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Notes:

GABA and $G_{i/o}$ 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 $G_{i/o}$ did not impair synchrony or rhythmicity. In contrast, inhibiting $G_{i/o}$ 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 rhythmicity to *VIP*^{-/-} SCN cultures during continuous GABA receptor antagonism but not during $G_{i/o}$ blockade. Pertussis toxin did not affect circadian cycling of the liver, suggesting that $G_{i/o}$ plays a specialized role in maintaining SCN rhythmicity. We conclude that endogenous GABA controls the amplitude of SCN neuronal rhythms by reducing daytime firing, whereas $G_{i/o}$ signaling suppresses nighttime firing, and it is necessary for synchrony among SCN neurons. We propose that $G_{i/o}$, not GABA activity, converges with VIP signaling to maintain and coordinate rhythms among SCN neurons.

luciferase | multielectrode array | *Period* gene | suprachiasmatic nucleus | vasoactive intestinal polypeptide

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 $G_{i/o}$ 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 μ M bicuculline and 100 μ 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 μ M; $n = 9$) or an inhibitor of $G_{i/o}$ proteins, 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 neurotransmission in the SCN, and PTX irreversibly ADP-

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Abbreviations: AC, adenylyl cyclase; BIC+SAC, bicuculline and saclofen; MEA, multielectrode array; PTX, pertussis toxin; SCN, suprachiasmatic nuclei; TTX, tetrodotoxin; VIP, vasoactive intestinal polypeptide; *Vipr2*, VIP receptor gene; VPAC₂, VIP receptor.

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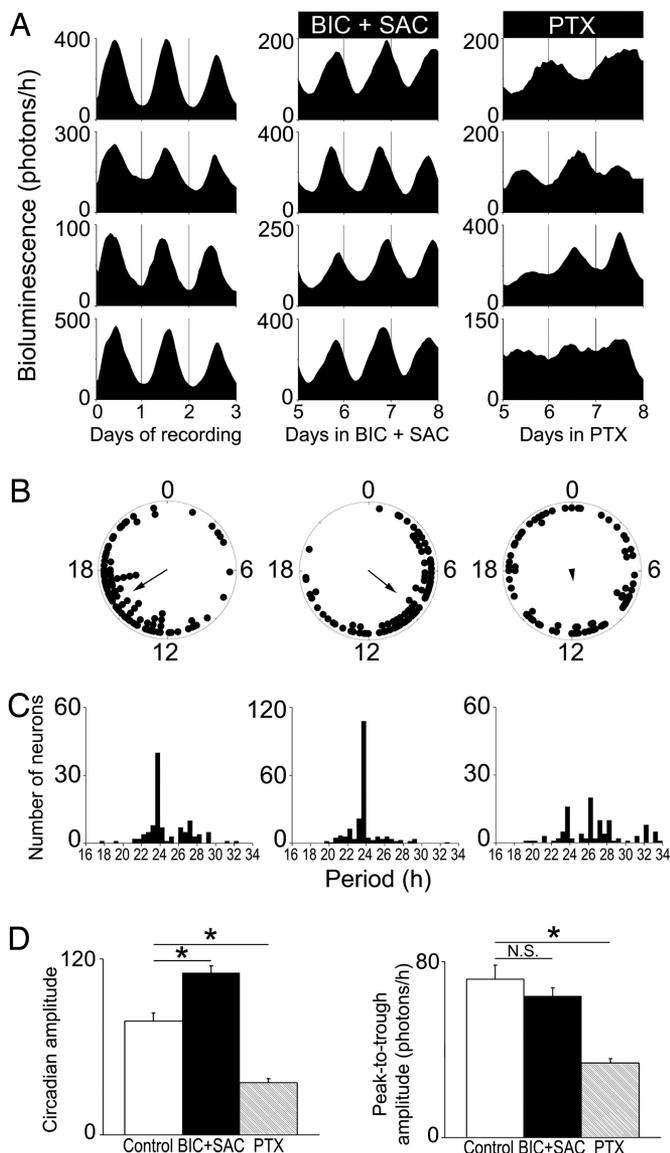


Fig. 2. PTX, not GABA receptor antagonism, disrupts rhythms and synchrony of *PER2::LUC* rhythms in SCN neurons. (A) Representative *PER2::LUC* traces from individual neurons within mouse SCN slices under control conditions (Left), on days 5–8 of treatment with BIC+SAC (Center), and on days 5–8 of treatment with PTX (Right). (B) Representative Rayleigh plots of all rhythmic neurons within a SCN slice from each treatment group. The bioluminescence acrophase of each neuron (filled circles) and the mean phase of all neurons (arrow) are plotted for the last 24 h of each treatment. The arrow length is proportional to the magnitude of the phase clustering (r), ranging from 0 (randomly phased) to 1 (peaking at the same time). Whereas control and BIC+SAC-treated neurons maintained phase synchrony ($n = 89$, $r = 0.63$, and $n = 85$, $r = 0.56$, respectively; $P < 0.001$, Rayleigh test), PTX-treated neurons peaked randomly ($n = 73$, $r = 0.16$; $P > 0.1$). (C) Period distributions for all neurons recorded in each treatment group. PTX significantly broadened the period distribution of neurons compared with control and BIC+SAC-treated neurons ($P < 0.00005$, Brown–Forsythe and Levene test). (D) BIC+SAC increased, and PTX decreased the daily precision (as measured by circadian amplitude) of *PER2::LUC* bioluminescence rhythms in individual SCN neurons relative to controls (*, $P < 0.05$, ANOVA with Scheffé post hoc test). (E) Compared with controls, PTX decreased ($P < 0.05$), and BIC+SAC ($P > 0.05$) did not affect, the peak-to-trough amplitude of bioluminescence rhythms.

plitude of bioluminescence rhythms ($P > 0.05$), suggesting that GABA blockade increases circadian amplitude through an increase in precision of clock-gene rhythms rather than a change in expression levels.

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; $P < 0.00001$), 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 $G_{i/o}$ 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 ± 5.0 h vs. 24.0 ± 2.8 h before treatment, mean \pm 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 $G_{i/o}$ 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. 3 D 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

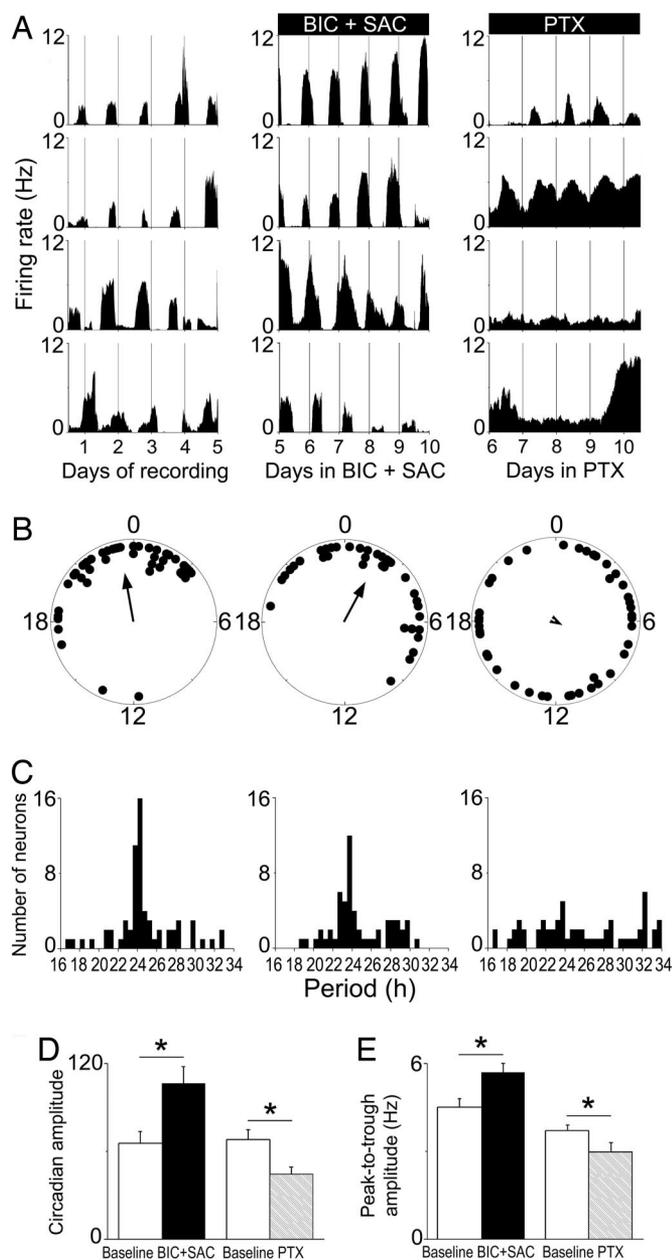


Fig. 3. PTX, not GABA receptor antagonism, impairs firing rhythmicity and synchrony between neurons. (A) Representative firing-rate rhythms for individual SCN neurons within a control culture (Left), over the last 5 days of a 10-day BIC+SAC treatment (Center), and on the last 5 days of a 10-day PTX treatment (Right). Neurons reliably peaked at similar times during control and BIC+SAC treatment, but they were less likely to maintain rhythms, they showed lower-amplitude rhythms, and they had unstable phase relationships during PTX treatment. (B) Peak phases of all rhythmic neurons within representative SCN cultures on the last day of baseline recording (Left), on the 10th day of BIC+SAC treatment (Center), and on the 10th day of PTX treatment (Right). Rayleigh distributions of peak phases before treatment ($n = 45$, $r = 0.66$) and during BIC+SAC treatment ($n = 37$, $r = 0.59$) were statistically nonrandom ($P < 0.001$), but they were random during PTX treatment ($n = 41$, $r = 0.09$; $P > 0.6$). (C) Period distributions for all rhythmic neurons were similar before (Left) and during (Center) BIC+SAC treatment ($P > 0.4$), and they were significantly broadened by PTX (Right; $P < 0.00001$). (D) Circadian amplitudes were greater during BIC+SAC treatment than during baseline recording (*, $P < 0.05$) and reduced during PTX treatment relative to baseline ($P < 0.05$). (E) As a result of increased daily peak firing rate ($P < 0.05$), the peak-to-trough amplitude increased significantly during BIC+SAC treatment ($P < 0.05$). As a result of increased daily minimum firing rate ($P < 0.00001$), the peak-to-trough amplitude decreased significantly during PTX treatment (Right; $P < 0.05$).

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 (VPAC₂) 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 VPAC₂ 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 ≈ 2 –4 h (17), we expect that daily applications result in 24-h rhythms of VPAC₂ 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 (VPAC₂) Signaling Is Insufficient for Rhythms and Synchrony in the Absence of $G_{i/o}$ Signaling. Because VPAC₂ is thought to activate G_s rather than $G_{i/o}$ in SCN neurons (12), we also tested whether daily VPAC₂ activation alone could restore rhythms and synchrony to $VIP^{-/-}$ neurons in the absence of $G_{i/o}$ function. We applied VPAC₂ 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 \pm 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 VPAC₂ 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 GABA 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-gene 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-gene 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.

G_{i/o} Proteins Mediate Rhythmicity and Synchrony in SCN Neurons.

Blocking G_{i/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 ≈30%, a decrease in rhythm amplitudes, and a loss of circadian synchrony between the remaining rhythmic neurons (5). Effects of PTX on *PER2::LUC* expression rhythms in SCN neurons also mirror circadian desynchrony and gradual damping of *Per1::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 G_{i/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 G_s (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 G_s and G_{i/o} 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 G_s (driven by circadian release of VIP from pacemaking neurons) and nighttime activation of G_{i/o} are both critical mediators of circadian rhythmicity in SCN neurons.

It is tempting to speculate on the source of G_{i/o} activity in the SCN. G_{i/o} could be regulated by extracellular or intracellular signals within the SCN. One candidate, *Dexas1*, is a nonreceptor-associated activator of G_{i/o} which inhibits AC and cAMP response element-binding protein activity in the SCN (33). The gene encoding *Dexas1* is highly and rhythmically expressed in the SCN, in antiphase to the *Period* genes (34). Mice lacking *Dexas1* show abnormal desynchronization of behavioral rhythms in constant light (35). Future studies should address which specific G_{i/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 (3, 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 *Per1::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- μ m-thick coronal sections of bilateral SCN from *Per1::luc* rats or *PER2::LUC* mice (or 1-mm-thick slices of *PER2::LUC* liver) were cultured on Millipore membranes (Millipore, Billerica, MA) as described in ref. 37. For recording single-neuron *PER2::LUC* rhythms, 150- to 300- μ m-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 μ l 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- μ m slices and dispersed by using papain (15). Viable 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 μ M 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).

Multi-electrode Array Recording. We used 60-electrode MEAs (30- μ m tips, 200- μ m 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 μ M; Sigma) and GABA_B antagonist SAC (100 μ M; 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 μ M; Sigma) after a 4- to 6-day baseline recording period.

VPAC₂ agonist Ro 25-1553 (38), provided by P. Robberecht (University of Brussels), was applied every 24 h for 6 days to *VIP^{-/-}* SCN as described in ref. 5 in the continuous presence of BIC+SAC or PTX. BIC+SAC or PTX was applied 5 min before the initial Ro 25-1553 application, and it remained in the recording medium throughout the 6 days of agonist application without further medium changes.

Data Analysis. All bioluminescence recordings were detrended by subtracting a 24-h running average (37). Cycle-to-cycle ampli-

tude 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 χ^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 χ^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 χ^2 periodogram analysis. We chose to report χ^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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