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Daniel Granados-Fuentes
*Washington University in St. Louis*

Peter Lambert
*Washington University School of Medicine in St. Louis*

Tatiana Simon
*Washington University in St. Louis*

Steven Mennerick
*Washington University School of Medicine in St. Louis*

Erik D Herzog
*Washington University School of Medicine*

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GABA<sub>A</sub> receptor subunit composition regulates circadian rhythms in rest–wake and synchrony among cells in the suprachiasmatic nucleus

Daniel Granados-Fuentes<sup>1,2</sup>, Peter Lambert<sup>3</sup>, Tatiana Simon<sup>4</sup>, Steven Mennerick<sup>3</sup>, and Erik D. Herzog<sup>1</sup>

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The mammalian circadian clock located in the suprachiasmatic nucleus (SCN) produces robust daily rhythms including rest–wake. SCN neurons synthesize and respond to γ-aminobutyric acid (GABA), but its role remains unresolved. We tested the hypothesis that γ2- and δ-subunits of the GABA<sub>A</sub> receptor in the SCN differ in their regulation of synchrony among circadian cells. We used two approaches: 1) shRNA to knock-down (KD) the expression of either γ2 or δ subunits in the SCN or 2) knock-in mice harboring a point mutation in the M2 domains of the endogenous GABA<sub>A</sub> γ2 or δ subunits. KD of either γ2 or δ subunits in the SCN increased daytime running and reduced nocturnal running by reducing their circadian amplitude by a third. Similarly, δ subunit knock-in mice showed decreased circadian amplitude, increased duration of daily activity, and decreased total daily activity. Reduction, or mutation of either γ2 or δ subunits halved the synchrony among, and amplitude of, circadian SCN cells as measured by firing rate or expression of the PERIOD2 protein, in vitro. Surprisingly, overexpression of the γ2 subunit rescued these phenotypes following KD or mutation of the δ subunit, and overexpression of the δ subunit rescued deficiencies due to γ2 subunit KD or mutation. We conclude that γ2 and δ GABA<sub>A</sub> receptor subunits play similar roles in maintaining circadian synchrony in the SCN and amplitude of daily rest–wake rhythms, but that modulation of their relative densities can change the duration and amplitude of daily activities.

Daily cycles in physiology and behaviors such as sleep–wake depend on a central circadian clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus (1, 2). In mammals, nearly all cells, including SCN neurons, contain a transcription–translation negative feedback loop of core clock genes and proteins (e.g., Period1 and 2 genes and their products, PER1 and PER2) that generates circadian oscillations and synchronizes to local time and to other cells (3). Unlike most mammalian neurons, SCN neurons can be depolarized or hyperpolarized directly by γ-aminobutyric acid (GABA) depending on cell type, time of day, and photoperiod (4–7). Although GABA is the primary neurotransmitter in the SCN mediating many functional connections that can vary in strength with time of day, its roles appear diverse depending on experimental conditions and timescales and methods of measurement (8, 9).

Both ionotropic GABA<sub>A</sub>- and metabotropic GABA<sub>B</sub>-receptor types mediate responses to GABA within the SCN (8, 10). GABA<sub>A</sub> receptors pass chloride and are significant drug targets for anesthesia and disorders including insomnia, anxiety, and epilepsy (11). Notably, the SCN is rarely considered in the context of GABA<sub>A</sub> receptor therapy or the genetic basis of diseases. Most GABA<sub>A</sub>Rs are composed of two α subunits, two β subunits, and one γ subunit, although many isoforms are possible with the 19 known subunits: six α, three β, three γ, three ρ, and one each of the δ, ε, π, and θ. For example, the γ2 subunit is essential for post synaptic clustering of GABA<sub>A</sub> receptors and phase responses to GABA (12–15) and δ subunits can localize to extrasynaptic sites where they mediate tonic inhibition to low GABA concentrations (16).

The γ2 and δ subunits, among the most highly expressed GABA receptor subtypes in the central nervous system, can be found in the same neurons and variable in their expression across cell types (11). In the SCN, pharmacological, immunohistochemical, and molecular biology studies have reported the presence of subunits α1–α6, β1–β3, γ1–γ3, δ, ε, π, and θ in postnatal and adult SCN neurons, but it is still not clear whether all subunits are present at early postnatal ages (CircadDB, http://circadb.hogeneschlab.org/ (10, 17–24). The relative roles of synaptic and extrasynaptic GABA<sub>A</sub> receptors have not been studied in the SCN. We therefore postulated that receptors containing the γ2 subunit mediate fast, synaptic responses to GABA and the δ subunit mediate tonic, extrasynaptic responses to GABA mediate synchrony in the SCN that affects wheel-running behavior.

Significance

The hypothalamic suprachiasmatic nucleus (SCN) synthesizes, releases, and responds to γ-aminobutyric acid (GABA), but the role of their GABA receptor subtypes is unknown. We recorded clock gene expression or firing rate from SCN neurons after either knocking down or mutating the γ2 or δ GABA<sub>A</sub> receptor subunits because they classically mediate tonic or phasic inhibitory neurotransmission, respectively. We found that either of these GABA<sub>A</sub> receptor subunit types was necessary to maintain synchrony among circadian neurons and daily rhythms in rest–wake behavior. Overexpression of either of these receptor subunits compensated for the loss of the other. We conclude that the density of GABA<sub>A</sub> receptors sets the phase relationships among circadian SCN neurons to coordinate daily behaviors.
Recent evidence for a tonic GABA current in the SCN (22, 25) has renewed interest in a long-standing hypothesis that extrasynaptic \( \text{GABA}_A \) receptors drive circadian changes in [Cl\(^-\)] (26), and play a role in daily rhythms in behavior. Computer simulations suggest that a GABA-mediated tonic current in the SCN could excite, and facilitate synchrony among, circadian cells in the SCN (26–29). Consistent with this, knockdown of the \( \delta \) subunit reduced circadian amplitude and synchrony in the SCN (28). Using shRNA to specifically knockdown expression in the SCN and mice with mutations in the genes coding for \( \gamma_2 \) or \( \delta \), we tested the hypothesis that \( \text{GABA}_A \) receptors containing \( \gamma_2 \) or \( \delta \) subunits differ in their regulation of daily rhythms. Using these two methods to independently reduce the density of either receptor subunit along with overexpression assays, we found, surprisingly, that both \( \gamma_2 \) and \( \delta \) subunits are required to regulate daily rhythms in physiology and behavior.

**Results**

**\( \gamma_2 \) and \( \delta \) \( \text{GABA}_A \) Receptors Regulate Daily Rhythms in Rest and Locomotor Activity.** To test whether subunits of the \( \text{GABA}_A \) receptor differentially regulate daily rhythms, we recorded locomotion of mice with genetic knock-down (KD) or knock-in (KI) of \( \gamma_2 \) or \( \delta \) subunits. Specifically, we compared running-wheel activity in mice with shRNA-mediated KD of \( \gamma_2 \) or \( \delta \) in neurons of the SCN, mice with functional mutations in \( \text{Gabrd} \), the gene encoding \( \delta \) receptor subunits, and control mice. Mice with mutant \( \text{Gabrd} \), encoding \( \gamma_2 \) subunits, had persistent seizures or died, apparently as a result of reduced channel open probability (30) and, consequently were excluded from locomotor analysis. In LD, mice ran in the dark more than in the light, but those with SCN-targeted knockdown of \( \gamma_2 \) or \( \delta \) subunits had reduced nocturnal activity (Fig. 1 A–D and \( K \)). In constant darkness (DD), knockdown of \( \gamma_2 \) or \( \delta \) subunits significantly decreased nocturnal activity (Fig. 1 E and \( L \)), increased the ratio of time spent active to inactive (activity/rest ratio; SI Appendix, Fig. S2C), and decreased the amplitude of daily wheel running (SI Appendix, Fig. S2D). Similarly, knock-in mice with the mutant \( \delta \) subunit showed decreased nocturnal activity (Fig. 1 F–J), increased activity/rest ratio (SI Appendix, Fig. S2 E and \( G \)), and decreased running wheel amplitude in LD and DD (Fig. 1 M and \( N \)).

**Circadian PER2 Expression of SCN Cells Depends on \( \gamma_2 \) and \( \delta \)-\( \text{GABA}_A \) Receptor Subunits.** To test whether the reduced daily rhythms in behavior with \( \gamma_2 \) or \( \delta \) deficiency relate to central circadian pacemaker function, we monitored expression of the clock protein, PER2, in SCN explants infected at P4. Two weeks after in vitro infection with targeted shRNA, we found that knockdown of \( \gamma_2 \) or \( \delta \) reduced the peak-to-trough amplitude of daily PER2 rhythms (Fig. 2 A and \( C \), \( \gamma_2 \) 51.6 ± 6.8%, \( \delta \) 65.4 ± 4.8%, compared to nontargeted, NT, 84.2 ± 3.5%, mean ± SEM on recording day 3). Similarly, SCN harvested from mice homozygous for the KI mutations of \( \gamma_2 \) or \( \delta \) subunits approximately halved PER2 circadian amplitude (\( \gamma_2 \) KI 45.5 ± 4.7%, \( \delta \) KI 59.9 ± 6.2% compared to WT 82.2 ± 6.6%,

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Fig. 1. Knock-down or mutation of \( \gamma_2 \) or \( \delta \) \( \text{GABA}_A \) receptor subunits reduced the amplitude of daily rhythms and decreased nocturnal activity in wheel-running behavior. Representative actograms for mice treated with shRNA nontargeted controls (\( A \), NT, \( n = 17 \)) or targeting either \( \gamma_2 \) (\( B \), KD, \( n = 19 \)) or \( \delta \) (\( C \), KD, \( n = 12 \)) \( \text{GABA}_A \) receptor subunits and mutation experiments from WT (\( F \), \( n = 14 \)) and \( \delta \) KI (\( G \) and \( H \), \( n = 17 \)) recorded in LD for ~10 d and then released in DD for ~11 to 20 d. Activity profiles for group mean ± SEM in LD for KD (\( D \)) and mutants (\( J \)) and in DD for KD (\( E \)) and mutants (\( L \)). Locomotor analysis for group mean ± SEM in LD (\( K \) for KD, and M, for KI) and DD (\( L \) for KD, and \( N \) for KI) showed a decrease in activity during the dark phase independent of light conditions. Panel \( K \), \( F(2,40) = 4.2, P = 0.02 \) in LD, panel \( L \), \( F(2,40) = 5.1, P = 0.01 \) in DD for KD, one-way ANOVA and panel \( M \), \( t(28) = 4.9, P < 0.0001 \) in LD and panel \( N \), \( t(30) = 2.6, P = 0.01 \) in DD for mutations, \( t \) test.
mean ± SEM on recording day 3, Fig. 2 B and D). Circadian period of PER2 expression increase in SCN explants following depletion of γ2 or δ subunits by knockdown (NT 24.2 ± 0.2 h, γ2 KD 25.1 ± 0.2 h, δ KD 24.9 ± 0.1 h, one-way ANOVA, F(2,33) = 9.7, P = 0.0005) or mutations (WT 24.6 ± 0.1 h, γ2 KI 25.2 ± 0.2 h, δ KI 25.2 ± 0.1 h, one-way ANOVA, F(2,56) = 8.3, P = 0.0007).

To evaluate whether the decrease in the ensemble PER2 amplitude resulted from desynchrony among SCN cells or reduced amplitude at the single-cell level, we imaged SCN explants following perturbation of γ2 or δ GABA_A receptor subunits. Based on the time of daily peak PER2 in each cell within an explant, we calculated the Synchrony Index (SI) where 0 indicates cells peaking at random times of day and 1 indicates all cells peaking at the same time of day. We found knockdown of either γ2 or δ decreased SI among cells in SCN explants by approximately two-fold (0.44 ± 0.1 for γ2 KD, 0.42 ± 0.1 for δ KD, mean ± SEM measured on recording day 3) compared to NT controls (0.95 ± 0.01, Fig. 3 A–E), with decreased daily PER2 amplitude in the population (Fig. 3D) and single SCN cells (SI Appendix, Fig. S3A) following γ2 KD (0.3 ± 0.1; mean ± SEM measured on recording day 3) or δ KD (0.2 ± 0.07) compared to NT (0.6 ± 0.1). Increased period distribution of single SCN cells (1.5 SD) for γ2 KD, 1.9 h for δ KD, and 1.1 h for NT group, Fig. 3F) and fewer circadian cells (1% arrhythmic in NT; 8% in γ2 KD and 20% in δ KD; SI Appendix, Fig. S4A) were found. Knockdown of γ2 or δ also disrupted the typical dorsal-to-ventral wave of daily PER2 expression and the enrichment of lower amplitude, shorter period cells in the ventral SCN (Movie S1).

Similarly, mutation of the genes encoding either γ2 or δ subunits reduced synchronous PER2 daily rhythms among SCN cells (SI of 0.7 ± 0.1 for γ2 KI, 0.6 ± 0.1 for δ KI, 0.95 ± 0.02 for WT; Fig. 3 G–K; mean ± SEM measured on recording day 3), increased their period distributions (0.3 h SD for WT, 2 h for γ2 KI and 1.1 h for δ KI, Fig. 3L), decreased single-cell circadian amplitudes (0.8 ± 0.04 in WT, 0.4 ± 0.02 in γ2 KI and 0.5 ± 0.08 in δ KI, SI Appendix, Fig. S3B) and increased the fraction of arrhythmic cells (1% in WT, 19% in γ2 KI, 21% in δ KI, SI Appendix, Fig. S4B). Again, circadian phase, amplitude, and period maps showed an altered distribution of these variables in the mutant SCN (Movie S2). These results, while surprising compared to prior results using GABA_A receptor antagonists in the SCN, indicate that targeted knockdown or mutation of GABA_A receptor subunits, γ2 or δ, impairs amplification and synchronization of circadian clock gene expression in SCN cells.

**γ2 or δ GABA_A Receptor Subunits Compensate for Each Other in the SCN.** With evidence that γ2 and δ GABA_A receptor subunits are required to coordinate daily rhythms in the SCN, we next tested whether they act independently by overexpressing (OV) one while impairing the function of the other. Overexpressing γ2 subunits rescued the deficits induced by KD or mutations targeting δ subunits in circadian amplitude, fraction of circadian cells, phase and period synchrony, to control levels. When we overexpressed δ subunits, we rescued the deficits induced by KD or mutations targeting γ2 subunits (Figs. 4 and 5 and Movies S3 and S4). These data reveal that the endogenous γ2 or δ GABA_A receptors can compensate for each other in the SCN.
Knock-Down of γ2 or δ GABA<sub>A</sub> Receptor Subunits Increases Excitability in SCN Neurons. We next tested the hypothesis that γ2 and δ GABA<sub>A</sub> receptor subunits independently regulate SCN neuronal firing patterns. Two weeks after in vitro shRNA-mediated knockdown (neurons were infected at P4) of either γ2 or δ GABA<sub>A</sub> receptor subunits, we recorded firing rates of dispersed SCN neurons on multielectrode arrays for at least six days. We found that depletion of γ2 or δ increased the daily peak (mean ± SEM, 8.1 ± 0.5 Hz for γ2 KD; 8.1 ± 0.5 Hz for δ KD and 3.6 ± 0.1 Hz for NT, P < 0.001) and trough firing rates (0.6 ± 0.06 Hz for γ2 KD period SD 1.5, period CV 5.7; δ KD period SD 1.9, period CV 7.2, Bartlett’s test P < 0.0001. For panel I, F(2, 68) = 41, one-way ANOVA, WT period SD 0.3, period CV 1.3; γ2 KD period SD 1.9, period CV 7.9; δ KD period SD 1.1, period CV 4.4, Bartlett’s test P = 0.0001. SD = standard deviation, CV = coefficient of variance.

Knock-down or mutation of γ2 or δ GABA<sub>A</sub> receptor subunits reduced synchrony among circadian cells in the SCN. (A, B, and C) Raster plots depicting PER2 expression over 6 d from cells in a representative SCN explant treated either with NT, γ2 KD, and δ KD (n = 6) or cultured from WT, γ2 KD, or δ KD mice, respectively (G, H, and J). Ensemble (mean ± SEM) PER2 expression from SCN explants treated with NT (n = 4), γ2 KD (n = 4), and δ KD (n = 6, panel D) or from WT (n = 4), γ2 KD (n = 5), or δ KD (n = 7, panel J) mice. Loss or mutation of γ2 or δ GABA receptor subunits reduced daily synchrony indexes (E and K; mean ± SEM) and increased the period distributions among circadian cells (F and L) in the isolated SCN. For panel E, Group F(2, 60) = 47, P < 0.0001; Days (5, 60) = 1.7, P = 0.1; Group*Days F(10, 60) = 0.4, P = 0.9. For panel K, Group F(2, 48) = 20.5, P < 0.0001; Days F(5, 48) = 1.8, P = 0.1; Group*Days F(10, 48) = 0.5, P = 0.8, two-way mixed ANOVA. For panel F, F(2, 65) = 45.3 one-way ANOVA; NT period SD 1.3, period CV 5.4; γ2 KD period SD 1.5, period CV 5.7; δ KD period SD 1.9, period CV 7.2, Bartlett’s test P < 0.0001. For panel L, F(2, 68) = 41, one-way ANOVA, WT period SD 0.3, period CV 1.3; γ2 KD period SD 1.9, period CV 7.9; δ KD period SD 1.1, period CV 4.4, Bartlett’s test P = 0.0001. SD = standard deviation, CV = coefficient of variance.
Overexpression of GABA receptors restores period distributions in the KD (Fig. 5) and mutations (Fig. 6) groups. Panel D and J columns of five representative SCN neurons for NT, γ2 KD, and δ KD showed decreased GABA receptor density rather than loss of GABA signaling. Because blocking GABA receptors with bicuculline or gabazine has been reported to convert SCN neurons from irregular to regular firing patterns (36), we analyzed their firing patterns. We found that all recorded neurons (NT, γ2 KD, and δ KD) showed increased excitability of individual neurons as a result of decreased GABA receptor density. Given that Gabazine has been reported to minimally alter SCN firing rates while increasing the fraction of neurons with tonic firing (22, 35, 36), this knockdown-induced increase in firing suggests increased excitability of individual neurons as a result of decreased GABA receptor density rather than loss of GABA signaling.

Fig. 5. Both γ2 and δ GABA subunits are regulating synchrony among SCN cells. Cell raster plots from a representative single SCN explant for δ OV + γ2 KD and γ2 OV + δ KD (A and B) or δ OV + γ2 KD and γ2 OV + δ KI (F and G). Ensemble mean ± SEM PER2 expression traces from the same color-coded single SCN explants for KD (C; δ OV + γ2 KD, n = 2 and γ2 OV + δ KD, n = 2) and mutations (H; δOV + γ2 KI, n = 3 and γ2 OV + δ KI, n = 3). (D and J) Group mean ± SEM sync indices showing that overexpression of one GABA receptor rescues desynchrony induced by KD or mutations of the complementary GABA receptor. (E and J) Overexpression of γ2 or δ receptors restore period distributions in the KD (D) and mutations (J) groups. Panel D; Group F(2,36) = 1.6, P = 0.2; Days F(5,36) = 0.1, P = 0.9; Group*Days F(10,36) = 0.1, P = 0.9. Panel J, Group F(2,25) = 2.4, P = 0.3; Days F(4,25) = 0.2, P = 0.9; Group*Days F(8,25) = 0.04, P = 0.9, two-way mixed ANOVA.

Fig. 6. Knock-down of γ2 or δ GABA receptor subunits increased excitability in SCN neurons. (A, B, and C) Columns of five representative SCN neurons for NT, γ2 KD, or δ KD recorded for 6 continuous days. (D and E) KD of γ2 or δ receptors increased firing rate at the peak (D = 0.5, P < 0.0001) or the trough (D = 0.7, P < 0.002, Kolmogorov–Smirnov test), respectively. (F) KD of γ2 or δ receptors also increased the peak-trough amplitude, F(2,267) = 43.5, **** P < 0.0001, one-way ANOVA.
irregular firing. We conclude that whereas GABAR antagonists promote tonic firing, a decrease in GABA γ2 or δ subunits does not change spike patterning.

Whole-cell patch-clamp results from SCN neurons from γ2 or δ KI mice recorded during the day supported the conclusion that either receptor subunit substitutes for the other in the SCN. Specifically, we found that GABA<sub>A</sub> receptor antagonists, picrotoxin or gabazine, reduced the tonic current by three-fold and abolished spontaneous inhibitory postsynaptic currents (sIPSCs) in neurons cultured SCN from WT, γ2 KI or δ KI mice. In addition, neurons from γ2 KI, δ KI, or WT SCN did not differ in their sIPSC amplitudes, frequencies, or decay rates (SI Appendix, Fig. S5).

Discussion

We found that decreasing density of either γ2 or δ GABA<sub>A</sub> subunits in the SCN increases the daily duration of time spent running while reducing the amount of nocturnal activity in mice. Consistent with these behavioral consequences, we found that KD of γ2 or δ subunits dramatically broadens the times when SCN cells reach their daily peak of PER2 expression and increases their day and night firing levels. These results are consistent with recent reports that deletion of the vesicular GABA transporter (VGAT) in SCN neurons increases locomotor activity duration and decreases nocturnal locomotion (37, 38). Surprisingly, loss of GABA receptor subunits had much larger effects on SCN firing rate and synchrony than blocking GABA neurotransmission (e.g., with bicuculline or gabazine) or genetic deletion of GAD or VGAT (rate-limiting steps in GABA production or vesicle loading) (35, 37–40). These results are consistent with a recent computational model (28) that predicted, while blocking GABA signaling has modest effects, the density of γ2 or δ subunits can modulate circadian synchrony and amplitude among SCN neurons. The underlying mechanism may relate to daily rhythms in intraneuronal Cl<sup>−</sup> which peak in the SCN around midday even during GABA<sub>A</sub> receptor blockade (41). It may be that, in a 12:12 light:dark cycle, abundant GABA<sub>A</sub> receptors and Cl<sup>−</sup> transporters drive intracellular daytime Cl<sup>−</sup> high, without need for extracellular GABA, resulting in GABA being excitatory in the SCN during the day and early night (4, 29, 42, 43). It is likely, however, the SCN is further complicated by a nightly increase in extracellular GABA mediated IPSCs or tonic currents, we posit that receptor-mediated Cl<sup>−</sup> or Ca<sup>2+</sup> signaling contributes to this increase in excitability but not to firing pattern changes. Regardless, this mechanism for increased excitability and impaired circadian synchrony and amplitude in the SCN contrasts with the increased synchrony seen during pharmacological GABA receptor blockade (35) and desynchrony seen with reduced neuronal firing [e.g., TTX, (45)] or neuropeptide signaling [e.g., Vip<sup>−/−</sup>, (46)].

These results showed that there is no difference between local KD of γ2 or δ subunits and global mutations of the same, the effects on PER2 amplitude and synchrony and even locomotor behaviors were indistinguishable. The global γ2 or δ subunit mutations used in present experiments have been reported to leave the subunits expressed but insensitive to picrotoxin (31). Here, we find that, at least in the SCN, these mutations can modify the functionality of these subunits inducing desynchrony among SCN neurons and changes in locomotor behavior. Although there are no reports of developmental compensation for the mutations used here, KD or mutations could result in changes in subunit expression level. Future studies should test whether the M2 mutation of GABA<sub>A</sub> subunits has greater effects in the SCN than in other brain regions. Because tonic inhibition plays an important role in synaptic plasticity, neurogenesis (47, 48) as well as cognitive functions (49, 50). Any disturbance in phasic or tonic inhibition is associated with many neurological and psychiatric diseases. Thus, modulating these signals has become the basis of drug therapy as well as anesthesia (51–54). Future studies will help to understand how changes in density of GABA<sub>A</sub> receptors modulate excitability, specifically how Cl<sup>−</sup> conductance and Ca<sup>2+</sup> levels are changing, circadian amplitude and synchrony and whether changes in day length regulate γ2 or δ levels or other subunits forming part of the GABA<sub>A</sub> receptors.

Materials and Methods

Animals. Animal procedures were approved by the Animal Care and Use Committee at Washington University and conducted in accordance with the NIH guidelines for the care and use of laboratory mice. We used homozygous Per2<sup>−/−</sup> male and female mice in which the Luciferase gene was fused to the endogenous mouse Per2 gene, resulting in the production of a PERIOD2·LUCIFERASE fusion protein as a bioluminescent reporter of PER2 protein abundance (55), founders generously provided by Dr. J.S. Takahashi, Univ. Texas-SW. Two additional knock-in mouse strains were used where point mutations of the endogenous mouse γ2 (γ2 KI) or δ (δ KI) subunit reduced picrotoxin sensitivity of the receptor without changing sensitivity to GABA (56, 57). These mice (originally on a C57BLx6CBA background) were crossed with Per2<sup>−/−</sup> mice to produce male and female mice homozygous for the mutation γ2 or δ KI Per2<sup>−/−</sup> homozygous. All mice were maintained on the C57BL/6JN genetic background for at least 4 generations before use in experiments.

Screening for GABA<sub>A</sub> γ2 and δ-Targeted knockdown. We developed an interfering RNA strategy to allow the acute KD of the γ2- or δ- GABA<sub>A</sub> receptor subunits in the in vivo or in vitro SCN. The initial screening consisted of overexpressing (OXV) Gabrg2; the GABA<sub>A</sub> γ2 (Addgene, #49170) gene, or Gabrd, the gene encoding δ (Applied Biological Materials, #AAB07Z2429) receptor subunits in HEK-293 cells (ATCC). The Gabrg2 and Gabrd constructs were cloned into a pcDNA 3.1 backbone with a GFP fluorophore and a neomycin/kanamycin selection site.
by standard cloning techniques (58). We transfected HEK-293 cells (60 to 80% confluent) in 60 mm dishes (Greiner, CellStar) with 16 µl of Fugene6 and 1 µg of plasmid diluted in OptiMEM (Life Technologies) up to a final volume of 250 µl. We cultured the cells in 2 ml DMEM supplemented with 5% fetal bovine serum (FBS; Gibco/Life Technologies) and 1% penicillin/streptomycin (Gibco/Life Technologies) for two days. On day three, penicillin/streptomycin was replaced with 50 µg/ml of G418 (ThermoFisher). Selected HEK-293 cells overexpressing γ2 or 6 receptor subunits were then transfected with one of five shRNA clones (Broad Institute, St. Louis) against either the γ2 (NM_008073.1-1932s1c1; NM_008073.1-1527s1c1), γ1 (NM_008073.1-479s1c1; NM_008073.1-984s1c1; NM_008073.1-1520s1c1) or δ subunit (NM_008072.1-1565s1c1; NM_008072.1-164s1c1; NM_008072.1-73s1c1; NM_008072.1-1361s1c1; NM_008072.1-397s1c1).

Quantification of Knockdown by qPCR. We measured the efficacy of each shRNA 48 h after transfection. We precipitated and isolated total RNA from changes were further probed using iTaq™ Universal SYBR® Green Supermix with RealScript® III First-Strand Synthesis System (Thermo Fisher). Gene expression changes were further probed using iTaq™ Universal SYBR® Green Supermix with RealScript® III First-Strand Synthesis System (Thermo Fisher).

Virus Injections in the SCN. Per2LUC male and female mice (ages 60 to 90 d; P60 to 90) were anesthetized with 2% isoflurane and secured in a stereotaxic head holder (David Kopf Instruments). For knockdown experiments, 0.4 µl of the AAV8 carrying non-targeting shRNA (59) and samples lacking reverse transscriptase or template. Based on their cycle thresholds, we chose γ2 clone NM_008073.1-1527s1c1 and δ clone NM_008072.1-397s1c1 which reduced expression of γ2 by 79% and of δ by 68%, respectively (SI Appendix, Fig. S1 A and B).

Generation of shRNA-Expressing Adeno-Associated Viruses. The selected γ2 (5’GAGAAGACACCTCTTCAGGAA-3’), δ (5’GGAGACACGAGACTAAGCTGACAGG-3’), and NT shRNA (5’CCACAGATGACCATCATTACGAG-3’), and NT shRNA (5’CCACAGATGACCAGACACAA-3’) nucleotide sequence sensors were synthesized (Integrated DNA Technologies) into the corresponding 97-nucleotide microRNA-adapted shRNA oligonucleotides, containing sense and antisense sequences linked by a 19-nucleotide hairpin loop. shRNAs were cloned into AAV backbone with a U6 promotor and a fluorescent protein (m-Cherry) and packaged using the AAV8 packaging system by the Hope Center Virus Core Facility at Washington University Medical School.

Extracellular Multielectrode Array Recordings. Dispersed SCN neuron cultures were prepared as previously described (64). Briefly, SCN explants were obtained from postnatal day 4 (P4) Per2LUC mice and coronal slices (300 µm) using a Vibratome (OCT-5000, Electron Microscopy Sciences). Six unilateral SCN punches (1 mm diameter) were obtained and enzymatically dissociated using papain. Dissociated SCN neurons in a volume of 10 µl were incubated with 0.4 µl of the NT, or γ2 shRNA or δ shRNA and plated at a density of ~10,000 neurons/mm² on poly-D-lysine/laminin coated multielectrode arrays (sixty 30-µm diameter electrodes, Multichannel Systems). After 1 h, 1.2 ml of DMEM supplemented with 10% fetal calf serum was added to each dish and maintained at 36 °C in a 95% O₂ / 5% CO₂ incubator. Extracellular action potentials were recorded continuously from dispersed SCN neurons for at least 5 d as previously described (65). Action potentials were digitized in real-time (MC-Rack Software, Multichannel Systems) and single-neuron activity discriminated offline using principal component analysis and a refractory period of greater than 1 ms (Offline Sorter). Firing rate was binned in 10-min intervals (NeuroExplorer). Firing patterns with periods between 18 and 30 h were scored as circadian using MetaCycle analysis (63). The average spontaneous firing rate of each neuron at its daily peak and trough was calculated from the five days of recording. Interspike interval histograms (ISI) for each recorded SCN neuron were obtained (NeuroExplorer) to look for changes in firing patterns after the depletion of γ2 or δ subunits using similar parameters reported by Kononenko (36). Briefly, ISI histograms were created using bins of 1 or 10 ms over 10-min recordings and analyzed for asymmetry of the ISI distributions.

Whole-Cell Electrophysiology. Cultured SCN slices (3 to 6 d in vitro) from WT, δ, and γ2 KI mice were transfected to a recording chamber with continuous perfusion (2 ml/min, 32 °C) of oxygenated artificial cerebrospinal fluid (in mM: 125 NaCl, 25 glucose, 2.5 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, 2 CaCl₂, and 1 MgCl₂, equilibrated with 95% oxygen/5% CO₂). Whole-cell recordings were performed with borosilicate glass pipettes (World Precision Instruments) with open tip resistances of 3 to 6 MΩ. Pipettes were filled with internal solution containing the following (in mM): 130 CsCl, 2 NaCl, 0.5 CaCl₂, 5 EGTA, 10 HEPES, 2 MgATP, and 0.5 Na,GTP, pH 7.39 with CsOH. Currents were confirmed to be GABAergic at the conclusion of recordings based on their response to application of 100 µM gabazine (GBZ, Tocris).

Voltage clamp recordings were performed at −70 mV, and spontaneous phasic GABAergic and tonic currents were recorded at baseline and in the presence of a photomultiplier tube (PMT, H93319-11; Hamamatsu, 10-min bins) or an ultraviolet-sensitive CCD camera (Andor iKon or iKon; 1 × 1 binning, 1 h exposures) at 36 °C (In Vivo Scientific incubator) on an inverted microscope (Nikon TE2000 or Leica DMi8 fitted with a 20× objective and a 0.5× coupler) in a sealed petri dish with media supplemented with 0.1 mM betleuciferin (Biosynth).

From the recorded bioluminescence, we measured peak-to-trough amplitude and circadian period using Chronostar (60), for whole SCN explant experiments. SCN explants were considered circadian if Cosinor analysis (Chronostar) found a period between 18 and 30 h and a coefficient of correlation greater than 0.7. To locate and track cellular bioluminescence, we used a custom Python code (45) which identified cells in each frame using a standard difference of Gaussian blob detector, in addition, videos underwent pixel-based analysis using a custom Python script to measure instantaneous phase, amplitude, and synchronizaiton index (SI). We measured intensities by implementing a multidimensional Gaussian filter in SciPy tools using established methods (61) to generate time series of pixel intensities. Generated time series of pixel intensities were then detrended and smoothed using Sinc filter and the instantaneous phase and amplitudes were calculated using a continuous wavelet transformation analysis implemented in pyBOAT (61). Synchro index values were calculated as the first-order Kuramoto (45) parameter R(t) = ∑ r |S(t) - S(t - j)|/vectorial or directional averaging of instantaneous phases on the complex plane (62). We used MetaCycle (63) to quantify the circadian phase, amplitude, and period of each pixel and calculated the percentage of circadian pixels as [number of pixels reported as significantly circadian (BH.Q < 0.01)]/number of pixels with nonzero pixel intensities × 100. The period range to consider a cell circadian was established between 18 and 30 h and with a P < 0.05 value from MetaCycle.
of picrotoxin (PTX, 100 μM). Tonic GABA\textsubscript{R} currents were recorded at baseline and measured as the SD of the baseline holding current in event-free portions of the recording and expressed relative to the GBZ condition.

**Wheel-Running Recordings.** Wheel-running activity was recorded in 6-min bins (ClockLab Actimetrics) for ~10 d in a 12:12 h light-dark (LD) cycle (lights ON 6:00 am, lights OFF 6:00 pm) followed by ~10 d in DD from shRNA-injected and knock-in mice. Bilateral injections aimed at the SCN (0.4 μl × side) of NT shRNA, γ2 shRNA, or δ shRNA-expressing AAV8 were made in adult Per\textsubscript{2}β\textsubscript{1} mice (postnatal days 60 to 90; P60 to P90). Injected mice recovered for 5 d before locomotor assays. All mice, including δ-mutant mice, were placed individually in cages equipped with a running wheel in light-tight chambers illuminated with fluorescent bulbs (~2.4 × 10\textsuperscript{18} photons/cm\textsuperscript{2}S; General Electric). The period and amplitude of behavioral rhythmicity of each mouse were determined by \( x^2 \) periodogram analysis (66) from the last 5 continuous days in LD and in DD.

**Statistical Analyses.** Unless otherwise specified, comparisons between control, KD, or KI groups were performed by a one-way or two-way mixed ANOVA (post hoc Tukey’s or Bartlett’s test). We used the Kolmogorov–Smirnov test for firing rate comparisons. Unless otherwise specified, data are presented as mean ± SEM or %. Statistical significance was set to \( P < 0.05 \).

**Data, Materials, and Software Availability.** All study data are included in the article and python scripts data have been deposited in github (https://github.com/herzog-lab/daniel-granados-et-al-2024 (67)).

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