Picrotoxin dramatically speeds the mammalian circadian clock independent of Cys-loop receptors

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Freeman GM Jr, Nakajima M, Ueda HR, Herzog ED. Picrotoxin dramatically speeds the mammalian circadian clock independent of Cys-loop receptors. J Neurophysiol 110: 103–108, 2013. First published April 10, 2013; doi:10.1152/jn.00220.2013.—Picrotoxin is extensively and specifically used to inhibit GABA_A receptors and other members of the Cys-loop receptor superfamily. We find that picrotoxin acts independently of known Cys-loop receptors to shorten the period of the circadian clock markedly by specifically advancing PERIOD2 protein. We show that this mechanism is surprisingly tetrodotoxin-insensitive, and the effect is larger than any known chemical or genetic manipulation. Notably, our results indicate that the circadian target of picrotoxin is common to a variety of human and rodent cell types but not Drosophila, thereby ruling out all conserved Cys-loop receptors and known regulators of mammalian PERIOD protein stability. Given that the circadian clock modulates significant aspects of cell physiology including synaptic plasticity, these results have immediate and broad experimental implications. Furthermore, our data point to the existence of an important and novel target within the mammalian circadian timing system.

Circadian clocks drive near 24-h rhythms in >10% of the genome and modulate daily changes in cellular metabolism and physiology (Herzog 2007; Panda et al. 2002). The period of circadian oscillations is temperature-compensated and remains remarkably resistant to pharmacological challenge. For example, a recent chemical screen of 1,280 active compounds reported no agents capable of dose-dependently shortening circadian periodicity by ≥4 h (Hirota et al. 2008). Here, we find that the classic GABA_A receptor antagonist, picrotoxin, dramatically shortens circadian rhythms in single cells and across multiple tissues. Surprisingly, this effect is independent of known Cys-loop receptors, including GABA_A receptors. Importantly, we find that picrotoxin significantly decreases the period of circadian oscillations at concentrations routinely used in cellular and systems neuroscience.

MATERIALS AND METHODS

Animals. Period2-luciferase (PER2::LUC) knock-in mice (Yoo et al. 2004; founders generously provided by J. S. Takahashi) and Period1-luc rats were housed in a 12:12-h light-dark cycle and bled as homozygous pairs in the Danforth Animal Facility at Washington University. Timeless-luciferase (Tim::Luc) Drosophila melanogaster (Stanewsky et al. 1997), generously provided by P. H. Taghert, were grown on standard molasses and yeast medium at room temperature and on a 12:12-h light-dark cycle. All procedures were approved by the Animal Care and Use Committee of Washington University and followed National Institutes of Health (NIH) guidelines.

Cell lines. Primary human astrocytes (generously provided by J. B. Rubin) were maintained at 37°C in DMEM supplemented with 10% FBS and penicillin/streptomycin.

Gene reporter construct. We performed lentiviral infections of pure human astrocyte cultures using a lentiviral construct expressing the Per2::LUC reporter (Liu et al. 2008; Zhang et al. 2009). Astrocytes were incubated with the viral particles for 12 h, washed, and passaged twice over a 2-wk period before plating and imaging.

Mammalian bioluminescence recording. For suprachiasmatic nucleus (SCN) explant recordings, 300-μm coronal SCN slices from PER2::LUC mice (age 7–14 days) were cultured at 34°C on Millicell CM membranes (Millipore, Billerica, MA) in sealed 35-mm culture dishes (BD Biosciences, San Jose, CA) with prewarmed air-buffered medium and 100 μM beetle luciferin (Promega, Madison, WI). Bioluminescence was recorded using a photomultiplier tube (PMT; HC135-11 MOD; Hamamatsu, Shizuoka, Japan) as previously described (Abe et al. 2002). Bioluminescence counts were integrated and stored at 1-min intervals for up to 18 days of recording. For single-cell recordings, explants were cultured for 4 days on Millicell CM membranes, inverted onto polylsine/laminin-coated glass coverslips in prewarmed air-buffered medium with 100 μM beetle luciferin, and imaged (VersArray 1024 cooled CCD camera; Princeton Instruments). Photon counts were integrated over 10–30 min with 2 × 2 binning and quantified using ImageJ software. For lung explant recordings, lung tissue from 14-day-old PER2::LUC mice was dissected and placed onto Millicell CM membranes. Tissue was cultured with air-buffered medium, 100 μM beetle luciferin, nystatin (20 μg/ml), and amphotericin B (0.25 μg/ml; Sigma). Bioluminescence was recorded using a PMT, and counts were integrated at 6-min intervals.

Drosophila bioluminescence recordings. Abdomens and wings of male Tim::Luc flies were dissected and placed individually in 96-well plates containing Schneider’s Insect Medium supplemented with 12% FBS, 1% penicillin/streptomycin, and 100 μM beetle luciferin (Giebultowicz et al. 2000). The plate was imaged at 30 frames per second using an XR/MEGA-10Z CCD camera placed in a light-tight, temperature-controlled box at 25°C (Stanford Photonics, Palo Alto, CA). Images were then integrated every 60 min using ImageJ software.

Electrophysiology. Long-term recordings of firing rate from high-density dispersed SCN neurons were made using multielectrode arrays (Multi Channel Systems, Reutlingen, Germany). To disperse cells, SCN were punched from 300-μm-thick slices, and cells were dissociated using papain (Herzog et al. 1998). Viable cells (from 10–14 SCN) were plated on 60 30-μm-diameter electrodes (200-μm spacing) and maintained in CO_2-buffered medium for 3 wk before recording. Culture chambers fixed to arrays were covered with a fluorinated ethylene-polypropylene membrane (Potter and DeMarce 2001) before transfer to recording incubator. The recording incubator was maintained at 36°C with 5% CO_2 throughout all experiments. Extracellular voltage signals were recorded for a minimum of 10 days from 60 electrodes simultaneously. Action potentials exceeding a
defined voltage threshold were digitized into 2-ms time-stamped cutouts (MC_Rack software; Multi Channel Systems). Spikes from individual neurons were discriminated offline using a principal component analysis-based system (Offline Sorter; Plexon), and firing rates were calculated over 1-min recording epochs (NeuroExplorer; Plexon). Correct discrimination of single-neuron activity was ensured by the presence of a clear refractory period in autocorrelograms of firing records (NeuroExplorer).

**Measurement of CK1ε activity.** Casein kinase 1ε (CK1ε) activity was measured by coinubation of the purified enzyme with a synthetic peptide substrate derived from the TrCP-binding region of mouse PER2, and CK1ε kinase activity was assayed using the P81 phosphocellulose assay (Isojima et al. 2009).

**Drug treatments.** Picrotoxin, picrotoxinin, bicuculline, strychnine, U73122, TBPS (Dillon et al. 1995), NPPB, MDL-12,330A, TTX, and TPMPA (generously provided by P. D. Lukasiewicz) were purchased from Sigma (Saint Louis, MO). SR-95531 (gabazine), (+)-tubocurarine (Yan et al. 1998), and 9-anthracene-carboxylate (9-AC) were purchased from Tocris (Ellisville, MO). All drugs were diluted in deionized water, 95% ethanol, or DMSO and stored at either −20 or 4°C depending on manufacturer instructions. A volume of concentrated stock solution (<0.5% of total volume) was applied to each culture.

**Data analysis.** Continuous recordings of gene expression or firing records lasting at least 4 days were used for analysis of rhythmicity. Circadian period of explant rhythmicity was calculated using 2-way ANOVA vs. vehicle; means ± SE, n = 4–6 SCN explants per concentration). Line represents a sigmoidal fit to the data. D: picrotoxin (100 μM) shortens periodicity of PER2::LUC expression in single neurons within an SCN explant (inset; n = 64 neurons; *P < 0.001, Student’s t-test). E: picrotoxin speeds circadian rhythms in firing activity of neurons recorded on a multielectrode array (inset; n = 10 neurons; *P < 0.001). F: pharmacological block of Cys-loop receptors and Cl− channels does not affect period. Picrotoxin and picrotoxinin shortened PER2::LUC rhythms, however, alternative inhibitors of Cys-loop receptors or Cl− channels do not affect period (means ± SD, the number of SCN explants is indicated for each treatment). Asterisk indicates significant difference compared with vehicle (P < 0.001; 1-way ANOVA). Dashed line indicates mean of vehicle treatment. LGICs, ligand-gated ion channels; TBPS, tert-butylbicyclophosphorothionate; TPMPA, (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; 9-AC, 9-anthracene-carboxylate.
periodogram analysis (Sokolove and Bushell 1978); period for single-cell gene expression and Drosophila was calculated by FFT-NLLS (Plautz et al. 1997). Confidence intervals were set to 99 and 95% for χ²-periodogram and FFT-NLLS, respectively. Additional statistical tests were done with Origin 7 software (OriginLab, Northampton, MA). Mean periods (for Figs. 1C, 1F, and 3A) were compared by one-way ANOVA and Scheffé post hoc tests. Mean periods for other analyses were compared by two-tailed t-tests. Distributions of periods were compared using both Levene’s and Brown-Forsythe’s tests for equal variance.

RESULTS

Picrotoxin speeds the circadian clock within the central mammalian pacemaker. To measure the effect of picrotoxin on circadian rhythmicity, we monitored a bioluminescent reporter of PERIOD2 (PER2) protein expression from SCN of Period2Luciferase mice. SCN explants generate sustained and stable daily rhythms and are therefore ideally suited for testing period effects of drugs (Fig. 1A). We found that within one cycle, a frequently used experimental concentration of picrotoxin (100 μM) decreased the period of PER2 rhythms from 23.9 ± 0.3 to 19.8 ± 0.1 h (n = 4; Fig. 1B) but did not affect the peak-to-trough amplitude (ratio of 3rd day of picrotoxin treatment to the 3rd day of baseline was 1.1 ± 0.2, n = 4). Circadian period shortened dramatically with increasing picrotoxin concentration, saturating at ~500 μM with a period of 17.4 ± 0.4 h (Fig. 1C). Picrotoxin was similarly effective on rat SCN, significantly accelerating rhythmicity of a luciferase reporter for Period1 transcriptional activity (vehicle, 25.5 ± 0.6 h; 100 μM picrotoxin, 19.2 ± 0.1 h; n = 3–5; P < 0.00001).

The SCN consists of a broad range of neural subgroups, differentiated according to neuropeptide content (van den Pol and Tsujimoto 1985), oscillatory behavior (Moore et al. 2002; Shinohara et al. 1995; Webb et al. 2009), and connectivity (Leak and Moore 2001; Moga and Moore 1997). To test whether picrotoxin decreases periodicity in all SCN cells or only a subset, we monitored PER2 expression in individual neurons within an SCN explant. We found that picrotoxin decreased periodicity in all measured SCN neurons (baseline, 23.5 ± 0.1 h; picrotoxin, 19.8 ± 0.1 h; n = 64 neurons; P < 0.00001; Fig. 1D). Importantly, picrotoxin also shortened rhythms in firing rate of individual SCN neurons dispersed on a multielectrode array (baseline, 23.9 ± 0.1 h; picrotoxin, 20.4 ± 0.2 h; n = 10 neurons; P < 0.00001; Fig. 1E).

Picrotoxin alters clock function independent of Cys-loop receptors. These effects of picrotoxin, although highly reliable and reversible, appeared to contradict a prior report that blockade of GABA_A receptors had no effect on circadian period (Aton et al. 2006). To examine the mechanism of the effect of picrotoxin on circadian rhythmicity, we first tested the necessity of inhibiting Cys-loop receptor picrotoxin-binding sites. Whereas picrotoxin or its constituent, picrotoxinin, reliably decreased the period of circadian oscillations, the chemically distinct competitor for the picrotoxin-binding site, TBPS (Squires et al. 1983), did not (Fig. 1F). Furthermore, pre- and cotreatment with equimolar TBPS did not abrogate the period-reducing effect of 50 μM picrotoxin (vehicle + picrotoxin, −1.91 ± 0.19 h; TBPS + picrotoxin, −1.91 ± 0.12 h, n = 5 per group; P = 1). Given that picrotoxin can inhibit other members of the Cys-loop receptor superfamily (Erkkila et al. 2004), we systematically tested antagonists of known picrotoxin targets on SCN rhythmicity (Fig. 1F). Blockade of the anionic Cys-loop receptors, GABA_A receptors (with gabazine or bicuculline), GABA_AR receptors (with TPMPA), and glycine receptors (with strychnine) all failed to alter periodicity (P > 0.05).

Antagonism of the cationic Cys-loop receptors, nicotinic acetylcholine receptors, and 5-HT3 receptors (with d-tubocurarine) was also ineffective (P > 0.05). Simultaneous application of gabazine (500 μM), strychnine (50 μM), and d-tubocurarine (250 μM) also did not alter SCN periodicity (P > 0.05). Together, these results indicate that picrotoxin acts independently of known Cys-loop receptor targets to shorten the period of the circadian clock.

Picrotoxin specifically accelerates PER2 protein accumulation without altering CK1ε activity. To gain insight into the mechanism by which picrotoxin speeds the molecular clock, we analyzed the waveform of PER2 expression in individual cells recorded in an SCN explant. Period shortening could result through uniform compression of the waveform, however, we found that 100 μM picrotoxin selectively advanced the accumulation of PER2 protein by ~3.7 h without affecting PER2 stability or degradation as measured by peak half-width (baseline, 8.7 h; picrotoxin, 8.6 h; calculated from averaged traces of 50 randomly selected neurons; Fig. 2A). Additionally, we directly monitored the effect of picrotoxin on CK1ε, a key posttranslational regulator of PER2 stability in vivo (Takahashi et al. 2008). We found that neither picrotoxin (100 μM) nor picrotoxinin (100 μM) altered CK1ε kinase activity (Fig. 2B). Taken together, these data suggest that picrotoxin acts inde-
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Picrotoxinin (1-way ANOVA, with vehicle or picrotoxin (500 µM, n = 3), Gq-phospholipase C (U73122, 10 µM, n = 3), or Gβγi/o signaling (pertussis toxin, 5 nM, n = 3) did not lessen the effect of picrotoxinin (1-way ANOVA, P > 0.05 vs. picrotoxinin). Data represent means ± SE. Dashed line represents mean period of vehicle-treated cultures. B: TTX (2 µM) does not abrogate picrotoxin-induced period shortening of PER2::LUC rhythmicity in SCN explants. *P < 0.0001. Data represent means ± SE, n = 3–4 independent SCN explants per treatment.

Period shortening does not require GPCR or action potential-dependent signaling. Advances in PER2 accumulation could occur through a variety of candidate pathways requiring G-protein-coupled receptor (GPCR) activation (Golombek and Rosene 2010). We found, however, that inhibition of Gq-adenylyl cyclase (MDL-12,330A, 2.5 µM), Gq-phospholipase C (U73122, 10 µM), or Gβγi/o signaling (pertussis toxin, 5 nM) all failed to block picrotoxin-induced period reduction (P > 0.05; Fig. 3A). In retina of the marine gastropod *Bulla gouldiana*, shortened circadian periods and phase-advanced rhythms in compound action potential frequency result from blockade of nonspecific chloride conductances (Khalsa et al. 1990; Michel et al. 1992). Given that picrotoxin antagonizes ligand-gated chloride conductances, we hypothesized that its effect on PER2 was due to chloride channel/transporter blockade. However, inhibition of Ca2+-activated and voltage-gated chloride channels by 9-AC or NPPB did not alter period length (P > 0.05; Fig. 1F). Since picrotoxin ubiquitously shortens period of single neurons independent of Cys-loop receptors and GPCR activation, we tested whether its actions required intercellular signaling. Application of the voltage-gated sodium channel blocker TTX to SCN explants did not block the effect of picrotoxin (TTX+vehicle, 24.1 ± 0.2 h; TTX+picrotoxin, 19.8 ± 0.4 h; n = 4 and 3, respectively; P < 0.0001; Fig. 3B). Together, these data indicate that picrotoxin acts cell-autonomously to accelerate the intracellular molecular mechanisms regulating PER2 accumulation.

Picrotoxin shortens periodicity in human and murine non-neuronal cells. Clock genes are rhythmically expressed in multiple mammalian tissues including brain, liver, and lung (Takahashi et al. 2008). To test the generality of the effect of picrotoxin on mammalian tissue, we applied picrotoxin to primary human astrocytes expressing a lentivirus-delivered luciferase reporter of Per2 transcription. We found that picrotoxin shortened the period of Per2::dLuc rhythmicity in astrocytes (vehicle, 26.0 ± 0.2 h; 100 µM picrotoxin, 23.9 ± 0.3 h; P < 0.0001; Fig. 4, C and D). Additionally, we applied picrotoxin to mouse lung explants and found it shortened period (vehicle, 22.5 ± 0.1 h; 500 µM picrotoxin, 19.4 ± 0.1 h; n = 4 and 3, respectively; P < 0.0001; Fig. 4, A and B). These results are also consistent with the period shortening found in the drug screen by Isojima et al. (2009) on NIH/3T3 and U2OS cells. Given that tritiated picrotoxin, [3H]α-dihydropicrotoxinin, does not specifically bind lung membrane fractions (Ticku

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Fig. 3. Picrotoxin speeds circadian rhythmicity independent of G-protein-coupled-receptor- or action potential-dependent signaling. A: picrotoxinin (PTX; 100 µM, n = 7) reduced circadian period (19.3 ± 0.7 h) of SCN explants. Cotreatment with picrotoxinin and inhibitors of Gq-phospholipase C (20.6 ± 0.2 h, U73122, 10 µM, n = 3), Gq-adenylyl cyclase (19.9 ± 0.7 h, MDL-12,330A (MDL), 2.5 µM, n = 3), or Gβγi/o (19.9 ± 0.3 h, pertussis toxin, 5 nM, n = 3) did not lessen the effect of picrotoxinin (1-way ANOVA, P > 0.05 vs. picrotoxinin). Data represent means ± SE. Dashed line represents mean period of vehicle-treated cultures. B: TTX (2 µM) does not abrogate picrotoxin-induced period shortening of PER2::LUC rhythmicity in SCN explants. *P < 0.0001. Data represent means ± SE, n = 3–4 independent SCN explants per treatment.

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Fig. 4. Picrotoxin speeds the circadian clock in murine and human nonneuronal tissue but not *Drosophila*. A: top: PER2::LUC rhythms from representative lung explants cultured with vehicle or picrotoxin (500 µM); bottom: quantification of the period of lung cultures under each condition (means ± SE, n = 3–4 per treatment; *P < 0.001). B: top: PER2::dLuc rhythms from representative primary human astrocytes cultured with vehicle or picrotoxin (100 µM); bottom: quantification of the period of human astrocyte cultures under each condition (n = 5 per treatment; *P < 0.001). C: top: Timeless:Luciferase (Tim::Luc) rhythms from representative *Drosophila* abdomens cultured in vehicle or picrotoxin (500 µM); bottom: *Drosophila* Tim::Luc abdominal rhythms were unaffected by picrotoxin (n = 26 per treatment; P > 0.05). n.s., Not significant.
et al. 1978), our results suggest that the target of picrotoxin may be intracellular.

**Picrotoxin does not alter periodicity in Drosophila.** Since picrotoxin speeds rhythmicity in nonneural tissue and across human, mouse, and rat, we tested whether it could also modulate period across phyla. We developed a high-throughput assay to monitor *Timeless* gene expression from *D. melanogaster*. Interestingly, rhythms in *Drosophila* abdominal clock gene expression are not altered by 500 μM picrotoxin (vehicle, 23.4 ± 0.1 h; picrotoxin, 23.7 ± 0.2 h; n = 26 per treatment; *P* > 0.05; Fig. 4, *E* and *F*). Similar results were also obtained from individual wings (vehicle, 25.4 ± 0.8 h; 100 μM picrotoxin, 25.4 ± 0.5 h; n = 20–25 per treatment, *P* > 0.05; vehicle, 23.3 ± 0.3 h; 500 μM picrotoxin, 22.8 ± 0.3 h; n = 23 per treatment, *P* > 0.05). Given that picrotoxin-sensitive Cys-loop receptors are conserved in *Drosophila* (Ortells and Lunt 1995), these results further indicate that picrotoxin is acting via a distinct and novel mechanism in mammalian cells.

**DISCUSSION**

Our results are consistent with observations that cell-autonomous regulation of PER2 protein accumulation can accelerate circadian rhythms (Blau 2008). For example, in humans, mice, hamsters, and flies, it has been argued that mutations in homologs of Per2 or CK1 speed circadian periodicity by altering PER phosphorylation, stability, nuclear localization, and transcriptional activation, although the specifics remain unclear (Takahashi et al. 2008). Notably, however, our results suggest that the target of picrotoxin is found in mammal but not flies, thereby ruling out all conserved Cys-loop receptors and known regulators of mammalian PER stability. We conclude that picrotoxin alters PER2 dynamics through a novel, mammalian-specific mechanism.

The effects of picrotoxin on circadian timing in vivo remain untested. Because picrotoxin can induce epileptiform activity and seizures (Sierra-Paredes and Sierra-Marcuno 1996a,b), most behavioral experiments have been limited to acute injections (Berretta et al. 2004; Ikemoto et al. 1997; Kodsi and Swerdlow 1995; Shin and Ikemoto 2010). For example, acute picrotoxin administration into the supramammillary nucleus transiently increased locomotor activity for several minutes (Shin and Ikemoto 2010). Although the effects of chronic picrotoxin exposure remain unknown, we predict that in vivo manipulation of the picrotoxin target would speed circadian rhythms in physiology and behavior. Unfortunately, until its circadian target is identified, the toxic side-effects of picrotoxin will prevent its use for in vivo tests on circadian timing.

Although picrotoxin is commonly used to inhibit Cys-loop receptors including GABA<sub>A</sub> receptors, our results show that it could impact experimental results through two additional routes. First, thousands of genes and proteins oscillate in abundance or activity over circadian time, as do features of LTP (Hirota T, Lewis WG, Liu AC, Lee JW, Schultz PG, Kay SA). A chemical biology approach reveals period shortening of the mammalian circadian clock by 10.220.33.5 on July 1, 2017 http://jn.physiology.org/ Downloaded from

 acute CK2 inhibition alters LTP by antagonizing activity-dependent NR2B phosphorylation and endocytosis (Sanz-Clemente et al. 2010). Given that picrotoxin surprisingly impacts protein dynamics over circadian time independent of known Cys-loop receptor targets, unknown targets may have important and novel short-term actions as well.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

G.M.F., H.R.U., and E.D.H. conception and design of research; G.M.F. and M.N. performed experiments; G.M.F. and M.N. analyzed data; G.M.F. and M.N. interpreted results of experiments; G.M.F. prepared figures; G.M.F. drafted manuscript; G.M.F., H.R.U., and E.D.H. edited and revised manuscript; G.M.F., M.N., H.R.U., and E.D.H. approved final version of manuscript.

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