

CIRCADIAN RHYTHMS

Cell-autonomous clock of astrocytes drives circadian behavior in mammals

Marco Brancaccio^{*†}, Mathew D. Edwards[‡], Andrew P. Patton, Nicola J. Smyllie, Johanna E. Chesham, Elizabeth S. Maywood, Michael H. Hastings^{*}

Circadian (~24-hour) rhythms depend on intracellular transcription-translation negative feedback loops (TTFLs). How these self-sustained cellular clocks achieve multicellular integration and thereby direct daily rhythms of behavior in animals is largely obscure. The suprachiasmatic nucleus (SCN) is the fulcrum of this pathway from gene to cell to circuit to behavior in mammals. We describe cell type-specific, functionally distinct TTFLs in neurons and astrocytes of the SCN and show that, in the absence of other cellular clocks, the cell-autonomous astrocytic TTFL alone can drive molecular oscillations in the SCN and circadian behavior in mice. Astrocytic clocks achieve this by reinstating clock gene expression and circadian function of SCN neurons via glutamatergic signals. Our results demonstrate that astrocytes can autonomously initiate and sustain complex mammalian behavior.

The transcription-translation negative feedback loop (TTFL) mechanisms responsible for intracellular circadian (~24-hour) time-keeping in animals are understood in molecular detail (1). The TTFL of mammals involves transcriptional activation by Clock/Bmal1 heterodimers, which drive daytime expression of *Period* (*Per*) and *Cryptochrome* (*Cry*) genes through E-box regulatory sequences. After dimerization and transport to the nucleus, Per-Cry complexes repress Clock-Bmal1 activity during circadian night, until progressive degradation of Per-Cry allows initiation of a new cycle. This self-sustaining cell-autonomous TTFL is universally active across mammalian tissues, so how cellular clocks interact to achieve multicellular integration and ultimately direct daily rhythms of behavior is a matter of considerable interest. The suprachiasmatic nucleus of the hypothalamus (SCN) is the fulcrum of this pathway from gene to cell to circuit to behavior. Its tightly coordinated multicellular oscillations can continue indefinitely to direct internal synchronization of cellular clocks across the body. The conventional view is that robust pacemaking relies on the intrinsic interneuronal connectivity of the SCN, albeit with principles still largely unknown (2). However, circadian time-keeping in the SCN is also influenced by a sophisticated interplay between its neurons and astrocytes (3). In common with other cell types, astrocytes have a TTFL that is assumed to be maintained by input from SCN neurons (4, 5). In light of the intimacy of astrocyte-neuron interactions in the SCN, however, we wondered

whether SCN astrocytes really are “slaves” to their neuronal partners, or whether the SCN pacemaker might instead be considered a bipartite cellular system in which astrocytes can also direct neuronal time-keeping and behavior.

To address this, we used adeno-associated virus vectors (AAVs) and genetics to characterize the distinctive properties of the TTFLs of SCN astrocytes and neurons. We assessed cell type-specific TTFL function with an AAV encoding a Cre recombinase-dependent reporter (6) in which firefly luciferase is driven by a minimal mouse *Cry1* promoter (*Cry1*) containing E-boxes (*Cry1*-Flex-Luc; Flex represents Cre-dependent flip-excision) (7, 8). Cotransduction of SCN slices with AAVs driving Cre by the glial fibrillary acidic protein (GFAP) or human synapsin 1 (Syn) promoters restricted *Cry1*-Flex-Luc expression to astrocytes or neurons, respectively (3) (Fig. 1, A to F). Bioluminescent recording revealed sustained circadian oscillations of *Cry1*-Luc in both SCN neurons and astrocytes. Although these oscillations had the same period and robustness, measured by the relative amplitude error (RAE), their waveforms differed (Fig. 1F), reminiscent of the distinctive waveforms of intracellular calcium rhythms observed in astrocytes and neurons (3). These SCN slices were also cotransduced with AAVs encoding the calcium reporter GCaMP3 driven by the Syn promoter (Syn-GCaMP3) to track circadian concentrations of neuronal intracellular calcium ($[Ca^{2+}]_i$). We used this reporter, which peaks during the mid-circadian day [circadian time 6.5 hours (CT6.5)] (3, 9), to internally register the circadian phase of the detected *Cry1*-Luc expression in SCN astrocytes and neurons. This showed that the peak of expression of astrocytically restricted *Cry1*-Luc was phase-delayed by ~6.5 hours (~CT17) when compared with that of the neurons, which peaked at ~CT11 (Fig. 1, B, C, and F). Thus, neurons and astrocytes of the SCN exhibit cell type-specific functionally distinct

Cry1-Luc reporter TTFLs characterized by different phases and waveforms.

To test the potential contribution of the astrocytic TTFL to SCN time-keeping, we used cell type-specific genetic complementation in SCN of mice lacking both *Cry* genes (*Cry1/2*-null mice) (10). In the absence of the Cry repressors, the endogenous TTFL does not function, so molecular circadian oscillations, monitored by the *Per2::Luc* reporter, are compromised (11) (Fig. 1G). Generalized (pan-cellular) expression of *Cry1* can initiate circadian molecular rhythms in Cry-deficient SCN slices (8). Using Cre-dependent AAVs encoding a *Cry1::EGFP* (enhanced green fluorescent protein) fusion protein driven by the *Cry1* promoter (*Cry1*-Flex-*Cry1::EGFP*), we expressed *Cry1* specifically in either neurons or astrocytes of *Cry1/2*-null SCN restricted by Syn-Cre or GFAP-Cre. As anticipated, expressing *Cry1* in neurons was sufficient to initiate self-sustained circadian oscillations of *Per2::Luc* in the SCN. Expression of *Cry1::EGFP* solely in astrocytes was also effective, however, highlighting astrocytes as pacemakers within the SCN circuit (fig. S1 and Fig. 1, G and H). Nevertheless, there were appreciable differences in both the early and the late phases of *Cry1* expression between the two cell type-specific manipulations. The effects on *Per2::Luc* oscillations of neuronally restricted *Cry1::EGFP* became apparent within ~2 days posttransduction (dpt), whereas astrocytically restricted *Cry1* took appreciably longer (>7 dpt) to initiate rhythms. In the later stages (11 to 15 dpt), *Cry1* maintained stable oscillations longer than 24 hours (appropriate to a *Cry2*-null background) (10) when expressed in either neurons or astrocytes, although astrocytically dependent rhythms had a significantly shorter period than neuronally driven rhythms (Fig. 1H). Thus, not only SCN neurons but also astrocytes can autonomously initiate and sustain stable oscillations of clock gene expression in the SCN, and their instructive, rather than simply permissive, role is evidenced by the observed period differences.

As shown by SCN transplantation between animals with contrasting genetically specified circadian periods (12, 13), the defining property of the SCN as the master circadian pacemaker is its ability to initiate circadian patterns of behavior, imposing its intrinsic periodicity to the rest of the body. We therefore tested whether the cell-autonomous astrocytic TTFL could drive circadian locomotor activity rhythms in otherwise “clockless” adult mice and compared them to rhythms of mice with similarly restricted manipulations of the neuronal TTFL (Fig. 2). The SCN of *Cry1/2*-null mice were stereotactically injected with Cre-conditional AAV-*Cry1*-Flex-*Cry1::EGFP* together with (i) AAV-GFAP-mCherry::Cre, (ii) AAV-Syn-mCherry::Cre, or (iii) AAV-GFAP-EGFP, as a Cre negative control group (Fig. 2, A to C, and fig. S2A). We confirmed high specificity and efficiency of Cre-dependent expression of *Cry1::EGFP* by evaluating post hoc the histological colocalization of the *Cry1::EGFP* signal with GFAP-mCherry::Cre or Syn-mCherry::Cre, respectively (Fig. 2, D and E). We further confirmed that the GFAP-driven Cre recombinase

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efficiently restricts expression of Cry1 to astrocytes by colocalizing the Cry1::EGFP signal with the astrocytic marker AldH1L1 (3, 4) (fig. S2B). Locomotor activity of mice was recorded before and after surgery under constant dim red light (DD) to assess the intrinsic free-running circadian rhythmicity. Before surgery, Cry1/2-null mice did not show any consistent circadian rhythmicity in DD (DD1). However, after surgery (DD2), and in contrast to Cre-negative control mice, both AAV-GFAP-mCherry::Cre-treated and AAV-Syn-mCherry::Cre-treated mice showed sustained circadian patterns of locomotor behavior (Fig. 2F and fig. S2A). The periods of the induced rhythms were consistent with the molecular cycles of Per2::Luc observed in SCN explants being >26 hours and with the astrocytically driven rhythm being ~1 hour shorter than that of mice with neuronally expressed Cry1 (Figs. 2F and 1H). Furthermore, across animals the number of Cry1::EGFP⁺ neurons correlated positively with the behavioral period, whereas when Cry1 was expressed in SCN astrocytes there was a negative relationship (Fig. 2G), supporting the view that increasing numbers of targeted neurons and astrocytes can drive the locomotor rhythm to a period progressively closer to that of the corresponding cell-autonomous TTFLs. Nevertheless, the daily profiles of circadian behavior were equivalent whether neuronally or astrocytically controlled (Fig. 2H and fig. S2C). Thus, SCN astrocytes can specifically instruct new circadian behavior in an otherwise-arrhythmic mouse.

Given that astrocytes are not directly connected to motor centers, we hypothesized that they rely on recruiting the (TTFL-incompetent) SCN neuronal circuitry to engage behavioral output. To test for such indirect mechanisms, we imaged single cell- and circuit-level TTFL dynamics in Cry1/2-null SCN slices during the early phases of neuronal or astrocytic Cry1 expression (Fig. 3). Neuronal expression of Cry1 immediately generated robust cellular Per2::Luc oscillations, consistent with a direct effect on the neuronal TTFL and similar to that observed after nonrestricted expression of Cry1 (8) (fig. S3). In contrast, although expression of Cry1 in astrocytes also initiated stable long period oscillations, it took >7 days to do so (movie S1 and Fig. 3, A and B). Analysis of individual Per2::Luc⁺ cells in the SCN revealed that Cry1 expression in astrocytes produced a progressive strengthening of Per2::Luc cellular rhythms, with periods initially differing by >16 hours and slowly converging to a single ~28.5-hour period (Fig. 3, C and D). This progressive effectiveness of astrocytes is consistent with an indirect engagement of the wider neuronal circuit.

To monitor neuronal activity directly, SCN slices expressing Cry1 only in astrocytes were supertransduced with AAVs encoding the synapsin-driven red genetically encoded calcium indicator RCaMP1h (Syn-RCaMP1h) (3). This revealed astrocytically driven circadian oscillations of neuronal $[Ca^{2+}]_i$ that were phase-advanced to Per2::Luc by ~6 circadian hours, as observed in wild-type SCN (Fig. 3, E to I, and movie S2). Circadian

peaks of $[Ca^{2+}]_i$ and clock gene expression travel across the SCNs in a stereotypical spatiotemporal wave, with neurons in the dorsal SCN phase-leading the ventral ones in a pattern strictly dependent on the SCN circuit proper-

ties (14, 15). To confirm that astrocytically restricted Cry1 expression also established appropriate spatio-temporal patterns of neuronal $[Ca^{2+}]_i$ across the SCN, we compared the calcium signal in wild-type SCN and SCN with

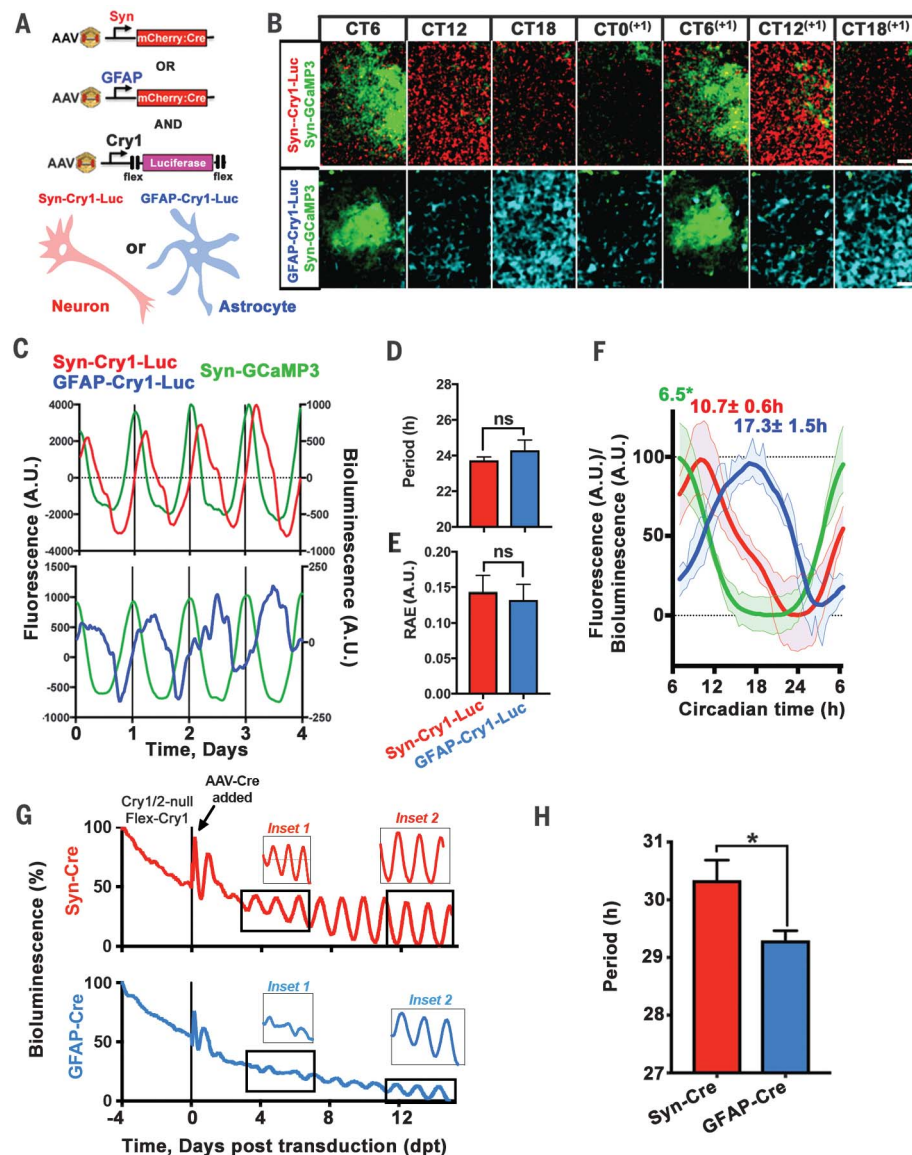


Fig. 1. An astrocytic clockwork can autonomously drive circadian clock gene expression in the SCN.

(A) Experimental design to restrict expression of Cry1-Flex-Luc to neurons or astrocytes by AAVs cotransduced with Syn-mCherry::Cre or GFAP-mCherry::Cre. (B) Stills from live-image recordings of SCN slices cotransduced with Cry1-Flex-Luc, alongside Syn-mCherry::Cre or GFAP-mCherry::Cre, showing circadian variation of the bioluminescent Cry1-Luc signal, phase-aligned to Syn-GCaMP3. Signals are false lookup table colors. (C) Representative detrended traces of neuronally or astrocytically restricted Cry1-Flex-Luc circadian oscillations, phase-aligned to Syn-GCaMP3. A.U., arbitrary units. (D and E) Period and RAE values of Cry1-Luc oscillations, restricted to neurons or astrocytes. Data are means ± SEM, $n = 5$ per group. (F) Waveform traces of neuronal and astrocytic Cry1-Flex-Luc expression phase-aligned to Syn-GCaMP3. Data are means ± SEM, $n = 5$ for each experimental group. The asterisk indicates that the circadian phase is based on previous data (3, 9). (G) Representative Per2::Luc traces from SCN slices of Cry1/2-null pups sequentially transduced with Cry1-Flex-Cry1::EGFP and then either Syn-mCherry::Cre or GFAP-mCherry::Cre AAVs to restore Cry1 expression in neurons or astrocytes, respectively. Insets show amplitudes of Per2::Luc in the early (inset 1) and late (inset 2) stages of neuronally and astrocytically restricted Cry1 expression. (H) Period values after neuronally or astrocytically restricted Cry1 expression in the late phases of the treatment. Data are means ± SEM, $n = 4$. Statistical test was an unpaired two-tailed t test. * $P < 0.05$. Scale bars, 50 μ m.

astrocytically restricted *Cry1* expression and found comparable dorsal-to-ventral organization of neuronal $[Ca^{2+}]_i$ (16) (Fig. 3, J and K, and movie S2). Given that *Per* gene promoters harbor calcium-responsive elements that phase-lock *Per* expression to neuronal $[Ca^{2+}]_i$, astrocytes may engage the E-box-based TTFL of neurons by driving neuronal $[Ca^{2+}]_i$ (9), sustaining intracellular oscillations of clock gene expression across SCN space and circadian time. Critically, this happens in the absence of *Cry* genes in neurons, thus revealing that the neuronal E-box-based TTFL may be dispensable for circuit-level circadian time-keeping. Thus, genetic complementation of *Cry1* in SCN astrocytes can initiate and sustain mammalian circadian function by recruiting the latent SCN neuronal circuit.

To investigate the relevant mechanisms, we tested the role of connexin 43 (Cx43), a major component of gap junctions and hemichannels specifically expressed in astrocytes that coordinates astrocytic networks and was recently implicated in hypothalamic regulation of sleep-wake cycles (17, 18). Cx43 is highly expressed in the SCN, extensively decorating astrocytic processes, as shown by colocalization with the GFAP-EGFP tag from control surgery mice (Figs. 2D and 4A). We then assessed the effects of Cx43 inhibition on circadian oscillations of clock gene expression in SCN slices by using the mimetic peptide TAT-Gap19 (19, 20). TAT-Gap19 elicited a dose-dependent and reversible reduction in the amplitude and period lengthening of *Per2::Luc* oscillations (Fig. 4B and fig. S4), confirming the role of astrocytes in circadian function of wild-type SCN. We then showed that Cx43 inhibition by TAT-Gap19 significantly compromised *Per2::Luc* oscillations driven by astrocytically restricted expression of *Cry1* in *Cry1/2*-null slices (Fig. 4, C and D). TAT-Gap19 specifically inhibits the hemichannel form of Cx43 that is involved in paracrine astrocytic release of gliotransmitters, including ATP and glutamate (19, 21). Astrocyte-released glutamate is a major gliotransmitter in the SCN (3); therefore, we tested its key role in driving circadian rhythmicity in *Cry1/2*-null mice where *Cry1* was expressed in astrocytes. Extracellular glutamate levels of *Cry1/2*-null SCN slices, measured using the AAV-encoded glutamate indicator iGluSnFR driven by *Syn* (3, 22), exhibited no detectable circadian oscillations, but GFAP-Cre-restricted expression of *Cry1* initiated robust circadian oscillations of glutamate. Moreover, these were strongly impaired by a Cx43 inhibitor (TAT-Gap19) (Fig. 4, E and F). These data support the role of astrocyte-derived circadian oscillations of glutamate in mediating astrocytic control of circadian oscillations in *Cry1/2*-null SCN.

To determine whether glutamate is specifically responsible for astrocyte-dependent circadian time-keeping in *Cry1/2*-null SCN, slices received DQP-II05, an antagonist for *N*-methyl-D-aspartate glutamate receptor assemblies containing the NR2C/D subunit (NMDAR2C) (23). NMDAR2C inhibition by DQP-II05 reversibly damps circadian rhythms of membrane potential and clock gene expression in wild-type SCN neurons (3).

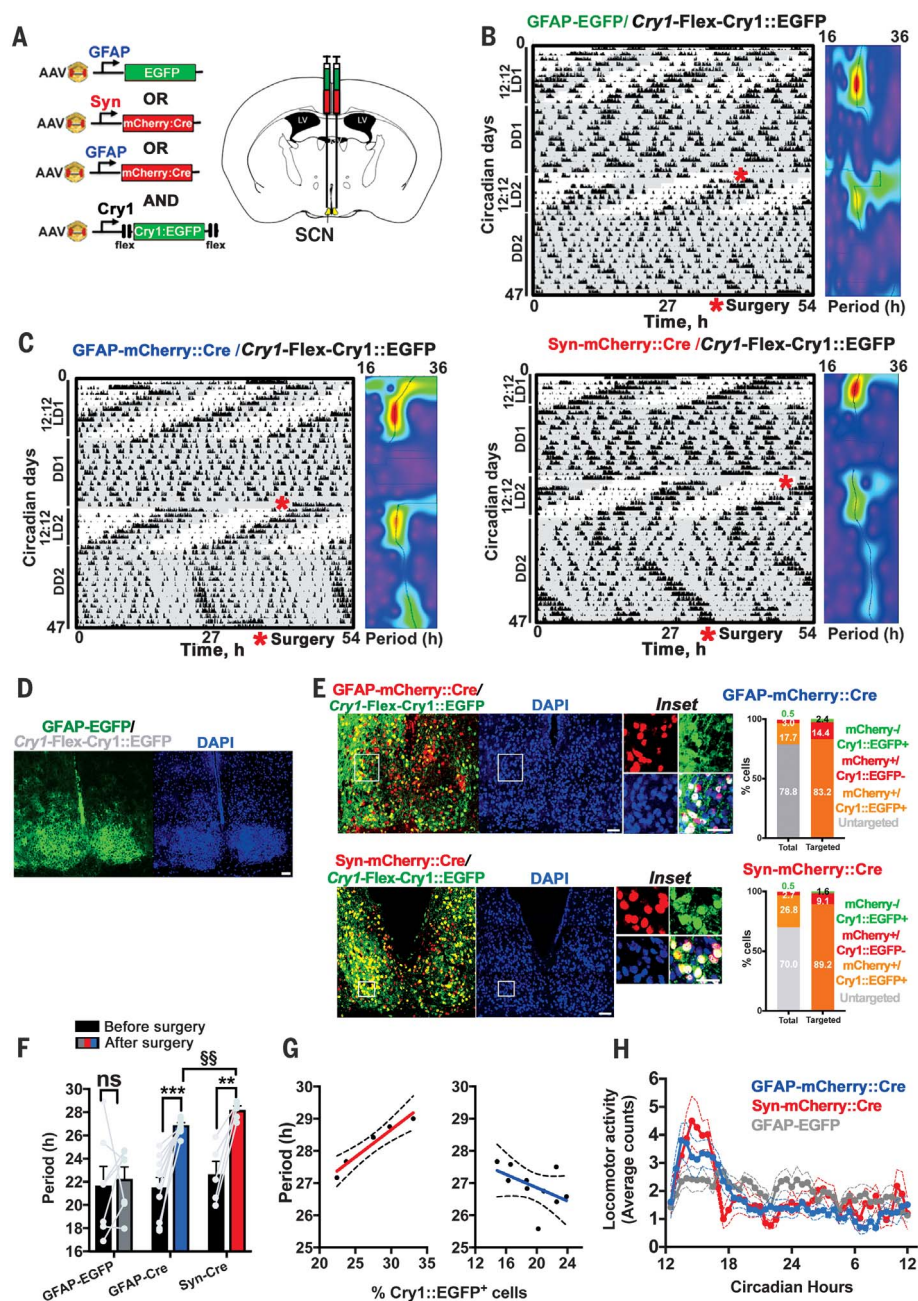
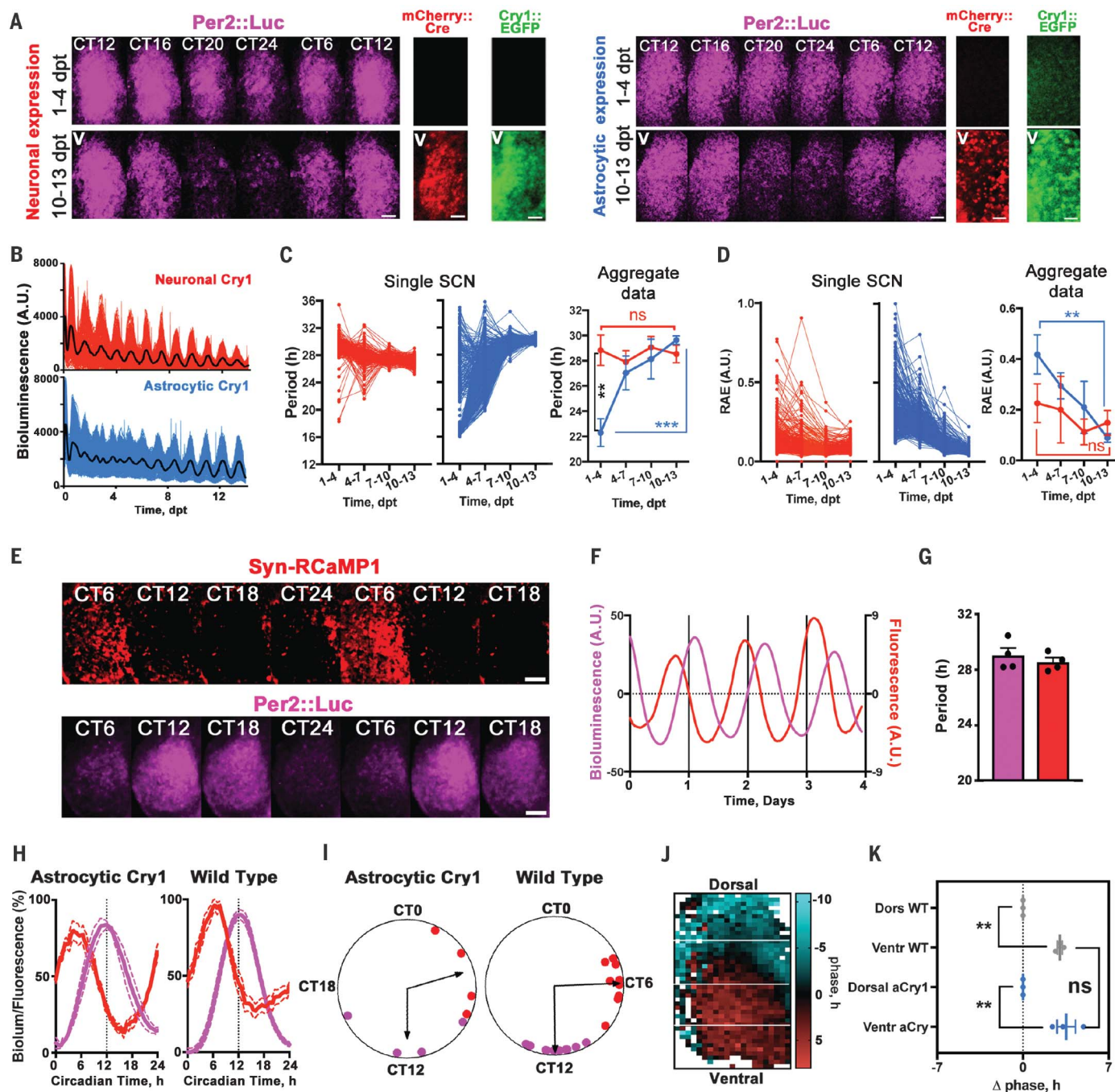


Fig. 2. Genetic complementation of *Cry1* in SCN astrocytes initiates and sustains robust circadian patterns of locomotor activity in circadian-incompetent *Cry1/2*-null mice. (A) Experimental design of in vivo expression of Flex-*Cry1::EGFP* restricted to SCN astrocytes or neurons by *Syn*- or GFAP-driven Cre, respectively. (B and C) Representative actograms and wavelet analyses of *Cry1/2*-null mice targeted with *Cry1-Flex-Cry1::EGFP* together with AAVs expressing GFAP-EGFP (control) (B) or Cre (C). Rhythmicity in LD1 and -2 is due to a masking effect of the light-dark cycle. (D and E) Representative confocal tiled microphotographs of SCN sections from control and Cre-treated mice evaluated post hoc to assess effective targeting of the SCN. Histograms represent colocalization of fluorescence signals from mCherry::Cre and *Cry1::EGFP* in Cre-treated mice (insets). Total number of cells counted: GFAP-Cre, $N_{(DAPI^+)} = 5491$, $n = 5$ targeted mice; Syn-Cre, $N_{(DAPI^+)} = 6037$, $n = 5$ targeted mice. DAPI, 4',6-diamidino-2-phenylindole. (F) Periods of circadian activity rhythms of control and Cre-treated mice before (DD1) and after (DD2) stereotaxic surgery. (G) Correlation analysis of number of *Cry1::EGFP*⁺ astrocytes or neurons and behavioral period (Syn-mCherry-Cre: $r = 1$, $n = 5$, $P = 0.02$; GFAP-mCherry-Cre: $r = -0.70$, $n = 10$, $P = 0.03$, two-way Spearman test). (H) Locomotor activity plotted across the circadian day (means \pm SEM). Group sizes were $n_{(GFAP-EGFP)} = 7$, $n_{(GFAP-Cre)} = 10$, and $n_{(Syn-Cre)} = 5$. The statistical test was a two-way repeated measures analysis of variance (RM-ANOVA) with Bonferroni correction. ** $P < 0.01$; *** $P < 0.001$; §§ $P < 0.01$ (ad hoc unpaired two-tailed *t* test with Sidak-Bonferroni correction). Scale bars, 50 μ m.



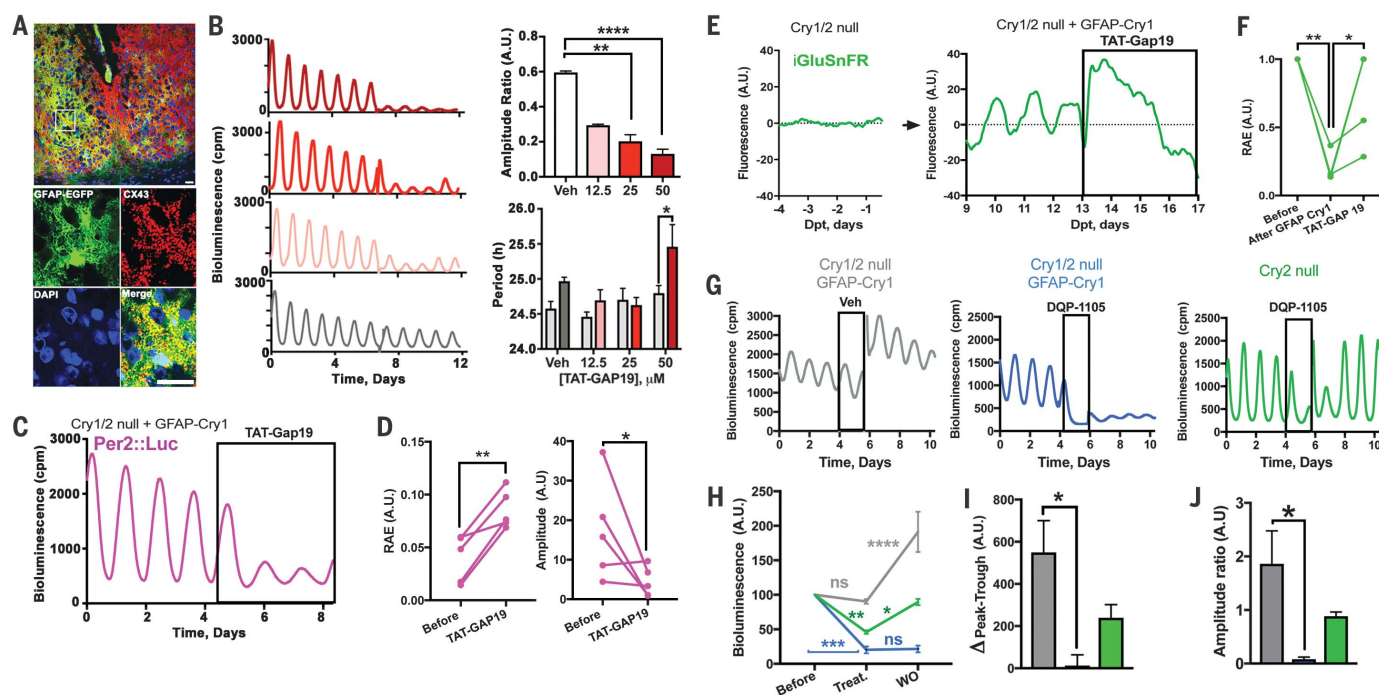


Fig. 4. Astrocytically released glutamate mediates astrocytic control of circuit-level circadian time-keeping in *Cry1/2*-null SCN expressing GFAP-restricted *Cry1*. (A) Confocal tiled microphotographs of adult SCN showing colocalization of GFAP-EGFP and Cx43, detected by polyclonal antiserum (results are representative of findings with three independent brains). (B) Representative *Per2::Luc* PMT traces and group data (mean + SEM), showing dose-response effects of TAT-Gap19 on the amplitude ratio (with drug/before drug) and period in wild-type SCN slices. The statistical test for the amplitude ratio was an unpaired ANOVA with Bonferroni correction. Analysis for period employed a two-way RM-ANOVA with Bonferroni correction [$n = 3$ for each group, except vehicle (Veh), $n = 4$]. (C and D) Representative *Per2::Luc* PMT trace (C) and paired scatter plot of RAE and amplitude (D) of *Cry1/2*-null SCN slices transduced with GFAP-mCherry::Cre and *Cry1-Flex-Cry1::EGFP* and treated with TAT-Gap19 (50 μ M). The statistical test was a paired one-tailed t test, $n = 5$. (E and F) Representative iGluSnFR traces (E) and paired scatter plot of

RAE (F) of *Cry1/2*-null SCN slices before and after GFAP-mCherry::Cre and *Cry1-Flex-Cry1::EGFP* transduction and treatment with TAT-Gap19 (50 μ M). The statistical test was an RM-ANOVA with Bonferroni correction, $n = 4$. (G) Representative *Per2::Luc* PMT traces of *Cry2*-null and *Cry1/2*-null SCN slices transduced with GFAP-mCherry::Cre and *Cry1-Flex-Cry1::EGFP* and treated with DQP-1105 (50 μ M) or vehicle, with subsequent washout. (H) Group data (means + SEM) of bioluminescence baseline traces represented in (G) before, in the presence of, and after removal of DQP-1105. The statistical test was a two-way RM-ANOVA with Bonferroni correction. (I) Group data (means + SEM) showing peak-to-trough differences in the presence of DQP-1105 of traces represented in (G). (J) Group data (means + SEM) showing amplitude ratio (after drug/with drug) of data presented in (G). The statistical test for (H) was a two-way RM-ANOVA with Bonferroni correction. The statistical test for (I) and (J) was an unpaired ANOVA with Bonferroni correction ($n = 4$ for *Cry2*-null and Veh groups; $n = 3$ for DQP-1105 treatment in *Cry1/2*-null group). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ ($n = 4$). Scale bars, 20 μ m.

The effect of the drug was much more marked in *Cry1/2*-null SCN with rhythms driven by astrocyte-expressed *Cry1*, as shown by the immediate drop in the baseline of the *Per2::Luc* rhythms and abolition of the peak-to-trough difference. Moreover, rhythmicity was restored upon washout of the drug, but the amplitude was irreversibly reduced. We interpreted this as protracted misalignment of circadian activity of SCN neurons and astrocytes. The pronounced effects of DQP-1105 were not evident in *Cry2*-null SCN, which retained *Cry1* expression in both neurons and astrocytes, thus ruling out any confounding impact of *Cry2* deficiency in our astrocytic *Cry1* rescue model (Fig. 4, G to J). Thus, glutamate is a necessary mediator of astrocytic control of circadian function in the SCN, as shown by two independent pharmacological approaches: interference with glutamate release by astrocytes (via Cx43 inhibition) and with neuronal glutamate sensing (via NMDAR2C antagonism) (2, 3).

A growing body of evidence has challenged a neuron-centric view of the control of behavior in mammals by showing that astrocytes can modulate complex neural processes, including cognition, fear, sleep, and circadian rhythms (3, 4, 17, 24, 25). However, most studies rely on the presence of a preexisting neuronally encoded behavior and show that behavioral performances are affected when astrocytic function is modified (24). Thus, regardless of the specificity of the astrocyte-neuron interactions described (3, 25, 26), those studies only addressed the ability of astrocytes to modulate neuronally encoded behavior; they did not establish their sufficiency in controlling behavior. Here, we have shown that astrocytes of the SCN can autonomously encode circadian information and instruct their neuronal partners, which lack a competent TTFL clock, to initiate and indefinitely sustain circadian patterns of neuronal activity and behavior.

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ACKNOWLEDGMENTS

We thank LMB Biological Services Group and the Ares staff for technical support. **Funding:** The Medical Research Council UK (core funding MC_U105170643 to M.H.H.) supported this work. **Author contributions:** M.B. designed, performed, and analyzed all experiments. M.H.H. contributed to the experimental design. M.D.E. and N.J.S. developed and validated the Cry1-Flex-Luc and Cry1-Flex-Cry1::EGFP constructs. A.P.P., E.S.M., and J.E.C. conducted preliminary experiments. All authors contributed to

project discussions. M.B. and M.H.H. wrote the manuscript. **Competing interests:** The authors declare no competing interests. **Data and materials availability:** All data are available in the manuscript or supplementary materials.

SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/363/6423/187/suppl/DC1
Materials and Methods
Figs. S1 to S4
References (27, 28)
Movies S1 and S2

23 February 2018; accepted 2 November 2018
10.1126/science.aat4104

Cell-autonomous clock of astrocytes drives circadian behavior in mammals

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Science **363** (6423), 187-192.
DOI: 10.1126/science.aat4104

Astrocytes can drive the master clock in the brain

The neurons of the suprachiasmatic nucleus (SCN) of the hypothalamus function as a central circadian clock, coordinating mammalian physiology with the 24-hour light-dark cycle. Brancaccio *et al.* found that these neurons have help from neighboring astrocytes (see the Perspective by Green). In mice lacking the *Cry* gene, which encodes a critical clock component, restoration of *Cry* expression and molecular clock function in the astrocytes, but not the neighboring neurons, restored rhythmic transcriptional oscillations in the SCN and reestablished circadian behaviors in the mice.

Science, this issue p. 187; see also p. 124

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