar is 100 μm . (B) Acrophase distribution expressed in histogram on the pretreat, 1st and 3rd day of TTX application. Histograms are normalized relative to the mean phase of the whole slice. (C) Histogram of the acrophase distribution within the dorsal and ventral regions in all 4 slices examined. Y axis is normalized to the peak of each region in a slice. Note that dispersion of 2 peaks is clear following TTX application (arrow heads).

Fig. 6. Effect of calbenoxolone on the calcium rhythm and network synchronization.

(A) YC 3.60 signals in a representative SCN slice near the peak phase of calcium rhythm on the pretreat. (B) Maps of the circadian rhythm parameters before and during CBX application in an SCN. (C) Upper panel shows circadian calcium rhythms before (pretreat) and during CBX treatment (shadowed area). Circadian calcium rhythms of 4 individual cells, indicated in A with circles of the same color, demonstrate that gap-junction blocking has no significant effect on any circadian parameter of calcium rhythms. Lower panel shows raster plots of calcium rhythm from the dorsal tip to the end of ventral region as indicated by a red line in A. See the legend of Fig.1 for the details. A scale indicates 100 μm .

Fig. 7. Statistical comparison of calbenoxolone effects.

Effect of CBX on amplitude (A), trough (B), and the SD of acrophases (F) on the pretreat, 1st and

3rd day of CBX treatment are shown for the whole, dorsal and ventral SCN. Data are expressed as the mean $\pm$ SD of 3 SCN slices. * p < 0.05.

Supplemental Information

Materials and Methods

Animal care

Wild type and PER2::LUC knock-in mice of C57BL/6J background were used in this study. Mice were born and bred in our animal quarters under controlled environmental conditions (temperature: $22 \pm 2^{\circ}\text{C}$, humidity: $60 \pm 5\%$, 12-h light/12-h dark, with lights on from 06:00–18:00). They were reared in polycarbonate cages ($182 \times 260 \times 128$ mm, Clea Japan, Tokyo, Japan) with 1–4 litter mates after weaning at postnatal day 21. Light intensity was ~ 100 lx at the cage surface. They were fed commercial chow and tap water ad libitum.

SCN slice cultures

Decapitation was performed at the middle of the light phase. The brain of neonate mice (4–6 day old) was rapidly removed and dipped in ice-cold balanced salt solution comprising 87 mM NaCl, 2.5 mM KCl, 7 mM MgCl_2 , 0.5 mM CaCl_2 , 1.25 mM NaH_2PO_4 , 25 mM NaHCO_3 , 25 mM glucose, 10 mM HEPES, and 75 mM sucrose. A 200- μm coronal brain slice containing rostrocaudally middle-to-posterior part of the SCN was carefully prepared using a vibratome (VT 1200; Leica Instruments, Nussloch, Germany). 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, MA, USA) in a 35-mm Petri dish containing 1.0 mL of Dulbecco's modified Eagle's medium (Invitrogen, CA, USA) and 5% fetal bovine serum (Sigma-Aldrich, MO, USA). Prior to the recordings, the membrane with a cultured SCN slice was cut out, flipped over, and transferred to collagen-coated glass dishes (Cellmatrix type 1-c; Nitta Gelatin, Osaka, Japan) that were supplemented with 180 μl DMEM and 25% FBS. Glass-base dishes (35 mm, No.1-S; AGC Techno Glass, Japan) were sealed with O_2 -permeable filters (membrane kit, High Sens; YSI Incorporated, OH, USA) using silicone grease compounds (SH111; Dow Corning Toray Co., Tokyo, Japan) and placed on custom-made 6-well recording plate holders. Five % CO_2 in air was continuously supplied to keep the slices healthy. SCN slices were cultured for 2–3 weeks before imaging. We used 5 slices for the experiments in

Fig.1-2, 4 slices for TTX experiments in Fig.3-5, 3 slices for CBX experiments in Fig.6-7, and 3 slices for “vehicle-treated” experiments in Fig.S8. In total, 15 slices were used for calcium imaging in the present study.

Time-lapse calcium imaging

Fluorescence images (512 × 512 pixels, 0.8- μ m resolution, 16-bit intensity) were captured at an exposure of 0.5 s for CFP and Venus images. Images of 100- μ m depth in the z-axis were obtained at 2- μ m z-steps. The imaging system is composed of Nipkow spinning disk confocal unit (CSU10; Yokogawa Electric, Tokyo, Japan), EM-CCD camera (Evolve; Photometrics, AZ, USA), inverted microscope (Ti-E; Nikon, Tokyo, Japan), dry objectives (20 X, 0.75 NA, Plan Apo VC; Nikon), box incubator (TIXHB; Tokai-hit, Shizuoka, Japan), and MetaMorph software (Molecular Devices, CA, USA). FRET-based calcium probe, YC 3.60, was excited at 445 nm with laser diode (Cube; Coherent, CA, USA) and the duration of laser illumination was controlled with a mechanical shutter (VMM-D3; Uniblitz, NY, USA). In the measurement of FRET signals, fluorescence was visualized with 480/30 nm and 535/26 nm emission filters for CFP and Venus (Semlock, NY, USA), respectively. Optical filters are switched rapidly using a filter wheel changer and controller (MAC6000 DC; LUDL, NY, USA). For measuring Venus expression alone (Fig.S1D), YC 3.60-expressing SCN slice was excited with 512/25 nm teal light (Lumencor) and visualized with 550/49 nm emission filter at an exposure of 5 s. A light-emitting diode (LED)-based light source (Spectra 7 Light Engine; Lumencor, OR, USA) was used as an alternative light source for Nipkow-disk confocal (1, 2). A quartz optical fiber (OPTRAN UV Fiber, CeramOptec Industries, MA, USA) with 0.22 NA and a 2-m cable length was used to couple the LED-based light source with CSU10. Images were obtained at 60-min intervals for up to 7 days. Two or 4 CCD images were merged for visualization to cover the entire bilateral SCNs in culture. The laser intensity was adjusted below 30 micro-watts on the objective lens. The total number of pixels in the entire, dorsal, and ventral areas in the SCN slices was $169,009 \pm 38,354$, $72,736 \pm 22,385$, and $96,273 \pm 15,969$, respectively (5 slices). The size of single SCN neurons was $122 \pm 6 \mu\text{m}^2$ ($n = 50$, random cell selection), and the number of SCN neurons in an area of $100 \mu\text{m} \times 100 \mu\text{m}$ was 29 ± 2 cells ($n = 5$, random area selection). Thus, the estimated number of SCN neurons in the entire, dorsal, and

ventral areas in one hemisphere was 303 ± 69 , 130 ± 40 , and 158 ± 29 cells, respectively (all data were expressed as the mean $\pm$ SD).

rAAV-mediated gene transfer into SCN slices

rAAV vector harboring YC 3.60 under the control of the neuron specific human synapsin promoter was prepared as described previously (3) and Fig. 1S. Aliquots of the virus (300 nl) were inoculated onto the surface of the SCN cultures 7–8 days after the preparation of the slices. For facilitating transduction, 20% hypertonic D-mannitol was included in the inoculation solution. Infected slices were further cultured for at least 7–14 days before imaging. One to two weeks after rAAV infection, YC 3.60 expression plateaued, and the signals became bright enough for detection and stable recording (1, 3).

Estimation of intracellular calcium concentration

We calculated the calcium concentration as described previously (1, 4-5). Chelating with EGTA (20 mM) and BAPTA-AM (30 μ M) reduced the Venus/CFP ratio to its minimum value ($R_{\min} = 1.31 \pm 0.03$; 19 cells in 2 slices), while ionophores (10 μ M ionomycin) and CaCl_2 (20 mM) elevated the Venus/CFP ratio to its maximum value ($R_{\max} = 5.18 \pm 0.11$; 43 cells in two slices). The calcium concentration was estimated based on the Hill coefficient and the K_d of YC 3.60 (1.7 and 250 nM, respectively) (5).

Bioluminescence imaging

We performed the bioluminescence imaging as described previously (6). Briefly, bioluminescence images of a cultured SCN slice from PER2::LUC mice were obtained using an imaging system (Luminoview 200, Olympus, Tokyo, Japan) equipped with an EM-CCD camera (ImagEM, Hamamatsu photonics, Hamamatsu, Japan, or iXon3, Andor technology, UK). The images were collected every 60 min (59 min exposure) with the EM-CCD camera cooled at -80°C . For simultaneous recording of YC 3.60 and PER2::LUC, LED-based light source (Lumencor) was coupled with Luminoview 200. YC 3.60 was excited by blue light (438/24 nm) and fluorescence was visualized with 478/40 nm (CFP) and 534/42 nm (Venus) emission filters (Semlock).

Fluorescence images were captured following every bioluminescence imaging at an exposure of 1 s during 1 min break.

Immunohistochemistry

After calcium imaging, the cultures were fixed with 4% paraformaldehyde in 0.1 M PBS and permeabilized using 0.4% Triton X-100 for 60 min. Nonspecific antibody binding was blocked by 60-min incubation with 10% goat serum at room temperature. For labeling AVP and VIP, slices were stained using mouse anti-AVP monoclonal antibody (generous gift of H.Gainer) (1:1,000 dilution) and rabbit anti-VIP polyclonal antibody (1:10,000 dilution, Peptide Institute, Osaka, Japan), respectively. Alexa 405-conjugated goat anti-mouse IgG and Alexa 647-conjugated goat anti-rabbit IgG (1:200 dilution; Invitrogen) were used as the secondary antibodies for AVP and VIP, respectively. The distribution pattern of VIP and AVP positive cells were stereotyped in the SCN. Thus, we analyzed the imaging data by dividing the SCN by AVP/VIP expression patterns. For labeling neurons and glial cells, slices were stained with Nissl (deep-red NeuroTrace, 1:100 dilution; Invitrogen) and mouse anti-GFAP (1:500 dilution; Invitrogen), respectively. Alexa 647-conjugated mouse anti-mouse IgG (1:200 dilution; Invitrogen) was used as the secondary antibody for GFAP. The slices were mounted with Prolong Gold-DAPI (Invitrogen). Fluorescence was visualized using spectral 32-channel detectors under confocal laser scanning microscopy (A1R-FN1; Nikon) in a virtual filter mode.

Materials

We purchased TTX from Tocris Bioscience (Bristol, UK), EGTA from Wako (Osaka, Japan); BAPTA-AM, from Invitrogen; and CBX and ionomycin, from Sigma-Aldrich.

Data analysis: We developed a custom-made program as a plug-in for the ImageJ image processing software. Prior to the analyses, Venus/CFP ratio images were calculated, smoothed with the median filter (1 pixel), and converted to 8-bit intensity. Background signals were selected from a region where no cells were found (referred as the background region). The mean + 3 SDs of the signal intensity of the background region was set as the cut-off level of the signal and background. Pixels with time-averaged values below the threshold level were eliminated from

subsequent analyses. The time series of the ratio image in each pixel, $\{Y_j(t_i); t_i = 1, 2, \dots, N(h)\}$, was fitted to cosine curve $y_j(t) = y_j(t; M_j, A_j, C_j, T_j) = M_j + A_j * \cosine(2\pi i(t - C_j) / T_j)$ using a least-square regression method, where j , $y_j(t)$ is the Venus/CFP ratio 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 Venus/CFP ratio. We used the data over 3 days. The period T was searched within the range of 18–30 h. Significance of curve fitting was tested by Chi-square test ($p < 0.05$), where a uniform uncertainty of SD in the background region was assumed (7). To assess the drug effects, the rhythm parameters were compared with 24 h data before drug application (pretreat).

Statistics

One-way analysis of variance (ANOVA) was used to determine differences between the dorsal and ventral SCN regions. Two-way repeated ANOVA with post-hoc Tukey-Kramer test was used for group differences. Rayleigh test was also used to compare the observed phase distribution. Comparisons between Rayleigh vectors were made with the Watson-Williams f test. Statistical analyses were performed using Prism GraphPad (GraphPad Software, Inc., CA, USA) and Oriana software (KCS, Bangor, U.K.).

Figure Legends

Fig. S1. Detection of circadian calcium rhythm in SCN slices.

(A) A construct of recombinant adeno-associated virus (rAAV) harboring YC 3.60 under the control of human synapsin promoter (P_{hSYN}). ECFP: enhanced cyan fluorescent protein, CaM: calmodulin, cpVenus: circularly permuted Venus. (B) Orthogonal confocal views of Venus expressions in an SCN slice. Note that the expressions are primarily located on the slice surface. 3V: third ventricle, OC: optic chiasma. Arrows indicate the direction from slice surface to bottom. (C) YC 3.60 signals are plotted for 3 days in two representative neurons as indicated in B. Top: Venus and CFP fluorescence signals are individually expressed. Bottom: Changes in Venus:CFP ratio. Venus intensity increases concomitant with the CFP intensity decrease, and vice versa. Ratio between the Venus and CFP does not depend on the expression levels of YC 3.60. (D) Excitation/emission of FRET acceptor (Venus) alone is not rhythmic In YC 3.60-expressing SCN slices. Venus signals are plotted for 3 days in three representative neurons (as indicated in top panel) and in the whole area.

Fig. S2. Expression patterns in YC 3.60 and PER2::LUC.

(A) Time-lapse bioluminescence images of PER2::LUC in an SCN slice for 85 h. (B) Expression patterns of PER2::LUC at trough (left) and peak (middle). Note that PER2::LUC expressions are sparse in the ventral regions of the SCN, whereas YC 3.60 expressions (right) are detectable in the entire SCN regions. (C) Magnified view of PER2::LUC (peak) and Venus expression indicated with green rectangles in B demonstrates that YC3.60 is highly expressed where PER2::LUC was virtually lacking. (D) Left: Images of PER2::LUC and Venus/CFP ratio (at respective peak phases) in an SCN slice. Right: Circadian rhythms in YC 3.60 (yellow) and PER2::LUC (blue) recorded from a same SCN slice demonstrates phase advance of calcium rhythm relative to PER2::LUC in the whole area. In this particular SCN slice, the difference of peak phases was 5.5 h.

Fig. S3. Estimation of transduction efficiency.

(A) Expression pattern of YC 3.60 in an SCN slice. 3V: third ventricle, OC: optic chiasma. (B)

Magnified views of the dorsal (top) and ventral regions (bottom) which were indicated by rectangles in A. SCN neurons were identified by Nissl (neuron marker) and DAPI (nucleus marker). (C) Transduction efficiency of YC 3.60 in the dorsal and ventral regions.

Fig. S4. YC 3.60 signals are not from glial cells.

Expression patterns of YC 3.60 and GFAP at the surface (A) and bottom of an SCN slice. (B). Note that YC 3.60 locate only at the surface, whereas GFAP predominate at the bottom. They do not co-localize. 3V: third ventricle, OC: optic chiasma.

Fig. S5. Procedures for mapping rhythm parameters.

(A) Step-by-step data processing procedures for making a phase map. (B) Raw data (blue) was fitted a cosine curve (green) in each pixel of time-series images. Significance of curve fitting was tested by chi-square analysis ($p < 0.05$). (C) An averaged waveform of all pixels in a slice (204,930 pixels) shows a cosine-like configuration (mean $\pm$ SEM). Error bars are smaller than individual data points. Data in each pixel are standardized to their peak phase.

Fig. S6. Reproducibility of calcium rhythm.

Phase maps of all 5 SCN slices examined are shown to demonstrate reproducibility in topography. Estimated border of the SCN and the dorsal/ventral regions are indicated by broken lines. Colored area where data were successfully fitted to cosine curve coincided with the area of SCN in all 5 slices. Acrophase is depicted and normalized relative to the mean phase of the whole slice. Five of 5 slices examined are displayed.

Fig. S7. Dissociation of rhythm couplings “within” and “between” the regions by TTX.

Rayleigh plots of acrophase distribution (A) were examined separately in the dorsal (green) and ventral (red) regions of the SCN before (pretreat) and during days 1 and 3 of TTX application ($n = 4$ slices). Dotted lines and blue bars show the difference of phase angle (μ). Note the progressive loss of synchrony “within” and “between” the regions during TTX treatment. Mean length of vectors

(r), an extent of phase clustering, gradually decreased in both the dorsal (B) (0.65 ± 0.03 in pretreat, 0.50 ± 0.05 h in TTX at 1st day, 0.43 ± 0.05 h in TTX at 3rd day) and ventral regions (C) (0.82 ± 0.03 in pretreat, 0.65 ± 0.02 h in TTX at 1st day, 0.53 ± 0.03 h in TTX at 3rd day). The decrease was more pronounced in the ventral region. (D) On the other hand, difference of phase angle (μ) between the dorsal and ventral regions (D) increased during the TTX treatment (27.45 ± 4.67 in pretreat, 40.05 ± 8.41 in TTX at 1st day, 46.41 ± 4.05 in TTX at 3rd day), indicating decoupling of rhythms in the two regions. Data are expressed as the mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 4$ slices.

Fig. S8. Effect of medium exchange on the circadian calcium rhythm.

(A) Pseudocolor image of YC 3.60 signals in the SCN slice. The image was taken near the peak phase of the calcium rhythm. (B) Calcium rhythms in 5 representative cells indicated in A with the circle of the same color before and after medium change. Culture medium was exchanged to new one at the time indicated by a red arrow, which demonstrates no effects of medium exchange on cellular calcium rhythm. (C) Maps of the rhythm parameters. Maps were formed by calculating time-series data before and after the medium change using 72 h and 90 h of data, respectively. Four maps were basically the same before and after the medium exchange. (D) The effects of medium change on (1) period, (2) amplitude, and (3) trough level are separately displayed in the two SCN regions. Right abscissa in 1 and 2 indicates the estimated calcium concentration. (E) The effect of medium change on the SD of acrophases in whole (1), dorsal (2), and ventral (3) regions of the SCN. Note that medium exchange had no effect on any parameter of the calcium rhythm. Data are expressed as the mean $\pm$ SD ($n = 3$).

Video S1.

Time-lapse imaging of circadian calcium rhythm in SCN slices (72 h).

Images were obtained from the same SCN as displayed in Fig. 1.

References

1. Enoki R et al., (2012) Single-cell resolution fluorescence imaging of circadian rhythms detected with a Nipkow spinning disk confocal system. *J Neurosci Methods* 207(1):72-9.
2. Saito K et al., (2011) Conjugation of both on-axis and off-axis light in Nipkow disk confocal microscope to increase availability of incoherent light source. *Cell Struct Funct* 36: 237-46.
3. Lütcke H et al., (2010) Optical recording of neuronal activity with a genetically-encoded calcium indicator in anesthetized and freely moving mice. *Front Neural Circuits* 4:9.
4. Ikeda M et al., (2003) Circadian dynamics of cytosolic and nuclear Ca^{2+} in single suprachiasmatic nucleus neurons. *Neuron* 38(2):253-263.
5. Nagai T et al., (2004) Expanded dynamic range of fluorescent indicators for Ca^{2+} by circularly permuted yellow fluorescent proteins. *Proc Natl Acad Sci U S A* 101(29):10554-10559.
6. Inagaki N et al., (2007) Separate oscillating cell groups in mouse suprachiasmatic nucleus couple photoperiodically to the onset and end of daily activity. *Proc Natl Acad Sci U S A* 104(18):7664-7669.
7. Data reduction and error analysis for the physical sciences. 3rd ed., eds Bevington PR, & Robinson DK (2003) (McGraw-Hill Science, New York).

Table S1. Effect of TTX on parameters of circadian calcium rhythms

amplitude (Δ Venus/CFP)				trough level (Venus/CFP)		
	Pretreat	TTX day 1	TTX day 3	Pretreat	TTX day 1	TTX day 3
Dorsal	0.31 $\pm$ 0.04	0.27 $\pm$ 0.03	0.23 $\pm$ 0.02	1.84 $\pm$ 0.05	1.77 $\pm$ 0.02	1.76 $\pm$ 0.03
Ventral	0.41 $\pm$ 0.03	0.30 $\pm$ 0.01	0.25 $\pm$ 0.01	1.85 $\pm$ 0.06	1.80 $\pm$ 0.02	1.78 $\pm$ 0.02

amplitude in calcium concentration (nM)				trough level in calcium concentration (nM)		
	Pretreat	TTX day 1	TTX day 3	Pretreat	TTX day 1	TTX day 3
Dorsal	32.7 $\pm$ 1.2	28.6 $\pm$ 0.7	25.1 $\pm$ 0.6	84.6 $\pm$ 4.0	77.2 $\pm$ 1.5	75.9 $\pm$ 2.3
Ventral	43.6 $\pm$ 1.3	31.5 $\pm$ 0.2	27.0 $\pm$ 0.4	85.6 $\pm$ 4.8	80.3 $\pm$ 1.4	78.5 $\pm$ 1.9

SD of acrophase (h)			
	Pretreat	TTX day 1	TTX day 3
Whole	3.2 $\pm$ 0.3	4.2 $\pm$ 0.2	4.7 $\pm$ 0.2
Dorsal	4.0 $\pm$ 0.5	4.7 $\pm$ 0.3	4.9 $\pm$ 0.3
Ventral	2.3 $\pm$ 0.3	3.1 $\pm$ 0.2	3.8 $\pm$ 0.2

n = 4. Data are expressed as the mean $\pm$ SD

Table S2. Change in the parameters of circadian calcium rhythm by TTX treatment (% of pretreat level) in the dorsal and ventral SCN regions

	amplitude (%)		trough level (%)		SD of acrophase (%)	
	TTX day 1	TTX day 3	TTX day 1	TTX day 3	TTX day 1	TTX day 3
Dorsal	-12.7 ± 6.7	-23.9 ± 6.0	-3.6 ± 1.2	-4.3 ± 1.0	+20.7 ± 10.7	+24.0 ± 11.1
Ventral	-27.7 ± 3.3	-38.7 ± 3.7	-2.6 ± 1.1	-3.5 ± 0.8	+35.4 ± 6.3	+64.5 ± 13.9

n = 4. Data are expressed as the mean ± SEM

Table S3. Effect of CBX on parameters of circadian calcium rhythms

	amplitude (Δ Venus/CFP)			trough level (Venus/CFP)		
	Pretreat	CBX day 1	CBX day 3	Pretreat	CBX day 1	CBX day 3
Dorsal	0.31 $\pm$ 0.03	0.32 $\pm$ 0.03	0.27 $\pm$ 0.02	1.87 $\pm$ 0.01	1.85 $\pm$ 0.00	1.87 $\pm$ 0.02
Ventral	0.37 $\pm$ 0.07	0.37 $\pm$ 0.06	0.34 $\pm$ 0.07	1.87 $\pm$ 0.03	1.85 $\pm$ 0.02	1.87 $\pm$ 0.07

	amplitude in calcium concentration (nM)			trough level in calcium concentration (nM)		
	Pretreat	CBX day 1	CBX day 3	Pretreat	CBX day 1	CBX day 3
Dorsal	33.1 $\pm$ 1.0	34.0 $\pm$ 0.9	29.0 $\pm$ 0.6	88.1 $\pm$ 0.6	86.3 $\pm$ 0.2	88.6 $\pm$ 1.4
Ventral	39.5 $\pm$ 2.6	39.4 $\pm$ 2.3	36.3 $\pm$ 2.6	87.8 $\pm$ 3.0	86.4 $\pm$ 2.0	88.5 $\pm$ 5.9

	SD of acrophase (h)		
	Pretreat	CBX day 1	CBX day 3
Whole	4.2 $\pm$ 0.5	4.3 $\pm$ 0.5	4.5 $\pm$ 0.8
Dorsal	4.2 $\pm$ 0.3	4.4 $\pm$ 0.1	4.6 $\pm$ 0.4
Ventral	2.9 $\pm$ 0.6	3.0 $\pm$ 0.8	3.1 $\pm$ 1.1

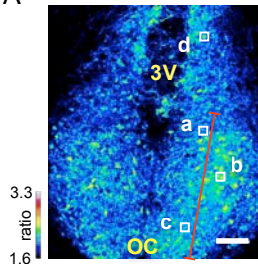
n = 3. Data are expressed as the mean $\pm$ SD

Table S4. Change in the parameters of circadian calcium rhythm by CBX treatment (% of pretreat level) in the dorsal and ventral SCN regions

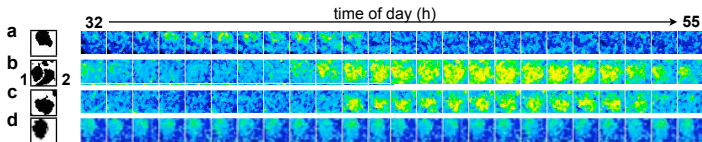
	amplitude (%)		trough level (%)		SD of acrophase (%)	
	CBX day 1	CBX day 3	CBX day 1	CBX day 3	CBX day 1	CBX day 3
Dorsal	+2.9 ± 3.6	−12.2 ± 2.3	−0.9 ± 0.3	+0.3 ± 0.7	+5.0 ± 5.4	+10.9 ± 3.1
Ventral	0.0 ± 2.9	−8.4 ± 2.6	−0.6 ± 1.4	+0.4 ± 1.8	+4.2 ± 3.9	+5.8 ± 8.0

n = 3. Data are expressed as the mean ± SEM

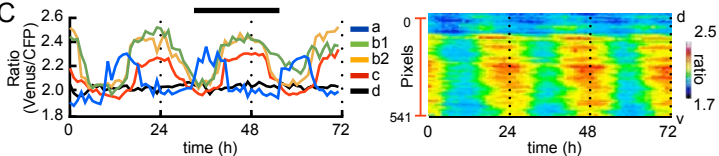
A



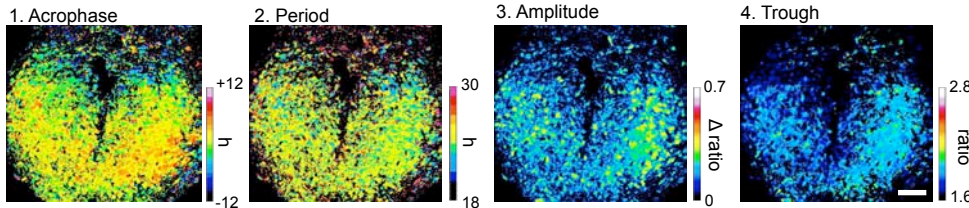
B



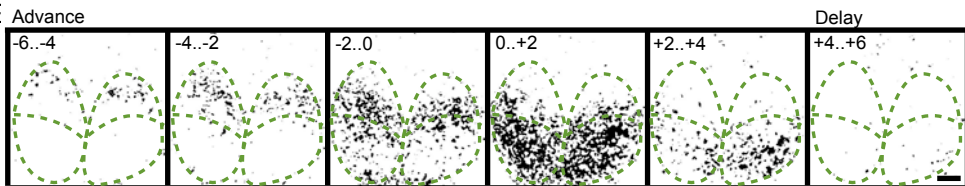
C

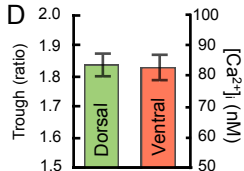
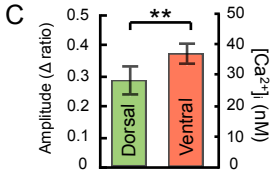
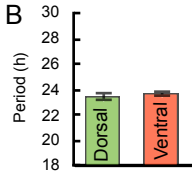
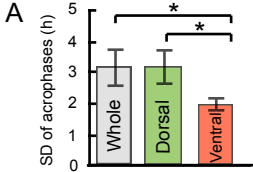


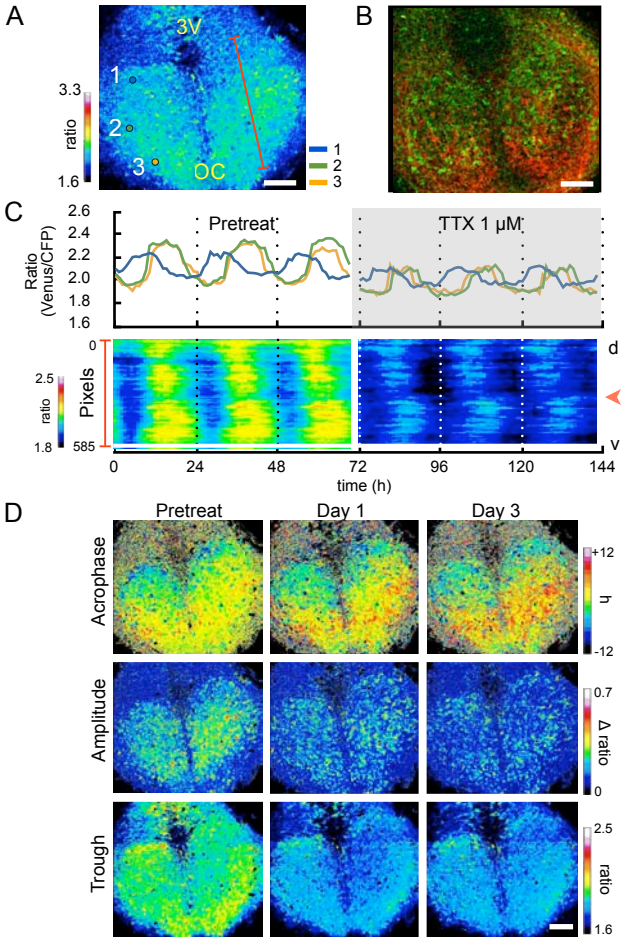
D

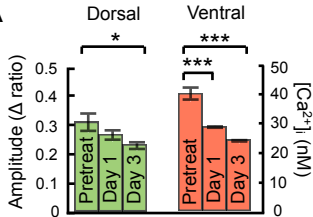
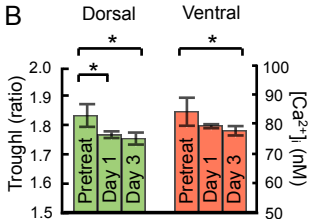
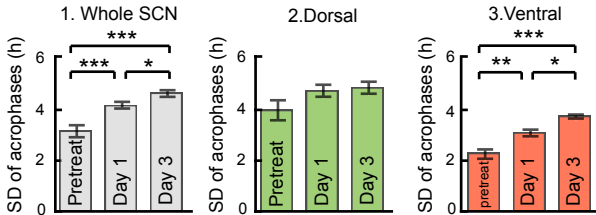


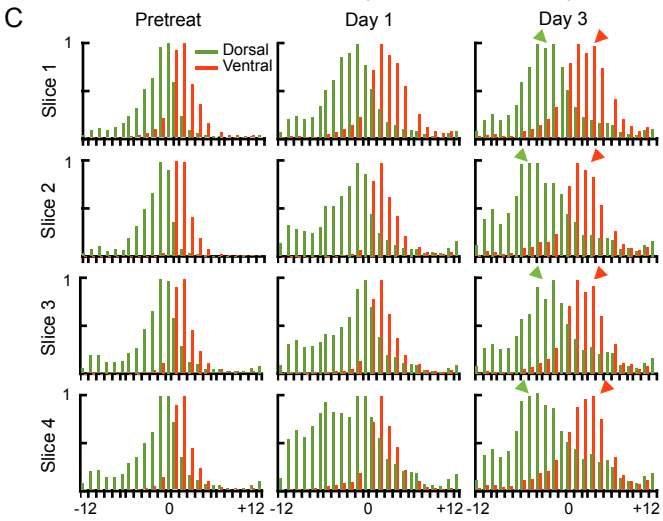
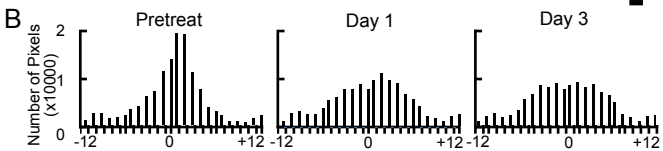
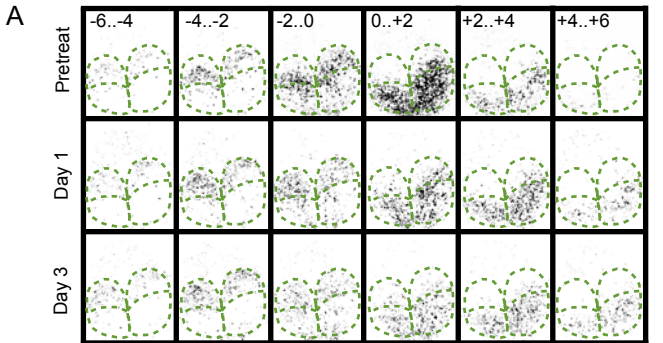
E

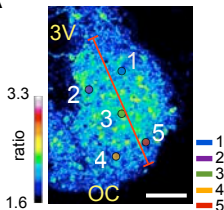
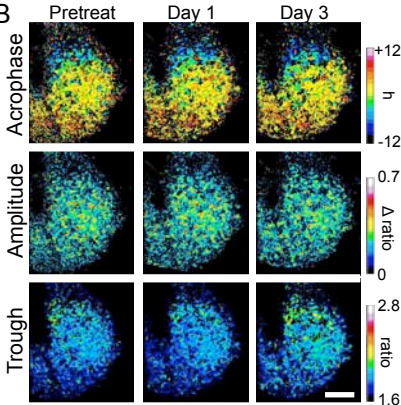
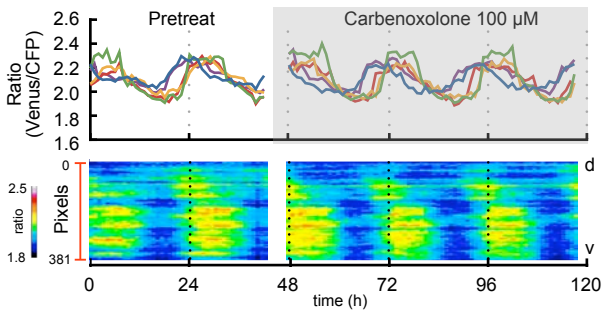


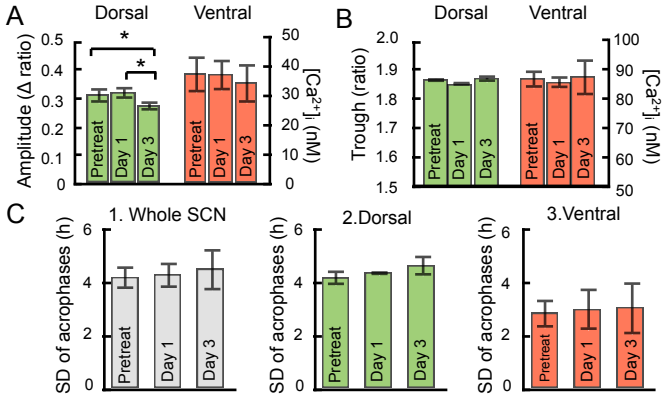


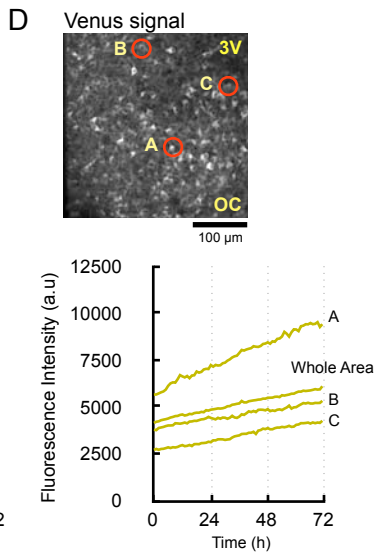
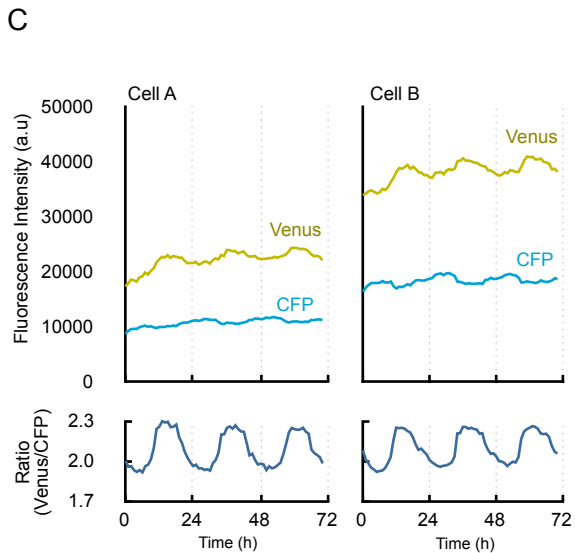
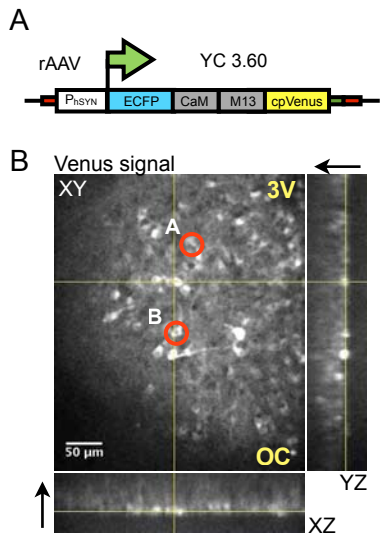


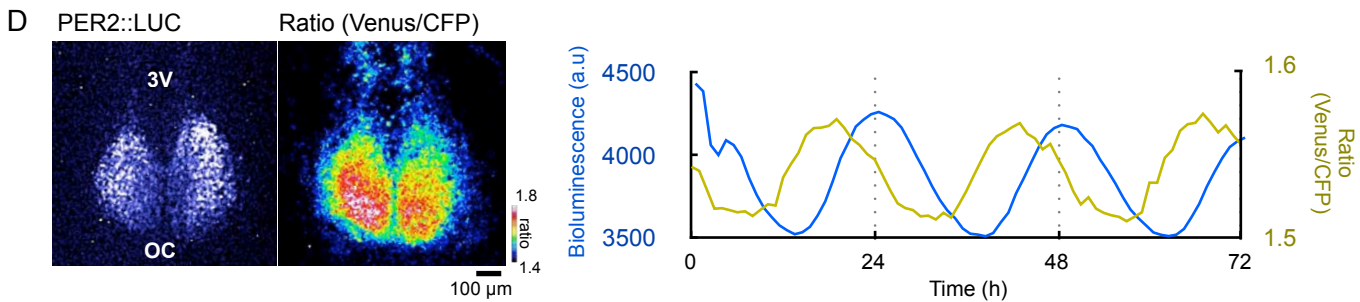
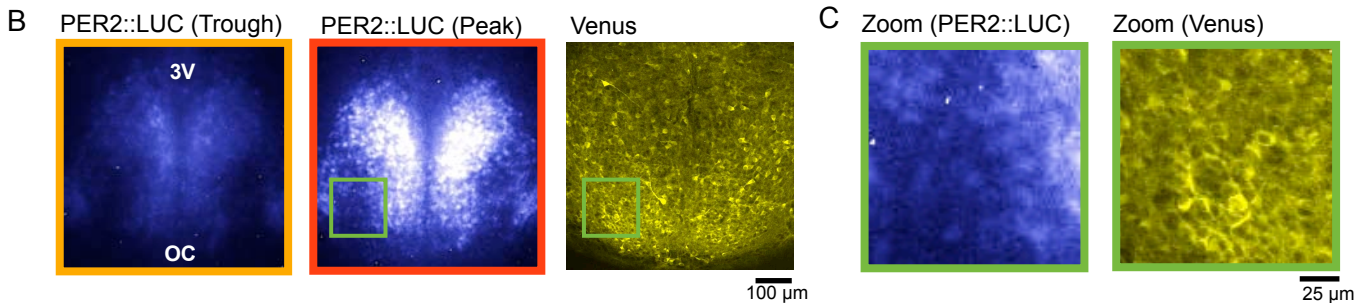
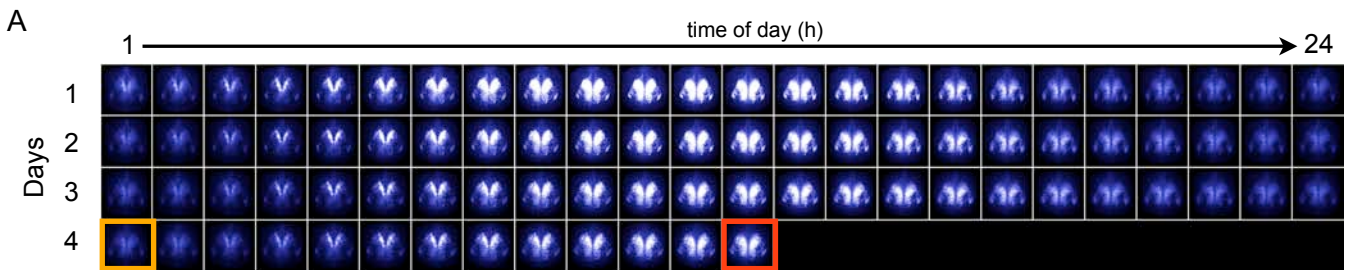
A**B****C**

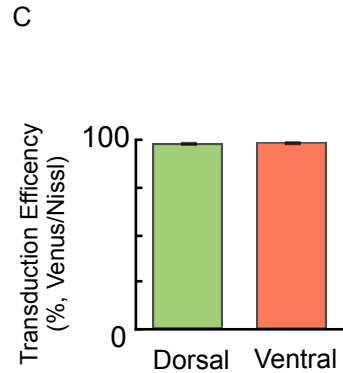
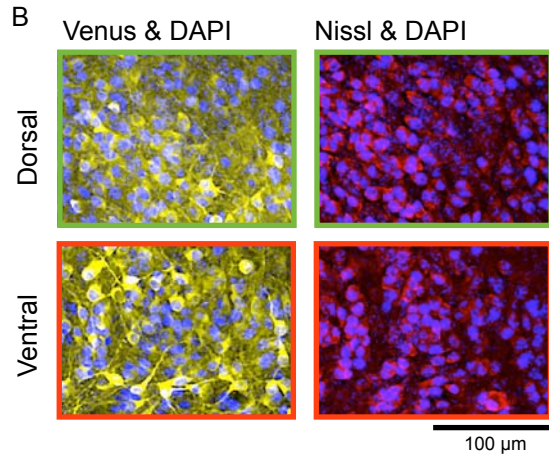
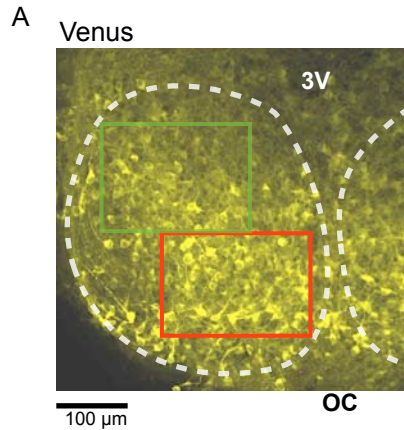


A**B****C**





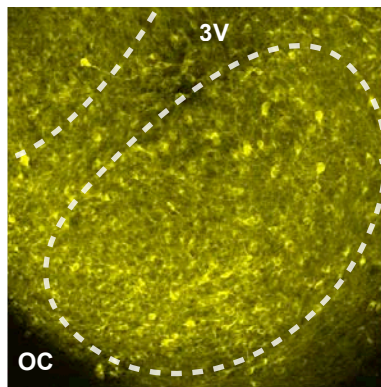




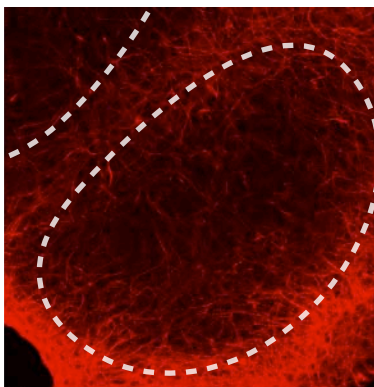
A

Slice Surface

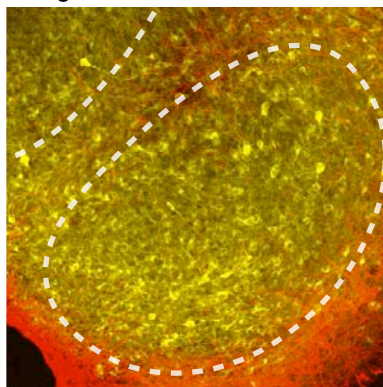
Venus



GFAP



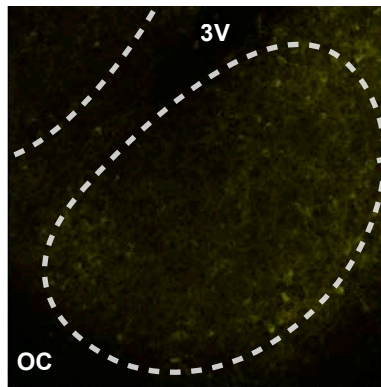
Merge

100 μ m

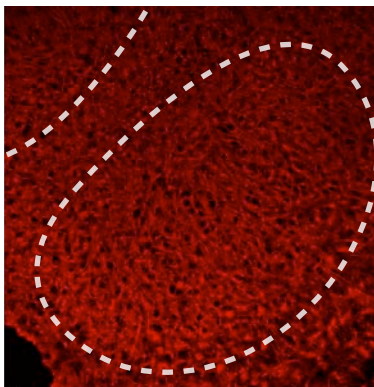
B

Slice Bottom

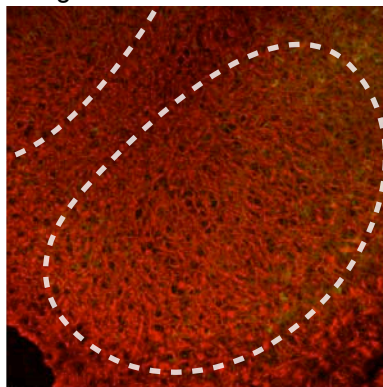
Venus



GFAP

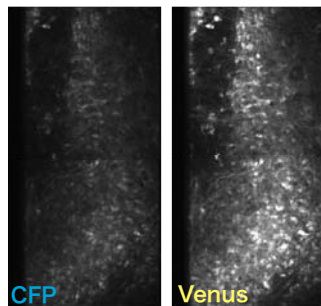


Merge

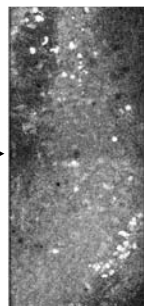
100 μ m

A

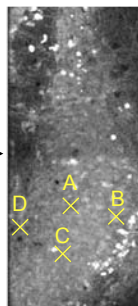
Fluorescent images



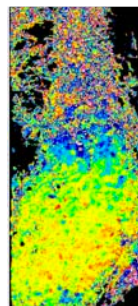
Step 1.
Make projection Images of
Venus and CFP at all time points



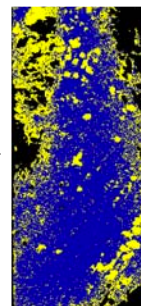
Step 2.
Calculate ratio



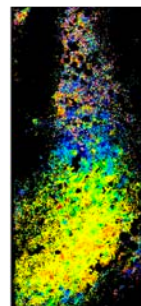
Step 3.
Median filter
(1 pixel)



Step 4.
Make maps

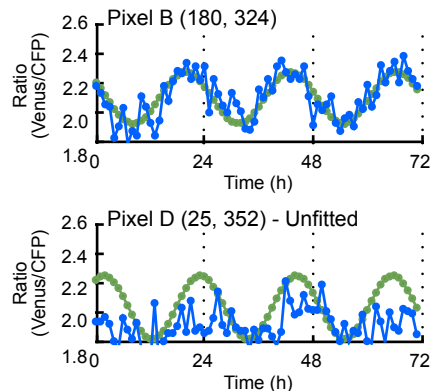
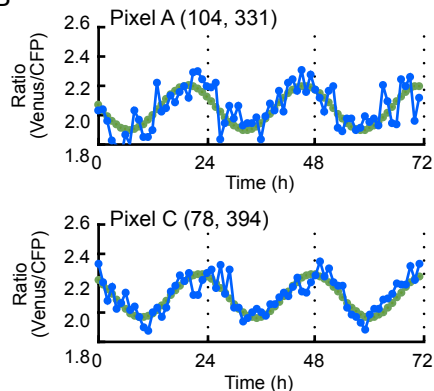


Step 5.
measure the
goodness of
the fitting

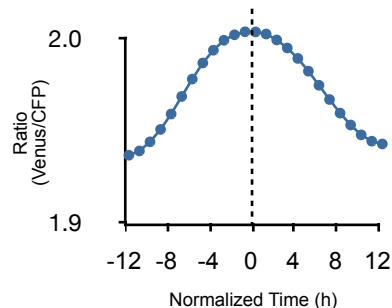


Step 6.
Remove unfitted
pixels

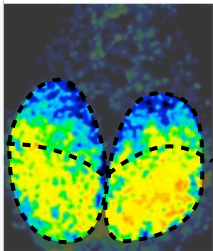
B



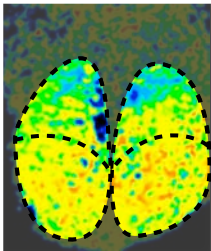
C



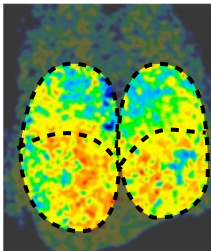
Slice 1



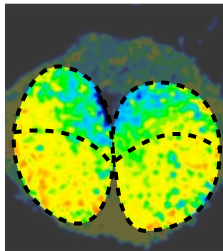
Slice 2



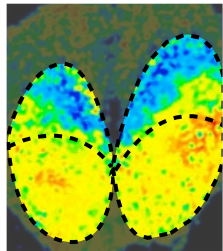
Slice 3



Slice 4



Slice 5



100 μm

A

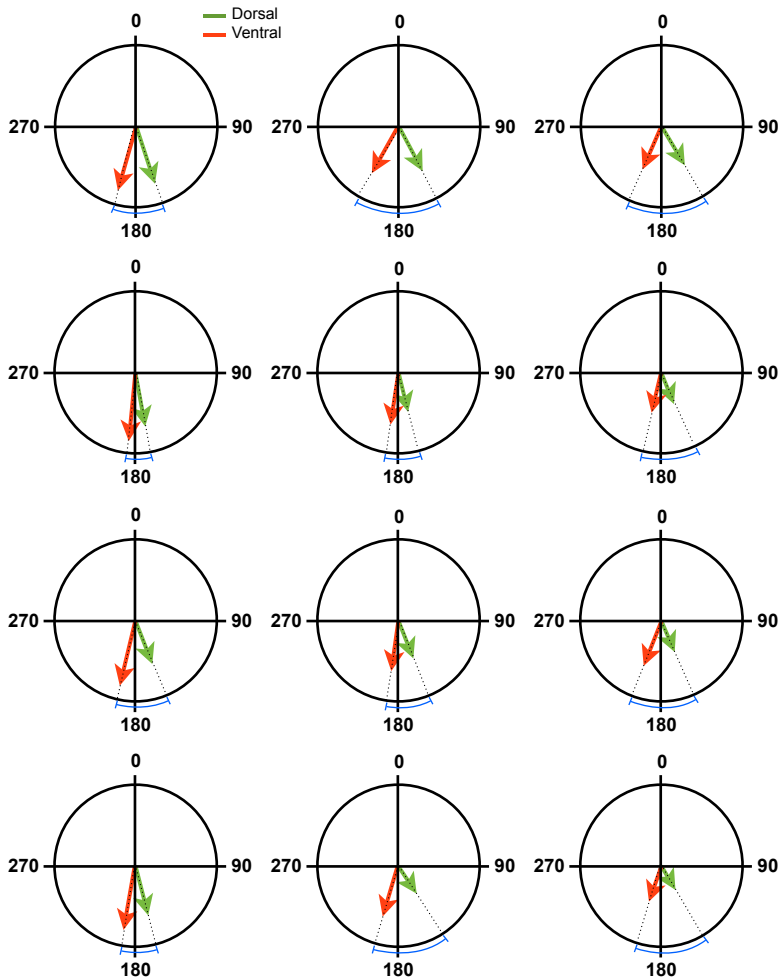
Slice 1

Pretreat

Day 1

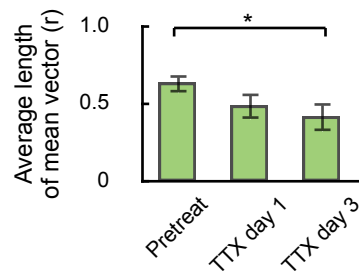
Day 3

— Dorsal
— Ventral



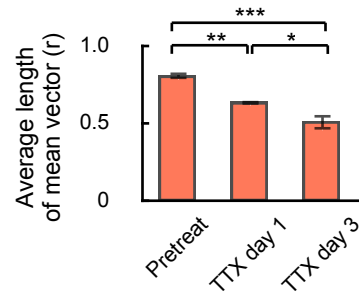
B

Dorsal

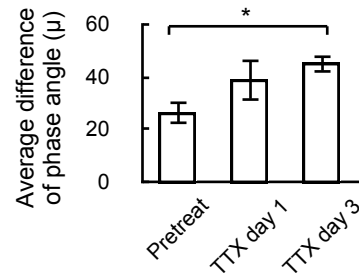


C

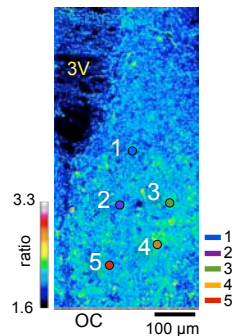
Ventral



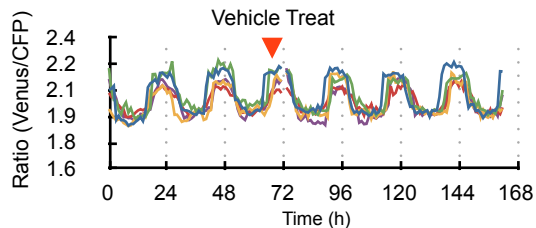
D



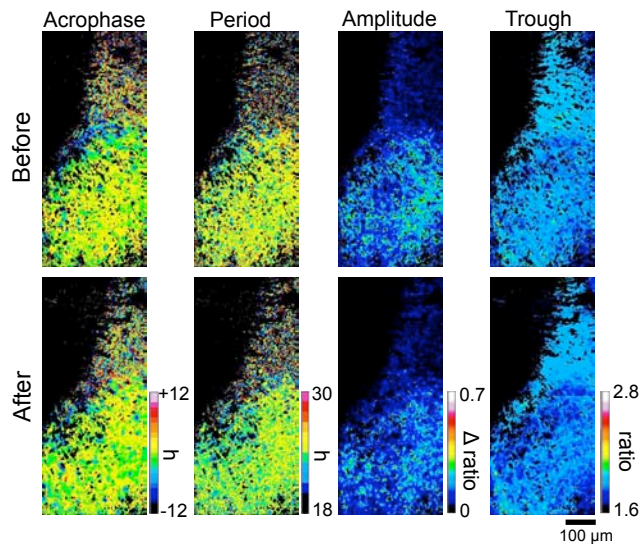
A



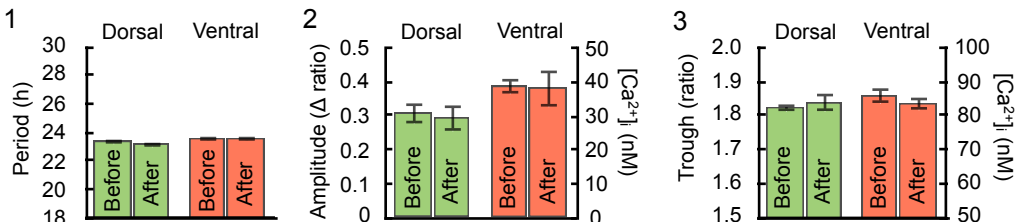
B



C



D



E

