Manipulating circadian clock neuron firing rate resets molecular circadian rhythms and behavior

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To examine the interaction between molecular, electrical and behavioral circadian rhythms, we combined optogenetic manipulation of suprachiasmatic nucleus (SCN) firing rate with bioluminescence imaging and locomotor activity monitoring. Manipulating firing rate reset circadian rhythms both ex vivo and in vivo, and this resetting required spikes and network communication. This suggests that SCN firing rate is fundamental to circadian pacemaking as both an input to and output of the molecular clockworks.

The SCN is the brain’s circadian clock, and it provides a model for studying the interaction between gene networks and behavior. The individual cellular oscillators that comprise the SCN network exhibit endogenous molecular and electrical rhythms. Additionally, a collection of intrinsic currents allows these neurons to fire action potentials in the absence of synaptic drive and to fire with higher frequency (up to 8–12 Hz) during the day while being nearly silent at night (typically <1 Hz)³–². Network communication by the neuropeptides vasoactive intestinal peptide (VIP) and arginine vasopressin (AVP) and the neurotransmitter GABA allow the oscillators to form a tissue-level clock, orchestrating daily changes in physiology and behavior³–⁶. Thus, interlocking molecular and electrical loops in the SCN interact to drive behavior; however, the precise interplay of these molecular, electrical and behavioral components of the brain’s biological clock remains unknown⁷–¹².

The inability to precisely manipulate firing rate in SCN neurons without confounding ionic or pharmacological stimuli has hindered the examination of these relationships. To address this problem, we used SCN-directed expression of the optogenetic constructs channelrhodopsin (ChR2) and halorhodopsin (NpHR) to drive or inhibit SCN neuron firing rate, respectively, both ex vivo and in vivo. Here we show that optogenetic induction or suppression of firing rate within SCN neurons is sufficient to reset the phase and alter the period of the molecular clockworks, that this resetting requires action potentials and VIPergic network communication, and that in vivo optogenetic stimulation of the SCN synchronizes behavioral rhythms. We therefore conclude that SCN firing rate is a key component in circadian rhythmicity and entrainment rather than solely an output of the molecular clock.

To manipulate firing rate in the SCN, we generated mouse lines that expressed either ChR2 or NpHR under an SCN-directed Cre driver (dopamine receptor D1α; Drd1α-ChR2 or Drd1α-NpHR mice) and confirmed transgene expression in the SCN using immunohistochemistry (Online Methods). Optogenetic constructs were highly expressed throughout the SCN in ~90% of AVP⁺ and VIP⁺ neurons, as well as a high proportion of AVP⁻ VIP⁺ SCN neurons (Supplementary Figs. 1 and 2). We were able to increase or decrease SCN neuron firing rate in vitro for 1 h or more with appropriate light input (8 Hz, 470 nm for Drd1α-ChR2 and continuous 590 nm for Drd1α-NpHR SCN neurons, respectively). Optogenetic stimulation of ex vivo SCN slices from Drd1α-ChR2 mice at the typical daytime peak of spontaneous firing rate in SCN neurons (470 nm, 8 Hz, 1 h) resulted in widespread cellular activation in the SCN, with ~90% of ChR2-expressing cells exhibiting gene activation as assayed by c-Fos immunohistochemistry (Supplementary Fig. 3).

By crossing Drd1α-ChR2 or Drd1α-NpHR mice with a PER2:Luc reporter line in which the clock protein PERIOD2 (PER2) is fused to luciferase, we were able to assay the effect of optogenetic manipulation on the molecular clockworks. Ex vivo Drd1α-ChR2 PER2::LUC SCN slices or Drd1α-NpHR PER2::LUC SCN slices were optogenetically stimulated or inhibited, respectively, at varying times relative to the peak of the PER2::LUC rhythm (defined as circadian time 12 (CT 12)). In Drd1α-ChR2 PER2::LUC SCN slices, optogenetic stimulation elicited delaying resets of the molecular clockworks from CT 12–18, advancing resets from CT 18–2 and no or minimal shifts from CT 2–12 (Fig. 1a,b). There were also changes in the period of the PER2 rhythm, with period lengthening resulting from delaying stimuli (CT 12–18), period shortening from advancing stimuli (CT 18–24) and no change in period from stimuli that did not result in phase shifts (CT 0–12; Supplementary Fig. 4). Identical optogenetic stimuli delivered to PER2::LUC SCN slices lacking ChR2 did not result in phase shifts or period changes. In Drd1α-NpHR PER2::LUC SCN slices, optogenetic inhibition resulted in a different pattern of resets, with delay resets from CT 0–6, advances from CT 6–12 and no resets from CT 12–24 (Fig. 1c,d). As with ChR2 stimulation, NpHR inhibition resulted in changes in period, with period shortening from delay-inducing treatments (CT 3–6), period shortening from advancing treatments (CT 9–12) and no change in period if shifts were not induced (CT 12–24; Supplementary Fig. 4). Identical optogenetic stimuli delivered to PER2::LUC SCN slices lacking NpHR did not result in phase shifts or period changes. In both Drd1α-ChR2 PER2::LUC and Drd1α-NpHR PER2::LUC SCN slices, we found effects on period and phase to persist for 5 to 6 d (n = 3 slices per genotype, data not shown).

To investigate the roles of action potentials and intercellular communication in ChR2-mediated changes in phase and period of the molecular clockworks, we used ex vivo optogenetic stimulation of Drd1α-ChR2 PER2::LUC SCN slices in the presence of the sodium
channel blocker tetrodotoxin (TTX) or the VIP receptor blocker [D-p-Cl-Phe6,Leu7]-VIP (VIPX). Stimulation during the delay zone (CT 12–18) in control medium resulted in phase delays and period lengthening as before; however, stimulating slices in medium containing TTX or VIPX inhibited these changes (Fig. 2a,b and Supplementary Fig. 5). Transfer to TTX, VIPX or control medium had no effect on unstimulated Drd1a-ChR2 organotypic slices (Fig. 2b and Supplementary Fig. 5). To determine the effects of TTX on ChR2-induced depolarization, we performed whole-cell current clamp recording on Drd1a-ChR2 neurons from acute SCN slices and measured the response to ChR2 stimulation in the absence or presence of TTX (Fig. 2c). In the absence of TTX, the average peak depolarization amplitude in response to ChR2 stimulation was 43.71 ± 0.47 mV, which included the action potentials riding on top of the ChR2-induced depolarization.

In the presence of TTX, however, depolarization by ChR2 stimulation persisted but was severely reduced in amplitude to 15.25 ± 0.17 mV. In other studies, neural plasticity has been induced by ChR2 stimulation even in the presence of TTX; however, our data suggest that in the SCN, the subthreshold depolarization induced by ChR2 stimulation is insufficient to reset the circadian clock.

Finally, to investigate the effects of manipulating SCN neuron firing rate on circadian behavior, we optogenetically stimulated the SCN of Drd1a-ChR2 mice in vivo over multiple days at a frequency similar to that of the daytime firing rate of SCN neurons. Although ChR2 stimulation allows exact temporal control of firing rate phase locked to pulsed illumination, NpHR inhibition requires continuous illumination and does not allow for such precise control (Supplementary Fig. 2). Thus, we chose ChR2 excitation over NpHR inhibition to test the specific role of clock neuron firing rate in vivo. SCN-targeted expression of ChR2 combined with stereotactic implantation of SCN-directed fiber optics and a limited range of blue light penetration in the brain allowed for specific activation of the SCN (Supplementary Fig. 6). Whereas control mice that lacked ChR2 expression showed no apparent response to repeated optogenetic stimulation of the SCN, the same stimulation entrained Drd1a-ChR2 mice, which aligned the onset of their locomotor behavioral rhythms to the time of stimulation and then continued free running after cessation of the stimulus (Fig. 3). Control mice continued to free run independently of the optogenetic stimulus, with their time of activity onset shifting away from the time of stimulation; conversely, Drd1a-ChR2

**Figure 1** Optogenetic manipulation of SCN neurons ex vivo produces changes in phase. (a) Representative PER2::LUC actograms demonstrating the effects of ChR2-mediated stimulation of Drd1a-ChR2 organotypic slices resulting in advances (left) and delays (right). Dashed gray lines, peak times of the undisturbed pretreatment rhythm extrapolated past the time of manipulation; dashed blue lines, peak times of the post-stimulation rhythm; blue arrows, direction of the resulting phase shift. ChR2+, n = 23 slices, 20 mice; black dots, ChR2−, n = 22 slices, 20 mice; black dots, ChR2−, n = 11 slices, 11 mice. (b) Phase response curve depicting changes of phase for PER2::LUC bioluminescence in Drd1a-ChR2 organotypic slices in response to ChR2-mediated stimulation at varying circadian times. Dashed blue line, Gaussian fit, $r^2 = 0.8510$; blue dots, ChR2+, n = 3 slices, 3 mice; black dots, ChR2−, n = 3 slices, 3 mice; and 3 slices, 3 mice, and 3 slices, 3 mice.

**Figure 2** Pharmacological blockade of action potential generation or VIP signaling ablates phase changes induced by ChR2 stimulation. (a) Representative PER2::LUC actograms of Drd1a-ChR2 organotypic slices subject to a control medium change followed by ChR2 stimulation (STIM, n = 4 slices, 3 mice), a change to medium containing 0.5 µM TTX followed by ChR2 stimulation (TTX, n = 6 slices, 5 mice) or a change to medium containing 1 µM VIPX followed by ChR2 stimulation (VIPX, n = 5 slices, 4 mice). Following the stimulation interval, all slices were switched back to control medium. Dashed gray lines, peak times extrapolated past the time of manipulation to project the timing of the undisturbed rhythm; dashed blue lines, peak times of post-manipulation rhythms; blue arrows, time of stimulation (470 nm, 1 h, 8 Hz); black arrows, direction of the resulting phase shift, if any. (b) Changes in phase of PER2::LUC rhythms in response to combinations of ChR2 stimulation (470 nm, 1 h, 8 Hz; STIM), TTX and/or VIPX. From left to right, n = 4 slices, 4 mice; 4 slices, 3 mice; 6 slices, 5 mice; 5 slices, 4 mice; 3 slices, 3 mice; and 3 slices, 3 mice. One-way ANOVA with post hoc Tukey’s honestly significant difference (HSD) test, $F = 21.47, ^*P < 0.0001$. (c) Representative voltage traces in response to ChR2 stimulation in the absence or presence of TTX (left). Dashed gray line, −50 mV. Peak depolarization amplitudes in response to ChR2 stimulation were significantly reduced in the presence of TTX (right). Mann-Whitney U, $^*P = 0.0286$; n = 4 cells. Means ± s.e.m.
mice entrained to the stimulus, with activity onset progressively moving toward the time of stimulation until activity onset locked on to the time of stimulation or shortly after.

The application of optogenetics to the SCN has allowed us to test the fundamental role of firing rate in influencing molecular and behavioral circadian rhythms. Artificial induction or suppression of firing rate across the SCN ex vivo has upstream effects on the phase and period of clock gene expression: the pattern of phase shifts elicited by ChR2 stimulation is essentially identical to that of light, which acts on the SCN through depolarizing glutamate release from retinal ganglion afferents15,16, whereas the pattern of phase shifts resulting from NpHR inhibition is similar to clock resetting by dark pulses or other nonphotic stimuli that are thought to act through inhibition of SCN neuron activity17,18. Induction of firing rate in vivo also has downstream effects on locomotor behavior consistent with its phase-shifting effects observed ex vivo, suggesting that increasing SCN firing rate per se is potentially behaviorally equivalent to light stimulation in its action on the circadian system. Additionally, our results show that pharmacological blockade of coupling or firing rate prevents phase shifts ex vivo, which suggests a stronger role for VIPergic network communication and for action potentials versus sub-threshold depolarizations in the resetting of the molecular clockworks. Together, these data indicate that manipulation of SCN firing rate is sufficient to produce lasting changes within the circadian clock. These results, and the stimulation system used to produce them, will serve as a foundation for future experiments in circadian neurobiology investigating the interaction between the molecular, electrical and behavioral components of the brain’s circadian clock.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

J.R.J., M.C.T. and D.G.M. designed the experiments. J.R.J., M.C.T. and D.G.M. performed all in vivo and ex vivo experiments. J.R.J. and M.C.T. performed all in vivo experiments. J.R.J., M.C.T. and D.G.M. prepared the paper. J.R.J. analyzed data. J.R.J. prepared the figures.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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**ONLINE METHODS**

**Animals.** *Drd1a-Cre* (B6.FVB(Cg)-Tg(Drd1a-Cre)EY266Gsat/Mmucd, GENSAT; Supplementary Fig. 1) mice were crossed with Cre-dependent *R2::lUC* (Ai27D, B6.Cg-Gt(ROSA)26Sortm27.1(CAG-COP4*H134R/tdTomato)Haze/J, Jackson Laboratories) or *NpHR* (Ai99, B6.129S5-Gt(ROSA)26Sortm39.9(CAG-hsp-EGFP/J)Hze/J, Jackson Laboratories) mice to yield *Drd1a-ChR2* or *Drd1a-NpHR* mice. A subset of *Drd1a-ChR2* and *Drd1a-NpHR* mice were crossed with PER2::lUC mice, as an AVP+ or VIP+ (i.e., green fluorescent) neuron completely surrounded by red *Drd1a-NpHR* plasma. Immunohistochemistry. *Drd1a-ChR2* mice were given a single injection of colchicine (2.5 mM; Sigma) in the lateral ventricle. –24 h later, colchicine-injected mice were deeply anesthetized and transected perfused with 4% (w/v) paraformaldehyde (PFA; Sigma). Brains were removed and post-fixed with 4% PFA overnight, and cryoprotected in 20% sucrose in PBS. A cryostat was used to obtain 40 µm coronal slices containing the SCN. Slices were then labeled for AVP using rabbit polyclonal anti-vasopressin (1:5000, ab1565, Millipore) or VIP using rabbit polyclonal anti-VIP (1:2500, ab38441, Abcam). For c-Fos experiments, membrane-attached organotypic SCN cultures were fixed for 1 h in 4% PFA post-chR2 stimulation and labeled for c-Fos using rabbit polyclonal anti-c-Fos (1:1000, ab7963, Abcam). For visualization, slices were incubated with Alexa Fluor 488 goat anti-rabbit IgG (1:500, Invitrogen A-11034 for secondary Ab). Slices were examined under a confocal microscope (LSM510; Zeiss) at 488 nm excitation. Imaging was performed using either a Leica TCS SP5 or a Zeiss LSM 510. Immunoreactive staining was analyzed using ImageJ. Pharmacological manipulation of PER2::lUC cultures. For some experiments, slices were cultured and recorded as above, but when they were removed from the incubator, slice cultures were either transferred to prewarmed recording medium, fresh recording medium containing 0.5 µM TTX or fresh recording medium containing 1 µM [p-(Cl-Phe6,Leu17)-VIP (Tocris cat. no. 3045)]. TTX concentration was selected from Brancaccio et al. and Kudo et al. with 0.5 µM VIP concentration was based on values from Evans et al. and Atkin et al. (2010). The original recording medium was sealed in its culture dish and kept warm inside the incubator. Slices were then placed in the incubator without stimulation for 1 h or stimulated as above, after which all slice cultures were rinsed, transferred to their original recording medium, and incubated with vacuum grease and returned to the LumiCycle for further bioluminescence recording.

In vivo optogenetic stimulation and locomotor activity monitoring. Anesthetized mice (100 mg/kg ketamine, 10 mg/kg xylazine) were placed into a stereotactic device and implanted with a fiber-optic cannula (5 mm in length, 400 µm diameter core, 0.39 NA; Thorlabs) sheathed to prevent light leak. The cannula was targeted to the SCN using the coordinates of +0.0 mm anterior and +0.0 mm lateral to bregma. After at least 3 d of recovery, mice were placed individually into litter-filled cages equipped with an infrared motion detector (Spy2, Visonic) inside light-tight boxes, and food and water was provided ad libitum. Animals were chronically tethered to a fiber-optic cable (400 µm diameter core, 0.39 NA; Thorlabs) attached to the implanted cannula and connected to a high-powered blue (470 nm) LED (Thorlabs) under the control of an LED driver (DC4100; Thorlabs). Mice were kept in constant darkness (DD) and allowed to free run for at least 4 d before stimulation. Locomotor activity was monitored in 5 min bins using ClockLab software (Actimetrics). Light pulses (470 nm, 8 Hz, 10 ms duration, 1 h) were generated at various CTs, with CT 12 defined as the start of locomotor activity, by a Grass SD-9 stimulus generator attached to the LED driver under the control of a light timer, and repeated daily at the same clock time. Light intensity at the cannula tip was determined to be 10.9 mW ± 0.1 mW when driven at 1,000 mA using a PM100D Optical Power Meter (Thorlabs). Calculated irradiances, along with the estimated ChR2 activation threshold, are shown in Supplementary Figure 6. Animals were excluded from analysis if they did not successfully free run in DD during the 4 d after surgery but before stimulation.

**PER2::lUC data analysis.** Sample sizes were chosen so that the data were adequate for statistical analysis based upon previous publications detailing similar measurements both *in vivo* and *in vitro*. Data analysis was performed blind to genotype, although there were no methods to randomize mice to experimental groups or to blind investigators to genotype for the duration of the experiment. Baseline subtracted bioluminescence data were obtained using a 24-h running average from the raw data using LumiCycle data analysis software (Actimetrics). Subtracted bioluminescence data were then loaded into Matlab (Mathworks) for 25 U/ml penicillin/streptomycin, 2% B27 and 0.1 mM beetle luciferin (Promega). Slice cultures containing the SCN were maintained in an incubator at 36.8 °C. Bioluminescence was monitored in real time with a LumiCycle (Actimetrics). After a minimum of two cycles of bioluminescence recording, slice cultures were removed from the LumiCycle and, while still in the incubator, placed under a custom-built high-power LED array consisting of blue Cree XP-E or yellow Luxeon Rebel LEDs (LEDSupply) soldered to an 0.25 µm aluminum heat sink heat sink and driven at 1,000 mA using an LED11B high-powered LED driver (Thorlabs) and subjected to either pulsed blue light (470 nm, 8 Hz, 10 ms duration) controlled by a Grass SD-9 stimulus generator (Grass Technologies) or continuous yellow light (590 nm) for 1 h. Light intensity inside the 35-mm culture dish was determined to be 20.1 mW ± 0.5 mW (470-nm LEDs) or 16.27 mW ± 0.3 mW (590-nm LEDs) when driven at 1,000 mA using a PM100D Optical Power Meter (Thorlabs). Control unstimulated slices were instead removed from the LumiCycle and kept in the incubator for an equivalent time without stimulation. Slice temperature before and after stimulation was measured with an infrared thermometer (Fluke) and was determined to not change (±0.1 °C) after stimulation (data not shown). Slice cultures were then returned to the LumiCycle, and bioluminescence was recorded for at least two additional cycles; data were excluded if bioluminescence did not persist for at least five total cycles.

**Slice preparation and electrophysiological recording.** Brains were removed and blocked in cold, oxygenated 95% O2/5% CO2 dissecting solution (in mM: 114.5 NaCl, 3.5 KCl, 1 NaH2PO4, 1.3 MgSO4, 2.5 CaCl2, 10 d-glucose and 35.7 NaHCO3). SCN slices (200 µm) were cut on a vibroslicer (WPI) at 4–10 °C and transferred directly to an open recording chamber continually superfused with warmed (35 ± 0.5 °C) extracellular solution (in mM: 124 NaCl, 3.5 KCl, 1 NaH2PO4, 1.3 MgSO4, 2.5 CaCl2, 10 d-glucose and 26 NaHCO3). Slices were allowed to recover for 1 h before recording. SCN neurons were visualized using a Leica DMLFS microscope (Leica Microsystems) equipped with near-infrared differential interference contrast and fluorescence optics. For cell-attached recordings, patch electrodes (4–6 MΩ) pulled from glass capillaries (WPI) on a multistage puller (DMZ; Zeitz) were filled with extracellular solution. For whole-cell current-clamp recordings, patch electrodes (8–10 MΩ) were filled with intracellular solution containing, in mM, 135 K-Gluconate, 10 KCl, 10 HEPES, 0.5 EGTA and 2 MgCl2. Recordings were obtained with an Axopatch 200B amplifier (Molecular Devices) and monitored online with pClamp 10.2 software (Molecular Devices). Slices were subjected to pulsed blue light (470 nm, 8 Hz, 10 ms duration) from a mounted high-power LED (Thorlabs) controlled by a Grass SD-9 stimulus generator (Grass Technologies) or continuous yellow light (590 nm) from a mounted high-power LED (Thorlabs). In some experiments, superfused extracellular solution was switched to extracellular solution containing 0.5 µM TTX (Sigma) after ~1.2 min of whole-cell current clamping recording.

*Ex vivo culture and PER2::lUC imaging.* Brains from mice killed without anesthesia by cervical dislocation were removed and blocked in cold HBSS supplemented with 100 U/ml penicillin/streptomycin, 10 mM HEPES, and 4.5 mM sodium bicarbonate. Hypothalamic coronal slices (200 µm) containing the SCN were cut on a vibroslicer (WPI) at 4–10 °C, trimmed to ~1.5 x 1.5 mm squares and transferred directly to culture membranes (Millipore) in vacuum grease-sealed 35-mm culture dishes with recording medium containing 1.0 ml of DMEM (D-2902; Sigma) supplemented with 3.5 g/L d-glucose, 10 mM HEPES,
further analysis. Data were smoothed using a Loess local regression filter and a damped sine wave was fit from the beginning of the smoothed data until the time of manipulation. The damped sine wave was then extrapolated past the time of manipulation to project the timing of the undisturbed rhythm. Phase shifts were determined by calculating the mean difference in the post-manipulation peaks and troughs of the actual rhythms and the extrapolated unstimulated rhythms on the first and second cycles after stimulation. Period changes were calculated in ClockLab analysis software (Actimetrics) by measuring the best fit line through the calculated acrophases before and after stimulation. For data visualization, smoothed baseline-subtracted bioluminescence rhythms were depicted as double-plotted actograms created using Matlab. CT 12 was defined as the peak of PER2::LUC bioluminescence.

**In vivo data analysis.** Sample sizes were chosen so as to be sufficient for statistical analysis based upon previous publications detailing similar measurements both in vivo and in vitro. Data analysis was performed blind to genotype, whereas there were no methods to randomize mice to experimental groups or to blind investigators to genotype for the duration of the experiment. A best fit line was drawn between the times of activity onset until the time at which the change in time of activity onset was less than ±0.1 h a day (i.e., when the animal “locked on” to the stimulus) or until cessation of the stimulus (such as in the case of control actograms). If the animal locked on to the stimulus as defined above, a second best fit line was drawn between these times of activity onset. The clock time of the best fit line(s) was then subtracted from the clock time of the daily optogenetic stimuli and the absolute value (i.e., activity onset in hours from time of stimulation) was plotted against days of stimulation for both ChR2⁺ and control animals.

**Statistical analysis.** All statistical analyses (Student’s t-test, one-way ANOVA, Tukey’s HSD, Mann-Whitney U) were performed in Matlab, with α defined as 0.05. A Bartlett’s multiple-sample test and a Kolmogorov-Smirnov test were used to confirm equal variance and normality. Curves were fit using Matlab’s Curve Fitting Toolbox (Mathworks). Data are presented as means ± s.e.m.

A Supplementary Methods Checklist is available.

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