THE ROLE OF P2X7 RECEPTOR SIGNALING IN REGULATION OF RHYTHMIC

ASTROCYTE PHYSIOLOGY IN MOUSE SUPRACHIASMATIC NUCLEUS CELLS

A Thesis

by

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ABSTRACT

Mammalian circadian rhythms are controlled by the suprachiasmatic nuclei (SCN) of the

hypothalamus. While neural circuits influence SCN entrainment, cells within the SCN also

act as individual oscillators. Research on mechanisms that regulate synchronization of

ensemble SCN rhythms has mainly focused on neuronal populations. However, astrocytes

are just as abundant in the SCN and display circadian rhythms in clock gene expression

and extracellular accumulation of ATP. Rhythmic accumulation of ATP in the rat SCN *in

vivo* has also been described to occur during the night. Nonetheless, it remains unclear

whether or not astrocytic ATP release functions as a synchronizing signal in the SCN and

contributes to ensemble neural activities such as inflammatory signaling. Preliminary

studies on SCN cell cultures and purinergic inhibitors disrupt ATP accumulation and clock

gene expression rhythms. Further, clock-defective astrocytes are unable to accumulate

ATP rhythmically. I tested the hypothesis that ATP signaling mediates rhythms in

astrocytic physiology through P2X7 receptor. First, I tested whether rhythmic ATP release

may synchronize individual oscillators in the SCN cells. I also tested the effectiveness of

BzATP, a P2X7R agonist, in inducing release of ATP in mouse SCN cells. I tested if

P2X7R, GFAP overall protein expression and astrocytic activation markers such as GFAP,

are rhythmic in mouse SCN cells. I demonstrate that ATP signaling among astrocytes is a

good candidate for a coordinating mechanism that synchronizes clock-controlled

physiological responses in the SCN and other brain regions. Furthermore, I present data

indicating that clock-controlled ATP signaling rhythms are involved in modulating

neuroinflammatory signaling. Since BzATP proved to be an effective P2X7R agonist with regard to potentiating ATP release from mouse SCN cells, further experiments with other agonists and antagonists can provide evidence for the role of ATP and P2X7R signaling rhythms, their circadian regulation, and their influence on astrocytic physiology and brain health.

DEDICATION

I dedicate this thesis to my grandmother Conchita "Little Shell" and all the Native American women that were not able to accomplish their goals and dreams because of colonialism and patriarchy.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Zoran, and my committee members, Dr. Thompson and Dr. Earnest, for their guidance and support throughout the course of this research.

Thanks also go to my friends in different colleges of the university for making my time at Texas A&M a tolerable experience. I also want to extend my gratitude to the National Science Foundation, which provided me with the funding to continue with my graduate studies.

Finally, thanks to my partner Christopher Sandoval.

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

Rhythmic gene expression has been described in organisms as diverse as animals, fungi, plants, bacteria and archaea. These gene expression rhythms and the biological clocks that underlie them are necessary in order to survive environmental fluctuations (Whitehead et al. 2009). Since temporal regulation of organismal physiology is necessary for each of these groups, they all possess common modulators that coordinate biological timing processes to anticipate environmental changes. These common modulators are highly conserved auto-regulatory feedback mechanisms, based on diverse molecular clock components. The way these internal molecular clocks keep time is by activating and/or gating transcription, translation or other modulatory processes that translate into rhythmic behavioral activities (Bell-Pedersen et al. 2005). Mammals, being multicellular organisms, depend on a master circadian pacemaker located in the brain to coordinate and synchronize other oscillators throughout the body. These central neural pacemaker regions located in the hypothalamus are the suprachiasmatic nuclei (SCN). This chapter will introduce the SCN, with a particular focus on rhythmic astrocyte physiology. Astrocytes in the SCN are known to have a peak in release of ATP every 24 hours (Womac et al. 2009). Since astrocytes also possess internal molecular clocks that are synchronized across an ensemble glial population, this thesis will describe experiments investigating the role of the purinergic receptor P2X7 in regulating the astrocyte synchronization and clock controlled ATP- accumulation in SCN-derived cell cultures.

Suprachiasmatic nucleus

Biological clocks establish physiological and behavioral rhythms synchronized at appropriate times of the day in order to secure organismal survival. In mammals, the suprachiasmatic nuclei (SCN) synchronizes these daily rhythms to the photoperiodic time of day. Since the SCN acts as a bridge between the environmental light-dark cycle and the rest of the body, it is directly connected to the eyes through the retinohypothalamic tract (RHT) via the optic nerve (Hastings & Herzog 2004). The eyes receive photic cues that are detected by melanopsin photopigment in specific cells of the retina (Nayak et al. 2001). Intrinsically photosensitive retinal ganglion cells (ipRGCs) that express melanopsin, project from the retina to the SCN and are responsible for circadian photoentrainment by releasing glutamate and pituitary adenylate cyclase-activating polypeptide (PACAP) onto the SCN neurons (Lowrey & Takahashi 2004). There are other neurotransmitters released by ipRGCs but glutamate and PACAP have been studied intensively since they have distinct roles in regulating entrainment. Studies have shown that exogenous application of glutamate mimics light-induced phase shifts in activity, and the use of N-methyl-D-aspartate receptor (NMDA) antagonists blocks those phase shifts (Golombek & Rosenstein 2010). Glutamate is also involved in further signal transduction events like activation of Ca^{2+}/calmodulin-dependent protein kinase type II (CaMKII), nitric oxide release, and CREB activated transcription (Cheng & Obrietan 2008). PACAP also activates signaling through transduction pathways like cAMP/PKA, PKC, MAPK cascades and triggers release of Ca^{2+} from intracellular stores (Michel et al. 2006). PACAP has a complex modulatory role since it has been found that its capacity to affect gene

expression is inversely related to its concentration. Very small amounts (pM) potentiate NMDA channel conductance, nanomolar amounts induce Per1 and Per2 expression, and micromolar concentrations of PACAP facilitate glutamate-induced transcription of Per1 and Per2 (Nielsen et al. 2001).

In terms of neuronal heterogeneity, the SCN has been separated into two distinct areas, the core region (ventrolateral) and the shell region (dorsomedial). The core receives direct input from the retina, intergeniculate leaflet and raphe nuclei (Moga & Moore 1997). The core also extensively innervates the shell, while lesser innervation occurs from the shell back to the core. While the SCN shell is characterized by high expression of arginine vasopressin (AVP) and metenkephalin the core neurons mainly express vasoactive intestinal polypeptide (VIP), gastrin-related peptide (GRP) and calbindin (Yan et al. 2007). GABAergic transmission is also prevalent in the SCN. The SCN core, upon receiving photic cues through the RHT, entrains neighboring neurons of the shell and other regions in the brain (Hastings & Herzog 2004). After the shell becomes entrained though core input, it then communicates with other target areas of hypothalamus (eg., medial subparaventricular zone, the preoptic area, and dorsomedial hypothalamus) and drives rhythmic outputs in neural circuits through its synchronous firing patterns. Rhythms in SCN neuronal firing are thought to underlie its ability to synchronize other brain areas. However, other mechanisms such as hormones or diffusible signals are required for entraining peripheral oscillators via synchronizing cues (Allen et al. 2001). Some candidates include the hormones such as cortisol and melatonin and the diffusible signals such as prokineticin 2, transforming growth factor-α (TGF- α) and cardiotrophin-like

cytokine (CLC) (Yan et al. 2007, Kraves & Weitz 2006).

Mammalian biological clock

The SCN's neuronal heterogeneity and its neural circuitry certainly contributes to how the SCN operates in terms of synchronization, but the cellular clock that keeps time within individual neurons is critical to this synchronization. Since the identification of the Circadian Locomotor Output Cycles Kaput (*Clock*) gene in 1997, other genes have been shown to be part of the mammalian core transcription-translation feedback loop. It is also known that the core molecular clock of the SCN is essential for entrained mammalian circadian rhythms (King et al. 1997, Herzog et al 1998). The "positive limb" of this loop is composed of two Period-Arnt-Single-minded (PAS) helix-loop-helix transcription factors, *Clock/Npas2,* and the brain and muscle ARNT-like protein 1 (*Bmal1/Mop3*) (Preitner et al. 2002). After CLOCK and BMAL1 hetero-dimerize in the cytoplasm and translocate into the nucleus, they activate transcription of other genes by binding E-box elements in their promoters. Therefore, they are positive regulators. Some of those activated genes are three *Period* (*per1*, *per2*, *per3*), two *Cryptochrome* (*cry1* and *cry2*), Rev-Erba and ROR-A genes (Lee et al. 2001, Sato et al. 2004). After PER and CRY are translated they also form a heterodimer and make up the "negative limb" of the loop, since they further translocate into the nucleus and inhibit CLOCK:BMAL1 transcriptional function. This causes PER:CRY levels to decrease due to low transcriptional activity and degradation of the proteins, after which the feedback loop is completed (Buhr & Takahashi 2013). A second negative feedback loop is orphan nuclear receptors REV-ERBα & REV-ERBβ and retinoic acid-related orphan receptor RORα, which inhibit and activate Bmal1

transcription respectively (Lowrey & Takahashi 2004). Both proteins bind to retinoic acid related orphan receptor response elements (ROREs) in the Bmal1 promoter (Preitner et al. 2002, Ko & Takahashi 2006). Other proteins involved in circadian regulation are the ones that carry out posttranslational regulation on core clock genes, such as casein kinase 1 epsilon (CK1ε), which have overlapping functions (Etchegaray et al. 2009). CK1ε, first identified as tau, and CK1δ are involved in the phosphorylation, destabilization and further degradation of PER while it is in the cytoplasm, as well as in translocating the PER:CRY into the nucleus (Ralph & Menaker 1998, Camacho et al. 2001).

Glial communication

Astrocytes are one of the most abundant cells in the central nervous system (CNS), especially in the hypothalamus and SCN. They have distinct morphology, biochemical properties and functions. Astrocytes are usually found surrounding brain capillaries and neurons near sites of synaptic connections. At the synapse, astrocytes can provide nutrients like glucose or lactate and regulate levels of neurotransmitters to avoid excitotoxicity (Tsacopoulos & Magistretti 1996, Rothstein et al 1996). Astrocytes also promote CNS synaptogenesis, while Schawnn cells promote it in the peripheral nervous system (PNS), as well as maintaining or eliminating those synapses during development and regeneration (Ullian et al. 2004, Sanes & Lichtman 1999, Nägler et al. 2001). Astrocytes can also release transmitters that act upon synapses modulating neurotransmission and electrical communication (Parpura & Haydon 2000). Through gliotransmission, astrocytes are able to communicate with neurons and other astrocytes to contribute to modulation of brain physiology (Araque et al. 1999). Although astrocytes are unable to generate action

potentials, they can communicate with other astrocytes via gap junctions, gliotransmitters and "excitable" Ca^{2+} waves (Araque et al. 1999). Calcium wave propagation among astrocytes is mediated by inositol-4,5-triphosphate (IP_3) pathway and release of Ca^{2+} from internal stores (De Pitta et al. 2009). Astrocytes respond to neuronal activity, as well as gap junctional or purinergic signaling from other glial cells (Guthrie et al. 1999). After IP_3 diffuses through gap junctions or ATP is elevated, IP_3 increases its binding to receptors on the endoplasmic reticulum (ER) that release intracellular Ca^{2+} into the cytoplasm. As intracellular calcium increases, it travels to neighboring astrocytes through gap junctions and activates further IP_3 receptors and more participating glial cells. Furthermore, with an intracellular rise in calcium, astrocytes are able to release gliotransmitters, and communicate with pre- and postsynaptic neurons. Thus, astrocytes participate in the tripartite synapse.

SCN astrocytes produce Ca^{2+} oscillations that respond to different neurotransmitters like glutamate and serotonin, suggesting a role in modulation of neurotransmission and clock regulation (van den Pol et al 1992). Since astrocytes also posses the canonical mammalian molecular clock, they have rhythmic gene expression as independent oscillators (Prolo et al. 2005). ATP evokes a Ca^{2+} rise among astrocytes and may be involved in neuron-astrocyte communication in the SCN (Guthrie et al. 1999). It has been shown that cultured SCN astrocytes display 24-hour rhythms in extracellular accumulation of ATP (Womac et al. 2009) (*Fig. 1*). Although the exact mechanism of clock-controlled ATP release from astrocytes has not been fully defined, it is thought to not be through vesicular release (Marpegan et al. 2011). It is also known that the rhythms

6

in ATP accumulation levels and mitochondrial calcium are in phase, and that ER calcium
stores are not required for the rhythmic extracellular ATP accumulation (Burkeen et al.
2011).
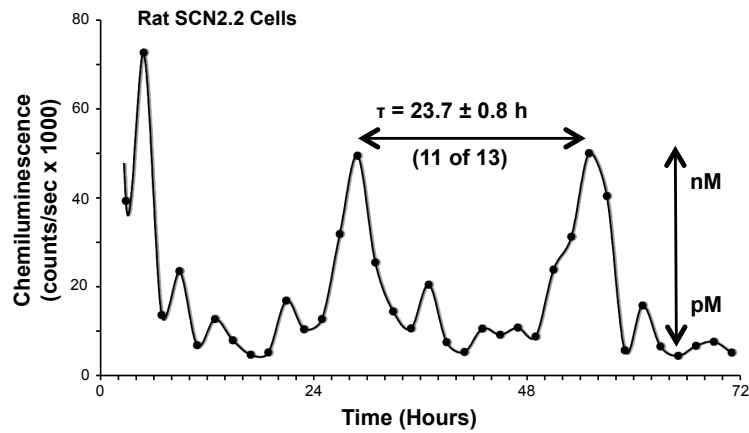
Figure 1. Circadian rhythm of extracellular ATP accumulation in rat
SCN2.2 cells. Rhythm continues for at least 3 cycles with a period (τ) of
23.7 h and a rhythm amplitude between 1 pM and 1 nM. (Adapted from
Womac et al. 2009).

Astrocytic activation

While ATP signaling among astrocytes is primarily known to be involved in intercellular calcium wave propagation, it is also involved in astrocyte-microglia communication. Microglia, the macrophages of the CNS, modulate and carry out diverse immune responses in the brain such as phagocytosis and release of cytokines. Both astrocytes and microglia can become reactive and enter a gliosis state that causes neuroinflammation. Although inflammation promotes beneficial processes like phagocytosis of debris and/or apoptotic cells and tissue repair processes, constant neuroinflammation causes a toxic environment for the brain. Neuroinflammation is commonly associated with neural trauma, neurodegenerative disorders and age-related neurological diseases (Steelman & Li 2014). Illnesses like Parkinson's disease (PD) and Alzheimer's disease (AD) are associated with increased numbers of reactive microglia and astrocytes, which play important roles in the development of neuroinflammation (Glass et al. 2010). Another common factor among these age-related diseases, besides enhanced neuroinflammation, is clock defects. *Bmal1* deletion in the SCN and other brain regions leads to upregulated glial fibrillary acidic protein (GFAP) expression in an age-dependent manner. This causes severe astrogliosis as well as accelerated aging and cognitive deficits (Musiek et al 2013, Duncan et al. 2013). In older mice, there is also increased sensitivity of astrocytes and microglia to cytokine exposure, highly marked in the SCN (Deng et al. 2010). The relationship between astrocyte activation and the biological clock suggest the existence of a homeostatic regulation of the brain with respect to purinergic signaling, since the P2X7

purinergic receptor of both brain inflammation and circadian ATP release rhythms is present in astrocytes.

Purinergic signaling

This project has attempted to discern if ATP signaling is involved in rhythmic oscillations of astrocytic activation. We have hypothesized that the purinoreceptor 2X7 (P2X7R) expressed in neurons, astrocytes and microglia, plays a key role in clock-controlled ATP accumulation rhythm (Jarvis & Khakh 2009). Previous data has suggested that the blockage of P2X7R with Brilliant Blue G (BBG) antagonist causes a decrease in the ATP accumulation rhythm in SCN2.2 cells, as well as dampening of PER2 expression rhythms on mPer2$^{\text{Luc}}$ cells (*Fig. 2A, 2B*).

A
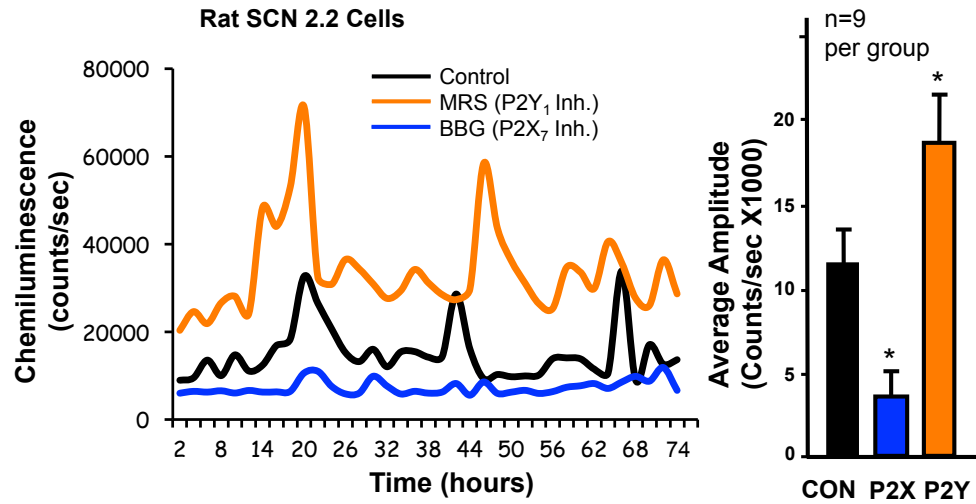
Figure 2. Effects of inhibition of purinergic signaling on extracellular ATP and expression of a Per2 reporter gene. **A)** Rhythms in extracellular ATP are inhibited with the application of P2X7 purinergic receptor inhibitor BBG, but facilitated by the P2Y1 receptor inhibitor MRS. **B)** The bioluminescence rhythm of Per2- luciferase reporter gene is dampened and its amplitude is significantly decreased with BBG disruption of ATP-P2X7R ligand-receptor interaction (Adapted from Womac et al. unpublished).
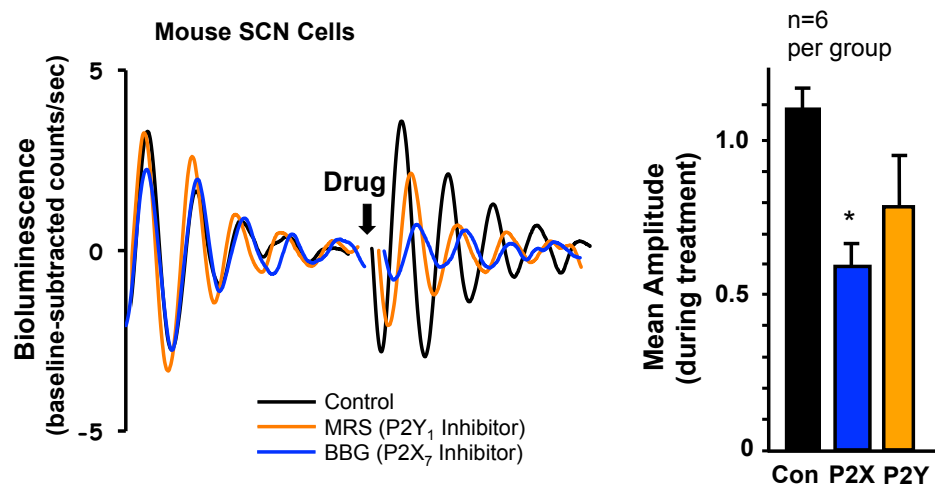
B

Figure 2 Continued

Nonetheless, research on P2X7R in glia has been mainly focused on its role in astrocytic activation by interleukin-1β (IL-1β) release especially from astrocyte-induced microglia *in vitro* and in association in the so called death complex with the hemichannel Pannexin 1 (PANX1) (Bianco et al. 2005, Jackson et al. 2014). ATP is released through anion-selective PANX1 channels activated by a cation-selective P2X7 receptor channel in astrocytes (Iglesias et al. 2008, Iglesias et al. 2009, Verkhratsky et al. 2012). P2X7R activation is known to trigger elevations in Ca^{2+} in neural cells and these increases in cytoplasmic calcium in turn activate PANX1 (Mitchell et al. 2008, Egan & Khakh 2004). Also, PANX1 auto-regulates its own ATP permeability through a negative feedback loop causing transient ATP release (Qiu & Dahl 2009) (*Fig. 3*). In addition, the astrocytic activation marker, GFAP, is rhythmic in SCN astrocytes and has been linked to morphological changes in astrocytes (La vialle & Serviere 1993).
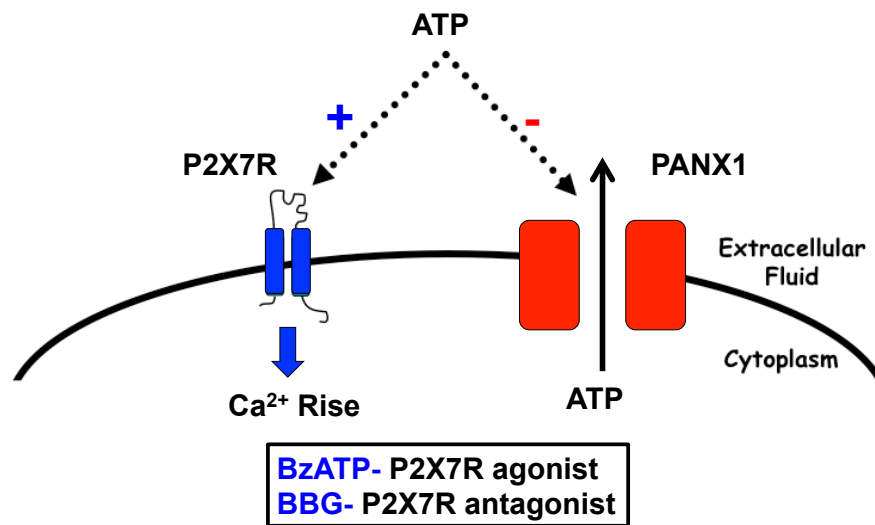
Figure 3. Schema suggested by the use of P2X7R agonist BzATP and antagonist BBG. ATP binding to P2X7R triggers calcium elevations in the cytoplasm of astrocytes, while PANX1 is an established channel for the release of ATP, particularly following P2X7R activation. BzATP and BBG drugs act by activating and inhibiting P2X7R, respectively.

Astrocyte activation in the SCN, as well as locomotor activity, can be phase-shifted with lipopolysaccharide (LPS) application, a bacterial endotoxin used to elicit a strong immune response (Marpegan et al. 2005). LPS causes changes in sleep/wake patterns in which susceptibility to the endotoxin depends on time of application (Marpegan et al. 2005). Also, the expression of transcription factor nuclear factor κB (NF-κB) is induced by LPS in SCN astrocytes (Leone et al 2006). Other studies have showed that the astrocytic activation marker, cytokine tumor necrosis factor α (TNF-α), alters phase and amplitude of PER2 rhythms in SCN astrocytes and PER1 rhythms in NIH/3T3 fibroblast cell line (Duhart et al. 2010). Together, these results suggest a role for SCN astrocytes in the circadian modulation of immune responses and brain cytokine levels. It is possible that ATP signaling through the P2X7R modulates clock controlled immune mechanisms. ATP may also be an important signaling molecule involved in circadian timing and synchronization between astrocyte-astrocyte or astrocyte-neuron communication in the SCN and other brain regions. Thus, I have tested the hypothesis that purinergic signaling, through P2X7R, mediates circadian astrocyte physiology, specifically rhythmic and synchronous activation of astrocyte populations.

Experiments in this project have used *in vitro* models of immortalized cell lines, rat SCN2.2 and mouse *Per2^{Luc}* SCN, which contain both neuronal and glial cell types (Earnest et al. 1999, Allen et al. 2001, Farnell et al. 2011). These SCN cell lines were created, characterized and graciously provided by Dr. David Earnest. SCN cell lines conserve endogenous circadian properties and provide a good model system to study mechanisms of clock-controlled physiology *in vitro,* such as rhythmic ATP accumulation

and astrocytic activation. The immortalized cell line with the mPERIOD2::LUCIFERASE fusion protein has been used as a real-time reporter of circadian rhythms as analyzed with the LumiCycle luminometer. I have performed these experiments to further understand brain inflammation and its circadian regulation through astrocytic signaling. I hypothesized that P2X7R mediates the rhythmic ATP accumulation rhythm as well as rhythmic and synchronous activation of SCN astrocytes. First, I determined that these rhythms in extracellular ATP accumulation are present in mouse SCN cells. Then I investigated the role of P2X7R in ATP rhythmic release by studying its activation by ATP and BzATP [2'(3')-*O*-(4-Benzoylbenzoyl) adenosine-5'-triphosphate], a specific P2X7R agonist (El-Moatassim & Dubyak 1992, Nuttle et al. 1993, Iglesias et al. 2008). Finally, I studied rhythmicity in astrocyte activation using GFAP expression and IL-1β signaling by P2X7R activation with BzATP.

MATERIALS AND METHODS

Cell culture conditions

Rat SCN2.2 cultures were grown in 60mm dishes (Corning, Corning, NY, USA) using Minimum Essential Medium (MEM; Invitrogen, Carlsbad, CA and MEM; Caisson, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS), glucose (3000μg/ml), L-glutamine (292μg/ml), and 1% penicillin-streptomycin-neomycin antibiotics mixture (PSN; Gibco/Invitrogen, Grand Island, NY, USA) (Earnest et al. 1999).   The culture dishes were maintained at 37°C and 5% CO_2, and were split every 2 days. The media was replaced at 48hr intervals. For all experiments SCN2.2 cells were derived from a single passage (Farnell et al. 2011). Cultures of SCN mPer2^{Luc} cells were grown under the same media conditions, temperature and CO_2 level as SCN2.2 cells. During cell propagation, the cultures were split every 3 days, and the media changed at 72-hour intervals. For all experiments mPer2^{Luc} cells were derived from a single passage and were propagated as described above. SCN mPer2^{Luc} cells grown for immunocytochemistry were plated onto 2-well Nunc glass-coverslip chamber slides (Lab-Tek, Thermo Fisher Scientific, Rochester, NY, USA) previously coated with Poly-D-lysine hydrobromide and laminin (Sigma Aldrich, St. Louis, MO, USA) and then allowed to grow to confluence (2 days). SCN mPer2^{Luc} cells grown for luminometry analysis were seeded onto 35mm dishes (Corning, Corning, NY, USA) and allowed to grow to confluence (2 days).

Chemiluminescence assay for analysis of ATP levels in culture media

To analyze ATP levels from SCN2.2 and SCN mPer2^{Luc} cells, chemiluminescence assays of cell-free media were performed by incubating aliquots (97µl) of media samples with 1 µl of luciferase (3 mg/ml) and 2 µl of luciferin (3 mg/ml) per well in black 96-well plates (Thermo, Milford, MA, USA). Internal controls consisting of unconditioned medium without ATP standard, luciferase, or luciferin were included on all analyzed plates. ATP-dependent chemiluminescence produced by medium samples was quantified using a Packard TopCount scintillation counter (Meriden, CT, USA).

Serum reduction protocol for chemiluminescence

 SCN2.2 and SCN mPer2^{Luc} cells used for analysis of ATP levels through chemiluminescence assays were subjected to a serum reduction (SR) procedure, since the chemiluminescence assay is dramatically disrupted by the presence of serum. After cells were previously grown and expanded from one single passage, all cell cultures were then plated onto 60mm dishes with 10% serum. After 24-hour period, the serum concentration was reduced to 5%. Another 24 hours later the media was replaced with serum-free neurobasal medium (supplemented with glucose, L-glutamine, and 1X B-27 serum- free supplement; Invitrogen). Finally, 2 hours later, the experimental analysis was initiated by collecting and replacing medium (500ul) from all culture dishes at 2- hour intervals for 72 hours. Samples were frozen and stored at -20°C and later analyzed for ATP accumulation using the chemiluminescence assay previously described. It is noteworthy that every step of serum reduction has to be done in exactly 24 hour intervals to keep all cells in synchrony

without serum shock or forskolin (FSK).

Acute effects of BzATP on extracellular ATP accumulation rhythms

To observe the immediate effects of BzATP (Tocris, Minneapolis, MN, USA) on ATP levels, cultures were allowed to reach confluence. After SR was performed, cultures were subjected to ATP (10nM), BzATP (10nM or 100μM) application, as well as dishes without cells and only neurobasal medium. The first medium sample (500μl) was collected before the drug application, then 5 min after of BzATP/ATP application, then 30 min, and finally 60 min later without replacing the medium. Samples were frozen and stored at -20°C and later analyzed for ATP accumulation using the chemiluminescence assay.

A dose response analysis was performed in order to determine the effectiveness of BzATP as an ATP analog in the luciferase/luciferin reaction by providing a direct substrate for chemiluminescence assay. Using different concentrations of ATP (10nM, 100nM, 1μM, 10μM, 100μM & 1mM) and BzATP (10nM, 100nM, 1μM, 10μM, 100μM & 1mM) in addition to luciferase/luciferin, a chemiluminescence assay was performed to compare the light intensity produced by ATP and BzATP. Note that only some aspects of this larger dose response analysis are presented in the results section of this thesis.

Effects of exogenous ATP application on extracellular ATP accumulation rhythms

ATP accumulation rhythms in SCN2.2 cells were analyzed during a 72-hour time course. After cells cultures were expanded, media samples were collected at intervals of 2 hours. At 6 hours and 16 hours, independent dishes received exogenous ATP (10nM), elevating

the ATP concentration of the endogenous rhythm. Samples were frozen and stored at -20°C and later analyzed for ATP accumulation using the chemiluminescence assay previously described.

Effects of BzATP application on extracellular ATP accumulation rhythms

The ATP accumulation rhythm in mPer2^{Luc} cells was analyzed during 72-hour time course. After cells were grown and underwent SR procedure, samples were collected at intervals of 2 hours. At 6 hours, independent dishes received BzATP (100μM). Samples were frozen and stored at -20°C and later analyzed for ATP accumulation using the chemiluminescence assay previously described.

Immunocytochemistry

For immunocytochemical analyses of P2X7 receptor and GFAP expression, SCN mPer2^{Luc} cells were seeded onto glass-coverslip chamber slides and allowed to grow to confluence (1-2 days). Individual cultures were fixed for 15 min with 4% paraformaldehyde in with phosphate buffered saline (PBS; Invitrogen) at 4-hour intervals for 48 hours. Cells were washed with PBS, and permeabilized with PBS containing 1% Tween-20 for 10 min. Cells were then incubated for 30 min with blocking solution containing PBS, 1% Tween and 10% fetal goat serum. Dishes were incubated 24 hours with primary antibodies (rabbit anti-P2X7R or rabbit anti-GFAP) both diluted 1:100 in blocking solution. Cells were washed and then incubated for 24 hours with goat anti-rabbit Alexa Fluor 488 IgG (Invitrogen) diluted 1:500 in blocking solution. Images were captured with a Olympus fluorescence microscope using 30x magnification with background subtraction. Then images were analyzed using grey level intensity

measurements with Simple PCI 4.0 imaging software (Compix, Cranberry Township, PA).

ELISA

For quantification of cytokine IL-1β, SCN mPer2^{Luc} cells were grown in 60mm dishes and subjected to the SR protocol. During the 24 hours following SR procedure, samples of medium were collected at intervals of 6 hours. Samples were stored at -20°C and later analyzed using a mouse IL-1β/ IL-1F2 immunoassay. The assay was performed according to manufacturer instructions (Quantikine ELISA, R&D Systems, Minneapolis, MN) and analyzed at 540nm on a Bio-Rad (Hercules, CA) Bio-Plex system employing Luminex technology.

Statistics

Raw chemiluminescence data (photons/sec) for SCN2.2 experiments were normalized relative to the maximum for each culture, which was arbitrarily set at 100 %. For raw chemiluminescence data in mPer2^{Luc} cells the data was not normalized but both were 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, with significant periods ranging from 22 to 26 hours in the case of experiments that used SCN2.2 cells . In most cases paired and pooled t-tests were performed to determine if changes in ATP levels were significantly different between peak and trough times. For

treatment groups one-way ANOVA was performed of fluorescence intensity and cytokine levels to find differences across time-points. Two-way ANOVA was used when testing differences between treatment groups across time. Tukey's HSD post hoc test was used when significant differences were found. For highest and lowest levels of fluorescence intensity and cytokine levels, a t-test was performed. All statistical analyses were done using R programming language and software. The value was set at 0.05 for significant differences for all statistical analyses.

RESULTS

Specific activation of P2X7R increases extracellular ATP levels in *mPer2^{Luc}SCN* cells

Previous studies performed in our lab demonstrated that astrocytes possess an endogenous rhythm in extracellular ATP accumulation in rat SCN cells *in vitro* and *in vivo*, as well as in mouse cortical glial cells *in vitro* (Womac et al. 2009). In the present study, I investigated the effect of purinergic receptor activation on the accumulation of extracellular ATP in a mouse SCN cell line (*mPer2^{Luc}SCN*). I used either ATP (10nM) or BzATP (10nM and 100μM) to activate all responsive purinergic signaling pathways or specifically the P2X7 purinergic receptor expressed in these mouse cells, respectively (Iglesias et al. 2008, Iglesias et al. 2009, Jackson et al. 2014). I observed that acute exposure of the mSCN cells to 100 μM BzATP produced a chemiluminescence signal (photons of light per second) orders of magnitude greater than either 10 nM ATP or 10 nM BzATP (*Fig. 4*). These results indicate that BzATP at the higher pharmacological concentration (100 μM) induces an increase in ATP release from SCN astrocytes. Furthermore, physiological concentrations of ATP (10 nM), or its analog, are not significantly different in their effects on the chemiluminescence assay. It is also important for my subsequent studies to note that 100 μM BzATP induced an increase in extracellular ATP, as reported by the chemiluminescence assay, that was detectable for greater than 60 min, although the intensity of the signal had significantly declined.

ATP 10nM
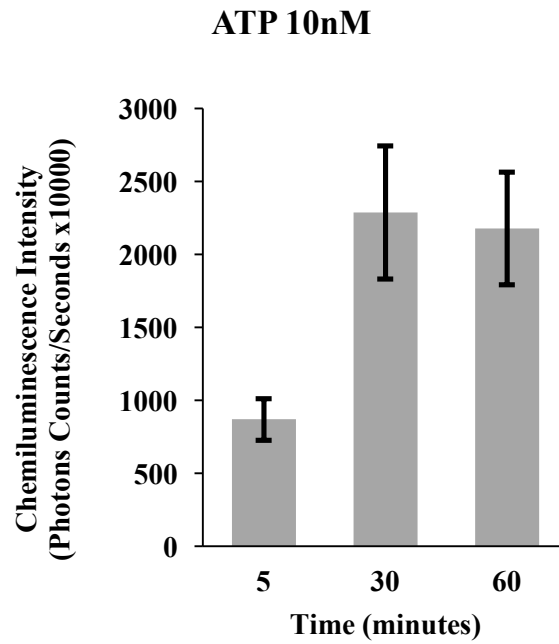
Figure 4. Acute effects of ATP and BzATP application on ATP dependent chemiluminescence using mPer2^{Luc} cells. After 5 and 30min, the average chemiluminescence produced after BzATP (100μM) is significantly higher than the one produced by ATP (10nM) (*p<0.05, n=4). Note the difference in scales. Error bars represent 95% confidence intervals.
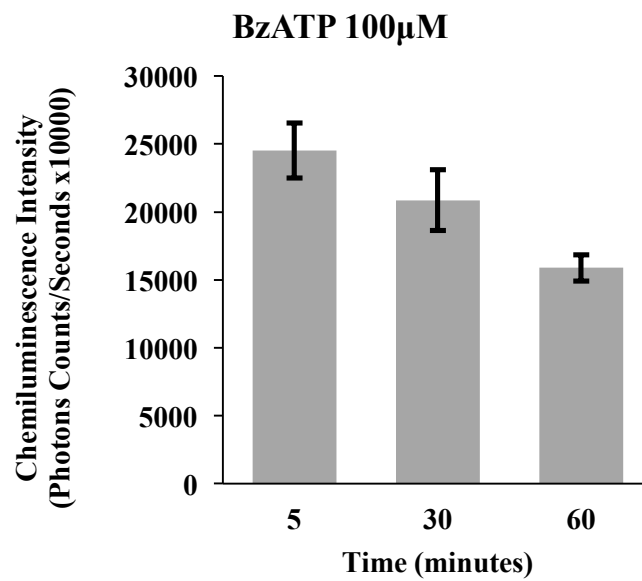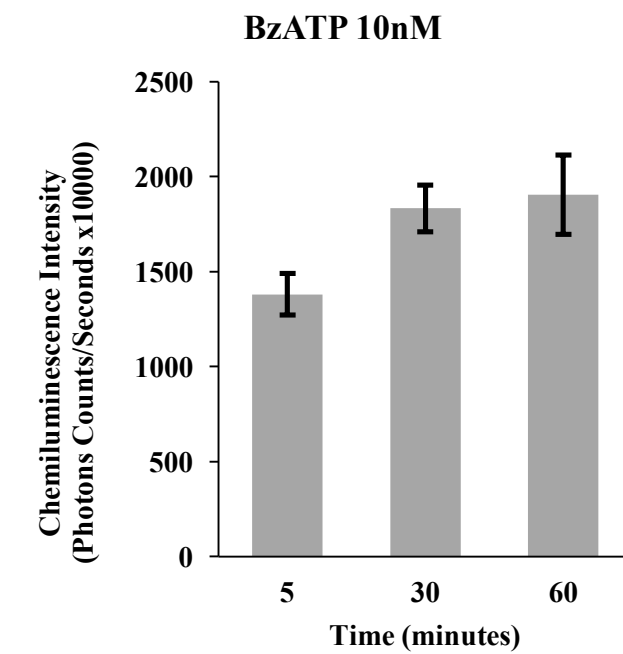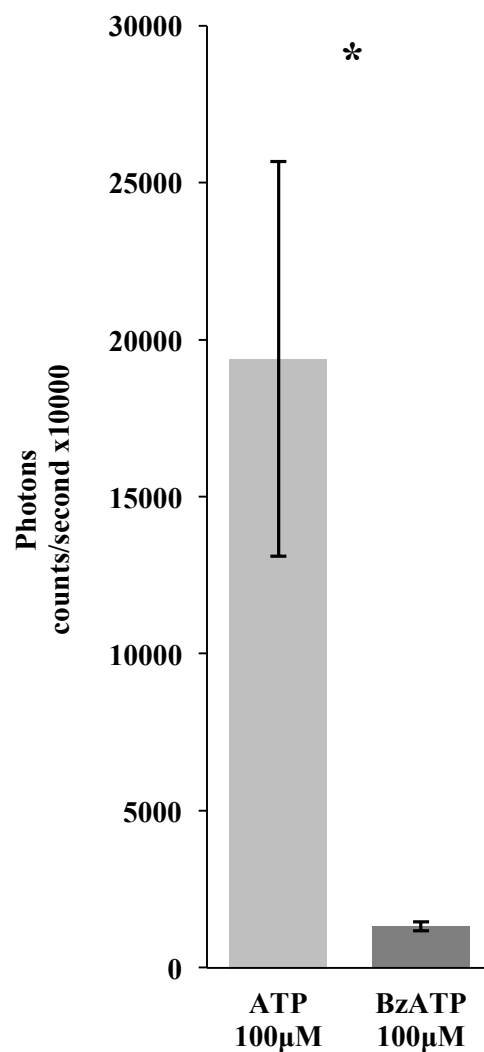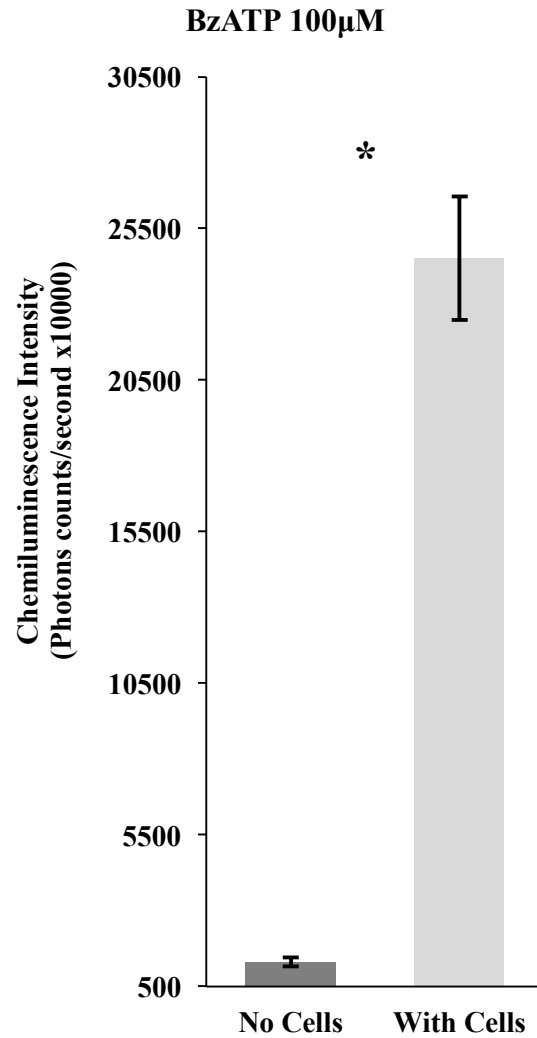
BzATP 10nM

BzATP 100μM

Figure 4 Continued

Since chemiluminescence assays have been used to determine extracellular ATP accumulation in cell culture medium, I performed experiments to determine if the light produced in these assays was a result of ATP release from the SCN cells, or a product of direct activation of the chemiluminescence reaction by the exogenous ATP or BzATP molecules. When comparing ATP and BzATP as direct substrates for the luciferase/luciferin reaction, the average chemiluminescence produced by ATP (100 µM) was 93% greater than the light generated by BzATP (100 µM; *Fig.5*), demonstrating that in the absence of medium from living cells BzATP is far less effective than ATP is driving the luciferin/luciferase reaction. Additionally, chemiluminescence assays conducted on medium sampled from culture dishes with and without SCN cells demonstrated that application of BzATP to those dishes only produced significant ATP-dependent signal when cells were present (*Fig. 6)*. Thus, significant elevations in the extracellular accumulation of ATP in *mPer2^{Luc}SCN* cell cultures, following BzATP application, is due to P2X7R activation and not due to direct BzATP-dependent activation of the chemiluminescence assay.

Figure 5. Direct chemiluminescence responses to ATP and BzATP. The capacity of both ATP and BzATP (100 µM) to drive the luciferase/luciferin chemiluminescence reaction was compared. ATP produced a 93% greater luminescence signal than BzATP (*$p < 0.05$, n=4). Note the different scales between the ATP and BzATP histograms. Error bars represent 95% confidence intervals.

BzATP 100μM

Figure 6. Chemiluminescence after BzATP (100μM) application to dishes with and without mPer2^{Luc} SCN cells. The average chemiluminescence intensity produced after BzATP (100μM) application in the presence of cells was significantly greater than the chemiluminescence produced by BzATP (100μM) alone (*p<0.05, n=4). Error bars represent 95% confidence intervals.

Exogenous ATP alters the amplitude of astrocytic ATP release rhythms

Rat SCN2.2 cells possess rhythms in extracellular ATP accumulation, which likely require the synchronous release from many astrocytes (Womac et al., 2009). Furthermore, antagonism of the P2X7 receptor dampens both ATP release and PER2 gene expression rhythms in mammalian SCN cell cultures (Womac et al., unpublished). Since my previous experiments demonstrated that ATP and its analog BzATP evoke robust elevations in extracellular ATP accumulation, I hypothesize that ATP, signaling through the P2X7 receptor, enhances the ensemble release rhythm of SCN astrocytes. To test this idea, I applied exogenous ATP (10nM) at two specific times that corresponded to the predicted peak and trough of the extracellular ATP rhythm in SCN2.2 cell cultures. ATP elicited opposing effects on the subsequent cycles of the endogenous ATP rhythm depending on the time of exposure.

At the peak (6hr) timepoint, application of ATP in 10 out of 12 cell cultures exhibited high amplitude rhythmicity in extracellular ATP accumulation in medium samples, with peaks occurring at approximately 24-hour intervals. These ATP release rhythms persisted for at least 3 cycles and, based on Fourier transform analyses, the mean (±SEM) period (τ) was 24.6 ± 0.2 hours (*Fig. 7A*), calculated from the 10 cultures with significant rhythmicity. In contrast to cultures treated with exogenous ATP, only 6 out of 12 naïve control cell cultures were rhythmic and had a mean (±SEM) period (τ) of 22.7 ± 0.3 h, which was significantly shorter than culture dishes exposed to exogenous ATP at peak (*Fig. 7C and 8A*). Furthermore, all 12 SCN2.2 cell cultures exposed to ATP (10 nM) application at the trough (16hr) timepoint were arrhythmic (*Fig. 7C*). With respect to rhythm amplitude, the mean extracellular ATP at the first cycle after ATP application (peak to trough comparison was significantly different in the peak-treated, but not trough treated cultures (*Fig. 8B)*.

Figure 7. Effects of exogenous ATP application on SCN2.2 cell culture ATP accumulation rhythms. ATP (10 nM) was applied at peak and trough levels of the endogenous extracellular ATP rhythm. **A)** Cell cultures were exposed to ATP at the peak (6hr) timepoint of the rhythm, when extracellular levels were predicted to be greatest (n=12). This time series represents the averaged and normalized ATP chemiluminescence for all cultures. **B)** ATP-dependent chemiluminescence time series in control cell cultures that received neurobasal medium at either 6h or 16h (vehicle; 500 μl) as treatment (n=12). **C)** ATP-dependent chemiluminescence time series in cell cultures exposed to ATP at the trough (16hr) timepoint, when ATP levels of the rhythm were at their predicted low (n=12). Dashed lines represent projected ATP peaks with a 24h rhythmicity.
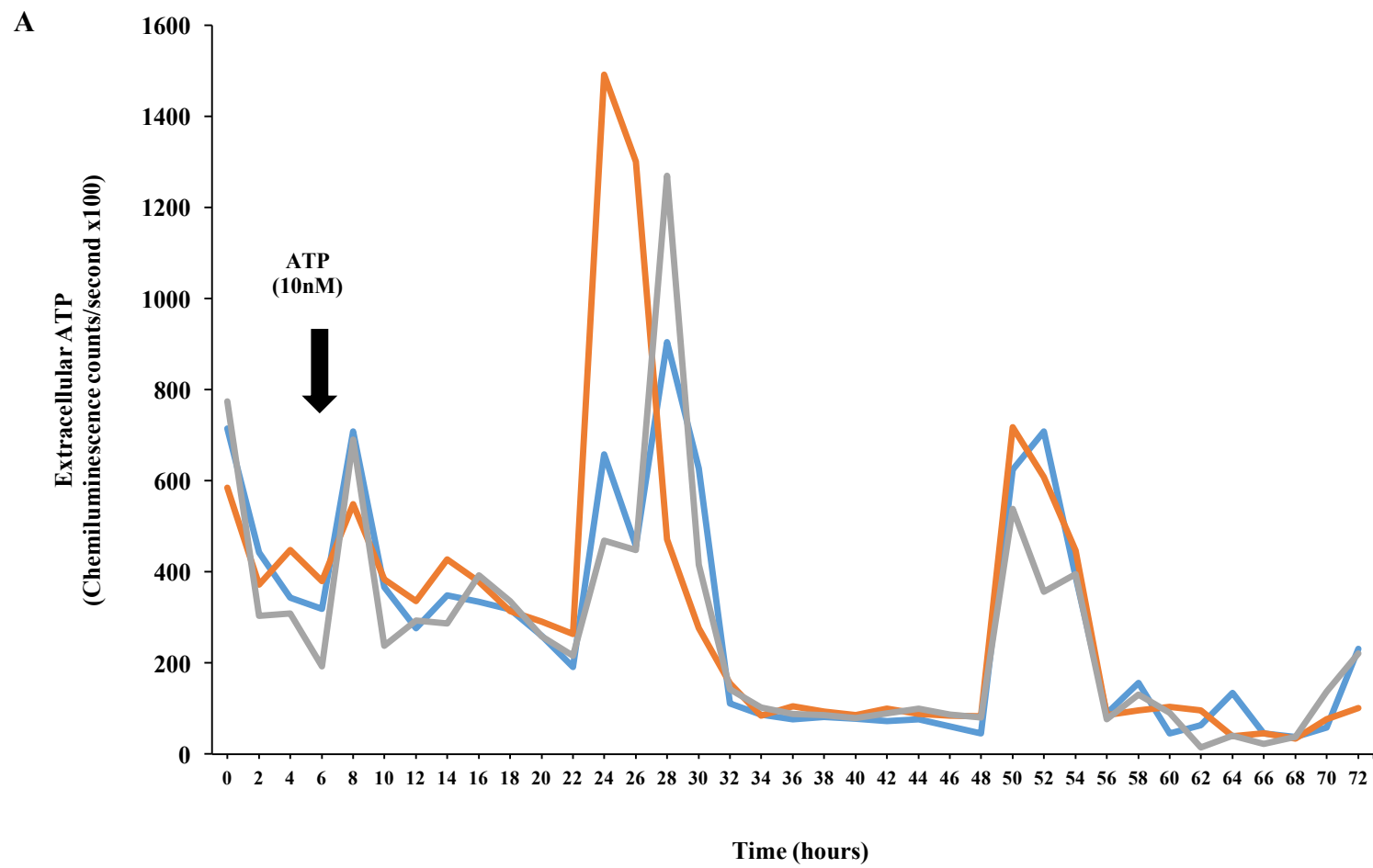
A

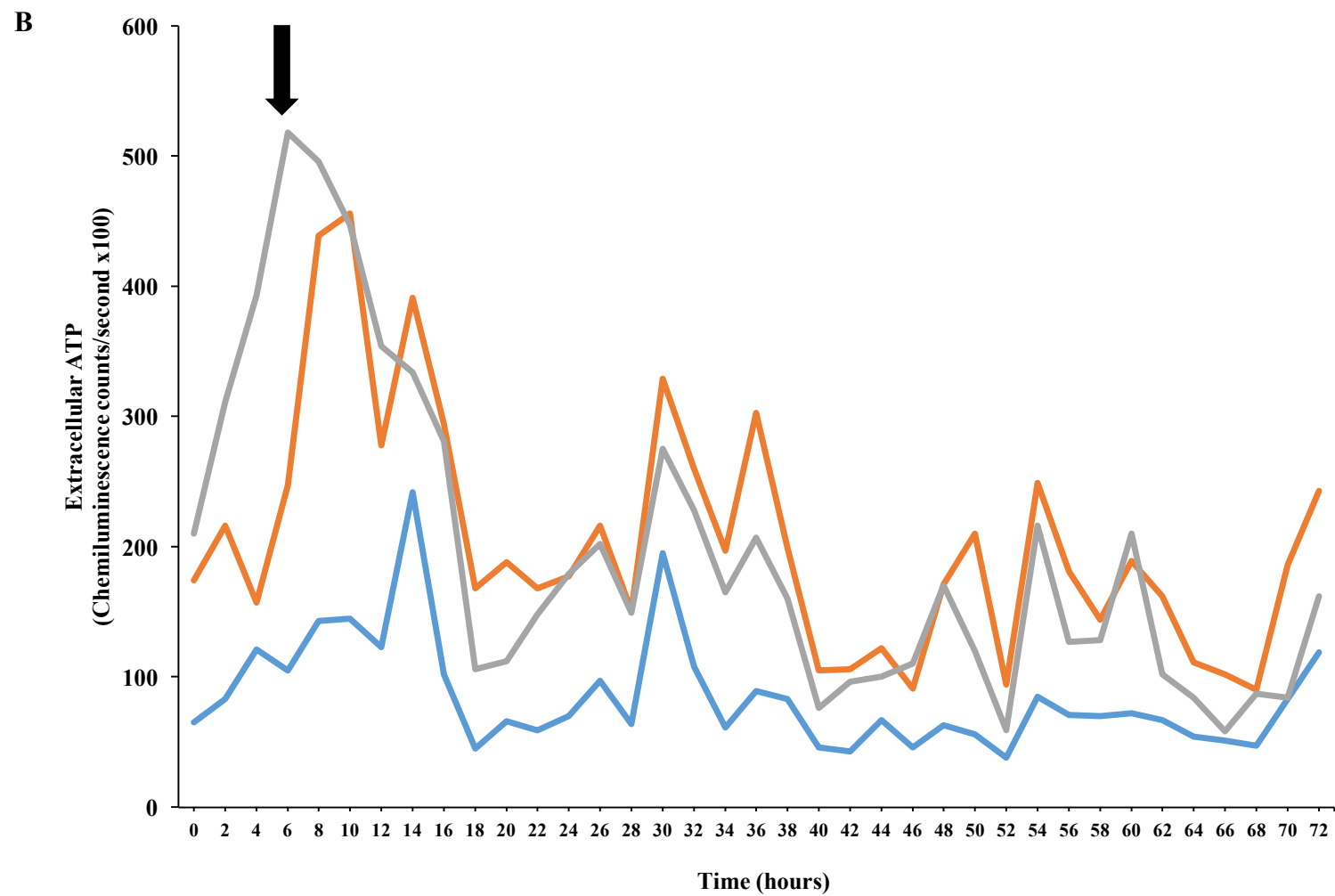Figure 7 Continued

31

B

Figure 7 Continued
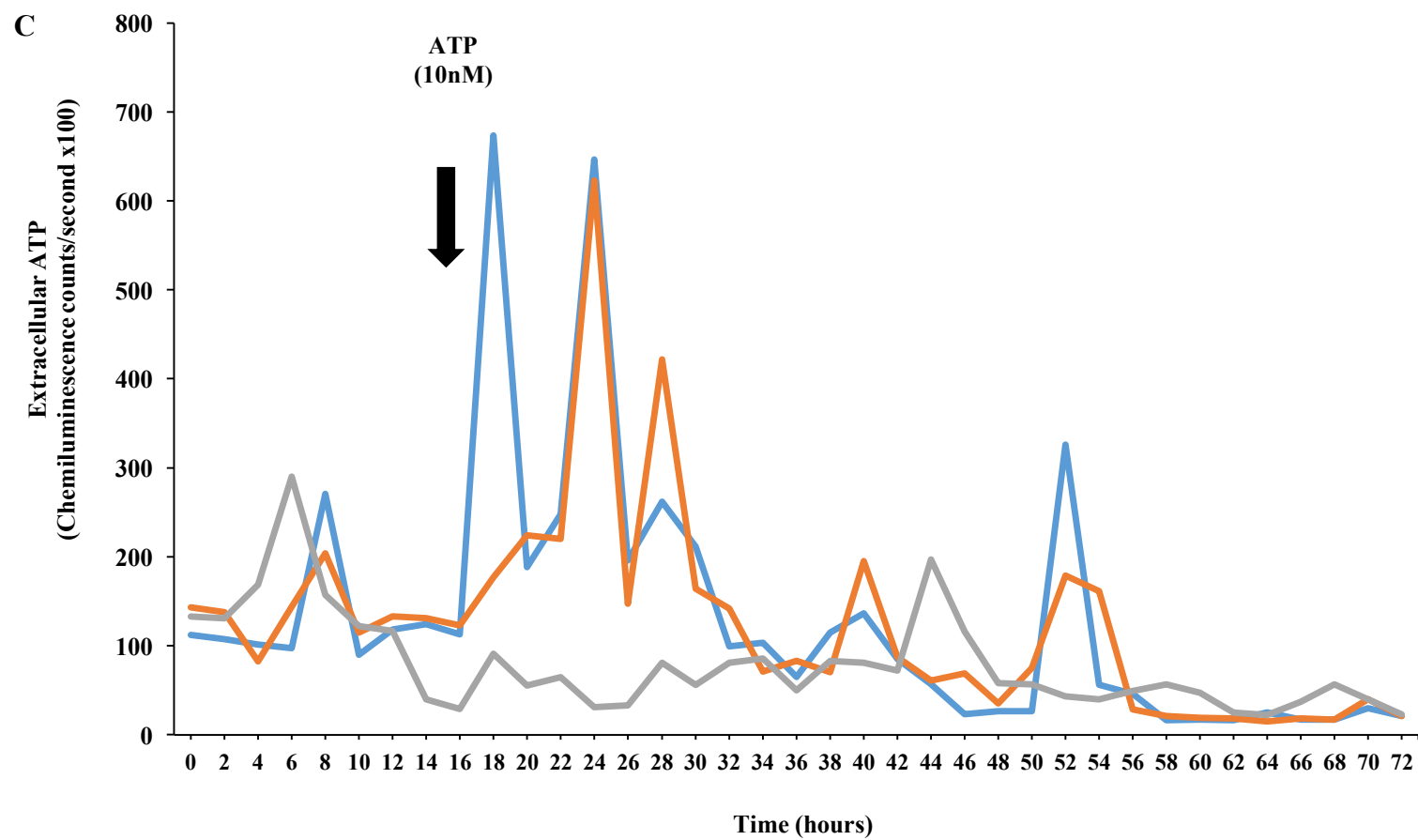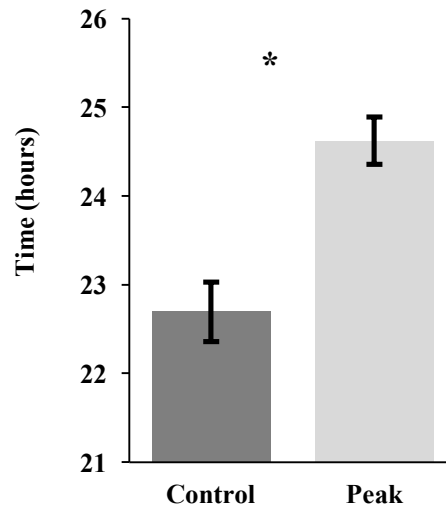
Figure 7 Continued

A   **Mean Period of Peak and Control Cell**

Figure 8. Circadian properties of the extracellular ATP accumulation rhythms. A) Comparison of mean (±SEM) period (τ) of cell cultures exposed to ATP at the peak of the ATP release rhythm (n=10) and control cell cultures (n=6). Analyses of the individual cultures revealed that the average rhythm frequency with power above critical limits in a Lomb-Scargle Fournier transform was 0.04065, indicative of a circadian rhythmicity with a period of 24.6h. The period in the 10 ATP-treated cultures was significantly longer than that observed in the 6 rhythmic control cultures (*p<0.05). **B)** Comparison of the mean ATP-dependent chemiluminescence in medium from peak and trough timepoints of cells at the first cycle following treatment with exogenous ATP (10 nM) at peak levels. Levels of extracellular ATP were significantly different (n=12; *p<0.05) between first cycle peak and trough. Error bars represent 95% confidence intervals.

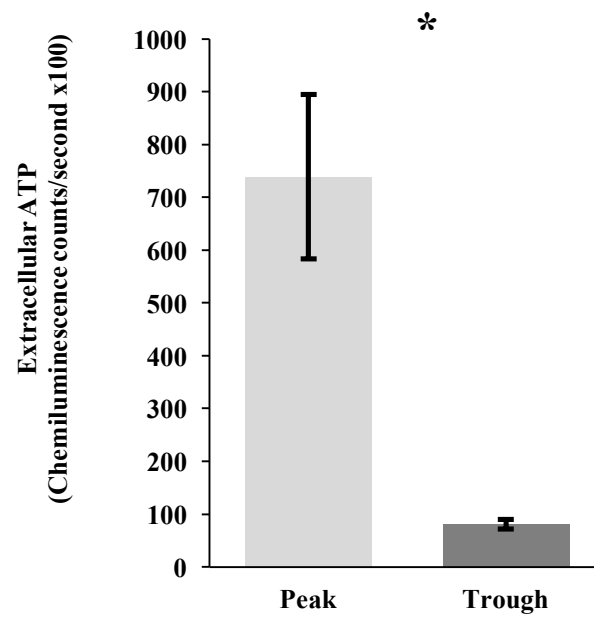**B**   **Peak and Trough in SCN2.2 cells that received ATP at Peak levels**

Figure 8 Continued

Since BzATP, a specific agonist of the P2X7R, induced a pronounced increase in ATP release from mouse mouse *mPer2^{Luc}SCN* cells, I investigate whether activation of the P2X7 receptor alone by this agonist could enhance ATP release rhythms in the mouse SCN cell line. Cultures exposed to BzATP (100uM) regularly possessed a peak in ATP release 24-28h following the exogenous treatment (*Fig. 9A).* Based on Fourier transform analyses, none of the 6 mSCN cell cultures were rhythmic. Still, most cultures possessed a frequency that was near circadian in rhythmicity, although not above significant critical limits, with a mean estimated period of 26.2 ± 0.7 h). Control mSCN cell cultures that received only vehicle treatment were not rhythmic either according to Fourier transform (*Fig. 9B*), nor did they exhibit a high amplitude peak in ATP release near the 24-28h timepoints. Thus, BzATP likely induced a synchronous release of ATP with a time delay of approximately 24h in mouse SCN astrocytes.

Figure 9. Effects of BzATP application on mPer2^{Luc} SCN cells. **A)** Cell cultures receiving BzATP (100 μM) treatment at the 6h time had a robust peak in ATP-dependent chemiluminescence at approximately 24h following the P2X7 agonist exposure (n=6). **B)** Control cell cultures exposed to only the neurobasal medium vehicle (500 μl) at the same time-point had no rise in ATP chemiluminescence at 24h following treatment (n=6).
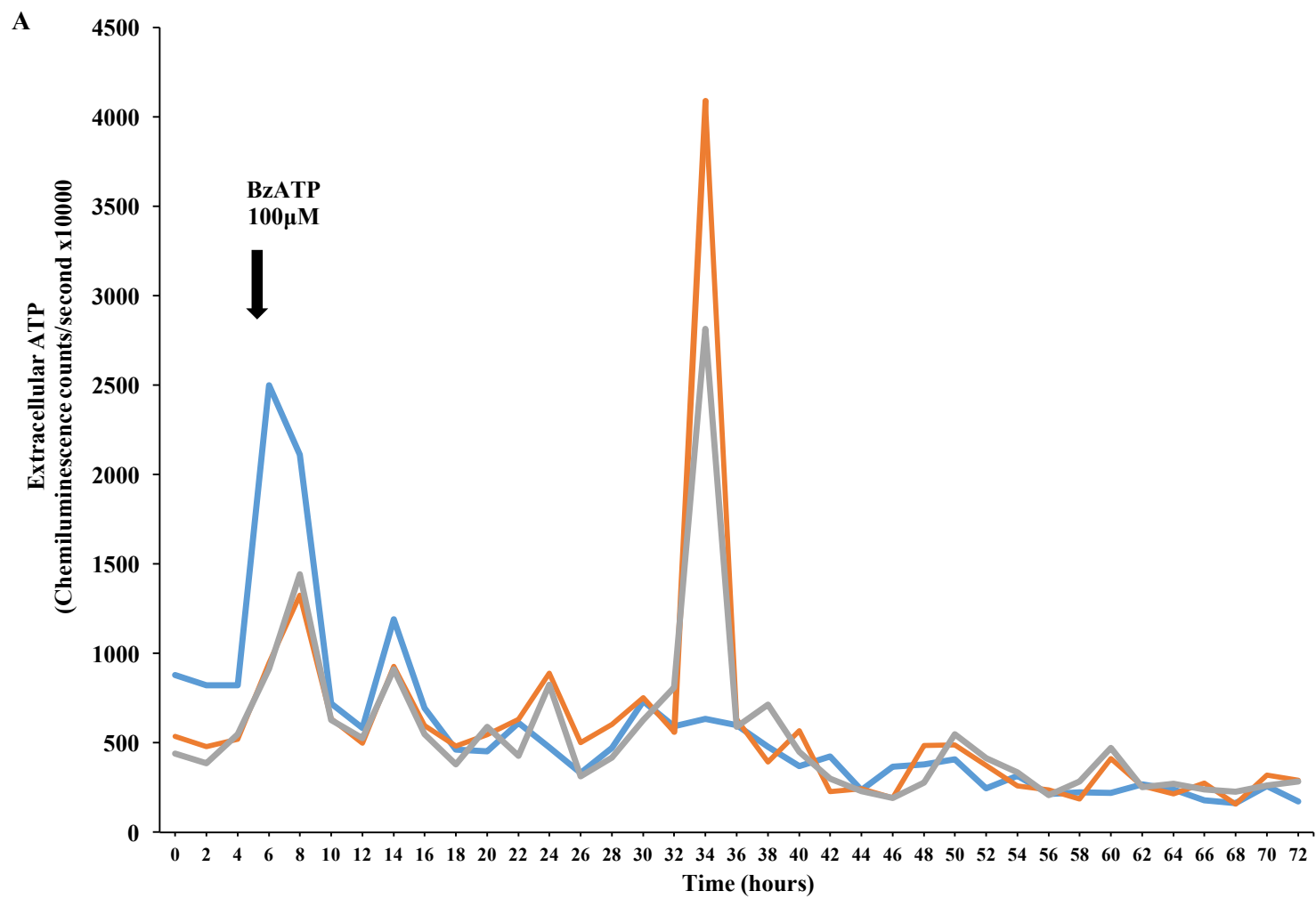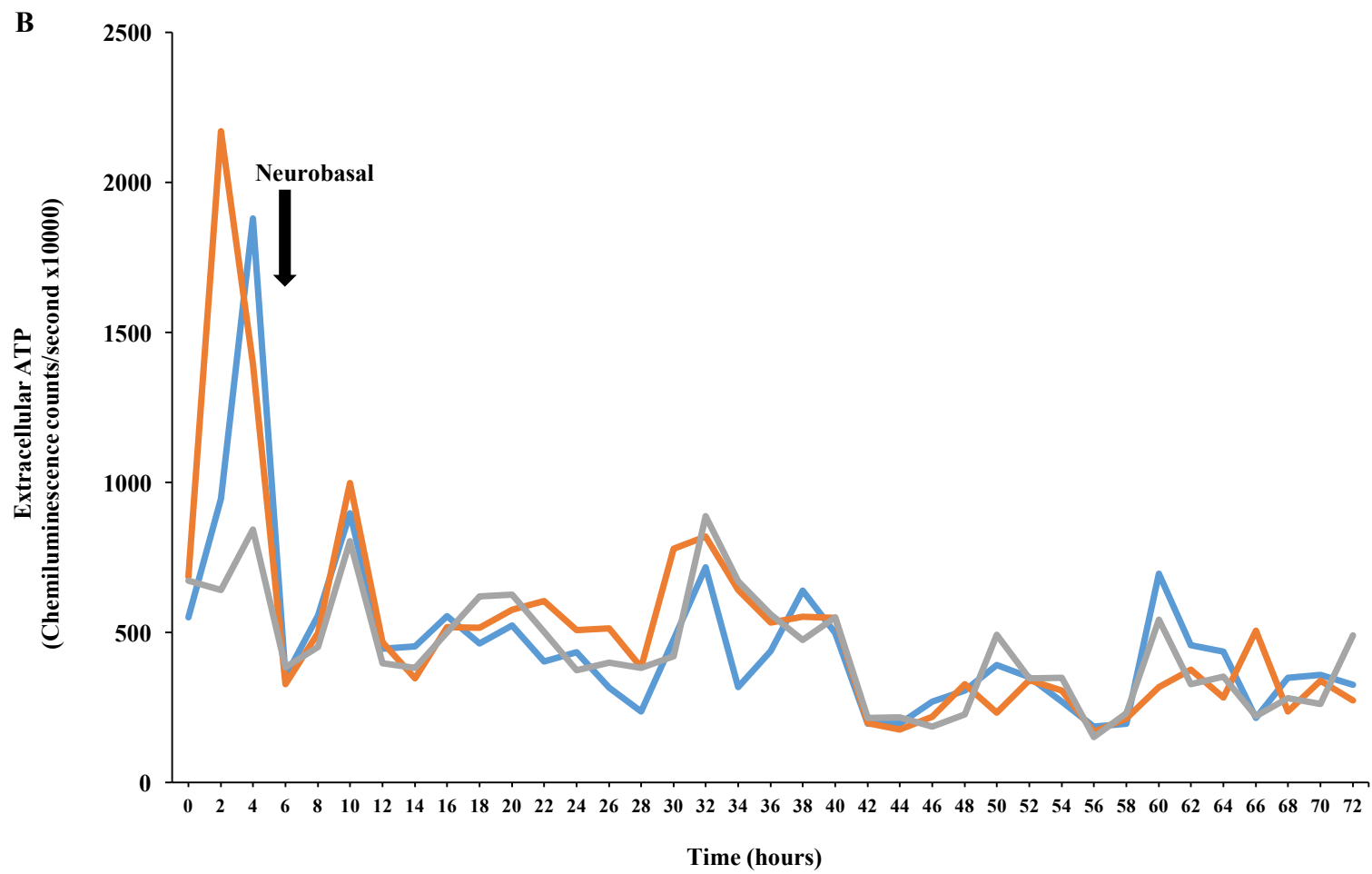
Figure 9 Continued

Figure 9 Continued

P2X7R activation potentiates amplitude of clock gene reporter rhythms

Since P2X7R antagonists disrupt clock gene expression rhythms in the mPer2^{Luc} SCN reporter cell line, I investigated whether a pharmacological dose of the P2X7R agonist, BzATP, would also alter clock gene expression rhythms. BzATP (100 µM), whether applied to mSCN cells at the peak of the endogenous ATP release rhythms significantly shortened the period of the PER2::LUC rhythm (21.9 ± 0.3; mean ± SD; n=4) by almost one hour, as compared to control (vehicle-treated) rhythm period of 22.5 ± 0.3 (n=4; p<0.05). Treatment with BzATP at the trough of the ATP rhythm has no significant impact on gene expression periodicity.

Interestingly, BzATP (100 µM) caused a marked increase in the amplitude of the PER2::LUC rhythm when applied at times corresponding to either peak or tough of the ATP release rhythm. This potentiation was likely due to activation of the P2X7R-mediated signaling pathway, and not direct enhancement of the luciferase reporter, since parallel cultures treated with ATP (10 nM) at a dose that drives chemiluminescence to a similar extent as the higher analog dosage, had no effect on the PER2::LUC rhythm (*Fig. 10A*). The increase in PER2::LUC rhythm amplitude was four-fold when BzATP was applied at the ATP release peak, compared to the ATP-treated cultures (p<0.05; *Fig. 10B*). A smaller, but also significant, increase in rhythm amplitude was induced by the P2X7R agonist when applied at the ATP release trough (p<0.05; *Fig. 10B*). Thus, activation of the P2X7R with BzATP causes both an increase in amplitude and a decrease in the period of PER2::LUC rhythms in mouse SCN astrocytes in culture.
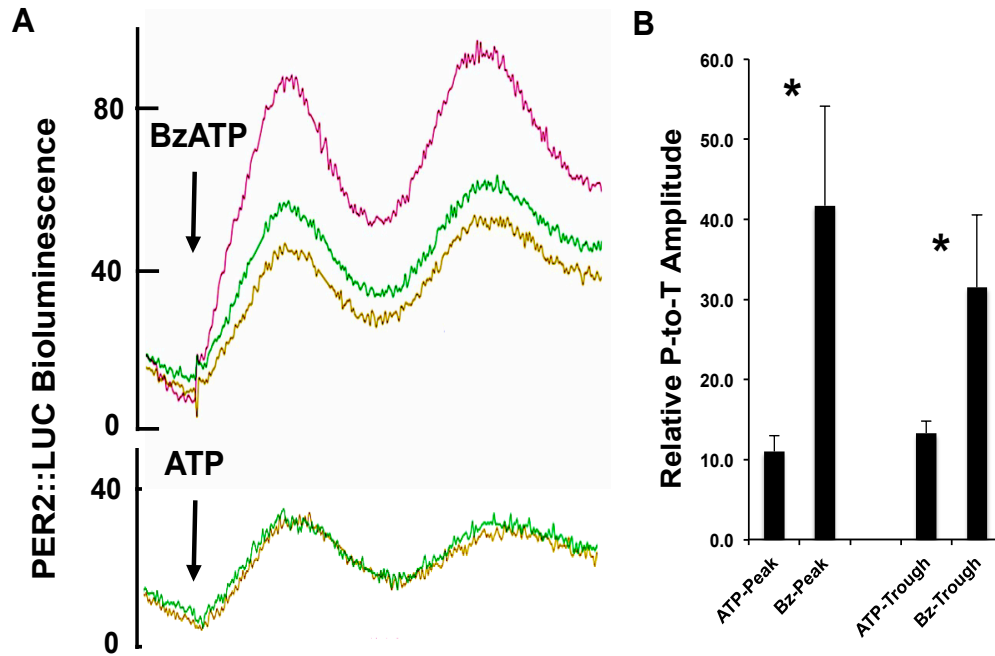
Figure 10. P2X7 receptor activation potentiates PER2::LUC rhythms in mPer2^{Luc} SCN cells. **A)** Bioluminescence data from mouse PER2::LUC SCN reporter cells show oscillations in clock gene expression. Application of 100 μM BzATP at the peak of the endogenous ATP release rhythm causes an increase in rhythm amplitude (3 representative cultures in top records). Treatment with 10 nM ATP had no effect on rhythm amplitude (2 representative bottom records). **B)** Comparison of mean bioluminescence amplitudes demonstrates that BzATP caused a significant increase at both peak and trough time of exposure, compared to ATP-treated cells (*$p < 0.05$; n=4). Error bars represent standard deviation.

P2X7R expression is rhythmic in mPer2^{Luc} cells

The rhythmic oscillation in ATP release from SCN cell lines, the modification of this rhythm's circadian properties by exogenous ATP, and the capacity of a P2X7 agonist to elicit acute and 24h-delayed rises in ATP release together suggest that ATP sensing in SCN astrocytes varies by time of day. To address this possibility, I conducted immunocytochemical analyses of P2X7R protein expression in mPer2^{Luc} SCN cells. P2X7 immunoreactivity was markedly rhythmic in cells permeabilized with Tween (polysorbate) 20 detergent (*Fig. 11A)*. Since P2X7 immunoreactivity was only performed for a 48 hours at 4-hour intervals, I cannot determine rhythmicity using Fourier transform, but levels of immunofluorescence changed depending on time. That is, average trough values of immunofluorescence intensity were observed 24 hours apart at T8 and T32 *(Fig. 11B)*. Average peaks in immunofluorescence were observed 20 hours apart at T20 and T40. Trough and peak protein expression differences, as indicated by the immunoreactivity, were statistically significantly ($p < 0.05$; *Fig. 11C*). Thus, P2X7R expression in mouse SCN astrocytes is rhythmic, likely in a circadian manner.
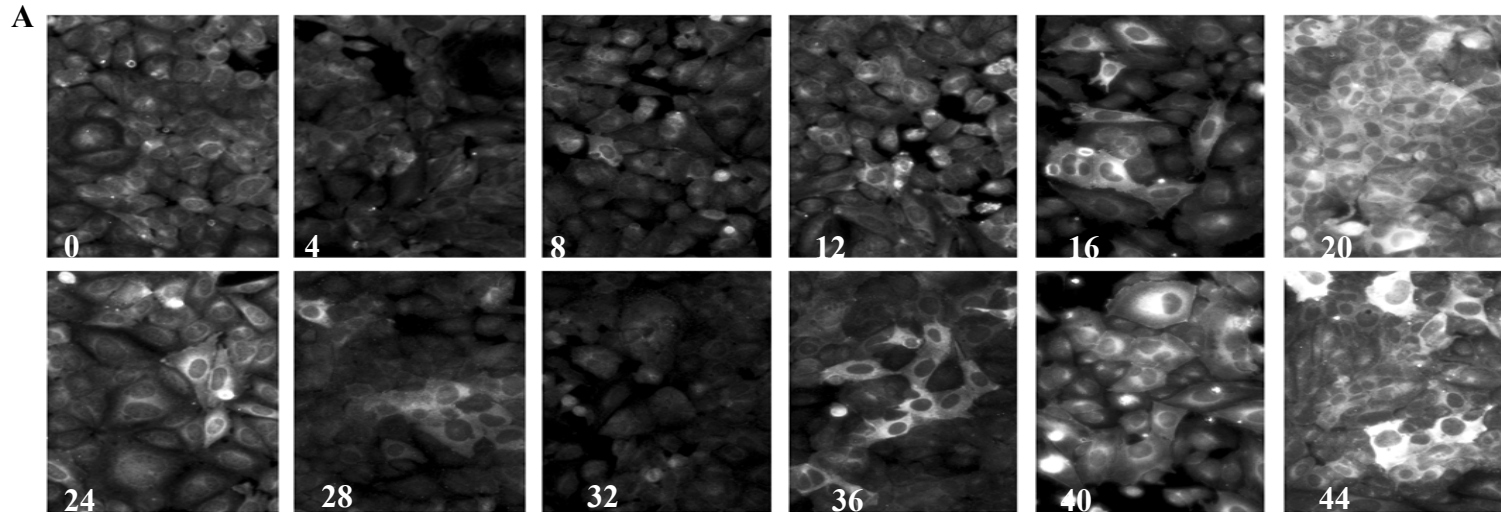
Figure 11. P2X7 receptor expression in mPer2^{Luc} SCN cells. **A)** Immunoreactivity was visualized with fluorescence microscopy at 4-hour timepoints over 48 hours of mPer2^{Luc} SCN cell culture. Images depict P2X7 antibody detection in confluent cell cultures. Scale bar is 10μm. **B)** Fluorescence intensity of the P2X7R immunoreactivity was rhythmic with peaks and troughs approximately 12h apart. **C)** Mean fluorescence intensity at timepoints T8 (trough) and T20 (peak) were significantly different (*p<0.05; n=4), with 68% higher fluorescence intensity at peak compared to trough. Error bars represent 95% confidence intervals.
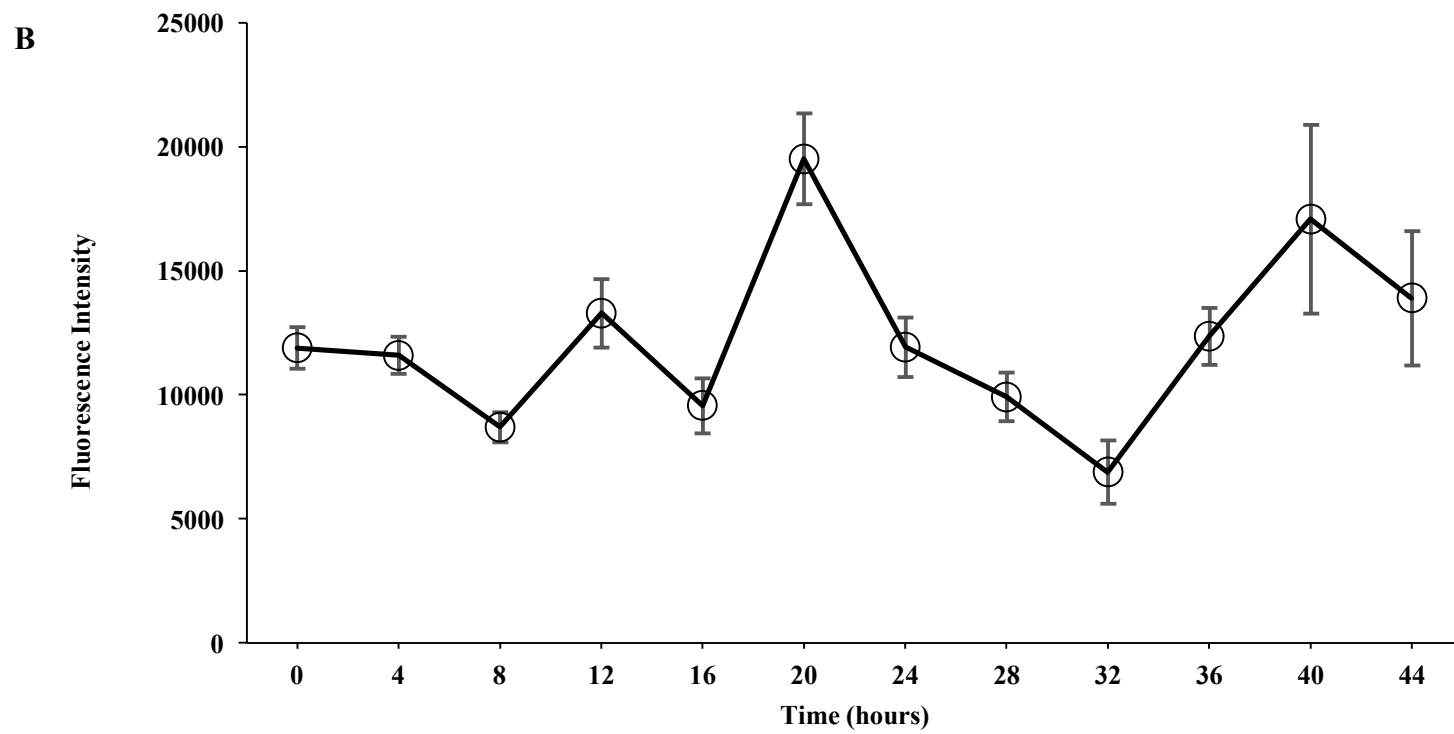
Figure 11 Continued

C
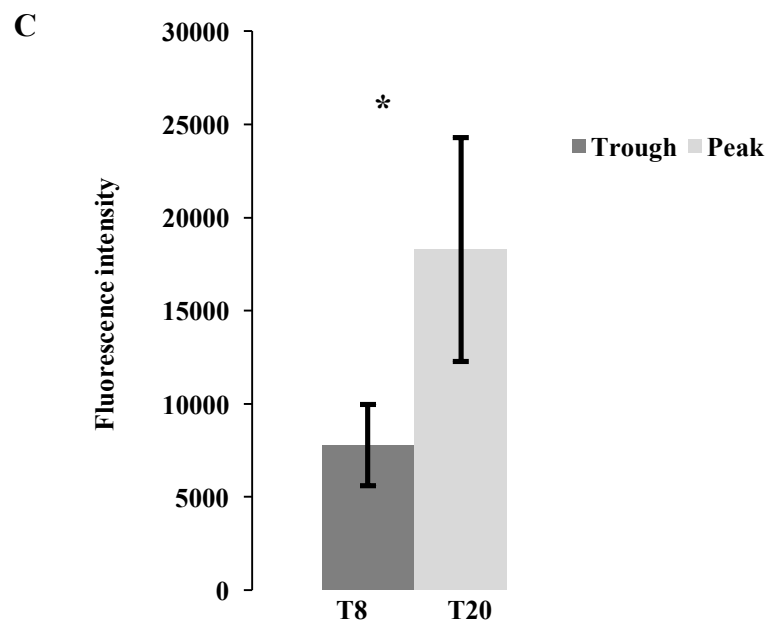
Figure 11 Continued

Time-dependent astrocytic activation and cytokine release

The P2X7 receptor is recognized as an important cell surface regulator of neural inflammatory signaling (Lister et al., 2007). ATP signaling, via the P2X7 receptor, mediates activation of astrocytes during brain trauma or disease (Rodrigues et al., 2015). This activation, or astrogliosis, is accompanied by upregulated expression of glial fibrillary acidic protein (GFAP), a process inhibited by P2X7R antagonism (Grygorowicz et al., 2016). Since rhythmic GFAP expression in rat SCN astrocytes has been observed *in vivo* (LaVialle & Serviere 1993) and I demonstrate here rhythmic P2X7R expression in mouse SCN cells, I tested whether GFAP expression was rhythmic in the mPer2^{Luc} SCN cell line. The GFAP immunoreactivity in these mSCN cells oscillated with peak (T36) and troughs (T24 and T48) and had time lags of approximately 12 hours between peak and trough (*Fig. 12A)*. The astrocytic expression of GFAP, as indicated by immunoreactivity, was cytoplasmic (*Fig. 12B*). Further, this cytoplasmic expression of the glial protein was significantly different between peak and trough timepoints ($p < 0.05$; *Fig. 12C*).

A
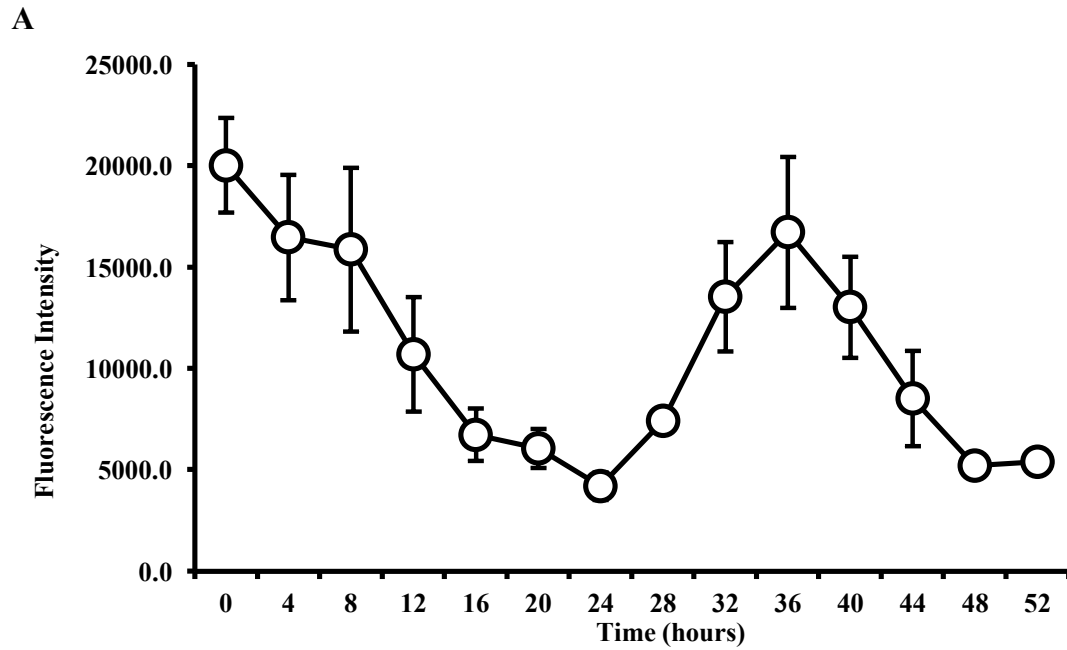
Figure 12. Immunoreactivity of GFAP antibody in mPer2*Luc* SCN cells. **A)** fluorescence microscopy at 4-hour timepoints over 48 hours of mPer2*Luc* SCN cell culture. **B)** Images depict GFAP antibody detection, which is cytoplasmic, at T24 (trough) and T36 (peak) timepoints. Blue fluorescence represents DAPI staining. Scale bar is 10μm. **C)** Mean fluorescence intensity of the GFAP immunoreactivity at time-points T24 and T36 (n=4) were significantly different (*$p < 0.05$), with 86% higher fluorescence intensity at peak compared to trough. Error bars represent 95% confidence intervals.
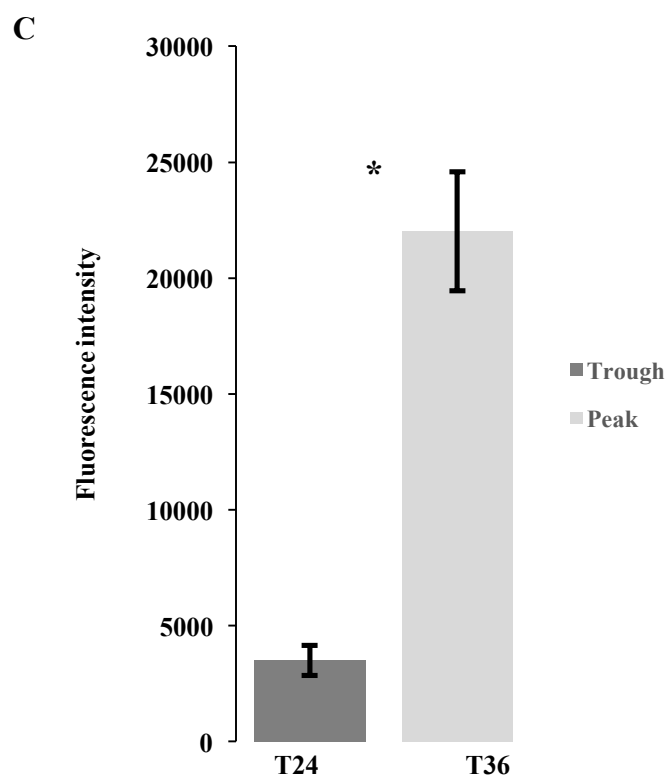
B

C
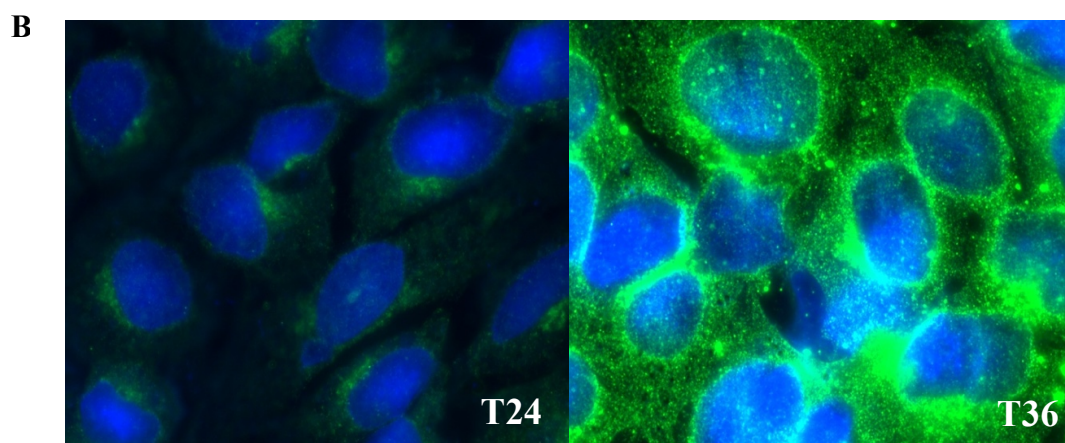
Figure 12 Continued

Since P2X7R is known to regulate neuroinflammatory signaling, including the release of the glial cytokine interleukin-1β (Il-1β; Lister et al., 2007), I tested whether treatment of mouse SCN astrocytes with the receptor agonist BzATP would induce Il-1β secretion in a time-dependent manner. Medium samples collected from mPer2^{Luc} SCN cells at 4 different time-points were analyzed by ELISA for the levels of the cytokine interleukin-1β (Il-1β) that accumulated in the cell culture medium. Media samples from control mSCN cells, which received neurobasal medium vehicle treatment only, exhibited variable levels of Il-1β depending on time of culture (*Fig. 13A*). The peak of Il-1β secretion was detected at T6 and was significantly greater than ($p < 0.05$) all other timepoints, including the Il-1β secretion trough at T18. BzATP treatment (100 μM), rather than elevating cytokine release, abolished the enhanced level of IL-1β at T6, and the associated significant differences in cytokine secretion across timepoints (*Fig. 13B*). Furthermore, when comparisons between control and experimental groups were conducted, a significant difference in Il-1β release was observed at the 6h timepoint ($p < 0.05$). Thus, astrocyte activation in SCN cell cultures is endogenously rhythmic with regard to GFAP expression and Il-1β release, both peaking at T6, and P2X7R activation with BzATP disrupts the ensemble secretion fluctuation in Il-1β release.
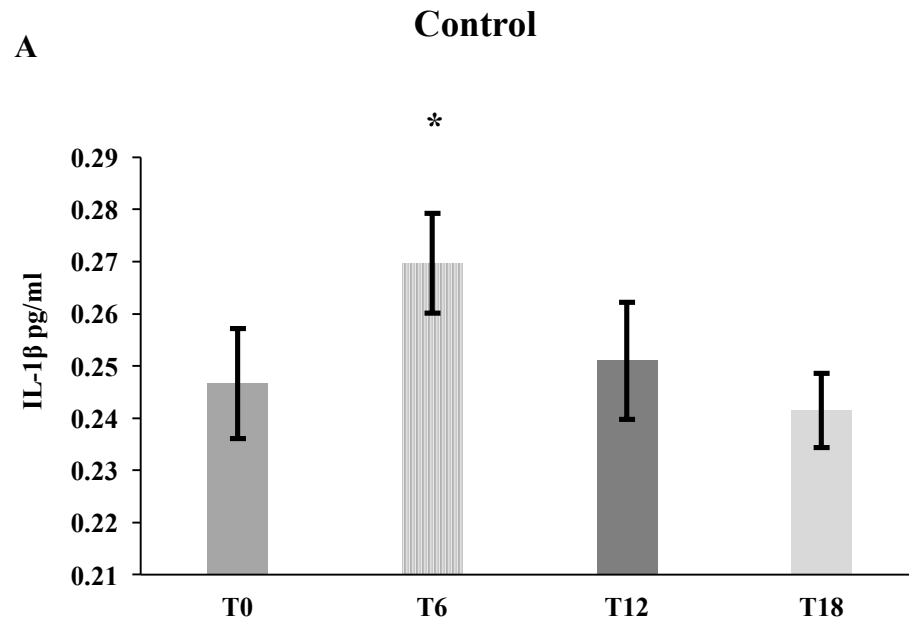
Figure 13. Extracellular IL-1β accumulation in mPer2^{Luc} SCN cell culture media is time-dependent. **A)** Mean IL-1β level at T6 in medium samples from control (untreated) mSCN cultures was significantly different from the other 3 collection time-points (n=12; *p<0.05). Error bars represent 95% confidence intervals. **B)** Following exposure to BzATP (100 µM), IL-1β levels were not significantly different across time-points (n=8). Error bars represent standard deviation. **C)** At time-point 6h (T6), the mean IL-1β level was significantly different between the control (n=12) and BzATP-treated cells (n=8; *p<0.05). Error bars represent 95% confidence intervals.

B BzATP

C 6hr
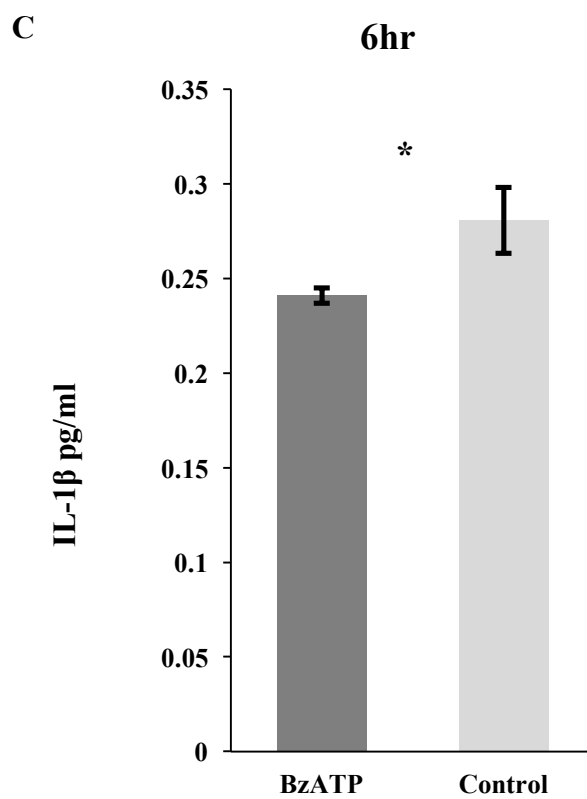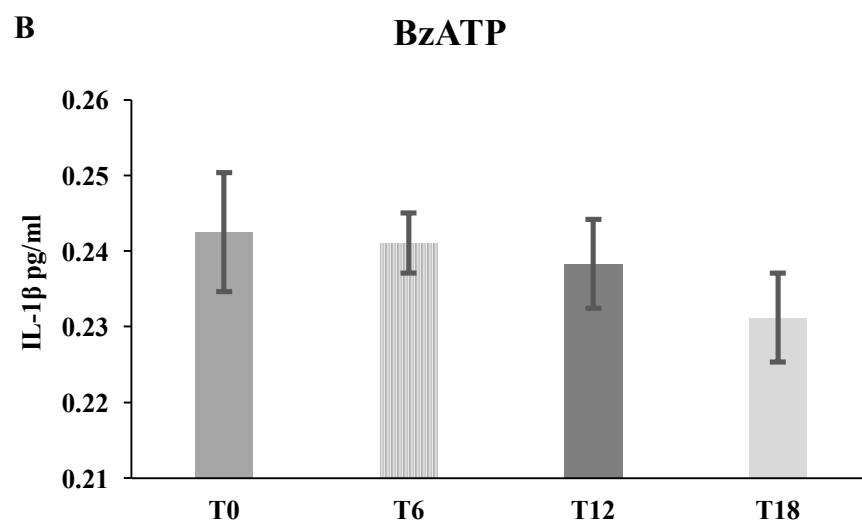
Figure 13 Continued

CONCLUSIONS

Circadian rhythms in neuronal physiology and gene expression are coordinated among SCN neurons (Hastings and Herzog, 2004). These SCN rhythms are necessary for expression of entrained circadian oscillation in animal physiology and behavior. Although some mechanisms regulating signaling among neurons are understood, mechanisms of glial signaling within the SCN are virtually unknown. Since a circadian rhythm in the accumulation of extracellular ATP was first described (Womac et al. 2009), it is thought that clock-controlled gliotransmission is not vesicular (Marpegan et al. 2011), but still requires calcium signaling (Burkeen et al. 2011). Furthermore, P2X7R inhibition abolishes the ATP release rhythms in rat SCN cells and dampens rhythms in PER2 gene expression in mouse SCN cells (Womac et al. unpublished). I used a potent P2X7R agonist, BzATP, to trigger an acute (less that 5 min) and robust (many orders of magnitude) increase in ATP release from immortalized mouse SCN cells.

A possible role of clock-controlled ATP release is in the coordination of ensemble astrocyte physiology in the SCN and other brain regions. ATP acts as an important signaling molecule between astrocytes and neurons (Parpura & Haydon 2000) and, therefore, is a good candidate for a synchronizing signal among individual oscillating astrocytes. Exogenous ATP alters the ATP release rhythm in rat SCN2.2 cells, where ATP application during peak times of the rhythm enhances subsequent release cycles. In contrast, when cell cultures received ATP during trough times the ATP accumulation rhythm was completely abolished. These results suggest that ATP may have served as a

signaling molecule that coordinates, and possibly contributes to the synchronization, of ensemble and clock-controlled astrocyte physiology. Interestingly, the period (τ) of the ATP release rhythm was significantly longer in the ATP peak-treated cells (24.6h), as compared to controls (22.7h). This result may set preliminary data for ATP as a synchronizer molecule that can cause phase shifts in the extracellular ATP accumulation rhythm and other physiological processes that require clock-controlled ATP signaling.

ATP release rhythms in SCN astrocytes are calcium-dependent (Burkeen et al. 2011) and P2X7R activation of calcium influxes is time-dependent (Womac et al. unpublished). Since P2X7R are necessary for these ATP release rhythms, BzATP was applied to mouse SCN cell cultures at the peak time of the ATP rhythm causing an additional peak of ATP release following a 24h delay. Additionally, BzATP at the peak of the ATP rhythm, but not the trough, causes a change in the period of the rhythm of PER2 gene expression (Camacho et al. data not shown). Thus, clock-controlled ATP signaling by SCN astrocytes might be modulated by a similarly clock-controlled ATP sensitivity rhythm in these glial cells. Another important thing to consider is that the application of ATP (10nM) had an effect on the extracellular ATP accumulation rhythm but not on the PER2 expression rhythm. This means the rhythmicity of the molecular clock is not disrupted by ATP, but the extracellular ATP accumulation rhythm is affected.

P2X7R expression is shown here to be rhythmic in mSCN astrocytes, shedding light on the mechanism of ATP-dependent communication among these glial cells., The trough of P2X7R expression at T8 was significantly different from the peak at T20. Therefore, these data demonstrate that the rhythm in P2X7R expression is anti-phasic with

the rhythm in extracellular ATP release. This result suggests that oscillations in extracellular ATP accumulation are not the only clock-controlled component of this glial signaling mechanism, since purinergic receptor availability may also be rhythmic in the SCN. It should be noted that no experiments were conducted here to determine if membrane localization of P2X7R or if *P2X7r* gene expression are rhythmic in these astrocyte cultures, and thus my interpretations are limited.

It was important in these studies using luciferase assays to rule out the possibility that BzATP merely drives the luciferase/luciferin reaction directly, rather than enhancing ATP-dependent rhythms. ATP-activated chemiluminescence, at same concentrations as BzATP, caused significantly higher levels of light production, suggesting that BzATP is not as efficient as ATP in driving the assay reaction. When the same concentration of BzATP (100 μM) was applied in the presence of cultured SCN cells, the chemiluminescence intensity was significantly greater than when performed in the presence of the luciferase/luciferin substrates alone. Thus, P2X7R activation is clearly mediating, as least in part, the ATP signaling underlying clock-controlled rhythms among SCN astrocytes.

Another important fact to consider is that BzATP has been widely used as a P2X7R agonist to study its role in partnership with PANX1 in astrocytic communication through ATP release and inflammatory responses (Qiu & Dahl 2009, Iglesias et al. 2008). The increased light production after BzATP application discovered here further suggests its efficacy in activating P2X7R modulation of the clock-controlled ATP release rhythm. Preliminary studies indicate that PANX1 expression may be also rhythmic in mSCN cells

and in phase with P2X7R (data not shown). This is important evidence in that the rhythmic regulation of P2X7R and PANX1 and their rhythmic regulation in mouse astrocytes may underlie circadian aspects of the brain's neuroinflammatory signaling.

ATP signaling in astrocytes, like many other cellular signaling mechanisms, is most likely involved in modulating several physiological processes in the brain. The extracellular ATP release rhythm is probably utilized for greater intercellular communication than just among astrocytes. That is, it may also be involved in astrocyte-microglia communication, critical in immune responses of the brain. One of the most widely used markers for astrocytic activation during neuroinflammation is increased GFAP expression. During this state of activation, astrocytes and microglia become reactive and promote neuro-inflammation. This neuroinflammatory response is characterized by astrocytes upregulating GFAP expression and microglia signaling for cytokine release from microglia or astrocytes. Thus, I was interested in determining if mouse SCN Per2^{Luc} cells express GFAP rhythmically, as has been reported for the rat SCN *in vivo* (La vialle & Serviere 1993). Mouse SCN cells showed variability in GFAP expression that was time-dependent. It is interesting to note that clock-defective SCN cells and astrocytes in other brain regions, through *Bmal1* deletion, have abnormal levels of GFAP expression that are maintained in an upregulated state (Musiek et al. 2013). This also supports the idea that neuroinflammation may be clock-controlled, since arrhythmicity causes increases in astrocytic activation. Preliminary data incubating mouse SCN cells with BzATP before performing immunocytochemical analyses with GFAP antibodies suggest that purinergic receptor activation causes either an increase or decrease

in GFAP expression in a time-dependent manner. It will be interesting to further continue that experiment and define more of the mechanistic features of these potential astrocyte-microglia interactions.

As mentioned previously, P2X7R in astrocytes and microglia is involved in immune responses that are ATP dependent (Jackson et al. 2014). One of those immune responses is the release of cytokine IL-1β (Bianco et al. 2005). ATP stimulation enhances release of IL-1β, which is blocked with P2X7R inhibitors. Most of the literature agrees that IL-1β release though modulated by P2X7R activation, is not physically released through the ionotropic receptor, but in fact through the pore forming PANX1 channel. This further suggests that ATP signaling through P2X7R is involved in several astrocytic physiological processes, such as eliciting immune responses in partnership with microglia. Therefore, I investigated if IL-1β can be detected in mouse SCN medium and, more importantly, if it is rhythmically accumulated. The mean levels of IL-1β had a peak in accumulation at T6. This peak was significantly higher than all other timepoints tested. I also incubated cells with BzATP and this purinergic receptor agonist abolished the cytokine oscillation. This means that P2X7R activation in SCN mPer2^{Luc} cells can elicit different responses in terms of IL-1β accumulation. It will be interesting to do an extended time course studies of this cytokine, since IL-1β transcript has been found to be rhythmic in mouse SCN (CircaDB, Circadian Expression Profiles Data Base). Other astrocytic activation markers, such as TNF-α can alter clock gene expression and possibly have a role in modulating circadian immune responses (Duhart et al. 2010). Therefore, it is possible that cytokine release, such as that of IL-1β by SCN glial cells, can be clock-

controlled and possibly regulated through circadian ATP release and P2X7R signaling complex.

In summary, ATP signaling among astrocytes is a good candidate for a coordinating mechanism that synchronizes clock-controlled physiological responses in the SCN and other brain regions. Furthermore, I present data indicating that clock-controlled ATP signaling rhythms are involved in modulating neuroinflammatory signaling. Since BzATP proved to be an effective P2X7R agonist with regard to potentiating ATP release from mouse SCN cells, further experiments with other agonists and antagonists could provide evidence for the role of ATP and P2X7R signaling rhythms, their circadian regulation, and their influence on astrocytic physiology and brain health.

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