GABA and Gi/o differentially control circadian rhythms and synchrony in clock neurons

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Neurons in the mammalian suprachiasmatic nuclei (SCN) generate daily rhythms in physiology and behavior, but it is unclear how they maintain and synchronize these rhythms in vivo. We hypothesized that parallel signaling pathways in the SCN are required to synchronize rhythms in these neurons for coherent output. We recorded firing and clock-gene expression patterns while blocking candidate signaling pathways for at least 8 days. GABA \(_A\) and GABA \(_B\) antagonism increased circadian peak firing rates and rhythm precision of cultured SCN neurons, but GABA \(_i/o\) did not impair synchrony or rhythmicity. In contrast, inhibiting GABA \(_i/o\) with pertussis toxin abolished rhythms in most neurons and desynchronized the population, phenocopying the loss of vasoactive intestinal polypeptide (VIP). Daily VIP receptor agonist treatment restored synchrony and oscillations of SCN neurons (13). How VIP mediates circadian rhythms and synchrony between SCN neurons (3, 4). Neurotransmitters likely mediating synchrony within the population, phenocopying the loss of VIP signaling converges with VIP signaling to maintain and coordinate rhythms among SCN neurons.

GABA and GABA \(_i/o\) differentially control circadian rhythms and synchrony in clock neurons

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Neurons in the mammalian suprachiasmatic nuclei (SCN) generate daily rhythms in physiology and behavior, but it is unclear how they maintain and synchronize these rhythms in vivo. We hypothesized that parallel signaling pathways in the SCN are required to synchronize rhythms in these neurons for coherent output. We recorded firing and clock-gene expression patterns while blocking candidate signaling pathways for at least 8 days. GABA \(_A\) and GABA \(_B\) antagonism increased circadian peak firing rates and rhythm precision of cultured SCN neurons, but GABA \(_i/o\) did not impair synchrony or rhythmicity. In contrast, inhibiting GABA \(_i/o\) with pertussis toxin abolished rhythms in most neurons and desynchronized the population, phenocopying the loss of vasoactive intestinal polypeptide (VIP). Daily VIP receptor agonist treatment restored synchrony and oscillations of SCN neurons (13). How VIP mediates circadian rhythms and synchrony between SCN neurons (3, 4). Neurotransmitters likely mediating synchrony within the population, phenocopying the loss of VIP signaling converges with VIP signaling to maintain and coordinate rhythms among SCN neurons.

The suprachiasmatic nuclei (SCN) of the mammalian hypothalamus serve as a master circadian pacemaker, mediating daily rhythms in behavior and physiology. SCN pacemaker function depends on near-24-h oscillations in expression of “clock genes” and in firing rate (1). Previous studies have shown that of the nearly 20,000 neurons in the bilateral SCN, one subset comprises cell-autonomous circadian clocks, and another subset requires vasoactive intestinal polypeptide (VIP) signaling to maintain daily rhythms (2).

For the SCN to coordinate coherent behavioral rhythms, SCN neurons must synchronize to one another in vivo. Blocking action potentials desynchronizes circadian rhythms among SCN neurons (3, 4). Neurotransmitters likely mediating synchrony within the SCN include VIP and GABA (2). VIP is necessary for synchrony between SCN neurons in vitro (5, 6) and for coherent behavioral rhythmicity in vivo (7, 8). GABA has been implicated because most (if not all) SCN neurons express GABA and its receptors (9–11). GABA is released in a daily rhythm within the SCN (12), and daily application of exogenous GABA synchronizes firing-rate rhythms of SCN neurons (13). How VIP mediates circadian synchrony and the necessity of GABA in this process have not been tested.

To examine the roles of endogenous GABA and G protein signaling, we recorded Period::luciferase (Per1::luc) expression from SCN slices and firing rate and Period2::luciferase (PER2::LUC) expression from individual SCN neurons. We find that long-term antagonism of GABA signaling increases the peak firing rate and precision of circadian rhythms, but surprisingly it does not impair SCN synchrony or oscillations. In contrast, inhibition of GABA \(_i/o\) activity with pertussis toxin (PTX) dramatically impairs coordination of daily rhythms among SCN neurons and abolishes rhythms in a subset of neurons. We conclude that GABA controls the amplitude of circadian rhythms in SCN neurons, and G protein-mediated signaling synchronizes these rhythms.

Results

PTX and Tetrodotoxin (TTX), Not GABA Receptor Antagonists, Damp SCN Ensemble Rhythms. To assess the roles of endogenous signaling pathways on SCN rhythms and synchrony, we first screened the effects of selective antagonists on Per1::luc rhythms of cultured SCN explants. Antagonism of pathways required for SCN pacemaking or intercellular coordination would be predicted to decrease the peak-to-trough amplitude of ensemble rhythms recorded from the explant. GABA \(_A\) and GABA \(_B\) receptor antagonists (200 \(\mu\)M bicuculline and 100 \(\mu\)M saclofen, respectively; BIC+SAC) gradually increased Per1::luc amplitudes compared with controls; this relationship was statistically significant after 10 cycles of BIC+SAC treatment (\(P < 0.05\); \(n = 6\) BIC+SAC explants and 11 controls, one-way ANOVA with Scheffé post hoc test; Fig. 1). Using whole-cell recordings, we confirmed that BIC+SAC effectively blocked GABA-evoked inhibitory postsynaptic currents for at least 10 days with no homeostatic increase in GABA signaling or sensitivity to GABA [see supporting information (SI) Fig. 4]. In contrast, treating SCN slices with either the voltage-gated sodium channel blocker TTX (2 \(\mu\)M; \(n = 9\)) or an inhibitor of GABA receptors, PTX (5 \(nM\); \(n = 9\)) significantly reduced the amplitude of Per1::luc rhythms relative to baseline. Normalized peak-to-trough amplitudes for both PTX- and TTX-treated SCN were significantly lower from the third cycle of treatment until the end of recording (\(P < 0.05\)). Importantly, normalized amplitudes recovered to control levels within 5 days after washout of both TTX and PTX. Cholera toxin, a constitutive activator of G protein subunit \(G_{s}\), produced a similar rate of damping to PTX- and TTX-treated treated cultures that was not reversible (\(n = 3\); data not shown). These results indicate that, unlike GABA, G protein signaling is necessary to maintain ensemble rhythm amplitude.

Damping of TTX- and PTX-Treated Slices Is Consistent with Damping and Desynchrony of Neuronal Rhythms. TTX blocks spike-induced neurotransmitter release in the SCN, and PTX irreversibly ADP-
Per1 damping of rhythms in individual neurons, or both on ensemble application of these drugs damps SCN ensemble rhythms, we hypothesized that PTX would have little effect on peripheral tissues, which lack neurotransmission. To test this hypothesis, we applied 5 nM PTX to mouse PER2::LUC SCN and liver explants. PTX caused bioluminescence rhythms from PER2::LUC SCN (n = 4) to damp at a rate similar to that of PTX-treated rat Per1::luc SCN, but it did not change the rate of damping for PER2::LUC liver rhythms (SI Fig. 6; n = 5; P > 0.2 for all cycles). Interestingly, PTX-treated SCN damped at the same rate as liver without PTX treatment (n = 5; P > 0.05 for all cycles). We conclude that PTX reduces the amplitude of circadian rhythms specifically by blocking pathways endogenous to the SCN but not represented in the liver.

PTX, Not GABA Receptor Antagonism, Impairs SCN PER2::LUC Rhythms and Synchrony in Individual Neurons. To test the roles of GABA and Gi/o in the SCN further, we recorded PER2::LUC rhythms from individual neurons in mouse SCN slices (Fig. 2). We found that 59% of all untreated neurons (111 of 188 from three slices) recorded over 3 days showed statistically significant circadian rhythms. GABA blockade for 8–10 days increased the percentage of rhythmic neurons to 79% (208 of 264). Consistent with predictions from Per1::luc and simulation results, individual neurons showed peak bioluminescence at similar times of day, both under control conditions and on days 8–10 of BIC+SAC treatment (P < 0.05, Rayleigh test, for all three SCN). BIC+SAC narrowed the period distribution (P < 0.01, Levene and Brown–Forsythe tests), and it shortened the mean period of PER2::LUC cycling (23.8 ± 1.8 h treated vs. 24.8 ± 2.5 h control, mean ± SD; P < 0.00005). These data suggest that GABA signaling is not required for SCN clock-gene rhythms or synchrony.

In contrast to BIC+SAC, long-term PTX treatment decreased the proportion of rhythmic neurons (to 45%; 106 of 235 neurons) and the synchrony between rhythmic neurons. Reduced synchrony was apparent after 8–11 days by disrupted coordination of peak bioluminescence timing among neurons within each slice (P > 0.1, Rayleigh test; n = 3) and a broadened distribution of periods (P < 0.005). PTX also significantly lengthened the mean period of PER2::LUC cycling (to 26.6 ± 3.4 h; P < 0.00001). These data, consistent with our Per1::luc and simulation results, indicate that Gi/o-mediated, not GABA, signaling is required for maintenance of clock-gene expression rhythms in SCN neurons and their synchronization.

PTX, Not GABA Receptor Antagonism, Reduces PER2::LUC Rhythm Amplitude. To assess the effects of drug treatments on the amplitude of PER2::LUC rhythms of individual neurons, we measured the circadian and peak-to-trough amplitudes for each rhythmic neuron. Whereas changes in peak-to-trough amplitude directly reveal changes in the level of clock-gene expression throughout the day, circadian amplitude (see Materials and Methods) is a function of cycle-to-cycle precision and peak-to-trough amplitude. Precision is the inverse of cycle-to-cycle period variation (16). Remarkably, BIC+SAC increased the circadian amplitudes of neuronal PER2::LUC rhythms (110.6 ± 4.7 compared with 77.6 ± 5.6 in untreated SCN slices; P < 0.00005; Fig. 2 D and E), likely leading to the increased proportion of neurons scored as rhythmic. Importantly, BIC+SAC treatment did not increase the peak-to-trough amplitude.
In contrast, treatment with PTX for 8–10 days significantly decreased circadian (to 35.6 ± 2.7; *P* < 0.00001) and peak-to-trough amplitudes of PER2::LUC oscillations (from 71.9 ± 6.4 to 33.8 ± 2.0 photons per h; *P* < 0.0001), suggesting that PTX directly affects the level of clock-gene expression across the circadian cycle.

**PTX, Not GABA Receptor Blockade, Impairs SCN Firing Rhythms and Synchrony.** To determine the roles of GABA and G<sub>i/o</sub> on a functional output of individual SCN neurons, we recorded the firing patterns of neurons in high-density dispersals before and during treatment with GABA receptor antagonists or PTX. Treatment with BIC+SAC for up to 10 days did not impair the ability of SCN neurons to generate daily firing rhythms (Fig. 3; 56% rhythmic before treatment vs. 60% during; *n* = 96 neurons from three cultures). In contrast, PTX decreased the proportion of rhythmically firing SCN neurons [from 60% (104 of 173) before treatment to 29% (60 of 210) of neurons recorded; *n* = 3 cultures] after 4–5 days of treatment, similar to its effects on PER2::LUC rhythmicity.

Circadian synchrony was unaffected by long-term treatment with BIC+SAC. Times of peak daily firing for neurons within a culture were significantly clustered both on the last day of recording in control conditions and after 10 days of antagonist treatment (*P* < 0.05, Rayleigh test; *n* = 3 cultures). PTX, however, disrupted the coordinated timing of peak firing among neurons within a culture (P > 0.4; *n* = 3 cultures). Similarly, the distributions of periods for all rhythmic neurons recorded were similar during BIC+SAC treatment and under control conditions (P > 0.4), but they were significantly broadened by PTX (*P* < 0.00001). The mean period of firing rhythms, like PER2::LUC rhythms, lengthened in PTX (to 25.7 ± 0.5 h vs. 24.0 ± 2.8 h before treatment, mean ± SD; *P* < 0.01), but it did not change in BIC+SAC (24.7 ± 2.9 h during vs. 24.7 ± 3.3 h before treatment; *P* > 0.99). Thus, firing and gene expression patterns suggest that G<sub>i/o</sub> signaling rather than not GABA is required to maintain rhythms and synchrony among individual SCN neurons.

**GABA Blockade Increases, Whereas PTX Reduces, Firing Rate Rhythm Amplitude.** GABA receptor antagonists augmented the strength and peak-to-trough amplitudes of firing-rate rhythms (circadian amplitude: 106.5 ± 11.4 compared with baseline without antagonists 65.3 ± 8.3; *P* < 0.005; peak-to-trough amplitude: 5.7 ± 0.3 Hz during, 4.5 ± 0.3 Hz before treatment; *P* < 0.05; Fig. 3D and E). This augmentation related to an increase in firing rates during the average daily peak of activity during BIC+SAC treatment (6.4 ± 0.4 Hz vs. 5.2 ± 0.4 Hz before treatment; *P* < 0.05) without a concomitant increase in firing during the daily trough of activity (0.8 ± 0.1 Hz during vs. 0.6 ± 0.1 Hz before treatment; *P* > 0.2). Mean firing rates were similar between baseline and BIC+SAC treatment when averaged over 4–5 days of recording (2.4 ± 0.3 Hz before treatment vs. 2.7 ± 0.3 Hz during BIC+SAC treatment; *P* > 0.4), which suggests that GABA receptor antagonists increase the firing rate primarily during the daily peak of firing, with little effect at other times of day. We conclude that endogenous GABA plays a critical role in regulating peak firing rates and rhythm amplitudes.

In contrast, PTX decreased the amplitudes of rhythmically firing neurons (circadian amplitude: from 68.1 ± 6.6 before treatment to 44.7 ± 4.5 during treatment, *P* < 0.05; peak-to-trough amplitude: 3.7 ± 0.2 Hz to 3.0 ± 0.3 Hz, *P* < 0.05). PTX had no effect on the peak firing rate (4.2 ± 0.2 Hz before vs. 4.6 ± 0.5 Hz during treatment; *P* > 0.3), but it significantly increased firing during the daily trough of activity (from 0.5 ± 0.1 Hz before treatment to 1.6 ± 0.3 Hz; *P* < 0.00001) and the mean firing rate (2.1 ± 0.1 Hz before treatment, vs. 2.9 ± 0.2 Hz during PTX treatment; *P* < 0.0005). These results indicate that...
chronic inhibition of $G_{i/o}$ activity reduces the amplitude of firing-rate rhythms and increases overall firing by selectively interfering with the silencing of neurons during the subjective night.

**Daily VIP receptor (VPAC$_2$) Signaling Suffices for Rhythms and Synchrony in the Absence of GABA Signaling.** Because VIP signaling modulates GABA release in the SCN (12), we tested whether VIP-induced amplification and synchronization of SCN neuronal rhythms are mediated indirectly through GABA signaling. We applied VPAC$_2$ receptor agonist Ro 25-1553 (150 nM) daily to VIP$^{-/-}$ SCN cultures in the continuous presence of BIC+SAC over 6 days (SI Fig. 7). Because the half-life of Ro 25-1553 activity is $\sim$2–4 h (17), we expect that daily applications result in 24-h rhythms of VPAC$_2$ receptor activation. In the absence of GABAergic signaling, daily Ro 25-1553 synchronized the times of peak firing among rhythmic neurons ($P < 0.05$, Rayleigh test), narrowed their distribution of periods ($P < 0.05$), and restored the proportion of rhythmic neurons from 31% (45 of 147 neurons from four VIP$^{-/-}$ cultures) to 60% (88 of 147).

**Daily VIP receptor (VPAC$_2$) Signaling Is Insufficient for Rhythms and Synchrony in the Absence of GABA Signaling.** Because VPAC$_2$ is thought to activate $G_{i}$ rather than $G_{i/o}$ in SCN neurons (12), we also tested whether daily VPAC$_2$ activation alone could restore rhythms and synchrony to VIP$^{-/-}$ neurons in the absence of $G_{i/o}$ function. We applied VPAC$_2$ agonist Ro 25-1553 daily to VIP$^{-/-}$ SCN cultures in the continuous presence of PTX over 6 days. Daily agonist failed to resynchronize the time of peak firing among rhythmic neurons treated with PTX ($P > 0.25$) or their periods (25.0 ± 5.3 h before, 24.5 ± 4.6 h during, mean ± SD; $P > 0.05$). PTX also prevented the restoration of the proportion of rhythmic VIP$^{-/-}$ neurons by daily Ro 25-1553 (62 of 194 neurons or 32% from three cultures vs. 45 of 194 neurons or 23%). We conclude that in the absence of $G_{i/o}$ signaling, daily VPAC$_2$ activation is insufficient to reinstate function to VIP$^{-/-}$ SCN neurons.

**Discussion**

GABA Modulates Peak Firing Rate and Rhythm Precision, Not Synchrony, in the SCN. Although GABA and GABAA receptors are ubiquitous among SCN neurons (for review, see ref. 18) and GABA is rhythmically released in the SCN (19), its function has been controversial (for review, see ref. 20). We found that chronic GABA receptor antagonism amplified firing-rate rhythms by specifically increasing firing rates during the daily peak. In contrast, some reports have suggested that GABA can acutely excite some SCN neurons at specific circadian times (21–23). Although we cannot exclude the possibility that a minority of SCN neurons are directly or indirectly excited during GABA receptor activation, our data support the findings of others who have found an inhibitory role for GABA signaling during the day (13, 20, 24). We conclude that endogenous GABA signaling restricts the daytime peak in firing, counterbalancing an increase in membrane excitability during the day, and having little effect on the already-low nighttime firing rate of SCN neurons.

GABA plays an additional role to reduce cycle-to-cycle precision of circadian rhythms in SCN firing and clock-gene expression. Two indirect measures (circadian amplitude and period distribution of individual neurons) indicate that blockade of GABA signaling modestly improves rhythm stability. These data are consistent with observations that GABA$_A$ antagonism increases the precision of firing of SCN neurons on a millisecond time scale (25) and does not modify day-night differences in membrane potential and input resistance of SCN neurons (26). Endogenous GABA also has been shown to play a role in adjusting SCN responsiveness to photic input (27, 28), and it may
play a role in coordinating entrainment of the dorsal and ventral SCN to large shifts in the light cycle (21). We conclude that GABA modulates circadian firing patterns by reducing maximal daytime firing rate, which may enhance sensitivity to depolarizing inputs.

A finding of equal or greater importance is what GABA does not do in the SCN. In contrast to its enhancement of firing-rate rhythms, GABA blockade did not affect the peak-to-trough amplitude of Period expression in individual neurons, which is evidence that a change in the amplitude of firing-rate rhythms does not necessarily coincide with a change in clock-genome expression. Furthermore, although daily application of exogenous GABA suffices to synchronize firing rhythms of dispersed SCN neurons (13), we find that it is not required for synchrony within the SCN slice. Finally, whereas the loss of VIP signaling desynchronizes circadian rhythms in the SCN (5, 6) and abolishes rhythms in GABA release (19), we found that VPAC2 agonist-induced synchrony is not mediated by GABA. Our data suggest that GABA release within the SCN does not normally produce phase shifts or affect clock-genome expression under steady-state conditions. These data strongly indicate that release of endogenous VIP rather than GABA coordinates pacemaking among SCN neurons. We conclude that intra-SCN GABA signaling is more important for entrainment of SCN neurons to environmental cycles than for their entrainment to one another.

**Gi/o Proteins Mediate Rhythmicity and Synchrony in SCN Neurons.**

Blocking Gi/o activity causes three changes in SCN rhythmicity, phenotypically similar to VIP or Vipr2 knockouts (6, 29): a decrease in the proportion of rhythmic neurons in the SCN from 60–70% to 16%, a decrease in rhythm amplitudes, and a loss of circadian synchrony between the remaining rhythmic neurons (5). Effects of PTX on Period/Luc expression rhythms in SCN neurons also mirror circadian desynchrony and gradual damping of Perl/luc rhythms in TTX-treated SCN (3). Importantly, we find that PTX similarly affects gene expression and firing-rate rhythms by reducing the proportion of rhythmic neurons, reducing the peak-to-trough and circadian amplitudes of rhythmic neurons, abolishing the phase clustering of their daily peaks in firing, and increasing their mean and variation in period. These results suggest that PTX interferes with circadian function at both the level of the core molecular clock and circadian output. Notably, PTX had no effect on gene expression rhythms in the liver, and untreated liver rhythms damped at a rate similar to PTX-treated SCN rhythms. We conclude that Gi/o plays a specialized role in the maintenance and coordination of rhythms in the SCN, enabling SCN tissue to sustain high-amplitude rhythmicity over time.

It remains unclear whether desynchrony within the SCN leads to lower-amplitude rhythms in individual neurons or vice versa; however, these two phenomena appear to go hand in hand within the SCN network (5). The similarities between effects of VIP+−/− and Vipr2+−/− mutations and PTX treatment on SCN function indicate a convergent mechanism for intercellular and intracellular regulation of circadian rhythms. Consistent with this interpretation, we found that daily VPAC2 activation fails to restore circadian function to VIP−/− SCN in the presence of PTX. We and others have suggested that VIP neurotransmission could entrain and amplify rhythms within the SCN by activation of AC (2, 30). Because VPAC2 activates AC through Gi/o (31) and PTX-sensitive G proteins inhibit AC activity (32), we hypothesize that both daily activation and inhibition of AC are required to entrain and amplify rhythms in SCN neurons (SI Fig. 8).

In support of the proposed convergent roles of Gi/o and GABA on circadian rhythm generation and synchronization in the SCN, one fundamental difference between Vipr2+−/− SCN neurons and PTX-treated SCN neurons is that, whereas Vipr2+−/− neurons show a decrease in the daily peak firing rate (29), PTX increases the daily minimum firing rate in rhythmic neurons. This observation suggests that daytime activation of Gi/o (driven by circadian release of VIP from pacemaking neurons) and nighttime activation of Gi/o are both critical mediators of circadian rhythmicity in SCN neurons.

It is tempting to speculate on the source of Gi/o activity in the SCN. Gi/o could be regulated by extracellular or intracellular signals within the SCN. One candidate, DExras1, is a nonreceptor-associated activator of Gi/o which inhibits AC and cAMP response element-binding protein activity in the SCN (33). The gene encoding DExras1 is highly and rhythmically expressed in the SCN, in antiphase to the Period genes (34). Mice lacking DExras1 show abnormal desynchronization of behavioral rhythms in constant light (35). Future studies should address which specific Gi/o activators are required for SCN synchrony and rhythmicity.

**SCN Neurons Fall into Functionally Distinct Classes.**

Early studies suggested that all 20,000 neurons of the bilateral SCN are cell-autonomous circadian clocks (14). More recent data suggest that blocking action potentials or VIP signaling abolishes synchrony among some SCN neurons and, critically, rhythms in the majority of cells (5, 6). It is likely that SCN neurons fall into at least two functional categories: those that require intercellular communication to maintain rhythmicity, and cell-autonomous clocks that require these signals to synchronize their daily rhythms with one another. Alternatively, these treatments may weaken the pacemaking mechanism such that a random subset of cells can sustain circadian cycling. The effects of PTX are consistent with either model, and they suggest that intercellular amplification and entrainment of SCN neuronal rhythms are mediated by alternating AC stimulation and inhibition by G protein signaling.

**Materials and Methods**

**Animals.** VIP−/− (gift from J. Waschek and C. Colwell, University of California, Los Angeles, CA), PER2::LUC knockin (gift from J. Takahashi, Northwestern University, Evanston, IL), and wild-type (C57BL/6; Charles River Laboratories, Wilmington, MA) mice and Perl::Luc rats (gift from H. Tei, Mitsubishi Kagaku Institute of Life Sciences, Tokyo, Japan) were maintained as homozygous lines in a facility at Washington University.

**Cell Culture.** We obtained SCN from 1- to 7-day-old mice or rats, housed in 12-h light/12-h dark cycles. Genotypes for VIP−/− and PER2::LUC mice were confirmed by PCR (8, 36). For bioluminescence recording of slices, 300-μm-thick coronal sections of bilateral SCN from Perl1::luc rats or PER2::LUC mice (or 1-mm-thick slices of PER2::LUC liver) were cultured on Milli-cell-CM membranes (Millipore, Billerica, MA) as described in ref. 37. For recording single-neuron PER2::LUC rhythms, 150- to 300-μm-thick coronal slices of bilateral SCN were cultured on membranes for 2–6 weeks, then they were inverted onto collagen-coated glass coverslips, as described in ref. 3. Slices were then maintained in 400 μl of CO2-buffered medium supplemented with 10% newborn calf serum (Invitrogen, Carlsbad, CA) for 1–2 weeks until recording. For dispersed cultures on multielectrode arrays (MEAs; Multichannel Systems, Reutlingen, Germany), SCN were punched from 300-μm slices and dispersed by using papain (15). Visible cells from four to eight SCN were plated at >10,000 cells per mm² on each MEA (5) and maintained in 1 ml of culture medium for 1–2 weeks before recording. After 7 days in vitro, dispersed cultures were treated with 20 μM cytosine arabinoside (Ara-C; Sigma, St. Louis, MO) to control glial proliferation.

**Bioluminescence Recording.** Recordings of bioluminescence from slices of SCN or liver were made in air-buffered medium.
supplemented with beetle luciferin (Promega, Madison, WI) at 37°C as described in ref. 37. Single-neuron PER2::LUC bioluminescence was imaged with a Versarray 1024 cooled-CCD camera (Princeton Instruments, Trenton, NJ) from SCN slices in air-buffered medium at 37°C. Photon counts were spatially (4 × 4 pixels) and temporally (1 h) integrated by using WinView software (Princeton Instruments). Bioluminescence from individual neurons was quantified over 72–96 h of recording, and background photon counts were subtracted by using Image (National Institutes of Health, Bethesda, MD).

**Multielectrode Array Recording.** We used 60-electrode MEAs (30-μm tips, 200-μm spacing) to record and discriminate neural activity at 37°C in 5% CO2 by using MC-Rack (Multichannel Systems) and Offline Sorter software (Plexon, Inc., Dallas, TX) as described in ref. 5. We plotted average firing rate per 10 min of each neuron (NeuroExplorer; Plexon, Inc.).

**Drug Treatments.** GABA\(_A\) antagonist BIC (200 μM; Sigma) and GABA\(_A\) antagonist SAC (100 μM; Sigma) (BIC + SAC) or Bordetella PTX (5 nM; Sigma) was diluted in deionized water and stored at 4°C or 20°C. Drugs were replenished in recording medium every 3–6 days for all single-cell recordings (PER2::LUC and firing rate). Per1::Luc SCN slices were treated once with BIC + SAC, PTX, or TTX (2 μM; Sigma) after a 4- to 6-day baseline recording period.

VPAC\(_2\) agonist Ro 25-1553 (38), provided by P. Robberecht (University of Brussels), was applied every 24 h for 6 days to determine independently the rhythmicity and period of firing and PER2::LUC patterns from the 3–5 days before and an equal duration during drug treatment. Periods between 16 and 32 h were considered statistically significant by \( \chi^2 \) periodogram if the amplitude exceeded the 99% confidence interval and by FFT-NLLS if they exceeded the 95% confidence interval. The two methods produced similar period estimates for individual neurons, differing on average by <1% for both firing rate and PER2::LUC expression rhythms. FFT-NLLS scored a larger proportion of neuron firing and gene-expression patterns as rhythmic, although the magnitude and direction of treatment effects were the same as \( \chi^2 \) periodogram analysis. We chose to report \( \chi^2 \) periodogram results for simplicity and consistency with previous reports. We measured circadian amplitude as the power above the confidence interval at the dominant period. We determined circadian synchrony within cultures by using Clock-Lab (Actimetrics, Wilmette, IL) to find the daily acrophase of each neuron, and we tested the resultant phase distribution for randomness with a Rayleigh test (41). We assessed differences in circadian period distributions with the Brown–Forsythe and Levene tests for equal variance.

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**Data Analysis.** All bioluminescence recordings were detrended by subtracting a 24-h running average (37). Cycle-to-cycle amplitude was measured over 4–5 days of baseline recording and then over 9–10 days after a medium exchange with or without (control) drug added. We normalized the amplitude of each cycle after the medium exchange to the amplitude of the last baseline cycle. The 2 days of recording after drug administration were not analyzed because of a transient, nonspecific increase in bioluminescence rhythm amplitudes associated with medium changes. We used \( \chi^2 \) periodogram (39) and fast Fourier transform–nonlinear least squares (FFT-NLLS) analyses (40) to determine independently the rhythmicity and period of firing and PER2::LUC patterns from the 3–5 days before and an equal duration during drug treatment. Periods between 16 and 32 h were considered statistically significant by \( \chi^2 \) periodogram if the amplitude exceeded the 99% confidence interval and by FFT-NLLS if they exceeded the 95% confidence interval. The two methods produced similar period estimates for individual neurons, differing on average by <1% for both firing rate and PER2::LUC expression rhythms. FFT-NLLS scored a larger proportion of neuron firing and gene-expression patterns as rhythmic, although the magnitude and direction of treatment effects were the same as \( \chi^2 \) periodogram analysis. We chose to report \( \chi^2 \) periodogram results for simplicity and consistency with previous reports. We measured circadian amplitude as the power above the confidence interval at the dominant period. We determined circadian synchrony within cultures by using Clock-Lab (Actimetrics, Wilmette, IL) to find the daily acrophase of each neuron, and we tested the resultant phase distribution for randomness with a Rayleigh test (41). We assessed differences in circadian period distributions with the Brown–Forsythe and Levene tests for equal variance.