Cellular/Molecular

Circadian Regulation of ATP Release in Astrocytes

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Circadian clocks sustain daily oscillations in gene expression, physiology, and behavior, relying on transcription–translation feedback loops of clock genes for rhythm generation. Cultured astrocytes display daily oscillations of extracellular ATP, suggesting that ATP release is a circadian output. We hypothesized that the circadian clock modulates ATP release via mechanisms that regulate acute ATP release from glia. To test the molecular basis for circadian ATP release, we developed methods to measure in real-time ATP release and *Bmal1::dLuc* circadian reporter expression in cortical astrocyte cultures from mice of different genotypes. Daily rhythms of gene expression required functional *Clock* and *Bmal1*, both *Per1* and *Per2*, and both *Cry1* and *Cry2* genes. Similarly, high-level, circadian ATP release also required a functional clock mechanism. Whereas blocking IP_3 signaling significantly disrupted ATP rhythms with no effect on *Bmal1::dLuc* cycling, blocking vesicular release did not alter circadian ATP release or gene expression. We conclude that astrocytes depend on circadian clock genes and IP_3 signaling to express daily rhythms in ATP release.

Introduction

Circadian pacemakers coordinate the daily timing of physiology and behavior with environmental cycles. These oscillators rely on transcription–translation feedback loops of canonical clock genes (e.g., *Bmal1*, *Clock*, *Period1*, *Period2*, *Cryptochrome1*, and *Cryptochrome2*) to generate overt circadian rhythms (Siepka et al., 2007). Clock genes are ubiquitous, and most mammalian cells and tissues express circadian rhythms both *in vitro* and *in vivo* (Yoo et al., 2004; Kowalska and Brown, 2007). Neuronal cultures from the hypothalamic suprachiasmatic nuclei (SCN) exhibit sustained oscillations in PERIOD2-driven luciferase activity (PER2::LUC) (Liu et al., 2007). Although single SCN neurons are intrinsically capable of generating sustained circadian oscillations, intercellular signals synchronize them to each other, sustaining and enhancing the precision of the rhythms at the population level (Liu et al., 2007; Webb et al., 2009). Individual fibroblasts also display sustained circadian oscillations in PER2::LUC (Welsh et al., 2004), but they do not synchronize to each other, leading to damping of the population rhythms (Welsh et al., 2004). Cultured astrocytes exhibit damped circadian oscillations that are sustained by SCN explants (Prolo et al., 2005) and can be entrained by the neuropeptide vasoactive intestinal polypeptide (VIP) (Marpegan et al., 2009). However, the biological significance of circadian oscillations in astrocytes is unknown.

Mammalian astrocytes regulate neuronal networks through the reuptake and release of various transmitters including glutamate (Perea et al., 2009; Halassa and Haydon, 2010). Glutamate reuptake is modulated by clock genes but is not circadian in cultured cortical astrocytes (Beaulé et al., 2009). In *Drosophila*, the glia-specific gene *ebony* is involved in the synthesis of biogenic amines and is required for circadian rhythms in locomotor behavior (Suh and Jackson, 2007), suggesting that glial cells can modulate neuronal networks responsible for complex behaviors through the circadian control of secreted neuroactive molecules.

A recent report showed that cultured mammalian astrocytes display circadian rhythms in extracellular ATP accumulation (Womac et al., 2009). It is possible that ATP is a circadian signal released by astrocytes. ATP and its dephosphorylated products, ADP, AMP, and adenosine, regulate synaptic communication (Burnstock, 2006) through a variety of purinergic receptors on astrocytes and neurons (Fields and Burnstock, 2006). Astrocytes release ATP through hemichannels (Cotrina et al., 1998), vesicle-dependent mechanisms (Coco et al., 2003; Pascual et al., 2005), the large pore of the $P2X_7$ purinergic receptor (Suadicani et al., 2006), and through ATP-binding cassette proteins (multidrug resistance protein and *p*-glycoprotein) (Declèves et al., 2000; Darby et al., 2003). There is also evidence that release of transmitters, including ATP, from glia is calcium dependent (for a recent review, see Parpura and Zorec, 2010). Little is known about circadian modulation of any of these mechanisms for ATP release.

We tested the hypotheses that key clock genes in astrocytes drive circadian oscillations in ATP release through either calcium- or vesicle-dependent mechanisms. We developed a novel, real-time assay and found that circadian ATP release depends on *Clock*, *Per1*, and *Per2* expression and IP_3-dependent calcium signaling in astrocytes.

Figure 1. ATP release from astrocytes is circadian. *A*, *B*, Representative circadian and arrhythmic ATP-induced bioluminescence from WT astrocytes. Numbers above the *x*-axis are the proportion of rhythmic cultures. *C*, Representative 6 min frame of bioluminescence induced by extracellular ATP in 32 wells of a 96-well plate. Each well was seeded with 10^4 wild-type astrocytes. Light flashes (arrow) and luminescence within each well are proportional to ATP concentration. *D*, Standard curve of bioluminescence as a function of ATP concentration performed immediately after the addition of ATP to a 96-well plate containing ATP reagent incubated at 32°C for 24 h. *E*, Addition of apyrase decreased ATP-induced bioluminescence to background levels. *F*, Only cultures with RelAmp values <0.2 were considered rhythmic. Graph shows the percentage of WT cultures scored as rhythmic as a function of RelAmp values determined by FFT-NLLS.

Materials and Methods

Animals. Mice were bred and maintained in the Danforth Campus Animal Facility under a 12 h light/dark cycle. All procedures were approved by the Animal Care and Use Committee at Washington University in St. Louis and conformed to National Institutes of Health guidelines. Unless noted, mice were maintained on a C57BL/6 genetic background by backcrossing annually with mice from Charles River.

Astrocytes were derived from mice of different genotypes to assess whether the molecular basis of daily timing in astrocytes is similar to other cells and to determine whether circadian rhythms in ATP release

are controlled by the expression of circadian genes. Founders for the circadian mutants used in this work were generously donated by Drs. J. Takahashi, University of Texas-Southwestern Medical Center (Dallas, TX) (*Clock/Clock)*, S. McKnight, University of Texas-Southwestern Medical Center (*Neuronal PAS domain 2 knockout, Npas2$^{-/-}$*), S. M. Reppert, University of Massachusetts Medical School (Boston, MA) (*Period1^{ldc}Period2^{ldc}* double mutant mice referred as *Per1^{m}Per2^m*), R. Van Gelder, University of Washington Medical Center (Seattle, WA) (*Cryptochome1* and *Cryprochrome2* homozygote double knock-out mice, *Cry1$^{-/-}$ Cry2$^{-/-}$*), and C. Bradfield, University of Wisconsin (Madison, WI) (*Bmal1* knock-out, *Bmal1$^{-/-}$*). *Cry1$^{-/-}$Cry2$^{-/-}$* mice were maintained on a mixed background of 129 × C57BL/6J, and *BMAL1$^{-/-}$* mice were obtained from heterozygote breeders.

To test possible mechanisms of circadian regulation of ATP release, we used two strains of mice with astrocyte-specific gliotransmission deficiencies. Both strains were obtained by crosses between a hemizygote hGFAP.tTA mouse ("tet-Off" tetracycline transactivator protein controlled by the human glial fibrillary acidic protein promoter) and either a hemizygote tetO.SNARE or tetO.VIPP. In the absence of tetracycline (or analogs), the hGFAP.tTA::tetO.SNARE mice [dominant-negative (dn) SNARE] express a fragment of the cytosolic portion of the SNARE domain of synaptobrevin 2, thus blocking vesicular release from astrocytes. dnSNARE mice were characterized previously and show a phenotype following sleep deprivation (Pascual et al., 2005; Halassa et al., 2009). In addition, tetO.SNARE mice contain a tetO.EGFP (enhanced green fluorescent protein) reporter used to validate transgene expression (Pascual et al., 2005; Halassa et al., 2009). The hGFAP.tTA::tetO.VIPP mice (VIPP) express high levels of type I Ins(1,4,5)P3 5-phosphatase (IPP), the enzyme that terminates IP$_3$ signaling (De Smedt et al., 1997), thus impairing IP$_3$-dependent calcium signaling pathways. VIPP mice contain a tetO. Venus yellow fluorescent protein (YFP) reporter fused to the IPP component to validate transgene expression. Complete characterization of the VIPP mouse is the subject of another study (S.-Y. Lee, M. M. Halassa, D. Hines, L. Gainey, Q. Deng, O. Pascual, C. Erneux, P. J. Atkinson, N. J. Brandon, P. G. Haydon, unpublished observations). For both genotypes, we confirmed transgene expression by measuring the expression of the fluorescent reporters EGFP for dnSNARE, and Venus-YFP for VIPP in our astrocyte cultures. To prevent transgene expression in the pups, female mice were fed the tetracycline analog doxycycline (40 mg/kg of rodent food, BioServ) from 2 weeks before mating until the astrocyte cultures were prepared.

Cell cultures. Cortical astrocytes were obtained from postnatal day 2–6 mice according to a previously published protocol (Beaulé et al., 2009). Each culture was made from 6–15 neonatal mice pooled from 1–3 litters of the same age and genotype. Replicates were performed with cultures made from the same and different litters. Astrocyte cultures were made from wild-type (WT), *Clock/Clock*, *Clock/$^+$*, *Npas2$^{-/-}$*, *Per1^{m}Per2^m*, *Cry1$^{-/-}$Cry2$^{-/-}$*, *Cry1$^{-/-}$Cry2$^{+/-}$*, *Bmal1$^{-/-}$*, *Bmal1$^{+/-}$*, dnSNARE,

and VIPP mice. NPAS2 is highly expressed in the cortical areas we harvested for astrocyte cultures (Garcia et al., 2000). Absence of NPAS2 in these areas results in behavioral deficits that are not compensated by the presence of its analog (CLOCK) that forms functional complexes with BMAL1 (Reick et al., 2001). For these reasons, we included astrocytes derived from $Npas2^{-/-}$ mice in our evaluation of genes required for circadian rhythmicity. For comparison with dnSNARE and VIPP astrocytes, we used astrocytes from wild-type mice or littermates expressing only the hGFAP.tTA transgene. All astrocytes were incubated at 37°C in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. Medium changes were performed twice a week. Cells grew to confluence before passaging into new flasks. Astrocyte cultures between passage 1 and 3 were used for all experiments.

Gene reporter constructs. We used *Bmal1::dLuc* and *Per2::dLuc* plasmids for transfection of pure astrocyte cultures (generous gifts from Dr. Hiroki R. Ueda, RIKEN Center for Developmental Biology, Kobe, Japan). Plasmids were each inserted into the 4818 bp pGL3 vector (Promega). The *Bmal1::dLuc* plasmid contained 530 bp of the *Bmal1* promoter driving luciferase. The *Per2::dLuc* plasmid contained 252 bp of the *Per2* promoter driving luciferase (Sato et al., 2004). Transfection was performed with *TransIT-LT1* reagent according to the manufacturer's instructions (Mirus). Briefly, 12 μl of the transfection reagent and 2 μg of plasmid DNA were diluted in DMEM up to a final volume of 100 μl. Transfection medium was added to 100 μl of a suspension containing between 9×10^4 and 1.8×10^5 astrocytes in DMEM with 10% FBS and penicillin/streptomycin. Transfection efficiency was 0.5–5% based on the number of cells expressing EGFP divided by the number of DAPI-stained nuclei in cultures cotransfected with pCMS-EGFP (Clontech). Astrocytes were immediately plated in 35 mm BD Primaria tissue culture dishes (BD Biosciences). The medium was replaced 48 h after transfection. Bioluminescence recordings were started ~3 weeks later.

We performed lentiviral infections of pure astrocyte cultures using a lentiviral construct expressing a *Bmal1::dLuc* 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 week period before plating and imaging. Viral transduction resulted in 30–50% of the cells expressing *Bmal1::dLuc*.

ATP reporter system. We used the BioThema ATP Reagent HS, which includes a more thermostable recombinant luciferase to measure real-time, extracellular ATP levels for up to 72 h. The ability of the ATP reagent to detect ATP over a 72 h period was greatly decreased as temperatures approached 37°C. We were thus required to lower the incubator temperature to 32°C to allow detection of ATP release for up to 72 h. This temperature also yielded high-amplitude oscillation in clock-gene driven bioluminescence.

Astrocytes were first plated into a 96-well plate (10^4 cells per well) and allowed to grow in a 5% CO₂ incubator for 48 h at 37°C. The culture medium was then changed to HEPES-buffered DMEM with B27, and plates were sealed with silicone grease, moved to the imaging chamber, and cultured for an additional 48 h at 32°C. After 48 h, 20 μl of ATP reagent (10% v/v final) and 5 μl of VIP (500 nM) were added to each well to induce high-amplitude oscillations as previously reported (see Marpegan et al., 2009 for the description of the effects of VIP on circadian reporter-driven bioluminescence rhythms).

We generated a standard curve of bioluminescence as a function of ATP concentration at 24 h to account for temperature-dependent deg-
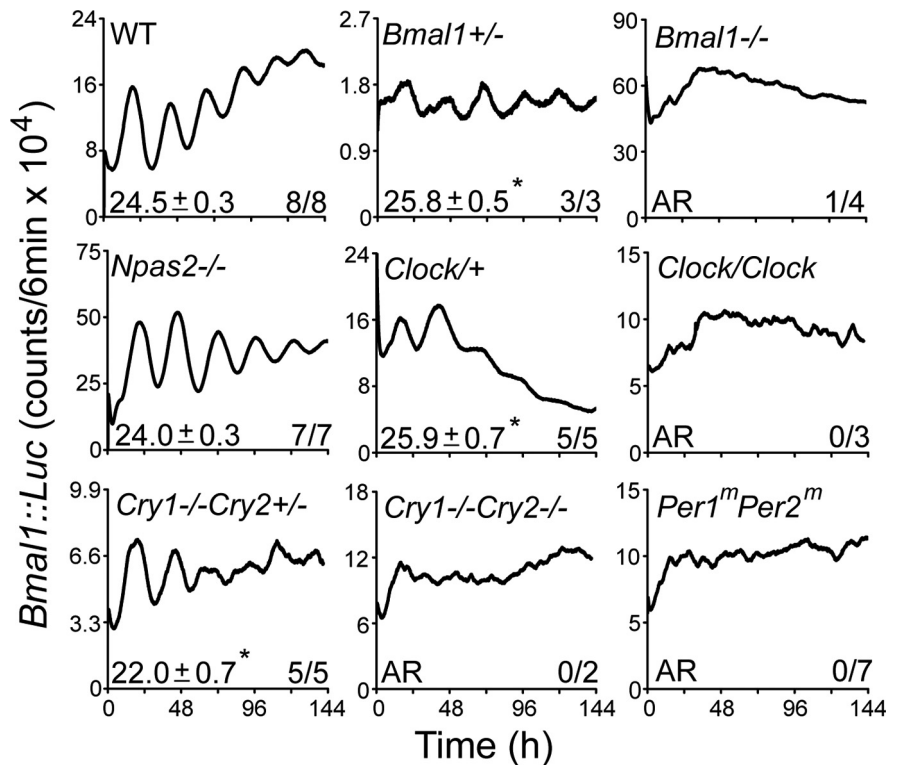

Figure 2. Clock genes regulate circadian rhythms in astrocytes. Representative raw bioluminescence traces for pure astrocyte cultures transfected with *Bmal1::dLuc*. Numbers above the x-axis provide the period (mean ± SEM) and the proportion of rhythmic cultures for each genotype. AR, Arrhythmic. *p < 0.05, significantly different from WT.

radation of ATP reagent (Fig. 1D). We confirmed that the bioluminescence measured was driven by extracellular ATP by supplementing cultures with the ATPase apyrase (Sigma, catalog #A6535–200UN). Apyrase was diluted in MilliQ water (Millipore, 1 mg/ml), sterile filtered, and stored at −20°C until use. Apyrase (5 μl/well) was first administered and allowed to act for 15 min before the addition of ATP reagent containing a second dose of apyrase (5 μl/well). Control cultures received 5 μl of sterile-filtered MilliQ water (Millipore) for 15 min, followed by ATP reagent with 5 μl of sterile-filtered MilliQ water (Millipore). ATP bioluminescence measurements were started immediately after the addition of the ATP reagent and lasted 72 h.

Bioluminescence recordings. Light from clock-gene reporters (*Bmal1::dLuc* or *Per2::dLuc*) and ATP release were detected in customized light-tight incubators equipped with either photomultiplier tubes (HC135-11; Hamamatsu Corp.) (Prolo et al., 2005; Beaulé et al., 2009; Marpegan et al., 2009) or with an intensified CCD camera (XR/Mega-10Z; Stanford Photonics Inc.) controlled by Micro-Manager imaging software (http://www.micro-manager.org). Single-cell bioluminescence imaging was performed with the same camera attached to an inverted microscope using a 10× objective (TE-2000, Nikon). Recordings of gene expression were conducted at 34°C and ATP release was recorded at 32°C. Bioluminescence was integrated every 6 min for whole cultures or every hour for single cells over 4–7 d after culture medium was exchanged with HEPES-buffered DMEM supplemented with B27 and 0.1 μM D-luciferin (BioThema).

Immunocytochemistry. Following bioluminescence data collection, astrocytes were stained to assess cell density, astrocyte purity, and transfection efficiency. Cells were rinsed once with 0.01 M PBS and fixed with 4% paraformaldehyde in 0.01 M phosphate buffer at room temperature for 15 min. Cells were then rinsed three times with PBS and stored at 4°C until staining. Astrocytes were blocked by incubating in 0.25% Triton X-100 in PBS (Triton-PBS) supplemented with 10% bovine serum albumin (BSA) for 30 min at room temperature. After blocking, cells were incubated with a rabbit polyclonal antibody raised against glial fibrillary acidic protein (GFAP) (Dako North America) diluted 1:1000 in Triton-
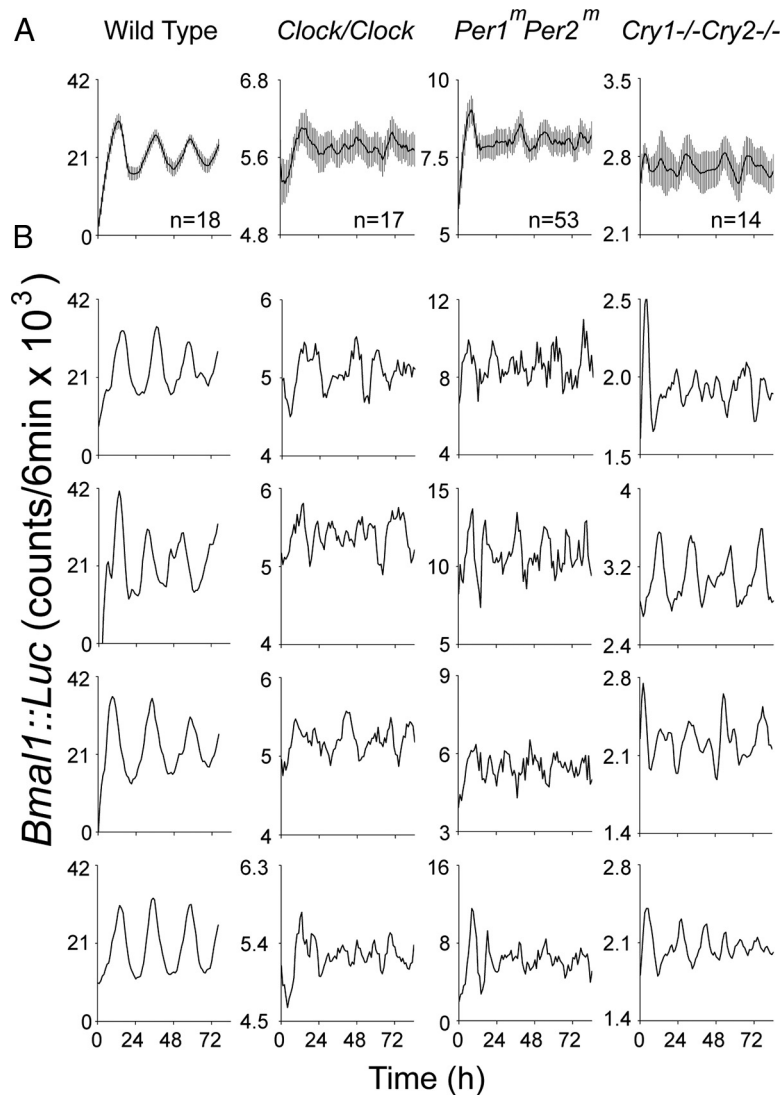
Figure 3. Individual astrocytes are circadian oscillators. Representative bioluminescence traces for astrocytes infected with lenti-*Bmal1::dLuc*. *A*, Mean (±SEM) bioluminescence for all single astrocytes recorded in one culture for each genotype. Numbers above *x*-axis represent the number of cells analyzed per culture. Note that the ensemble of WT astrocytes showed circadian cycling. *B*, Representative bioluminescence traces reveal reliable circadian rhythms in individual WT astrocytes and weak or arrhythmic expression in astrocytes of the other three genotypes.

was determined by fast Fourier transform, followed by nonlinear least-squares fitting (FFT-NLLS). Because FFT-NLLS will fit data with different goodness of fit levels, we selected a criterion relative amplitude (RelAmp) value ≤0.2 to score cultures as rhythmic. Data not fitted by FFT-NLLS or with RelAmp values >0.2 were scored as arrhythmic. The range of dominant periods allowed for FFT-NLLS was set between 16 and 32 h. Serial dilutions of ATP were used to generate ATP standard curve after fitting the data (grayscale intensity vs ATP concentration) with a linear regression model. This curve was used to approximate the concentration of ATP released by astrocytes. Using a standard curve generated after 24 h at 32°C, we compared ATP release between genotypes by converting bioluminescence values to ATP concentration, and normalizing this value by the number of cells in each well at the end of the experiments. These ATP values are reported as femtomolar (fM) per cell. One-way ANOVA with Scheffé *post hoc* test was used to compare ATP levels between genotypes. Chi-square (χ^2) was used to compare the proportion of cultures showing ATP release rhythms. Alpha was set at 0.05 for all analyses.

Results

ATP release from cultured astrocytes is circadian

We developed a real-time assay to continuously monitor ATP release using stabilized recombinant firefly luciferase and luciferin that allowed us to detect extracellular ATP for >72 h. We found 72% (23 of 32 cultures) of WT astrocyte cultures were circadian for at least 3 d based on strict period and amplitude criteria (Fig. 1*A*,*B*). Thus, ATP release from murine cortical astrocytes is circadian, consistent with findings in rat astrocytes, immortalized SCN 2.2 cells, and the *in vivo* rat SCN (Womac et al., 2009).

Cultured astrocytes showed diffused long-lasting bioluminescence as well as brief flashes (~0.75–2.5 mm in diameter) lasting up to 40 min (Fig. 1*C*). Light emission exceeded background levels by 1000-fold at 0.015 nM ATP and increased linearly to 2.5 nM at 32°C when measured 24 h after addition of the ATP reagent (Fig. 1*D*). The addition of apyrase, which catalyzes ATP hydrolysis, decreased bioluminescence levels to background values (Fig. 1*E*; $n = 16$ wells for vehicle- and apyrase-treated astrocytes, $n = 8$ for empty wells), demonstrating the specificity of the assay. Astrocytes released sufficient ATP so that bioluminescence exceeded background levels (wells without cells or without ATP reagent) for >72 h. The FFT-NLLS criterion we selected to score cultures as rhythmic is shown in Figure 1*F*.

PBS with 3% BSA. Cells were incubated for 24 h at 4°C in the primary solution. On the following day, cells were rinsed three times for 5 min each with PBS and incubated for 90 min at room temperature with a donkey anti-rabbit antibody conjugated to Cy3 (1:200 dilution in Triton-PBS and 3% BSA, Jackson ImmunoResearch). Cells were washed three times for 5 min with PBS, stained with 300 nM DAPI (Invitrogen) for 10 min, and rinsed in PBS. Fluorescence images were obtained with a Retiga-EX camera at 10× magnification using the Northern Eclipse software.

Cell density analysis. Six nonoverlapping photomicrographs were obtained for each culture at 10× magnification, and DAPI-stained nuclei were counted with the Nucleus Counter plugin in the NIH ImageJ image processing software (http://rsbweb.nih.gov/ij). The average cell density (cells/mm²) was calculated and the total number of cells estimated by multiplying the cell density by the total area covered by cells

Data analysis. Bioluminescence data were analyzed as previously published with modifications (Abe et al., 2002). Raw data were detrended by subtracting a 24 h running average and then smoothed using a 3 h running average. Each smoothed trace was baseline adjusted by adding the raw bioluminescence value at 24 h. The presence of circadian rhythms

Astrocyte circadian rhythms depend on clock genes

To assess the molecular basis of circadian timing in astrocytes, we tested the roles of canonical clock genes (*Bmal1*, *Clock*, *Npas2*, *Per1* and *Per2*, *Cry1* and *Cry2*). All WT astroglial cultures transfected with *Bmal1::dLuc* reporters displayed circadian oscillations in

bioluminescence (Fig. 2). High-amplitude rhythms were also present in $Npas2^{-/-}$ astrocytes. In contrast, $Bmal1^{-/-}$, $Clock/Clock$, $Cry1^{-/-}Cry2^{-/-}$, and $Per1^mPer2^m$ astrocyte cultures were arrhythmic. $Bmal1^{+/-}$ and $Clock^{+}$ cultures had longer circadian periods (25.8 ± 0.5 h and 25.9 ± 0.7 h, respectively), and $Cry1^{-/}$ $-Cry2^{+/-}$ astrocytes had shorter periods (22.0 ± 0.7 h) than WT (24.5 ± 0.3 h; $p < 0.05$, Dunnett's test). We found similar clock phenotypes with a transfected $Per2::dLuc$ reporter (data not shown).

Loss of rhythmicity in knock-out cells could reflect desynchrony or arrhythmicity of individual cells. To differentiate the two possibilities, we measured bioluminescence in individual astrocytes expressing a lentiviral $Bmal1::dLuc$ reporter, and found that the majority of $Clock/Clock$ ($n = 25$ of 26, 2 cultures), $Per1^mPer2^m$ (53 of 53, 1 culture), and $Cry1^{-/-}Cry2^{-/-}$ (24 of 26, 2 cultures) cells were not circadian, in contrast to the proportion of WT astrocytes that displayed rhythmicity (8 of 18, 1 culture; Fig. 3). We conclude that cultured individual astrocytes, like many other types of mammalian cells, rely on functional $Bmal1$, $Clock$, both $Per1$ and $Per2$, and both $Cry1$ and $Cry2$ for circadian oscillations.

Circadian rhythms in ATP release depend on clock genes

To test whether the circadian clock mechanism underlies circadian ATP release, we assayed extracellular ATP in astrocytes of different clock genotypes. We observed that daily cycling of ATP release was severely blunted in astrocytes from two representative clock mutants, $Clock/Clock$ (28% circadian; 9 of 32 cultures) and $Per1^mPer2^m$ mice (38% circadian; 9 of 24) compared with WT astrocytes (72% circadian; 23 of 32) (Fig. 4 A, B). The proportions of $Clock/Clock$ and $Per1^mPer2^m$ cultures that showed rhythmic ATP release were significantly different from WT ($\chi^2 = 13.403$, $p < 0.01$), but the mutant genotypes did not differ from each other ($\chi^2 = 0.533$, N.S.). Furthermore, the $Clock/Clock$ and $Per1^mPer2^m$ mutations more than halved the amount of ATP released compared with WT cultures (101.4 ± 3.6 fM per cell for $Clock/Clock$, 94.2 ± 1.7 fM for $Per1^mPer2^m$, 207.6 ± 5.3 fM for WT; when measured 24 h after the start of the recording; $p < 0.05$, Scheffé test; Fig. 4 B). Thus, the circadian pattern and overall magnitude of ATP release in astrocytes depend on functional $Clock$ and both $Per1$ and $Per2$ genes.
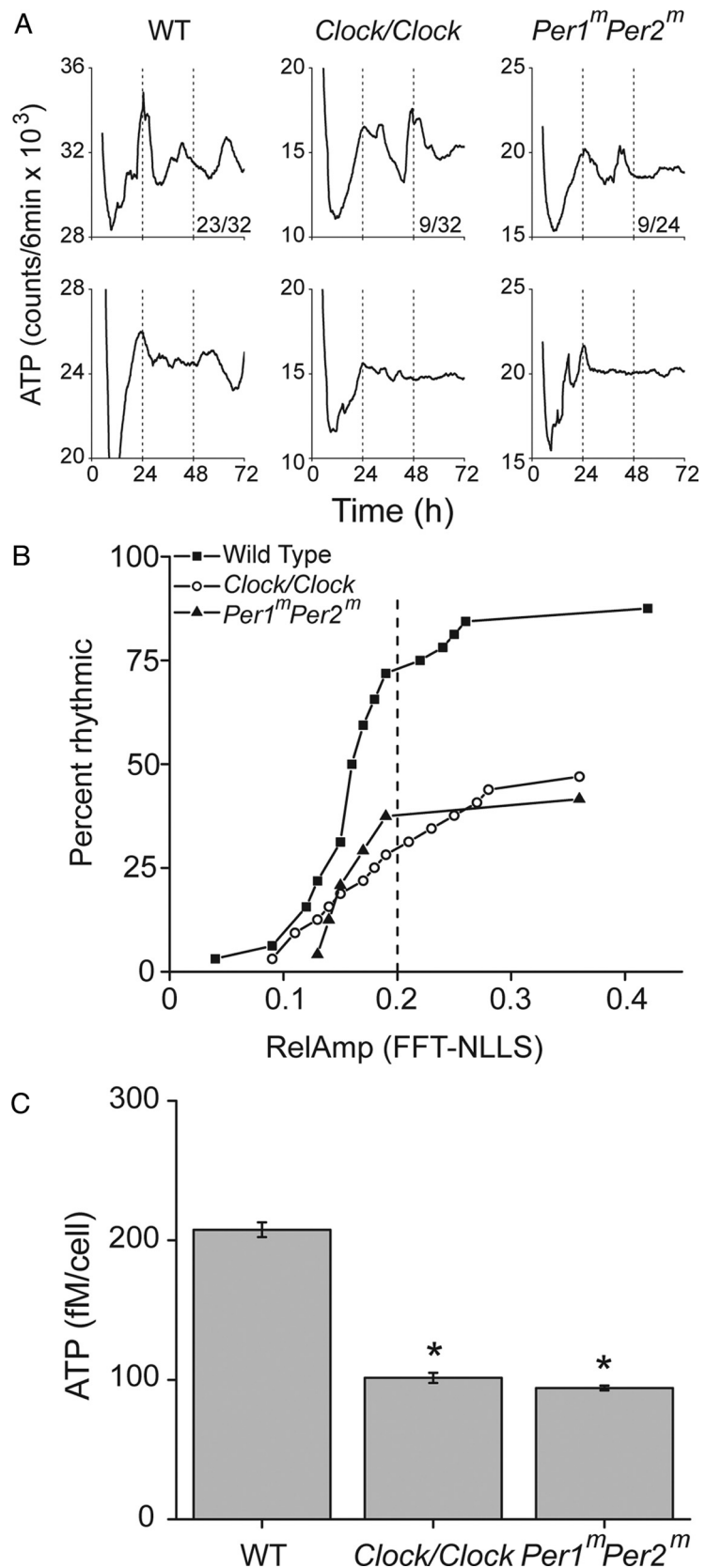
Figure 4. Rhythmic ATP release depends on a functional clock. A, Representative ATP bioluminescence traces for circadian (top) and arrhythmic (bottom) cultures of WT (left), $Clock/Clock$ (middle), and $Per1^mPer2^m$ (right) astrocytes. Numbers above x-axis represent proportion of rhythmic cultures for each genotype. B, The proportion of circadian cultures was calculated based on the RelAmp values determined by FFT-NLLS (rhythmic: RelAmp ≤ 0.2). C, ATP release per cell (mean $\pm$ SEM) in $Clock/Clock$ and $Per1^mPer2^m$ cultures was significantly less than that in WT astrocytes measured 24 h after the start of the recording (*$p < 0.05$, Scheffé test).
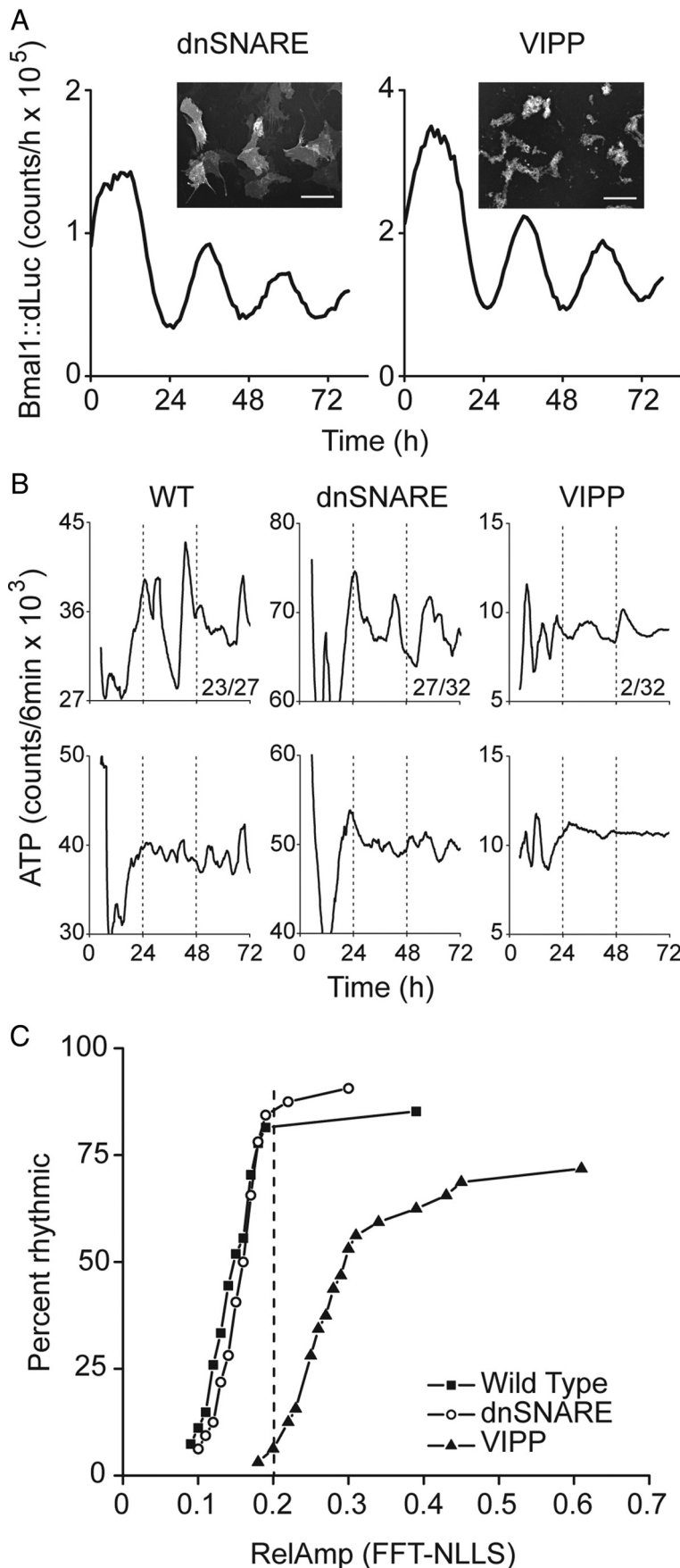
ATP release, but not *Bmal1* expression, depends on gliotransmission

Gliotransmitters can be released through mechanisms including vesicular release, hemichannels, and transporters. Using astrocytes cultured from two transgenic mouse models designed to interfere with astrocytic transmitter release, we studied possible targets for circadian regulation of ATP release. The dnSNARE mouse overexpresses a dominant-negative form of synaptobrevin 2 only in astrocytes, blocking vesicular gliotransmission (Pascual et al., 2005; Halassa et al., 2009). The VIPP mouse overexpresses type I Ins(1,4,5)P3 5-phosphatase and therefore lacks IP_3-dependent intracellular calcium signaling, a mechanism proposed for gliotransmission. Expression of dnSNARE was confirmed by the identification of EGFP-positive cells, and VIPP was confirmed by Venus-positive cells (Fig. 5A). Both dnSNARE ($n = 5$) and VIPP ($n = 8$) astrocyte cultures exhibited high-amplitude circadian rhythms in *Bmal1*-driven bioluminescence (Fig. 5A). In contrast, ATP release rhythms were found in only 6% of the VIPP cultures (2 of 32), compared with 82% of WT ($n = 23$ of 28) and 84% of dnSNARE ($n = 27$ of 32) cultures (Fig. 5B,C). This reduction in the proportion of rhythmic cultures for VIPP astrocytes differed significantly from the WT and dnSNARE cultures ($\chi^2 = 52.204$, $p < 0.0001$) and, importantly, WT and dnSNARE did not differ from each other ($\chi^2 = 0.007$, N.S.). We conclude that while vesicular release is not critical for circadian rhythms in ATP release in astrocytes, IP_3 signaling contributes to the amplitude of ATP release.

Discussion

We found that cultured mouse cortical astrocytes display circadian rhythms in extracellular ATP, in agreement with recent results from rat astrocyte cultures, SCN and SCN2.2 cells (Womac et al., 2009). We used a stabilized form of luciferase that allowed long-term recordings of extracellular ATP from the same cells without perturbations that can affect circadian

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proportion of rhythmic cultures for each genotype. EGFP (dnSNARE cells) and Venus (VIPP cells) fluorescence emitted from astrocyte cultures at the end of the recordings confirmed the phenotype of the cells. Insets, EGFP and Venus signals in grayscale. Scale bar, 100 μm. **B**, Representative ATP-induced bioluminescence traces for circadian (top) and arrhythmic (bottom) cultures of WT, dnSNARE, and VIPP astrocytes. **C**, The proportion of circadian cultures was calculated based on the RelAmp values determined by FFT-NLLS (rhythmic: RelAmp ≤0.2).

Figure 5. Gliotransmission affects ATP release but not *Bmal1::Luc* rhythms. **A**, Representative raw bioluminescence traces for dnSNARE and VIPP cortical astrocyte cultures infected with the lenti-*Bmal1::dLuc* reporter. Numbers above the *x*-axis are the

clock-gene expression in astrocytes (Prolo et al., 2005). We found that *Clock/Clock, Per1^m Per2^m, Cry1^{−/−} Cry2^{−/−}* and *Bmal1^{−/−}* astrocytes are arrhythmic, similar to the locomotor behavior deficits of these mice (Vitaterna et al., 1994; van der Horst et al., 1999; Bunger et al., 2000; Zheng et al., 2001). We found that *Bmal1, Clock^{−/+}* and *Cry1^{−/−} Cry2^{−/+}* glia have abnormal periods, much like the heterozygous mouse behavior. The correlations between rhythmicity in clock genes, extracellular ATP in glia and locomotor behavior suggest they may be tightly related.

Most of the astrocyte cultures deficient for functional CLOCK or PERIOD proteins lacked circadian rhythms in *Bmal1::dLuc* and *Per2::dLuc* reporter expression and in extracellular ATP. In addition, a low percentage of *Clock/Clock* or *Per1^m Per2^m* glial cultures displayed disrupted circadian rhythms in ATP release. It is unclear whether the mutant cultures scored as circadian were able to generate weak circadian cycling. Importantly, their ATP release was constitutively low, suggesting that the circadian clock functions to upregulate ATP release at specific times of day. Although circadian rhythms in extracellular ATP metabolism and intracellular ATP production may contribute to the rhythms we observed, the ATP-dependent flashes recorded here, the reduction of extracellular ATP levels, and the loss of (high-amplitude) rhythmicity following genetic disruption of IP_3 signaling provide evidence for a circadian control of ATP release. In contrast, genetic disruption of vesicular release had no effect on circadian ATP release suggesting that vesicular release of ATP is not under circadian control or that its deficiency can be compensated by other releasing mechanisms.

It is unknown how the circadian clock modulates pathways involved in purinergic signaling. We observed tonic and discrete changes in bioluminescence, suggesting that there are at least two mechanisms that contribute to glial release of ATP. Future studies examining the frequency of flashes as a function of genotype and circadian time may be able to resolve the role of the circadian clock in the discrete release events. Extracellular ATP concentration depends on diverse mechanisms including cell–cell interactions, activity of ectonucleotidases, cell-volume regulation and ionic homeostasis (Reetz et al., 1997; Arcuino et al., 2002; Corriden and Insel, 2010). There are also over 20 purinergic receptors sensitive to ATP or its metabolites, some of which can feedback on ATP release. Although none of these were studied for circadian variation, the activity of enzymes involved in the synthesis of adenosine display daily rhythms in areas of the brain related to sleep (Mackiewicz et al., 2003). ATP release could also be affected by the intracellular concentration of ATP. Although circadian and metabolic pathways can be interrelated (Yang et al., 2006; Lin et al., 2008; Nakahata et al., 2009; Ramsey et al., 2009), no circadian rhythms in intracellular ATP have been reported. Since purines are common signals between cells in the CNS and peripheral organs, future studies should address the relationship between circadian systems and ATP release.

The role of circadian rhythms in ATP release is unclear. Daily increases in extracellular ATP must not be critical for circadian rhythms in cortical astrocytes because VIPP astrocytes show normal circadian rhythms in *Bmal1::dLuc* expression despite having arrhythmic extracellular ATP. ATP or its metabolites may be a circadian output from cortical astrocytes that will affect other cells. Whether this is a universal property of astrocytes from all brain regions is unknown. Rhythms in ATP content and release from the SCN and SCN derived cells (Yamazaki et al., 1994; Womac et al., 2009), as well as retinal adenosine (Ribelayga and Mangel, 2005), also have no known function. Extracellular ATP, 5′-AMP and adenosine are associated with many brain functions

which are also circadian, including thermoregulation, hormonal secretion, and sleep (Hill et al., 1976; Scaccianoce et al., 1989; Gourine et al., 2002; Jones, 2009; Stojilkovic, 2009). ATP-induced activation of P2X receptors in the hypothalamus regulates both hormone secretion and body temperature while adenosine is associated with sleep need (Porkka-Heiskanen et al., 1997; Blanco-Centurion et al., 2006). Adenosine content in basal ganglia shows daily variations that were proposed to be related to changes in sleep–wake cycles during aging while A1 receptors and 5′-ectonucleotidase activity are rhythmic in cortex presumably playing a role in sleep (Virus et al., 1984; Florio et al., 1991; Rosati et al., 1993; Mackiewicz et al., 2003; Murillo-Rodriguez et al., 2004). Sleep deprivation can shift circadian rhythms (Antle and Mistlberger, 2000; Antle et al., 2001), possibly through adenosine that accumulates with sleep need, a mechanism supported by the findings that light-induced phase shifts can be blocked by both, A1 agonists and IP3 blockers (Watanabe et al., 1996; Marpegán et al., 2005; Leone et al., 2006; Krueger, 2008). Although ATP receptors are expressed throughout the brain in both neurons and glial cells (North, 2002), differential expression of the numerous subtypes of receptors can lead to different responses and roles for ATP in different areas. This is illustrated by the expression of P2X5 in the hypothalamus, where it is coexpressed with arginine vasopressin and neuronal nitric oxide synthase in several nuclei but not in the SCN (Xiang et al., 2006) indicating that circadian rhythms in ATP release could lead to multiple responses depending on the combinations of receptors, dynamics of release, and cell types involved. Corriden and Insel (2010) proposed that ATP release and the consequent activation of purinergic receptors could help establish the basal level of activation for signal transduction pathways, regulating a wide array of responses including blood flow, ion transport, cell volume regulation, neuronal signaling, and host-pathogen interactions. All these responses certainly require a temporal organization that could be provided by the circadian control of basal ATP release in astrocytes and other cell types.

Interestingly previous *in vivo* studies using dnSNARE mice have shown a critical role for the glial SNARE-sensitive purinergic pathway in the modulation of sleep homeostasis (Halassa et al., 2009). In these studies, sleep deprivation induced changes in slow-wave activity as well as compensatory behavioral changes including total sleep time, and these changes were shown to require an astrocytic dnSNARE-sensitive pathway of ATP/adenosine. Given that the current study shows that circadian changes in ATP are insensitive to astrocytic dnSNARE expression, we conclude that sleep homeostasis and circadian oscillations may be regulated by different signaling mechanisms.

In summary, continuous measurements of extracellular ATP levels reveal circadian release from mammalian astroglia which depends on key clock genes and IP3 signaling. These results indicate that extracellular ATP levels are augmented at specific times of day by CLOCK, PER and BMAL1 activity and suggest a clock-induced increase in energy metabolism and glial activity which may participate in sleep–wake changes in the brain.

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