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Circadian Rhythms of Extracellular ATP Accumulation in SCN Cells and Cultured Astrocytes

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Abstract

The master circadian pacemaker located within the suprachiasmatic nuclei (SCN) of the mammalian brain controls system-level rhythms in animal physiology. Specific SCN outputs synchronize circadian physiological rhythms in other brain regions. Within the SCN, communication among neural cells provides for the coordination of autonomous cellular oscillations into ensemble rhythms. Adenosine triphosphate (ATP) is a neural transmitter involved in local communication among astrocytes and between astrocytes and neurons. Using a luciferin-luciferase chemiluminescent assay, we have demonstrated that ATP levels fluctuate rhythmically within both SCN2.2 cell cultures and the rat SCN *in vivo*. SCN2.2 cells generated circadian oscillations in both the production and extracellular accumulation of ATP. Circadian fluctuations in ATP accumulation persisted with an average period (τ) of 23.7hr in untreated as well as vehicle- and forskolin-treated SCN2.2 cells, indicating that treatment with an inductive stimulus is not necessary to propagate these rhythms. ATP levels in the rat SCN *in vivo* were marked by rhythmic variation during exposure to LD12:12 or constant darkness, with peak accumulation occurring during the latter half of the dark phase or subjective night. Primary cultures of cortical astrocytes similarly expressed circadian oscillations in extracellular ATP accumulation that persisted for multiple cycles with periods of about 23hr. These results suggest that circadian oscillations in extracellular ATP levels represent a physiological output of the mammalian cellular clock, common to the SCN pacemaker and astrocytes from at least some brain regions, and thus may provide a mechanism for clock control of gliotransmission between astrocytes and to neurons.

Keywords

Suprachiasmatic Nucleus; Pacemaker; Oscillator; SCN2.2; ATP; Glia

INTRODUCTION

In mammals, the suprachiasmatic nuclei (SCN) of the hypothalamus function as the master pacemaker, orchestrating circadian rhythmicity in the brain and peripheral tissues. The SCN also generate circadian oscillations that persist in the absence of external input. SCN cells intrinsically produce circadian rhythms of neuropeptide secretion, cellular metabolism,

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electrical activity, and gene expression *in vivo* and *in vitro* (Lee et al., 2003). These circadian oscillations are not only an ensemble property of the SCN, but are autonomously generated by individual SCN neurons (Welsh et al., 1995; Hastings & Herzog, 2004). For example, rhythmic GFP-fluorescence driven by the clock gene *Per1* is a composite of the autonomous oscillations (Quintero et al., 2003). Other neural loci also contain cell-autonomous clocks similar to those found in the SCN. Individual olfactory bulb neurons exhibit circadian oscillations of *Per1* transcription and firing rate *in vitro* (Granados-Fuentes et al., 2004). Identification of the genes and signal molecules responsible for the coordination of oscillations among multiple cellular clocks within the SCN (Bell-Pedersen et al., 2005) and other brain regions is therefore of critical importance for understanding how SCN clock cells are coupled and how extra-SCN neural oscillators maintain local time for indigenous processes.

ATP, besides providing a critical energy source for driving cellular chemical reactions, is a signaling molecule involved in intercellular communication between astrocytes and neurons (Haydon, 2001; Scemes & Giaume, 2006). ATP released from astrocytes accumulates as extracellular adenosine in the brain and regulates synaptic transmission and neural integration (Pascual et al., 2005; Fellin et al., 2006). Furthermore, gliotransmission is thought to regulate aspects of brain metabolism (Bernardinelli et al., 2004; Magistretti, 2006). Therefore, ATP is a good candidate for a signal that mediates the local coordination of individual circadian clocks in the SCN and perhaps in other brain regions.

Because the expression of genes involved in the regulation of ATP oscillates in the SCN (Menger et al., 2005), we first examined ATP production by SCN cells for evidence of rhythmic fluctuations in levels of this gliotransmitter *in vitro* and *in vivo*. Immortalized rat SCN cells (SCN2.2) were used for our *in vitro* analysis because these cells retain the endogenous rhythm-generating and pacemaker properties of the SCN *in situ* (Allen et al., 2004). The cellular composition of the SCN2.2 line is similar to the rat SCN consisting of a heterogeneous population of neural cells that includes large numbers of astrocytes, among which ATP may provide an important signal for intercellular communication. In addition, *in vivo* microdialysis methods were used to determine whether the rat SCN is marked by diurnal and circadian oscillations in ATP levels. Because circadian oscillations and the underlying clockworks are common to extra-SCN neural cells (Granados-Fuentes et al., 2004; Guilding & Piggins, 2007), we next determined whether cortical astrocytes express circadian patterns of extracellular ATP accumulation *in vitro*. Evidence for the circadian regulation of extracellular ATP levels in SCN cells and in other neural oscillators suggests that ATP may be an important circadian output of the clockworks in the SCN and some neural oscillators in other brain regions.

MATERIALS AND METHODS

Cell Culture Conditions

SCN2.2 cells were cultured on laminin-coated dishes (60mm; Corning, Corning, NY) and maintained at 37°C and 5% CO₂ in Minimum Essential Medium (MEM; Invitrogen, Carlsbad, CA, USA), supplemented with 10% FBS, glucose (3000µg/ml), L-glutamine (292µg/ml), and 1% penicillin-streptomycin-neomycin (PSN) mixture (Invitrogen). Primary cortical astrocytes were purified from the forebrains of Sprague-Dawley rat pups on postnatal day 2 using a differential detachment method (Li et al., 2008) and cultured under similar conditions. During cell propagation, the medium was changed at 48hr intervals, and cultures were split every 2-3 days.

Experiment 1: Temporal profile of ATP production in SCN2.2 cultures

On three separate occasions, ATP levels were examined for evidence of rhythmic variation in living cultures of SCN2.2 cells that were derived from a single passage. Prior to experimental analysis, cells were propagated as described above, seeded onto a 24-well plate in culture medium with a decreased FBS concentration of 5% and 24hr later subjected to medium replacement with serum-free Neurobasal medium (supplemented with glucose, L-glutamine, and 1X B-27 serum-free supplement; Invitrogen). Pairs of individual cultures in a 24-well plate were used as replicates of 12 specific timepoints and the paired wells were exposed for 2hr to serum-free medium containing either dimethylsulfoxide (DMSO; Sigma, St. Louis, MO, USA) vehicle (0.1%) or 15 μ M forskolin (FSK; Calbiochem, La Jolla, CA, USA). This procedure was repeated every 2hr for the remaining pairs of cultures so as to provide for image analysis of 12 consecutive timepoints over a complete 24hr cycle on one plate. FSK, an adenylate cyclase agonist that increases cyclic AMP levels, has been used to coordinate rhythms of clock gene expression and glucose uptake across SCN2.2 cell cultures (Allen et al., 2001). Immediately after treatment, cells were rinsed and maintained thereafter in serum-free Neurobasal medium. Chemiluminescent imaging of ATP levels was performed 24hr later on living cultures incubated in fresh serum-free Neurobasal medium (1ml) containing 10 μ l luciferase (3mg/ml; Sigma) and 20 μ l luciferin (3mg/ml; Invitrogen) for 30min prior to analysis.

Experiment 2: Temporal profile of extracellular ATP accumulation in SCN2.2 cultures

To examine extracellular accumulation and its potential contribution to the profiles observed in the preceding experiment, three biological replicates derived from cells of a similar passage number (20-25) were performed in which ATP levels were analyzed in serial samples of the medium from SCN2.2 cultures (N=13). SCN2.2 cells derived from a single passage were propagated and treated as described in Experiment 1 except that cultures were maintained in 60mm dishes throughout this analysis. Following the reduction in serum concentration to 5%, SCN2.2 cultures were untreated (N=2) or exposed to either DMSO (N=6) or 15 μ M FSK (N=5) for 2hr. Thereafter, the medium was replaced with serum-free Neurobasal medium (3ml) containing the aforementioned supplements and 2hr later experimental analysis was initiated by collecting and replacing medium (1ml) from all cultures at 2hr intervals for 72hr. To assess the influence of sampling procedures on the temporal pattern of extracellular ATP accumulation, some experiments were performed so as to increase the time for total volume exchange from 6 to 16hr by collecting/replacing smaller sample volumes (500 μ l) every 2hr from cultures incubated in 4ml of medium. Media samples were frozen, stored at -20°C and later analyzed for ATP accumulation using a luciferin/luciferase (luc/luc) chemiluminescence assay.

Experiment 3: Temporal profile of ATP levels in the rat SCN

In vivo microdialysis methods were used to determine whether extracellular ATP levels also fluctuate rhythmically in the rat SCN. Experimental subjects were 8 Sprague-Dawley rats (250-350gm). These animals were born and reared in the vivarium at the Texas A&M University System Health Science Center under a standard 12h light:12h dark photoperiod (LD 12:12; lights-on at 0600 hr). Prior to experimental analysis, animals were housed 2-3 per cage. Access to food and water was provided *ad libitum* and periodic animal care was performed at random times. The procedures used in this study were approved by the University Laboratory Animal Care Committee at Texas A&M University. Chronic placement of a guide cannula for the microdialysis probe (CMA11, CMA Microdialysis, North Chelmsford, MA, USA) in the SCN region was accomplished using empirical stereotaxic techniques (Earnest et al., 1999; Liang et al., 2000). During the light phase of the LD 12:12 cycle, animals were anesthetized (xylazine 65mg/kg; ketamine 87mg/kg) and stereotaxic coordinates (0.9mm posterior to Bregma; 0.4mm lateral to midline; 5.8mm

ventral to the dura) were used to place the cannula assembly (guide with dummy stylet) along the lateral margin of the SCN. Three small screws were inserted in the skull (one anterior and two in posterior-lateral locations) and the exposed portion of the guide cannula was secured in place to these anchors with dental acrylic resin. After a recovery period of 24-30hr, animals were anesthetized with isofluorane (VEDCO Inc., St. Joseph, MO, USA) and following removal of the dummy stylet, a microdialysis probe (CMA 11, CMA Microdialysis; 240 μ m diameter; cuprophane membrane with 6kD cut-off) was inserted into the guide cannula. The microdialysis probes were designed to provide for extension of the probe tip $\approx$ 1mm beyond the guide cannula and for limited perfusion ($\approx$ 50 μ m) of the surrounding parenchyma. Probes were attached to micro-bore tubing traveling through a microdialysis swivel and head tether assembly (Instech, Plymouth Meeting, PA, USA) that allowed animal movement around the cage. Artificial cerebrospinal fluid (aCSF) was delivered to the probe via a KDS220 Infusion Pump (KD Scientific, Holliston, MA, USA) at a rate of 2 μ l/min and beginning at zeitgeber time (ZT) or circadian time (CT) 12, samples ($\approx$ 120 μ l) were collected in a cooled (8°C) fraction collector (820 Microsampler, SciPro Inc., Sanborn, NY, USA) at 2hr intervals for 24hr. During this analysis, animals were either maintained under LD 12:12 conditions (N=5) or exposed to constant darkness (DD) (N=3). Microdialysis samples were frozen and stored at -80°C until later assay of ATP levels. At the conclusion of microdialysis sampling procedures, animals were anesthetized (sodium pentobarbital 3mg/kg) and sacrificed by transcardiac perfusion with 50ml of 0.1M phosphate buffer (pH=7.3) containing heparin followed by 200-250ml of 4% paraformaldehyde. Immediately after perfusion, the brains were removed, post-fixed for 1-2 hours at 4°C and stored overnight in cryoprotectant solution (15% sucrose in 0.15M phosphate buffer). The tissue was then frozen and sectioned in the coronal plane at 30 μ m using a sliding microtome. Coronal sections containing the SCN were mounted on glass slides, air-dried overnight, stained with Cresyl violet and coverslipped with Permount®. Probe placement in relation to the SCN was determined by localization of the ventral extent of the cannula tract in mounted sections using brightfield microscopy.

Experiment 4: Temporal profiles of ATP accumulation in the culture medium from other neural cell types

For each of two biological replicates derived from separate animal tissue collections, cultures of primary cortical astrocytes were propagated on 60mm dishes and analyzed for evidence of rhythmic ATP accumulation in the medium. Similar to the analysis in Experiment 2, the serum concentration was reduced to 5% and then all astrocyte cultures were untreated (N=2) or exposed to DMSO (N=7) or 15 μ M FSK (N=7) for 2hr followed by sampling of culture medium (1ml) at 2hr intervals for 72hr.

Chemiluminescence assays for analysis of ATP levels

To analyze ATP levels in living cultures (Experiment 1), chemiluminescent imaging was performed on SCN2.2 cells that were maintained in a humidified incubator at 37°C and 5% CO₂ equipped with a liquid nitrogen-cooled CCD camera (Versarray, Photometrics, Tucson, AZ, USA). The CCD was cooled to -110°C and images were captured using 5min exposures in total darkness. Chemiluminescence images were captured in 3 consecutive exposures, and intensities of luminescence from the collected images were analyzed using MetaMorph4.6 imaging software (Universal Imaging Corporation, Downingtown, PA, USA).

Cell-free, chemiluminescence assays of extracellular ATP levels were performed by incubating aliquots (100 μ l) of media samples (Experiment 2 and 4) or aliquots (20 μ l) of microdialysis samples (Experiment 3) with 1 μ l of luciferase and 2 μ l of luciferin in wells of a black, 96-well plate (Thermo, Milford, MA, USA). ATP-dependent chemiluminescent activity produced by media or microdialysis samples was measured in constant darkness

using a multiplate Packard TopCount scintillation counter (Meriden, CT, USA). Based on the repeated analysis of the same samples across multiple assays, interassay variation in the determination of ATP levels was less than 10%.

To approximate ATP levels in living SCN2.2 cultures (Experiment 1) and in conditioned culture medium (Experiment 2 and 4), standard curves were generated for both the CCD-based imaging assay and the TopCount (TC)-based photomultiplier assay using known concentrations of ATP (Fig. 1A). Chemiluminescence derived from culture media samples (Experiment 2 and 4) was calibrated relative to assay standards ranging from 1pM to 100nM ATP in unconditioned medium. For microdialysis samples (Experiment 3), chemiluminescent activity was calibrated to ATP standards ranging from 1nM to 10nM. Comparison of the standard curves revealed that the sensitivity of ATP detection is similar between the imaging and photomultiplier assays. Internal controls consisting of unconditioned medium (Experiment 2 and 4) without ATP standard, luciferase, or luciferin were included on all analyzed plates. An important consideration in the implemented design of Experiments 1, 2 and 4 (i.e., the use of serum-free medium) was based on methodological analysis indicating that the luciferase reaction was dramatically disrupted by the presence of serum in the culture medium. In this analysis, ATP standards containing FBS exhibited a dose-dependent suppression of chemiluminescent signal and media samples from SCN2.2 cultures containing 10% FBS consistently produced lower signal intensities than those obtained from cultures maintained in serum-free medium (data not shown).

Technical analysis was also performed to confirm that chemiluminescent activity was dependent on ATP in the culture medium. Treatment with apyrase (50U/ml), an enzyme that degrades ATP, had a significant effect ($t_{20} = 7.29$; $P = 1.27 \times 10^{-11}$) in abolishing detectable chemiluminescence in the medium from SCN2.2 cultures (Fig. 1B), demonstrating that ATP is necessary to drive the Luc/Luc reaction in this assay.

Statistical analysis

Raw chemiluminescence data (photons/sec) were normalized in relation to the maximum for each culture, which was arbitrarily set at 100. The normalized data from Experiment 2 and 4 was subjected to a Lomb-Scargle Fourier Transform analysis using AutoSignal software (Systat Software Inc., Point Richmond, CA, USA). A least-square fitting of the data was applied with a sinusoidal parametric function. Through regression analysis at various frequencies, the period (τ) of recurrent oscillations was extracted from the time series data. For analysis of extracellular ATP accumulation in SCN2.2 cultures treated with apyrase and in the SCN *in vivo* (Experiment 3), paired and pooled *t*-tests were performed to determine if peak levels were significantly different from trough values. The α value was set at 0.05 for all statistical analyses.

RESULTS

Experiment 1: Temporal profile of ATP production in SCN2.2 cultures

To determine whether ATP levels in living cultures of SCN2.2 cells oscillate in a rhythmic fashion, cells were bathed in luciferin/luciferase-containing medium and images of ATP-dependent chemiluminescence were captured. Rhythmic fluctuations in ATP levels were observed in each of 10 independent SCN2.2 cultures. Dimethylsulfoxide- (DMSO; N=5) and forskolin-treated (FSK; N=5) cultures were similar with regard to the expression of these ATP rhythms (Fig. 2) and peak chemiluminescence ranging from 800-4000 raw pixel intensity values. Rhythms in ATP levels were marked by robust differences of greater than 17-fold between peak and trough values. Based on comparisons with standard curve determinations using known concentrations of ATP (Fig. 1A), ATP levels were

approximated at 10nM for the peak and at 10pM for the trough of the ATP rhythms in SCN2.2 cultures. The rhythmic peak in SCN2.2 ATP levels typically persisted for 2-8hr before declining to basal levels. In most of the cultures (9/10), the temporal profiles of ATP-dependent chemiluminescence exhibited a single peak over the 24hr time course. One DMSO- treated culture exhibited a bimodal pattern in which the primary peak in ATP levels was followed 10 hours later by a secondary, low-amplitude peak. The phase of the ATP rhythms in SCN2.2 cultures was variable within and between the DMSO and FSK treatment groups. This variability in rhythm phase is presumably related to comparisons founded on three experiments using cultures derived from separate passages. However, DMSO- and FSK-treated SCN2.2 cultures within a given experiment generated ATP rhythms in which peak levels were either concurrent or 12 hours out of phase. ATP-dependent luminescence from cultures lacking luciferin, luciferase, or both reagents in the assay medium was equivalent to background levels in blank wells with no evidence of rhythmicity.

Experiment 2: Temporal profile of extracellular ATP accumulation in SCN2.2 cultures

Because ATP accumulation in the medium presumably contributed to the profiles observed in the preceding image-based analysis of living cells, we next conducted chemiluminescence assays on cell-free samples of conditioned medium from SCN2.2 cultures to distinguish the extent and temporal pattern of extracellular ATP accumulation. Consistent with oscillations in ATP levels observed in chemiluminescent imaging of living cells (Experiment 1), untreated (N=2), DMSO- (N=6) and FSK-treated (N=5) SCN2.2 cultures exhibited rhythmic profiles of ATP accumulation in the medium with recurrent peaks at circadian intervals (Fig. 3). Similar to the values established for living SCN2.2 cultures in Experiment 1, standard curve estimates of ATP levels (Fig. 1A) were about 10nM for the peak and 10pM for the trough of SCN2.2 rhythms in extracellular ATP accumulation. These circadian rhythms in extracellular ATP levels persisted for 3 cycles in all SCN2.2 cultures (N=13) with peak-to-peak intervals of typically 20-24hr (10/13) and 4-57 fold differences between peak and trough levels of chemiluminescence. In the three remaining cultures, the extracellular ATP rhythms were distinguished by peak-to-peak intervals of 16hr (2 DMSO-treated) or 28hr (1 DMSO-treated). The phase of the ATP rhythms in SCN2.2 cultures differed across treatment groups and even exhibited a degree of variability among individual cultures exposed to the same treatment presumably because the data are derived from three biological replicates. Phase differences across treatment groups were especially evident during the first cycle such that the timing of initial ATP peaks in individual SCN2.2 cultures ranged from 2-20hr after the onset of analysis. Based on Fourier transform analysis, circadian frequencies were predominant in the temporal profiles of extracellular ATP accumulation for 11 of 13 independent cultures and the mean ($\pm$ SEM) period (τ) for these SCN2.2 rhythms was 23.7 ± 0.8 hr. It is noteworthy that when different sampling procedures were used so as to collect a smaller volume of media at 2hr intervals and increase the time for total volume exchange from 6 to 16hr, the rhythms of extracellular ATP accumulation and their underlying properties in SCN2.2 cultures (N=4) were similar to those observed using the standard protocol (Fig. 3). To examine the possible influence of low calcium treatment associated with exposure to CMF buffer at the onset of analysis, additional experiments were performed using Neurobasal medium to rinse cultures prior to the initiation of sampling. In SCN2.2 cultures rinsed with Neurobasal medium (N=5), the initial elevation in extracellular ATP levels was greatly diminished, but the amplitude of the extracellular ATP rhythms was similar relative to that found in CMF-exposed cells (Fig. 3). Collectively, these observations indicate that the rhythms in extracellular ATP accumulation are not a product of either sampling procedures or FSK- and low calcium-induced increases in ATP levels, but instead are endogenous to SCN2.2 cells.

Experiment 3: Temporal profile of ATP levels in the rat SCN

Post-experimental histological analysis confirmed probe placement in seven out of eight animals along the lateral margin of the left SCN and dorsal to the chiasm with no evidence of damage to the SCN. Similar to the rhythmic pattern expressed by SCN2.2 cells *in vitro*, extracellular ATP accumulation in the SCN of these rats showed overt signs of rhythmicity under LD 12:12 (N=4) and DD (N=3) conditions. During exposure to LD 12:12, SCN levels of ATP remained low throughout the daytime and the first half of the night, rapidly increased reaching peak values near the middle of the dark phase, and then declined over the remainder of the night returning to basal values just prior to the onset of the light phase (Fig. 4A). Peak levels of ATP in the SCN during the night were significantly ($t_{15} = 5.7$; $P = 0.00036$) and about 10-40-fold greater than those observed throughout the daytime. The SCN rhythms in extracellular ATP levels persisted during exposure to DD with comparable amplitudes (i.e., peak-to-trough differences of 20-40-fold) and peak levels occurring during the middle of the subjective night (Fig. 4B). This crest in the circadian regulation of ATP accumulation is coincident with the rhythmic peak in SCN cellular content of ATP that occurs during the middle of the subjective night (Yamazaki et al., 1994). In the remaining animal where the probe was located in the anterior hypothalamic area (AHA) about 400-600 μ m caudal to the SCN, ATP levels were consistently low and exhibited no evidence of diurnal, circadian or even regular rhythmic, fluctuations (Fig. 4). In contrast to this finding, Yamazaki and co-workers (1994) reported that ATP content fluctuates on a circadian basis in the AHA with peak levels occurring during the middle of the subjective day. It is unclear why the AHA is distinguished by the circadian regulation of cellular content, but not extracellular accumulation, of ATP. Cellular content presumably reflects ATP levels found in both neurons and glia within a given brain region whereas extracellular accumulation is derived from astrocytes. Thus, a potential explanation for the low ATP levels and lack of rhythmicity in the AHA is that astrocytes are much more prevalent within the SCN than the AHA. This explanation is compatible with the finding that the density of GFAP-immunoreactive cells in the SCN is much greater than that found in any other region of the hypothalamus, including the AHA (Morin et al., 1989). Nevertheless, the observed regional differences in extracellular ATP levels suggest that our microdialysis probes and analysis provide a good reflection of regional ATP levels so as to distinguish SCN profiles from those in surrounding areas.

Experiment 4: Temporal profiles of ATP accumulation in the medium from cultured cortical astrocytes

The SCN2.2 rhythm in ATP accumulation may reflect the functional activity of astrocytes, which represent a prominent component of this multipotent cell line. Therefore, we next examined extracellular ATP levels in astrocyte cultures derived from another brain region. Specifically, primary cultures of cortical astrocytes were analyzed for evidence of circadian fluctuations in extracellular ATP accumulation *in vitro*. Similar to the rhythmic patterns observed in SCN2.2 cells, ATP levels in the medium from primary cultures of untreated (N=2), DMSO- (N=7) and FSK-treated (N=7) rat cortical astrocytes oscillated with recurrent peaks at intervals of 20-24hr (Fig. 5). In all treatment groups, the amplitude of these circadian oscillations in extracellular ATP accumulation was robust, with 9-92 fold differences between peak and trough levels over 3-4 cycles. Fourier transform analysis of the temporal patterns of extracellular ATP accumulation revealed that predominant frequencies were circadian in all cortical astrocyte cultures and the mean ($\pm$ SEM) τ for these astrocyte rhythms was 23.1 ± 0.2 hr.

DISCUSSION

Chemiluminescence-based analysis of ATP levels revealed that SCN2.2 cells generate circadian oscillations in the production and extracellular accumulation of this gliotransmitter *in vitro*. Moreover, the rat SCN was similarly characterized by daily and circadian fluctuations in extracellular ATP levels *in vivo*. The rhythmic regulation of ATP levels was anticipated in SCN2.2 cultures and in the SCN *in vivo* for several reasons. First, transcriptional profiling studies indicate that the expression of some genes in ATP signaling and metabolic pathways is similarly clock-controlled in SCN2.2 cells and the SCN *in vivo* (Panda et al., 2002; Menger et al., 2005). The circadian clock in SCN2.2 cells influences mitochondrial energy transduction through the rhythmic expression of mitochondrial ATP synthase 8 (*mt-Atp8*) and Ca²⁺ transporting ATPase (*Atp2a3*), and impacts upon glucose metabolism by regulating oscillations in the expression of malic enzyme 1 (*Me1*), hexokinase 2 (*Hk2*), and glyoxylate reductase/hydroxypyruvate reductase, an enzyme that mediates the conversion of serine to glucose. Second, cellular content of both ATP and cAMP fluctuate on a circadian basis in the rat SCN. The cellular content of ATP in extracted SCN tissue reaches peak levels during the middle of the subjective night (Yamazaki et al., 1994) and SCN content of cAMP *in vitro* is marked by bimodal peaks during the late subjective day and late subjective night (Prosser & Gillette, 1991). Finally, circadian oscillations are a hallmark property of SCN metabolism. Both SCN2.2 cells and the rat SCN are distinguished by circadian regulation of 2-deoxyglucose (2DG) utilization (Allen et al., 2001; Schwartz, 1991) as well as rhythmic expression of *Glut-1* (*Slc2a1*), the primary facilitative transporter of D-glucose, and *Mct1* (*Slc16a1*), a major transporter of ketone bodies and lactate in glial cells (Menger et al., 2005).

ATP levels in the medium also oscillated with a periodicity of approximately 24hr in primary cultures of cortical astrocytes. It is interesting that circadian oscillations in extracellular ATP accumulation were similarly observed in astrocytes even when cultures were untreated or vehicle-treated because non-SCN cells are typically unable to sustain circadian rhythmicity as an ensemble *in vitro* in the absence of SCN pacemaking cues, serum shock, or activation of various signal transduction pathways (Allen et al., 2001). Thus, the circadian oscillations in ATP accumulation reported here may represent a pervasive physiological output of the mammalian cellular clock in SCN cells and astrocytes from at least some brain regions.

The mechanism responsible for generating these ATP oscillations in SCN cells and cortical astrocytes is unknown. Although our data have limited implications in this regard, it seems likely that ATP release, uptake, and degradation may individually or even collectively contribute to the observed circadian rhythms in extracellular ATP accumulation. The differential prevalence of circadian oscillations among genes regulating glucose metabolism, mitochondrial energy transduction, and metabolite transporters in SCN cells (Rutter et al., 2002; Menger et al., 2005) raises the possibility that the oscillations in extracellular ATP accumulation may represent a byproduct of rhythms in SCN cellular metabolism. However, this explanation is incompatible with the phase differences between the oscillation in ATP levels and these metabolic or cellular rhythms in the SCN. For example, the ATP oscillations in the rat SCN reach their apex during the night (Fig. 4) in advance of the daytime peaks in SCN neural activity and glucose utilization (Inouye & Kawamura, 1982; Schwartz, 1991). Alternatively, cell lysis, calcium influx via voltage-dependent calcium channels (VDCCs), neuron-like exocytotic release, or membrane passage via channels or transporters have been linked to the regulation of ATP release from cells (Pascual et al., 2005; Scemes & Giaume, 2006). In relation to our investigation, studies of cortical and hippocampal astrocytes are noteworthy in suggesting that calcium entry through VDCCs or release from intracellular stores may mediate a calcium-regulated exocytosis of ATP-

containing vesicles (Queiroz et al., 1999; Pascual et al., 2005). Thus, these calcium-dependent mechanisms may play a role in regulating extracellular ATP accumulation and its circadian profile in our astrocyte cultures and even in SCN2.2 cells because VDCCs are rhythmically expressed and inhibition of VDCCs disrupts clock gene oscillations in these cells (Nahm et al., 2005).

The functional implications of extracellular ATP rhythms are similarly equivocal, but the present findings raise the possibility that this nucleotide may play a role in intercellular signaling between circadian oscillators in the SCN and other brain regions. ATP released from astrocytes acts as an autocrine or paracrine messenger that regulates intercellular calcium waves (Scemes & Giaume, 2006) and intercellular communication via gliotransmission among astrocytes and neurons (Haydon, 2001). In turn, intercellular gliotransmission is thought to regulate brain metabolism (Bernardinelli et al., 2004; Magistretti, 2006). After its release, extracellular ATP is degraded by ectonucleotidases and its primary metabolites, adenosine and 5'-AMP, are involved in regulating hypothalamic mechanisms of sleep (Scammell et al., 2001) and metabolic processes in the liver (Zhang et al., 2006), respectively. Interestingly, the collective observations from other studies of cAMP-dependent signaling indicate that cAMP content in the SCN similarly fluctuates on a circadian basis with peak levels during the subjective day and that this SCN oscillation is accompanied by circadian regulation of cAMP response element (CRE) activity (Murakami & Takahashi, 1983; Obrietan et al., 1999; O'Neill et al., 2008). Thus, the SCN rhythm in extracellular ATP accumulation may represent a local signal that is involved in clock control of gliotransmission and synchronizing the rhythmic behavior of individual cellular oscillators. ATP may influence intercellular communication between autonomous SCN oscillators via a direct action or through the regulation of purinergic signaling by its metabolite, adenosine. The latter mechanism is compatible with electrophysiological evidence for adenosine A1 and A2 receptors in the SCN (Chen & van den Pol, 1997).

It is also noteworthy that during astrogliosis the activation of P2X purinergic receptors by elevated levels of extracellular ATP is coupled with increases in glial fibrillary acidic protein (GFAP) expression and process elongation (Neary et al., 1994, 1996). Consistent with this relationship between extracellular ATP accumulation and astrocytic properties, the observed peak of the ATP rhythm during the night precedes the time when both GFAP distribution and astrocytic process elongation in the SCN are at their maxima during the day (Lavialle & Serviere, 1993). ATP signaling and its rhythmic regulation in SCN oscillators may thus be important in the activation of neuroglial endfeet networks so as to modulate ion buffering, transmitter uptake, and energy transfer. Although further analysis will be necessary to determine the specific functions of extracellular ATP rhythms, their prevalence in the mammalian SCN and differential expression in cortical astrocytes suggest that ATP may represent an important signaling molecule for circadian timekeeping among astrocytes and between astrocytes and neurons in the SCN and some other brain regions.

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Figure 1.

Comparison of ATP levels in the medium of SCN2.2 cultures with standard concentrations of ATP generated with both charge-coupled device (CCD) camera and TopCount (TC) photomultiplier assays. **A)** Two standard curves were generated using known concentrations ranging from 1pM to 100nM ATP (CCD assay, solid circles; TC assay, open circles) and compared to experimentally determined averages for peak (P) and trough (T) levels of SCN2.2 rhythms in extracellular ATP accumulation. The estimated range of rhythmic ATP levels in living SCN2.2 cultures (dashed lines) and in media samples from SCN2.2 cultures (dotted lines) was between 10pM (trough) and 10nM (peak) ATP. Comparable levels of ATP were estimated from medium images (N=4 cultures) and media samples (N=4 cultures). **B)** Chemiluminescent activity in the medium from SCN2.2 cultures is dependent on ATP. Bars denote determinations of ATP levels in media samples collected from SCN2.2 cultures treated with vehicle (CON) or apyrase (APY), an enzyme that degrades ATP. Chemiluminescent signal was significantly reduced in APY-treated SCN2.2 cultures ($p < 0.05$; N=4) relative to that of control cultures (N=18).

Figure 2.

Circadian regulation of ATP levels in living SCN2.2 cultures. Images (top) and corresponding temporal patterns (bottom) of ATP-dependent chemiluminescence captured from representative SCN2.2 cultures exposed to DMSO (left panels, closed square) or FSK (right panels, closed circle) for 2hr immediately prior to this analysis. Symbols denote determinations of signal intensity at 2hr intervals by image-based analysis of luminescence produced by the luciferin/luciferase reaction. The plotted values correspond to chemiluminescent signal measurements that were normalized in relation to the maximum for each culture, which was arbitrarily set at 100.

Figure 3.

Circadian rhythms of extracellular ATP accumulation in representative SCN2.2 cultures that were untreated (top), or exposed to DMSO (middle) or FSK (bottom) for 2hr immediately prior to this analysis. For comparison, the extracellular ATP rhythms are depicted for an untreated SCN2.2 culture in which sampling procedures were modified so as to increase the time for total volume exchange (top panel; □, dashed line) and for a DMSO-treated SCN2.2 culture rinsed with Neurobasal medium (middle panel; □, dashed line) rather than CMF buffer prior to the initiation of sampling. Symbols denote normalized values for photomultiplier tube-based determinations of ATP-dependent chemiluminescence in the medium from SCN2.2 cultures at 2hr intervals for 72hr. Dotted lines demarcate 24hr intervals.

Figure 4.

Diurnal and circadian ATP rhythms in the rat SCN *in vivo*. **A)** Top panel depicts the representative temporal pattern of extracellular ATP accumulation in the rat SCN during entrainment to LD 12:12. Bottom panel represents the temporal profile of extracellular ATP levels in the anterior hypothalamic area (AHA) about 400-600 μ m caudal to the SCN. The bar at the top signifies the timing of the light (open) and dark (closed) phase in the LD 12:12 cycle. **B)** Representative temporal profile of ATP accumulation in the rat SCN during exposure to constant darkness (DD). Symbols denote the raw data for photomultiplier tube-based determinations of ATP-dependent chemiluminescence in microdialysis samples collected at 2hr intervals for 24hr.

Figure 5.

Circadian rhythms of extracellular ATP accumulation in primary cultures of cortical astrocytes that were untreated (top), or exposed to DMSO (middle) or FSK (bottom) for 2hr immediately prior to this analysis. Symbols denote normalized values for photomultiplier tube-based determinations of ATP-dependent chemiluminescence in the medium from astrocyte cultures at 2hr intervals for 72hr.