MELATONIN MODULATES INTERCELLULAR COMMUNICATION AMONG IMMORTALIZED RAT SUPRACHIASMATIC NUCLEUS CELLS

A Thesis

by

KIMBERLY YVONNE COX

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2007

Major Subject: Biology

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Approved by:

Chair of Committee,	Mark J. Zoran
Committee Members,	David J. Earnest
	Robert C. Burghardt
Head of Department,	Vincent Cassone

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ABSTRACT

Melatonin Modulates Intercellular Communication among Immortalized Rat Suprachiasmatic Nucleus Cells. (December 2007)
Kimberly Yvonne Cox, B.S., University of Houston-Clear Lake
Chair of Advisory Committee: Dr. Mark J. Zoran

The mammalian brain contains a regulatory center in the diencephalic region known as the hypothalamus that plays a critical role in physiological homeostasis, and contains a variety of centers for behavioral drives, such as hunger and thirst. Located deep within the hypothalamus is the suprachiasmatic nucleus (SCN), or the master biological clock, that organizes rhythmic physiology and behavior, such that critical events take place at the most appropriate time of the day or night and in the most appropriate temporal, phase relationships. Cell-to-cell communication is essential for conveying inputs to and outputs from the SCN. The goal of the present study was to use an immortalized neural cell line (SCN2.2), derived from the presumptive anlage of the rat suprachiasmatic nucleus, as an in vitro model system to study intercellular communication among SCN cells. I tested whether the pineal neurohormone melatonin could modulate cell-to-cell signaling, via both dye coupling (gap junctional communication) and calcium waves (ATP-dependent gliotransmission). I also tested whether extracellular ATP could influence the spread of calcium waves in SCN2.2 cells. Lastly, the ability of extracellular ATP to modulate SCN physiological responses to melatonin in SCN2.2 cells was examined.

I show that melatonin at a physiological concentration (nM) reduced dye coupling (gap junctional communication) in SCN2.2 cells, as determined by a scrape loading procedure employing the fluorescent dye lucifer yellow. Melatonin caused a significant reduction in the spread of calcium waves in cycling SCN2.2 cultures as determined by ratiometric calcium imaging with Fura-2 AM, a calcium sensitive indicator dye. This reduction was greatest when an endogenous circadian rhythm in

extracellular ATP accumulation, determined by luciferase assay, was at its trough or lowest extracellular concentration. In addition, melatonin and ATP interacted in the regulation of gliotransmission (calcium waves), and this interaction was also specific to particular phases of the endogenous SCN physiological rhythmicity. Thus, I have established that a complex interaction exists between established melatonin signaling pathways and this newly discovered ATP accumulation rhythm, with the mechanisms underlying this relationship linked to endogenous cycling of SCN cellular physiology.

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CHAPTER I
INTRODUCTION

Cellular processes governing how an animal's physiology compensates for daily changes in environmental conditions are largely the product of specific neural structures. In these tissues, neural cells integrate sensory inputs into appropriate effector outputs. The coupling of inputs and outputs is typically orchestrated by neural oscillators that set the pace of physiological processes in the ultimate target tissue. When these oscillatory mechanisms and their driven outputs are sustained in constant environmental conditions, i.e., in the absence of entraining sensory information, they are defined as biological clocks. Additionally, if these oscillations possess a period of about (circa) a day (dian) when monitored under constant conditions, they are called circadian rhythms. In this chapter, the critical features of circadian rhythm generation in mammals and the nervous system structures that govern them will be introduced. In particular, a part of mammalian brain, the hypothalamus, and a specific kind of brain cell, the astrocyte, are of central importance to the research described in this thesis. In addition, the cellular physiology regulating how these cells communicate and how that communication might be regulated by the biological clock lies at the heart of the hypotheses tested here.

Mammalian hypothalamus

The hypothalamus is a visceral control center located just beneath the thalamus in the diencephalic region of the mammalian brain and serves as the primary link between the nervous and endocrine systems (Martini et al., 2000a). Although the hypothalamus occupies less than 1% of the total human brain volume, it contains a variety of centers for behavioral drives, such as hunger and thirst, and plays an important role in physiological homeostasis. Neural outputs from the hypothalamus control many

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This thesis follows the style of The Journal of Neuroscience.

functions of the autonomic division of the nervous system as well as an assortment of endocrine functions. Functions of the hypothalamus include: activation of the sympathetic nervous system (as in fight-or-flight response), maintenance of body temperature, control of body osmolarity, control of reproductive functions, control of food intake, interaction with the limbic system to influence behavior and emotions, secretion of trophic factors that control the release of hormones from the anterior pituitary gland, and influence over the cardiovascular control center in the medulla oblongata. The hypothalamus receives input from multiple sources, which include various sensory receptors, the reticular formation, and the cerebral cortex. Its outputs project to the thalamus and ultimately to multiple effector pathways, which are mostly bidirectional. One exception is the unidirectional, ascending pathway that leads from the retina to the suprachiasmatic nucleus, or SCN (Silverthorn, 2001a).

In mammals, photoreception takes place only in the retinae of the eyes (Dunlap et al., 2004b). Light enters the eye and is perceived by photoreceptors in the retina. Visual information is sent via retinal ganglion cells' axons of the optic nerve, which synapse in the lateral geniculate nucleus of the thalamus. The neurons of the thalamus then project to the visual cortex of the occipital lobe (Silverthorn, 2001b), where visual information is integrated for association and cognition. Collaterals of this visual retinothalamic pathway connect to two nuclei located in the hypothalamus and midbrain, which include the suprachiasmatic nucleus and the superior colliculus, respectively. The superior colliculus integrates visual information with other sensory inputs (Martini et al., 2000b). The retinal ganglion cell pathway to the hypothalamus is called the retinohypothalamic tract (RHT) and terminates in the SCN. The suprachiasmatic nucleus is the chief circadian pacemaker and regulates physiological processes under biological clock control in mammals (Klein and Moore, 1979).

Suprachiasmatic nucleus

The mammalian SCN is located just above the optic chiasm, where the crossing optic tracts arrive at the anterior ventral hypothalamus of the brain. It consists of two small,

densely packed clusters of neural cells. Each bilateral cluster contains about 10,000 neuronal cells, along with an abundance of glial cells, primarily astrocytes, and fibroblasts. Photoperiod inputs to the SCN entrain or set the phase of the biological clock, thereby allowing an organism to anticipate environmental opportunities for behavior and physiology. The circadian clock organizes effector outputs to take place at the most appropriate time of the day or night and in appropriate phase relationships. Metabolism in diurnal mammals, for example, switches between processes that support psychological alertness and energetic demand (diurnal processes) and those involved in growth and repair of the body (nocturnal processes; Dunlap et al., 2004a).

Circadian physiology of the SCN can be studied using in vitro cell culture. Model cell culture systems, like the SCN2.2 system used here, have been developed through an immortalization technique. This process employs gene transfer techniques to mediate the introduction of an oncogene and expression of its immortalizing protein product in primary SCN cells (Dunlap et al., 2004b). The SCN2.2 cell line was created from primary cultured cells of the presumptive anlage of the rat suprachiasmatic nucleus, which were immortalized by infection with a retroviral vector encoding the adenovirus 12S E1A gene. The resulting neural cell line consisted of a heterogeneous (astrocytes, neurons, and fibroblasts) population of cells in various stages of differentiation, although a large portion (≈75-90% of colony total) of the SCN2.2 cell line was characterized by glial-like morphology (Earnest et al., 1999a). These immortalized SCN cells were capable of endogenously generating circadian rhythms in 2-deoxyglucose (2-DG) uptake (Earnest, et al., 1999b), canonical clock gene expression (Allen et al., 2001), and neurotrophin content (Earnest et al., 1999b). The phase relationship between 2-DG uptake and neurotrophin content in vitro was identical to that exhibited by the SCN in vivo. That is, a 12-hour antiphase relationship was exhibited between the rhythms of glucose utilization and brain-derived neurotrophic factor (BDNF) content in immortalized SCN cells. Similarly, the rat SCN in vivo has been characterized by maximal 2-DG uptake at circadian time (CT) 6, or subjective day (Schwartz, 1991), and peak BDNF content around CT 18, or subjective night (Liang et al., 1998). Furthermore,

transplantation of SCN2.2 cells into arrhythmic, SCN-lesioned rats restored circadian activity rhythms. Where as, transplantation of mesencephalic or fibroblast cell lines did not restore the circadian activity rhythm. Thus, these immortalized SCN progenitors retain the oscillator, pacemaker, and clock-control properties of the SCN and maintain these properties in appropriate circadian phase relationships (Earnest et al., 1999b).

One goal of the present study is to establish the SCN2.2 cell line as a suitable in vitro model system for the study of intercellular communication among mammalian neural, clock-control elements. Since individual cells of the SCN are competent circadian oscillators and together they function as an organismal physiology pacemaker, it is important to understand how these cells communicate with each other within the hypothalamus. Cell-to-cell communication is essential for integration of inputs to and outputs from the SCN. Signaling pathways by which the oscillatory cells of the SCN communicate may involve: 1) classical neurotransmitters, such as γ-aminobutyric acid (GABA; Decavel and Van den Pol, 1990), as intercellular coupling agents, 2) neuropeptides, such as arginine vasopressin and vasoactive intestinal peptide (AVP, VIP, respectively; Card et al., 1988), as coupling molecules, 3) gases, like nitric oxide (NO; Amir et al., 1995), that readily cross biological membranes and alter the biochemistry of surrounding cells, 4) electrical coupling via intercellular gap junctions (Colwell, 2000), and 5) complex glial-glial and glial-neuronal interactions involving gliotransmitters, such as glutamate and ATP (Michel and Colwell, 2001).

Over the past decade, many insights have been gained into the complex interactions that occur between neurons and glia in the nervous system (Volterra et al., 2002a). A large number of synapses in the SCN, like those found in many brain regions such as the hippocampus (Volterra and Steinhäuser, 2004), are enveloped by astroglial processes. Additionally, receptors for the pineal hormone melatonin are abundant on astrocytes of the SCN (Adachi et al., 2002; Rivera-Bermúdez et al., 2004). Consequently, glial cells, particularly astrocytes, may play a special role in regulating communication between populations of neurons in the SCN (Michel and Colwell, 2001). In the hypothalamus, astrocyte-neuron signaling is believed to regulate gonadotropin-

releasing hormone (GnRH) production and secretion (Dhandapani et al., 2003). The synaptic plasticity associated with GnRH neurons is modulated by hypothalamic astrocytes and may involve dynamic glial ensheathment, and the extension and retraction of glial processes (Witkin et al., 1995; Witkin et al., 1997).

Astrocytic communication

Hypothalamic astrocytes communicate intercellularly via gap junctions and gliotransmitter release (Welsh and Reppert, 1996; Batter et al., 1992; Blomstrand et al., 1999; Pascual et al., 2005; Araque et al., 1999a; Araque et al., 1999b; Fig. 1). The role that gap junctions play in intercellular communication has been well-documented in various tissues, including a wide range of functions in the brains of animals, where they act as gateways for the intercellular exchange of membrane currents, electrolytes, second messengers and metabolites. In mammals, gap junctions are found in virtually every cell type except mature skeletal muscle, spermatozoa, and erythrocytes (Dermietzel and Spray, 1993), and in these diverse tissues- in both developmental and adult contexts- govern equally diverse biological events.

Gap junctions are permeant to molecules as large as 1 kDa and are formed when two cells contribute a hemichannel composed of six membrane-spanning protein subunits called connexins. These hemichannels, or membrane pores, become juxtaposed to generate a hydrophilic channel that plays a major role in cellular homeostasis, differentiation, and growth control by virtue of permitting transfer of regulatory and physiologically significant metabolites (Barhoumi et al., 1993). However, gap junctional communication may not be an exclusively cell-to-cell process. Recent work suggests that unapposed hemichannels, especially those formed of pannexin proteins, may permit the passage of molecules between the cytoplasm and extracellular surroundings (Ebihara, 2003; Goodenough and Paul, 2003). Biochemical communication via gap junctions has been documented in epithelial cells (Sanderson et al., 1990) and neurons (Kandler and Katz, 1998). In epithelial cells, inositol (1,4,5)-trisphosphate (IP_3) generated in a stimulated cell was able to spread to neighboring

electrically coupled cells via gap junctions (see Fig. 1*A*), where it caused a release of Ca^{2+} from intracellular stores (Sanderson et al., 1990). Long-range signaling by Ca^{2+} waves, which involve elevations in intracellular Ca^{2+} concentration that propagate through a group of cells, has also been credited to astrocytic gap junctions (Nedergaard et al., 2003). Astrocytic gap junctional communication contributes to the classical homeostatic functions these glial cells, such as: 1) transport of nutrients from the bloodstream to neurons, 2) 'spatial buffering' of K^+ during neuronal excitation when K^+ is released into the extracellular space and taken up by astrocytes, plus, 3) gap junctional mediated glutamate uptake and dissipation among astrocytes (Anderson et al., 2003).

Hypothalamic astrocytes also communicate via gliotransmitter release (Fig. 1*B*). Astrocytes express calcium excitability, which means that they possess mechanisms for the Ca^{2+}-dependent release of chemical transmitters such as glutamate and ATP. Just as neurons express excitability as a function of plasma membranes equipped with many voltage-gated ion channels that allow membrane depolarization and the propagation of action potentials, glial cells express this Ca^{2+}-based form of excitability (Charles, 2005). Glial cells use an intracellular route to raise Ca^{2+} levels, employing intracellular organelles, specifically the endoplasmic reticulum (ER) and mitochondria. These organelles generate and maintain intracellular, as well as intercellular, Ca^{2+} waves (Volterra et al., 2002b). A characteristic property of astrocytes grown in cell culture is that a Ca^{2+} rise in one cell leads to a Ca^{2+} rise in neighboring cells, thus generating a multicellular Ca^{2+} wave. Activation of metabotropic receptors (e.g., purinergic receptors) in the astrocyte plasma membrane causes a G protein-dependent activation of phospholipase C, which leads to the generation of IP_3. This in turn triggers Ca^{2+} release from the ER, which underlies propagating intracellular and intercellular waves (Volterra et al., 2002b). Inhibition of ER Ca^{2+} accumulation with thapsigargin, which blocks SERCA pumps (sarcoplasmic endoplasmic reticulum Ca^{2+}-ATPase) that fill the ER with Ca^{2+}, blocks both types of calcium waves. Additionally, the blockade of IP_3 receptors by heparin blocks both types of waves as well (Kirischuk et al., 1995; Newman and Zahs, 1997). Additionally, blockade of IP_3 receptors by 2-aminoethoxydiphenylborate

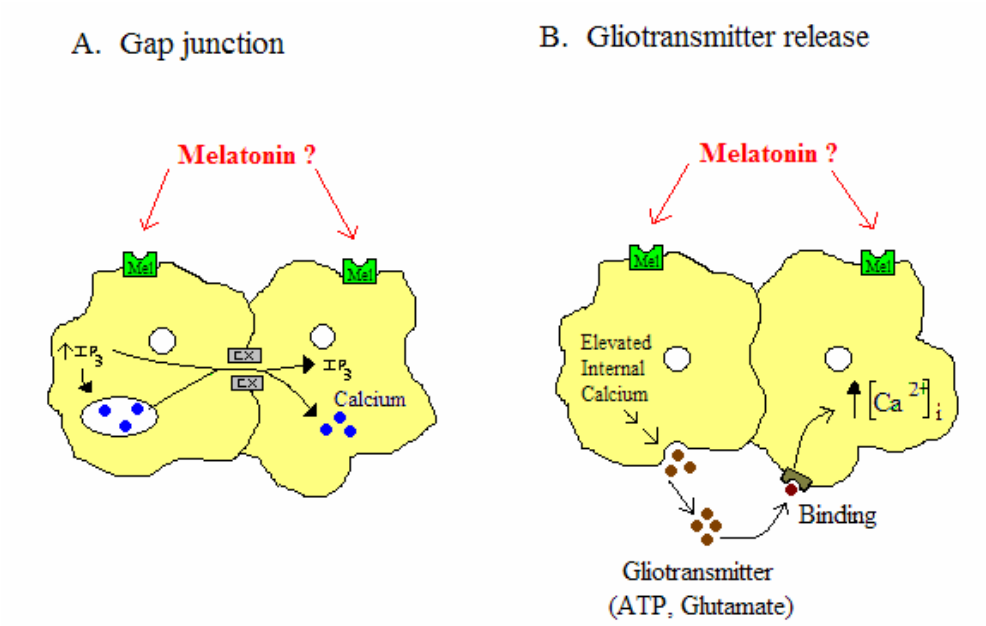

Figure 1. Model for astrocytic communication. Hypothalamic astrocytes, like those of many brain regions, communicate intercellularly via gap junctions and gliotransmitter release. Diencephalic structures, including the hypothalamus, express numerous types of G-protein-coupled melatonin receptors (Brooks and Cassone, 1992; Cassone et al., 1995; Reppert et al., 1995; Reppert et al., 1996; von Gall et al., 2002; Weaver et al., 1989) and melatonin reportedly modulates both gap junctional communication as well as gliotransmitter signaling among astrocytes, as seen in avian (diurnal) brains (Peters et al., 2005a). However, how melatonin modulates astrocytic communication among astrocytes derived from a rat (nocturnal) brain remains unclear. ***A***, An increase in inositol (1,4,5)-trisphosphate (IP_3) can trigger a release of Ca^{2+} from intracellular stores, and IP_3 and Ca^{2+} can spread to neighboring electrically coupled cells through gap junctions formed by connexin proteins. ***B***, Gliotransmitters, such as ATP and glutamate, are also released from glial cells, which bind to receptors on neighboring cells and activate signal transduction pathways leading to a rise in intracellular calcium in the neighboring cells. Calcium wave propagation is believed to involve both types of astrocytic communication (Nedergaard et al., 2003).

(2-APB) dramatically reduces the propagation of Ca^{2+} waves among chick diencephalic astrocytes (Peters et al., 2005a). Given that Ca^{2+} elevations in astrocytes lead to the release of these gliotransmitters that, in turn, modulate synaptic transmission among neighboring neurons (Araque et al., 1998a; Araque et al., 1998b; Araque et al., 1999b; Kang et al., 1998; Robitaille, 1998), the idea of the tripartite synapse (Araque et al., 1999a) has been proposed. This idea posits that a synapse should be thought of as having three critical elements, the presynaptic terminal and postsynaptic dendritic spine, and the astrocytic endfoot that encircles them. At this tripartite synapse, the associated astrocyte listens in to neuronal signaling with appropriate receptors and responds by modulating cell signaling at that synapse, that of other synapses in the surrounding area, or gliotransmission of neighboring astrocytes (Haydon, 2001).

Melatonin modulation of astrocytes

Melatonin (5-methoxy-*N*-acetyltryptamine) is a hormone that is involved in the regulation of circadian rhythms in mammals, reptiles, and birds (reviewed in Cassone, 1990). Melatonin modulates a variety of physiological functions including sleep, visual, cerebrovascular, reproductive, neuroendocrine, and neuroimmunological processes (Arendt, 2000; Brzezinski, 1997). Melatonin levels are dynamically regulated by the rhythmic synthesis and rapid degradation (e.g., half-life of melatonin in mammalian plasma is about 10 minutes) of melatonin (von Gall et al., 2002). It is synthesized primarily in the pineal gland from serotonin by the sequential actions of the enzymes serotonin N-acetyltransferase and 5-hydroxy-indole-O-methyltransferase (Underwood and Goldman, 1987). In mammals, melatonin is removed from the circulation in two steps: 1) 6-hydroxylation in the liver and 2) excretion in a sulfatoxy-conjugated form. The duration of nocturnal melatonin secretion changes in proportion to the length of the night. Hence, seasonal variations in the length of the solar day are encoded in this humoral signal (von Gall et al., 2002). For example, melatonin production is high during the hours of darkness in animals maintained on a light/dark cycle and persists to

be highest during the subjective night in animals maintained in constant darkness (Klein and Moore, 1979; Moore, 1996; Underwood and Goldman, 1987).

Melatonin is believed to entrain and synchronize circadian rhythms by activating melatonin receptors in the SCN (Dubocovich et al., 1996). In fact, melatonin production is dependent on circadian oscillators within the SCN. The daily administration of melatonin is thought to entrain the underlying rhythmicity of the circadian clock within the SCN through daily phase advances. The cellular mechanisms of circadian clock function can be studied by examining the sites and mechansims of melatonin action (Cassone, 1990). Studies examining melatonin's action on SCN physiology indicate that melatonin directly affects the SCN. For example, in vivo, subcutaneous injection of melatonin altered rhythms of 2-deoxy[^{14}C] glucose (2-DG) uptake in a phase- and dose-dependent fashion in the rat SCN (Cassone et al., 1988).

Functional Mel_{1A} and Mel_{1C} melatonin receptors have been found in astrocyte-rich chick diencephalic astrocyte cultures. In these glial cells, rhythmic administration of physiological levels of melatonin imposed a rhythm in 2-DG uptake and the rhythmic release of both lactate and pyruvate into the medium (Adachi et al., 2002). In similar primary cultures of chick astrocytes, 10 nM melatonin decreases gap junctional coupling and enhances the spread of calcium waves (Peters et al., 2005a). Similar enhancement of calcium waves was seen in mouse hypothalamic astrocytes (Peters et al., 2005b). Does melatonin have the same effect in rat suprachiasmatic nucleus cells? I hypothesize that melatonin modulates both gap junctional communication as well as calcium signaling among SCN cells (Fig. 1).

Diencephalic structures, including the hypothalamus, express numerous types of G-protein-coupled melatonin receptors (Brooks and Cassone, 1992; Cassone et al., 1995; Reppert et al., 1995; Reppert et al., 1996; von Gall et al., 2002; Weaver et al., 1989). Western blot analysis has demonstrated that MT_1 and MT_2 melatonin receptor proteins are expressed in both rat SCN tissue in vivo and rat SCN2.2 cells in culture. Melatonin feedback to the master clock, the hypothalamic SCN, acts to inhibit neuronal firing rates through activation of the MT_1 melatonin receptor (Gillette and McArthur, 1996; Gillette

and Mitchell, 2002; Liu et al., 1997). Activation of the MT_2 melatonin receptor is responsible for phase shifts in circadian rhythms (Dubocovich et al., 1998; Hunt et al., 2001). Since SCN2.2 cells express multiple functional melatonin receptors, these cultures have provided a valuable in vitro model for the study of melatonin signaling pathway(s) and cellular mechanism(s) by which melatonin regulates circadian activity (Rivera-Bermúdez et al., 2004).

ATP signaling

Intercellular communication between astrocytes and neurons is regulated, in part, by ATP signaling (Fellin et al, 2006). ATP released from astrocytes accumulates as extracellular adenosine and regulates synaptic transmission and neural integration (Pascual et al., 2005). However, a pivotal finding was the discovery that communication between astrocytes was mediated by ATP as well (Cotrina et al., 1998; Guthrie et al., 1999). ATP is thought to be released from astrocytes by multiple mechanisms, which may include vesicular exocytosis and release via gap junction hemichannels (Fields and Burnstock, 2006). Recent studies in our group have determined that ATP is accumulated in SCN2.2 cell cultures in a rhythmic and circadian fashion with peaks in extracellular ATP levels occurring about every 22-24 hours (Womac et al., 2006). Consequently, ATP may represent a circadian output signal of the mammalian SCN. Since ATP plays a critical role in the regulation of astrocytic calcium waves, I hypothesize that the circadian accumulation of ATP can influence the spread of calcium waves in immortalized SCN cells. Furthermore, additional studies in our lab have demonstrated that the spread of calcium waves in SCN2.2 cell cultures is mediated by a purinergic, ATP-dependent signaling pathway. The addition of apyrase, an ATP diphosphohydrolase, to forskolin-treated SCN2.2 cells completely abolishes calcium wave propagation (Burkeen and Zoran, 2006).

In the following chapters, I will present data that the circadian accumulation of ATP modulates intercellular communication among SCN cells. Interestingly, a melatonin-mediated phase shift of circadian rhythms occurs at two windows of

sensitivity that correspond to the hours around the day-night (dusk) and night-day (dawn) transition, and may be related to activation of PKC-dependent pathways (Hunt et al., 2001; McArthur et al., 1997). Hence, I have investigated the potential relationship between the circadian release of ATP from SCN cells and melatonin-mediated regulation of SCN cell signaling. I hypothesize that extracellular ATP modulates SCN physiological responses to melatonin in SCN cells.

The present study utilizes SCN2.2 cells to establish an in vitro model system to study intercellular communication among SCN cells, particularly astrocytes. I have used this system to investigate the relationship between melatonin signaling and ATP signaling in SCN2.2 cells. First, I hypothesized that melatonin modulates both gap junctional communication as well as calcium signaling among SCN2.2 cells. To test this hypothesis, dye coupling studies and calcium wave studies were conducted to assess intercellular communication among SCN2.2 cells. Second, I theorized that extracellular ATP influences the spread of calcium waves in SCN2.2 cells. Third, I hypothesized that extracellular ATP modulates SCN physiological responses to melatonin in SCN2.2 cells. As a test, the influence of melatonin on calcium waves was studied in the presence of endogenously accumulated or exogenously supplemented ATP.

CHAPTER II
MATERIALS AND METHODS

Cell culture

Cultures of SCN2.2 cells were grown on laminin-coated culture dishes (60 mm; Corning, Corning NY) and maintained at 37ºC and 5% CO_2 in Minimum Essential Medium (MEM; Invitrogen, Carlsbad, CA), supplemented with 10% FBS, glucose (3000 µg/ml), L-glutamine (292 µg/ml) and 1% PSN antibiotic (penicillin, streptomycin, neomycin; Gibco/Invitrogen, Grand Island, NY). During cell propagation, the medium was changed at 48 hr intervals, and the cultures were split every 2 days. For all experiments, SCN2.2 cells derived from a single passage were propagated as described above and then seeded onto cover-glass chambers (Lab-Tek) and allowed to grow to confluence (2 days).

Scrape loading dye transfer

For studies of intercellular dye coupling, confluent SCN2.2 cells were washed with calcium/magnesium free media (CMF; Invitrogen, Carlsbad, CA) and then subjected to medium replacement with supplemented MEM described above. Cells were subjected to a 30-min. incubation period with melatonin (10 nM; Sigma, St. Louis, MO) and/or 100 µM carbenoxolone (CBX; Sigma) at 37ºC and 5% CO_2. Carbenoxolone is a gap junctional blocker. Cells were washed with 1X Dulbecco's phosphate buffered saline (PBS; Invitrogen) and exposed to a dye solution containing lucifer yellow (0.05%; Sigma) and rhodamine dextran (10,000 Da; 0.0125 mM; Molecular Probes, Eugene, OR) dissolved in PBS. Lucifer yellow is a highly fluorescent dye that allows visualization of dye coupling, or gap junctional communication. Rhodamine dextran is a high molecular weight marker dye that is unable to cross gap junctions when introduced intracellularly. The dye solution was supplemented with melatonin or CBX as appropriate. Confluent cells on glass-bottom dishes were then damaged with a fresh, sterile scalpel blade or a pulled glass pipette tip. The scrape loading procedure involved dragging each tip gently

across the surface of the cells. As a result of the damage, cells along the edge of the scrape became loaded with lucifer yellow and the dye was transmitted to neighboring cells coupled via gap junctions. Cells were washed with PBS and placed in fresh MEM (without phenol red) medium with supplements (as described above). The MEM was also supplemented with melatonin or CBX as appropriate. Imaging was conducted at 37ºC with an Olympus IX70 inverted microscope connected to an Orca ER CCD camera (Hamamatsu). The 20× objective of the microscope and the camera were used to capture a field of cells loaded with lucifer yellow. Grey level intensity measurements were taken using Simple PCI 4.0 imaging software (Compix, Cranberry Township, PA) and dye coupling coefficients (ratios) were calculated by dividing each of the cells involved in a line of dye spread by the intensity of the first cell that took up the dye minus the background.

Ratiometric calcium imaging

Confluent SCN2.2 cultures were seeded onto cover-glass chambers (Lab-Tek) and allowed to grow to confluence (2 days). Cells were washed with CMF and placed in fresh MEM medium with supplements (as described above). Cells were loaded with 8 μM Fura-2 AM (diluted from a 1 mM Fura-2 AM stock in a 20% Pluronic F-127 in DMSO; Molecular Probes) in culture medium for 1 hr at 37ºC and 5% CO_2. In experiments using melatonin, CBX, or luzindole (Luz; Sigma), cells were treated for 1 h at 37ºC and 5% CO_2 prior to imaging. Luzindole is a melatonin receptor antagonist. In experiments using ATP (10 nM; Molecular Probes), cells were bathed in ATP for 2 hr prior to imaging. Treatments were added during or after Fura loading as needed. After Fura loading, cells were immediately washed with CMF and fresh culture medium (without phenol red) was added to the cells. Ratiometric calcium imaging was conducted at 37ºC with an Olympus IX70 inverted microscope connected to an Orca ER CCD camera (Hamamatsu). The 40× objective of the microscope and the camera were used to capture a field, and this full field of cells was considered a region of interest (ROI). Resting calcium levels were first obtained from an ROI, and then a single cell

was stimulated once using gentle mechanical stimulation with a micromanipulated glass micropipette to elicit an immediate calcium wave. The area of maximum wave spread was calculated (Simple PCI 4.0) for each ROI. The estimated calcium concentrations were calculated using conventional methods (Grynkiewicz et al., 1985). Images were collected for approximately 30-40 s, sufficient time to detect any calcium changes in cells within the ROI. Based on previous experiments, we were confident that a sampling time of 30-40 s was sufficient to detect all cells within the ROI involved in a calcium wave as calcium levels returned to resting values after this time. Since a 40× objective was used in these experiments, the calculations of maximum wave areas are undoubtedly underestimates of the extent of the wave, since they often propagated beyond the field of view (Fig. 3*D*).

SFCF protocol for calcium wave studies

In calcium wave experiments using the SFCF (serum reduction and forskolin-activation with calcium-free media washes) protocol, an entraining stimulus was used to coordinate individual cellular oscillations into ensemble rhythms allowing neurons and glial cells to become synchronized in vitro. Confluent SCN2.2 cultures were seeded onto cover-glass chambers in culture medium with a decreased FBS concentration of 5% and allowed to grow at 37ºC for 24 hr to reach 50% confluence. After 24 hr, cells were washed with CMF and subjected to medium replacement with serum-free Neurobasal medium (supplemented with glucose, L-glutamine, and 1X B-27 serum-free supplement; Invitrogen, Carlsbad, CA). Forskolin (15μM; FSK; Calbiochem, La Jolla, CA) was added to serum-free medium and cells were incubated at 37ºC and 5% CO_2 for 2 hr. FSK, an adenylate cyclase agonist that coordinates rhythms by raising concentrations of cyclic AMP after activating adenylate cyclase, has been used in previous studies of rhythmic clock gene expression and glucose uptake in SCN2.2 cell cultures (Allen et al., 2001). Immediately following treatment (time 0), cells were rinsed with CMF and maintained thereafter in serum-free Neurobasal medium (2 ml). Cells were loaded with Fura-2 AM as previously described and calcium waves were imaged (as described

above) at targeted time points, which were 20 hr and 32 hr from the end of FSK treatment.

SR protocol for calcium wave studies

In experiments using the SR (serum reduction) protocol, confluent SCN2.2 cultures were seeded onto cover-glass chambers in culture medium with a decreased FBS concentration of 5% and allowed to grow at 37ºC for exactly 24 hr to reach 50% confluence. The plating event served as time 0. After 24 hr, cells were washed with serum-free Neurobasal medium (with supplements as described above) and fresh neurobasal medium was added to each well (2 ml). Note that all subsequent washes were performed with serum-free Neurobasal medium instead of CMF. Cells were loaded with Fura-2 AM as previously described and calcium waves were imaged (as described above) at targeted time points, which were 40 hr and 50 hr from the plating event.

Chemiluminescence assay for analysis of ATP accumulation in culture medium

For analysis of ATP accumulation in the culture medium, media samples were taken prior to calcium wave imaging. Samples were frozen and stored at -20°C and later analyzed for ATP accumulation using a chemiluminescence assay. The chemiluminescence assay of extracellular ATP accumulation was performed by aliquoting 100 µl of each media sample, 1 µl of luciferase (3 mg/ml) and 2 µl of luciferin (3 mg/ml) per well in a black, 96-well plate (Thermo, Milford, MA). Chemiluminescent activity associated with ATP accumulation in the medium was measured in constant darkness using a multiplate Packard TopCount scintillation counter (Meriden, CT).

2-Deoxyglucose uptake

For studies of glucose uptake, SCN2.2 cells were subjected to the SR protocol described above. However, instead of Fura-2 AM loading, cells were bathed for 1 hr in serum-free

Neurobasal medium (with supplements as described above) containing 2-NBDG (0.1 mM; Invitrogen), a fluorescent glucose analog that allows glucose uptake to be monitored in live cells. The 2-NBDG incubation was started 30 minutes prior to the targeted time point. During incubations, cultures were paired with 1 well washed and exposed to culture medium supplemented with normal glucose (3000 μg/ml) and the 2nd well washed and exposed to culture medium containing no glucose. Immediately following 2-NBDG incubations, cells were washed with serum-free Neurobasal medium (with supplements) and imaged at 37ºC using an Olympus IX70 inverted microscope. Image analysis (Simple PCI 4.0) was performed, where images were analyzed for grey level intensity.

Statistics

In all experiments, multiple ROIs were sampled from multiple independent SCN2.2 cultures. The multiple culture wells used in any experiment were derived from the passage of a single culture. Thus, the experimental number (n) throughout every calcium wave study equals the total number of ROIs studied across multiple cultures. The n for dye coupling studies equals the total number of lines of dye spread, where dye can be traced from an initially loaded cell to neighboring, coupled cells to create a cellular line of spread made up of 4-5 cells. Multiple lines of dye spread were found in a given ROI, or field of view (Fig. 2*B*). Dye-coupling ratios and ratiometric calcium imaging data were analyzed using Microsoft Excel and SPSS 13.0 for Windows. Treatment groups were statistically compared using one-way ANOVA. Fisher's LSD post hoc test was used when significant differences were found. In all cases, $p<0.05$ level was used to determine minimum significance.

CHAPTER III
RESULTS

Melatonin modulates gap junctional and gliotransmitter signaling among SCN2.2 cells

Melatonin has been shown to decrease gap junctional communication (using dye coupling assays) and enhance gliotransmitter-mediated signaling (using calcium wave analyses) in primary cultures of chick diencephalic astrocytes (Peters et al., 2005a). Here, I tested the hypothesis that melatonin also modulates both gap junctional coupling and gliotransmitter signaling among SCN2.2 cell cultures. That is, does melatonin have the same effects on these nocturnal rodent-derived suprachiasmatic nucleus cells, as it did previously on diencephalic cells from a diurnal avian species?

Effects of melatonin on SCN2.2 cell dye coupling

Melatonin caused a significant decrease in dye coupling at a physiological concentration previously shown to have a potent effect in a chicken (10 nM; Peters et al., 2005a). The average dye coupling coefficient (DCC) between SCN2.2 cells, as determined by a scrape loading procedure employing the fluorescent dye lucifer yellow (Fig. 2*A*,*B*), was reduced by 28% compared to control DCC values obtained from vehicle (DMSO)-treated cultures (Fig. 2*C*). Carbenoxolone (100 μM), a gap junction blocker, caused a much greater reduction in the average dye coupling of SCN2.2 cells, where the average DCC was 95% of that in control cultures. As in previous studies of chick diencephalic astrocytes (Peters et al., 2005a), melatonin decreased dye coupling among SCN2.2 cells. Therefore, melatonin appears to have a similar influence (approximately a 30% decrease) on dye coupling among SCN astrocytes derived from the SCN of a rat as it did on avian diencephalic astrocytes.

Dye coupling among SCN2.2 cells was not as extensive as that seen previously in other avian (Peters et al., 2005a) and mammalian neural cell cultures (Welsh and

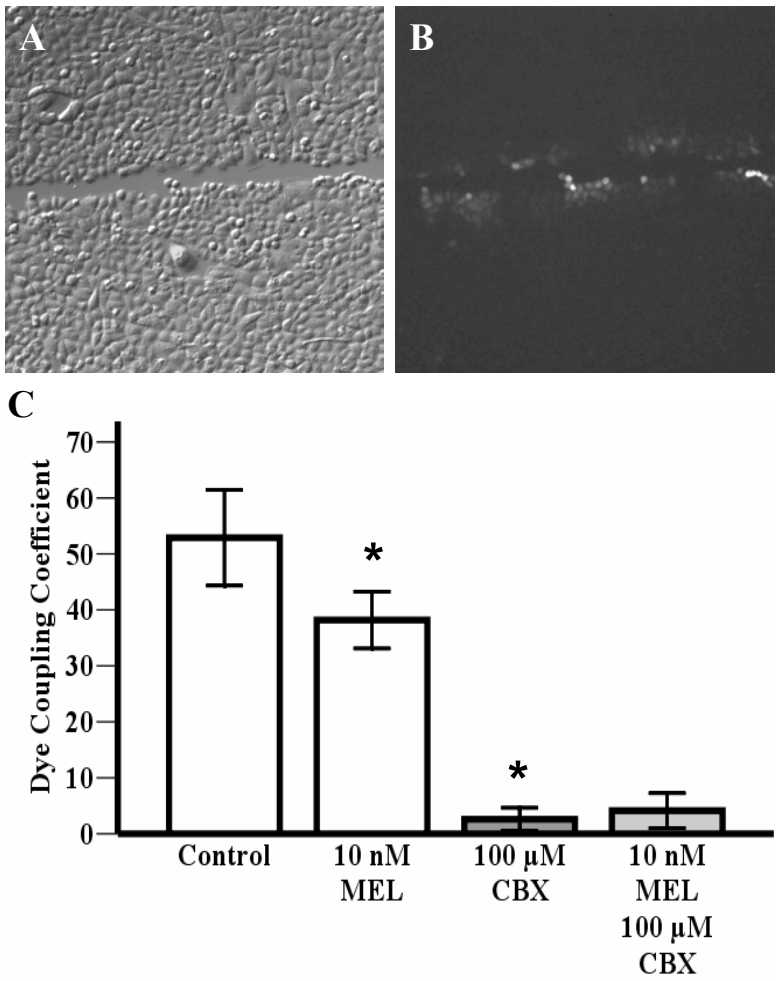

Figure 2. Melatonin decreases dye coupling among SCN2.2 cells. ***A***, A representative DIC image of vehicle-treated cells showing a visible line of damage produced by the scrape loading procedure. ***B***, A representative image showing the fluorescence of cells containing the dye lucifer yellow. The brightest cells along the damage line show the initial uptake of the dye. Fainter fluorescence of neighboring cells indicates dye transfer via gap junctions. Note the general lack of dye coupling among the majority of cells along the scrape. ***C***, Melatonin (10 nM) significantly reduced (*$p<0.05$, n=106) the average dye coupling by 28% compared with controls (n=30) as determined by calculating a dye coupling coefficient. Carbenoxolone (100 µM), a gap junctional blocker, significantly reduced (*$p<0.05$, n=17) dye coupling compared with controls. Error bars represent 95% confidence intervals.

Reppert, 1996). That is, only small clusters of cells along the scrape exhibited dye coupling with surrounding cells and many cells were not coupled to neighbors (Fig. 2*A*,*B*). Additional approaches were used to assess gap junctional communication, such as single cell injections with lucifer yellow and fluorescence recovery after photobleaching (FRAP). Both of these approaches confirmed the limited nature of dye coupling in these cultures (data not shown). Therefore, although specific cells in dye coupled clusters of SCN2.2 cells had coupling coefficients of approximately 50%, global gap junctional communication was low in these cultures. Nonetheless, melatonin significantly reduced this coupling when present.

Effects of melatonin on calcium waves

Contrary to gap junctional coupling, paracrine communication (i.e., monitored by the propagation of intercellular calcium waves) among SCN2.2 cells was robust (Fig. 3*A*-*D*). Gliotransmitter signaling was examined by mechanical stimulation of calcium waves and subsequent measurement of the areas of wave spread. Following wave activation in SCN2.2 cells, the average calcium wave area was not significantly different among control and melatonin-treated (10 nM) cultures (Fig. 3*E*). On average, melatonin had little effect on the magnitude of calcium wave propagation, as it enhanced the spread of some waves and reduced the spread of others, often in the same culture well. This large variation in maximal area of wave spread within individual cultures (and consequently the large error in the data set; Fig. 3*E*), indicated that the source of this variation was not differences between cell culture passages. In previous studies of chick diencephalic astrocytes (Peters et al., 2005a) and mouse hypothalamic astrocytes (Peters et al., 2005b), melatonin potentiated intercellular calcium waves. The lack of significant calcium wave modulation by melatonin in rat SCN2.2 cells and the highly variable nature of wave propagation may have resulted from a lack of synchrony in these cell cultures. The mammalian SCN is known to be a composite of independent oscillatory cells that, when synchronized, exhibit rhythms in gene expression, metabolism, and other physiological parameters such as neuronal activity patterns. Therefore, variation in

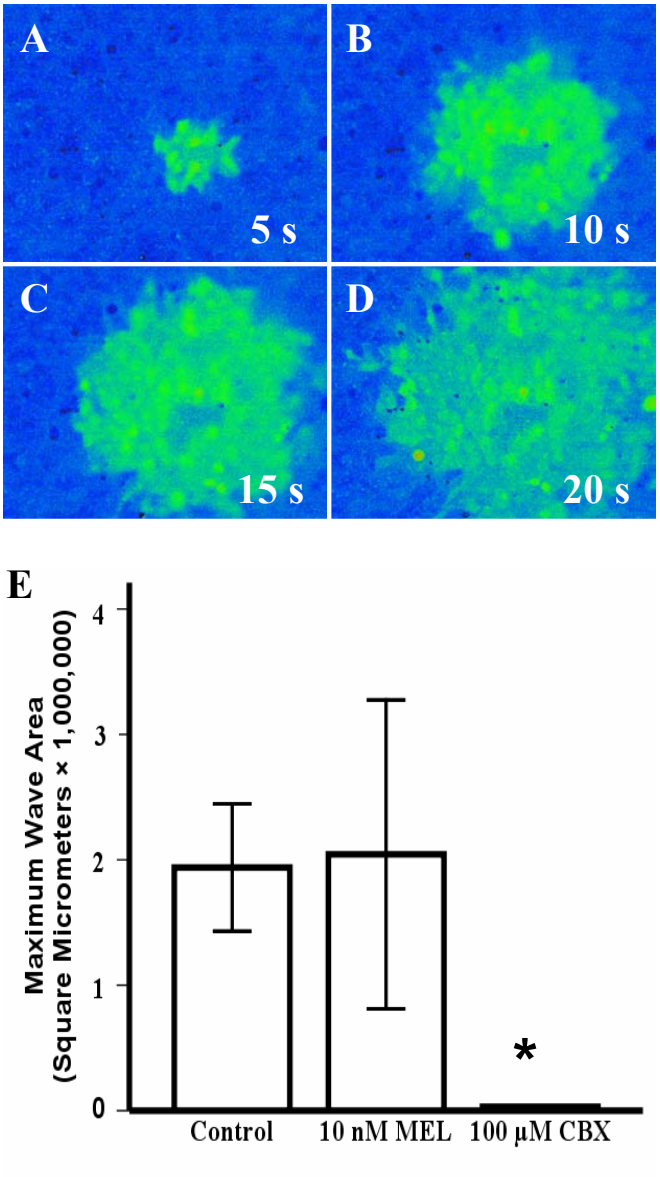

Figure 3. Melatonin does not affect the spread of calcium waves among SCN2.2 cells. ***A-D***, Representative calcium wave elicited by mechanical stimulation of SCN2.2 cells with a glass micropipette. Stimulation produced an increase in intracellular calcium in the stimulated cell followed by subsequent increases in intracellular calcium levels in surrounding cells. The use of a calcium sensitive probe, Fura-2 AM (8 µM), allowed the spread of the wave to be monitored using ratiometric calcium imaging. The spread of a calcium wave in a vehicle-treated (control) culture was monitored over a 20 second time period. ***E***, The average calcium wave area was not significantly different among control (n=12) and melatonin-treated (10 nM; n=11) cultures. Carbenoxolone (100 µM; n=12), a gap junctional blocker, completely blocked the spread of calcium waves. Error bars represent 95% confidence intervals.

calcium wave propagation, a physiological event that likely requires coherent cellular activities, will confound analysis unless controlled by experimental culture condition.

To test whether gap junctional coupling was a requirement for calcium wave propagation, cells were treated as before with the gap junctional blocker, carbenoxolone (CBX). Interestingly, carbenoxolone (100 μM) completely blocked the spread of calcium waves in SCN2.2 cultures (Fig 3*E*). This data indicates that functional gap junctions might be critical for calcium wave generation. However, carbenoxolone like many uncoupling agents can affect changes in multiple membrane channels and receptors, as well as gap junctions. For example, CBX can still exert an effect on purinergic signaling pathways even when astrocytic gap junctions are absent (Cx43 knockout mice) or have been pharmacologically rendered nonfunctional (Rouach et al., 2003). Moreover, CBX has been shown to affect neuronal membrane properties (e.g., reductions in firing properties and input resistance) in respiratory neurons of the preBötzinger complex (Rekling et al., 2000). Therefore, our CBX results demonstrate that functional, membrane-based cell-to-cell signaling is necessary for calcium wave propagation in SCN2.2 cells, but whether or not gap junctional coupling is necessary for this intercellular signaling, remains unclear.

Melatonin reduced resting calcium levels

SCN2.2 cells possessed an average resting cytosolic calcium concentration of 48 ± 2.5 nM under control conditions, as estimated by ratiometric image analysis with the calcium indicator Fura-2 AM (Fig. 4). Melatonin (10 nM) significantly reduced the average resting calcium level by 20% compared to control (vehicle-treated) cultures. When the cells were treated with carbenoxolone (100 μM), a significant increase (42%) in the average resting cytosolic calcium concentration was observed as compared to controls.

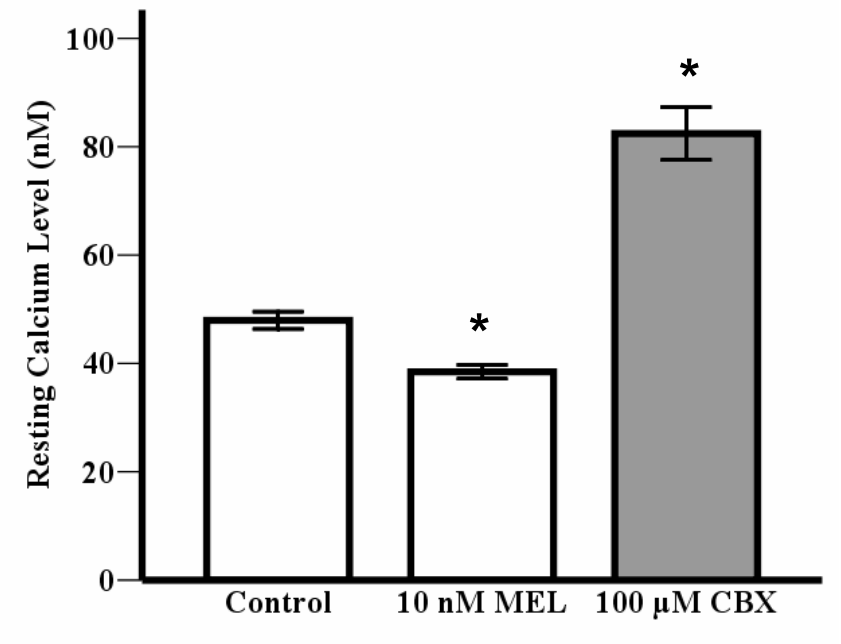

Figure 4. Melatonin reduced the resting cytosolic calcium concentration in SCN2.2 cells. At physiological concentrations (10 nM), melatonin significantly reduced (*$p<0.05$, n=11) the average resting calcium level by 20% compared with the control (n=12) as estimated by ratiometric calcium image analysis with the calcium-sensitive indicator Fura-2 AM (8 µM). Carbenoxolone (100 µM), a gap junctional blocker, significantly enhanced (*$p<0.05$, n=12) the average resting calcium level by 42% over the average control concentration (48 ± 2.5 nM). Error bars represent 95% confidence intervals.

Extracellular adenosine triphosphate (ATP) influences the spread of calcium waves

The spread of intercellular calcium waves in astrocytes derived from cortical and hippocampal astrocytes are mediated by the gliotransmitter ATP. Likewise, in SCN2.2 cultures, calcium waves have recently been shown to be mediated by a purinergic, ATP-dependent signaling pathway (Burkeen and Zoran, 2006). In addition, extracellular ATP is released from SCN2.2 cells in a rhythmic and circadian fashion, with peak extracellular levels of ATP accumulation occurring about every 22-24 hours (Womac et al., 2006). I tested the hypothesis that this extracellular ATP influences the spread of calcium waves in SCN2.2 cells. To monitor and quantify the circadian release of ATP in SCN2.2 cultures, cells were manipulated and processed in a specific, thoroughly tested, and carefully controlled protocol. This approach involved the use of an entraining stimulus (see Materials and Methods) and passage onto glass-bottom dishes to coordinate individual cellular oscillations into ensemble rhythms, allowing SCN2.2 cells to acquire sustained and reproducible synchronization of ATP accumulation in vitro. A previously reported, and similar protocol, is known to induce synchronization of cellular events similar to those seen in the intact mammalian SCN (Allen et al., 2001).

Effects of ATP on calcium waves

The level of endogenous extracellular ATP, as determined by a luciferin-luciferase chemiluminescence assay, was not correlated with calcium wave area (Fig. 5). Since SCN2.2 cultures display peaks in extracellular ATP content with approximate 24h rhythmicity, a circadian phase can be established and multiple peaks and troughs can be targeted to examine purinergic signaling (i.e., calcium waves) at given times over the course of multiple days. Initial experiments targeted a trough at 20h and a peak at 32h. No correlation between the level of extracellular ATP and the area of the calcium wave spread was detected (Fig. 6*A*,*C*). Nevertheless, a robust difference in accumulated ATP was present between time points. In these experiments, the entraining stimulus used to coordinate individual cellular oscillations consisted of a combination of: (1) a serum

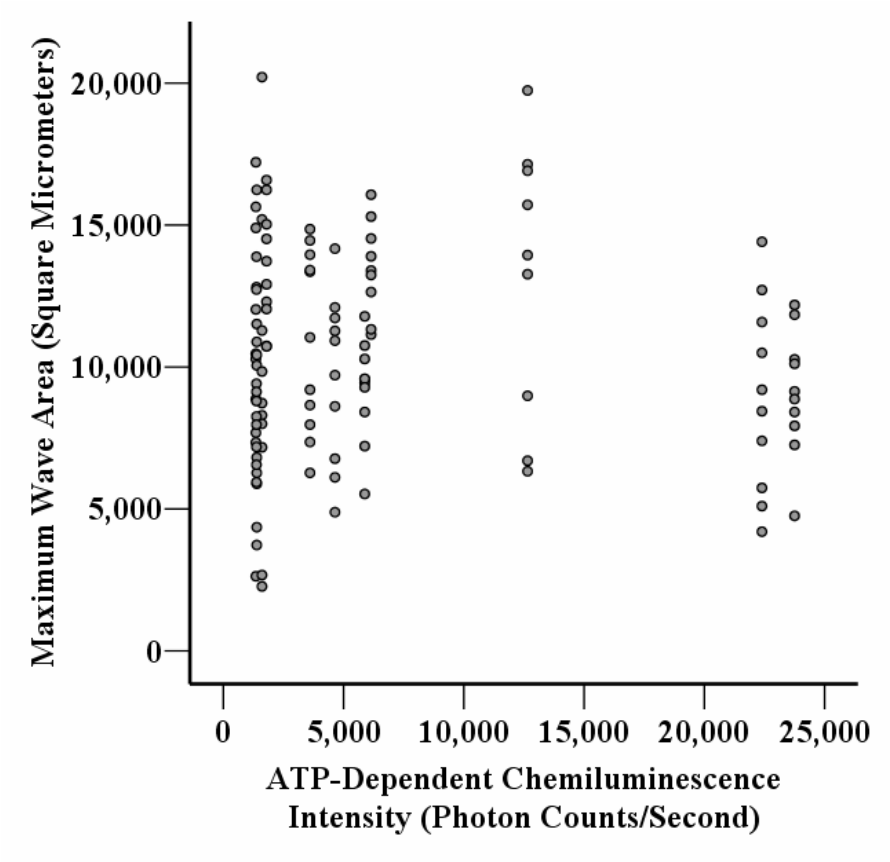

Figure 5. The level of endogenously accumulated extracellular ATP had no correlation to the calcium wave area. A scatter plot of the maximum area of wave spread (Pearson correlation = -0.081, n=120) is shown with the accompanying extracellular ATP content found in the well in which the calcium wave was elicited. Extracellular ATP levels were determined by luciferin-luciferase chemiluminescence assay.

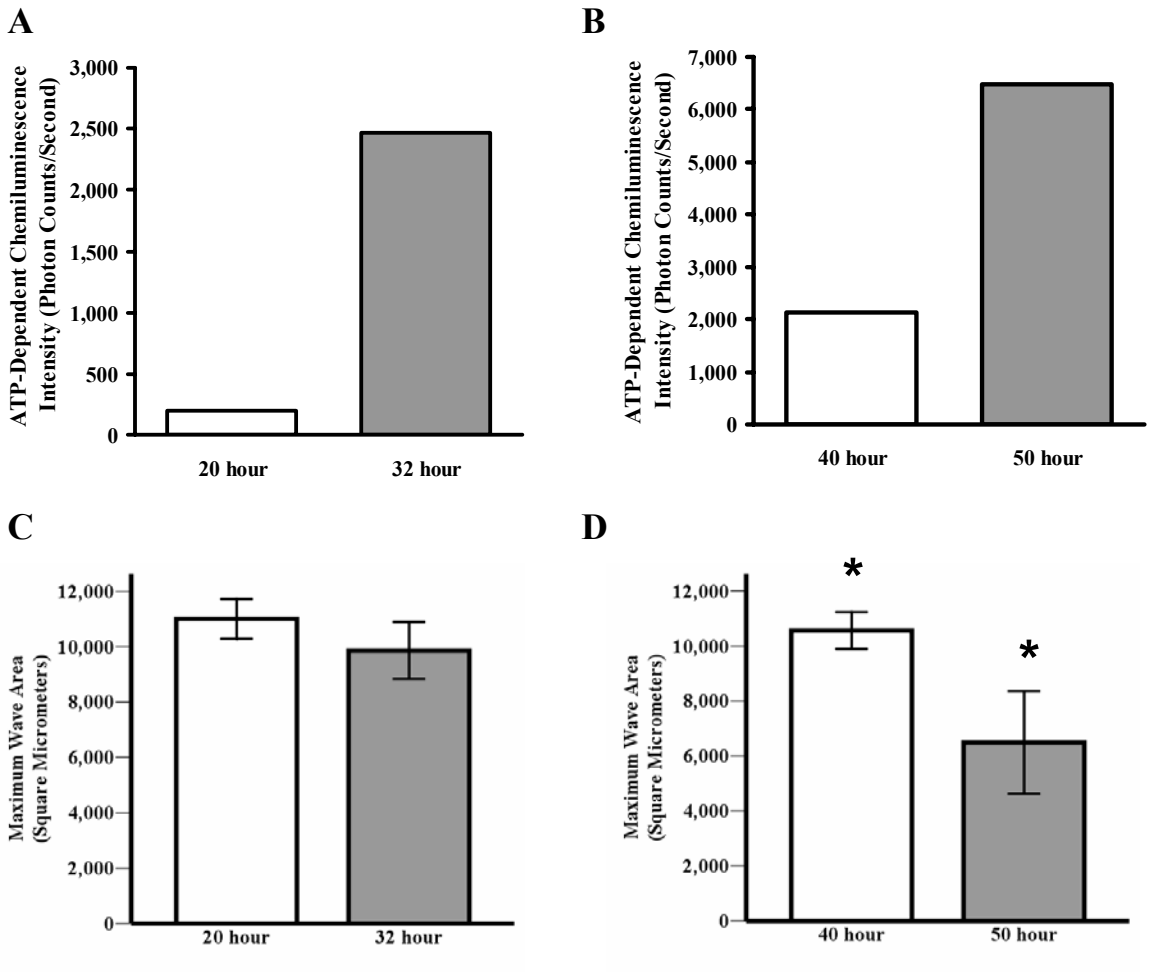

Figure 6. Endogenous extracellular ATP accumulation and calcium wave propagation are rhythmic and inversely correlated. ***A***, ***B***, Endogenous levels of extracellular ATP at two targeted troughs (20h, 40h; shown in white) and two targeted peaks (32h, 50h; shown in grey), as determined by luciferin-luciferase chemiluminescence assay, were different, with higher levels at 32h and 50h time points. The levels of extracellular ATP were consistently higher at targeted peaks. Each bar displays the level of ATP in a single representative well. ***C***, ***D***, The average area of wave spread across all wells was not significantly different between the 20h and 32h time points. In contrast, the average area of wave spread was reduced significantly (*$p<0.05$, $n=10$) by 39% at the 50h time point compared to the 40h time point. The SFCF protocol was used for the 20h and 32h time points in ***A*** and ***C***, where as the SR protocol was used for the 40h and 50h time points in ***B*** and ***D***. Error bars represent 95% confidence intervals.

reduction followed by (2) a forskolin-induced activation of cAMP signaling, and subsequently media exchanges (cell washes) using (3) a calcium-magnesium free buffer (CMF). Upon final establishment of the cell cultures in neurobasal medium, the SCN2.2 cells were considered for time series analysis to be at time 0 (T0). We have defined this three-part entraining protocol as the SFCF treatment, for serum reduction and forskolin-activation and calcium-free medium. Figure 6 shows the level of endogenous extracellular ATP at a targeted time point from a representative culture and the corresponding average wave areas for calcium waves stimulated in that well. The media samples from the representative wells shown had similar extracellular ATP values to other wells sampled at the same time point in this experiment.

One caveat of these experiments was the difficulty in targeting troughs and peaks of ATP accumulation using the SFCF protocol, which may have been due to cells not adhering as well to the glass dishes as they had to the plastic dishes used in our previous studies. Therefore, the stimulation protocol was changed to allow the cells to grow on glass for at least 24h in the presence of serum-containing medium, thus providing better cell adhesion to the culture dishes. The new protocol involved plating the cells to glass dishes and retaining 5% FBS in the culture medium for the first 24h on glass. In addition, previous experiments demonstrated that exposure to a low Ca^{2+} medium caused an immediate and substantial increase in the level of extracellular ATP in SCN2.2 cells (data not shown). Also, a clear rhythm in extracellular ATP accumulation was evident in cultures not treated with FSK (data not shown). Therefore, the forskolin treatment and CMF washes were removed and we termed this treatment, simply, the serum reduction or SR protocol. Thus, the serum reduction was performed exactly 24h after the initial plating to glass dishes and this plating event was defined as time 0 (T0). Figure 6*B* and *D* shows an experiment performed with the SR protocol. A robust trough (40h) and peak (50h) in extracellular ATP was again determined by chemiluminescence assay. However, a significant difference in calcium wave propagation was now obvious between the peak and trough time points. The level of extracellular ATP varied across multiple experiments (i.e., cell culture wells) due to the nature of the circadian

oscillations in extracellular ATP seen in these SCN2.2 cultures. Our lab has demonstrated that ATP rhythms involve sharp peaks in ATP accumulation, lasting often for only 2-4h (Womac et al., 2006), thereby making the absolute peak in ATP levels difficult to target in single time point experimental protocols. Note that the level of endogenous extracellular ATP in representative wells was higher at both the 40h and 50h time points using the SR protocol, as compared to the 20h and 32h time points using the SFCF protocol. Nonetheless, the 32h and 50h (peak) time points had higher levels of ATP accumulation than the 20h and 40h (trough) samples. Thus, the level of extracellular ATP accumulation differed in magnitude between the trough and peak time points; however, significant differences in wave propagation were only present using the SR protocol at the 40h and 50h time points. Interestingly, the average area of calcium wave spread was reduced significantly by 39% at the 50h (peak ATP) time point compared to the 40h (trough ATP) time point. Therefore, although no correlation between the endogenous level of extracellular ATP accumulation and calcium wave area was found in the original SFCF induction experiments, the SR protocol led to the resolution of the difference in both ATP accumulation and calcium wave magnitude between time points. In addition, there was an inverse relationship between the levels of endogenous extracellular ATP and area of calcium wave spread. It remains to be determined whether this relationship is causative or merely correlative.

Effects of melatonin are circadian phase dependent

Previously, melatonin had no effect on calcium wave signaling among SCN2.2 cells (Fig. 3*E*) where rhythmicity and timing of culture treatments were not controlled. However, melatonin may have an effect on calcium wave signaling if the SCN2.2 cultures express a metabolic rhythmicity. For example, a combination of glucose utilization and ATP accumulation may create an underlying metabolic rhythm to modulate intercellular communication differentially, depending on the time of day and whether or not melatonin is present. Therefore, I tested the idea that rhythmic metabolic processes, particularly rhythmic accumulation of extracellular ATP, regulate

physiological responses of SCN cells to melatonin. Melatonin (10 nM) affected the spread of calcium waves at an endogenous trough in the extracellular ATP rhythm (Fig. 7*A*), but had no effect on calcium wave spread at the peak time point in the ATP rhythm (Fig. 7*B*). Different concentrations of melatonin were tested at the trough time point (40h), where both 10 pM and 10 nM melatonin significantly reduced calcium wave area (Fig. 8). Luzindole (100 nM or 10 μM), a competitive Mel_{1b} melatonin receptor antagonist, did not compete away the effect of melatonin on calcium wave spread among SCN2.2 cells.

Interactions between ATP and melatonin signaling in SCN2.2 cells

Applying exogenous extracellular ATP at a physiological concentration (10 nM) reduced the average calcium wave area at both the endogenous trough (40h) and peak (50h) in the extracellular ATP rhythm (Fig. 7*A*,*B*). Exogenously applied ATP significantly reduced the average calcium wave area by 27% at the trough and by 36% at the peak, compared to the average control wave area for each time point. Surprisingly, although both melatonin and exogenous ATP independently reduced the spread of calcium waves at the trough time point (40h), they did not have an additive effect on calcium wave area when combined. For example, the combination of 10 nM ATP and 10 nM melatonin at 40h had essentially the same effect (i.e., a 32% reduction) on the spread of calcium waves as ATP and melatonin alone (27% and 31% reductions, respectively). Interestingly, melatonin blocked the effect of exogenously applied ATP at the endogenous peak of ATP accumulation (Fig. 7*B*). The combination of 10 nM ATP and 10 nM melatonin at 50h had no effect, but ATP alone significantly reduced the area of wave spread at this time point. Therefore, a dynamic interaction between melatonin and ATP is evident, at least with regard to the spread of calcium waves.

Melatonin affects extracellular ATP accumulation

The previous experiments demonstrated that melatonin disrupts the effect of exogenous ATP to reduce the spread of calcium waves. This interaction between melatonin and

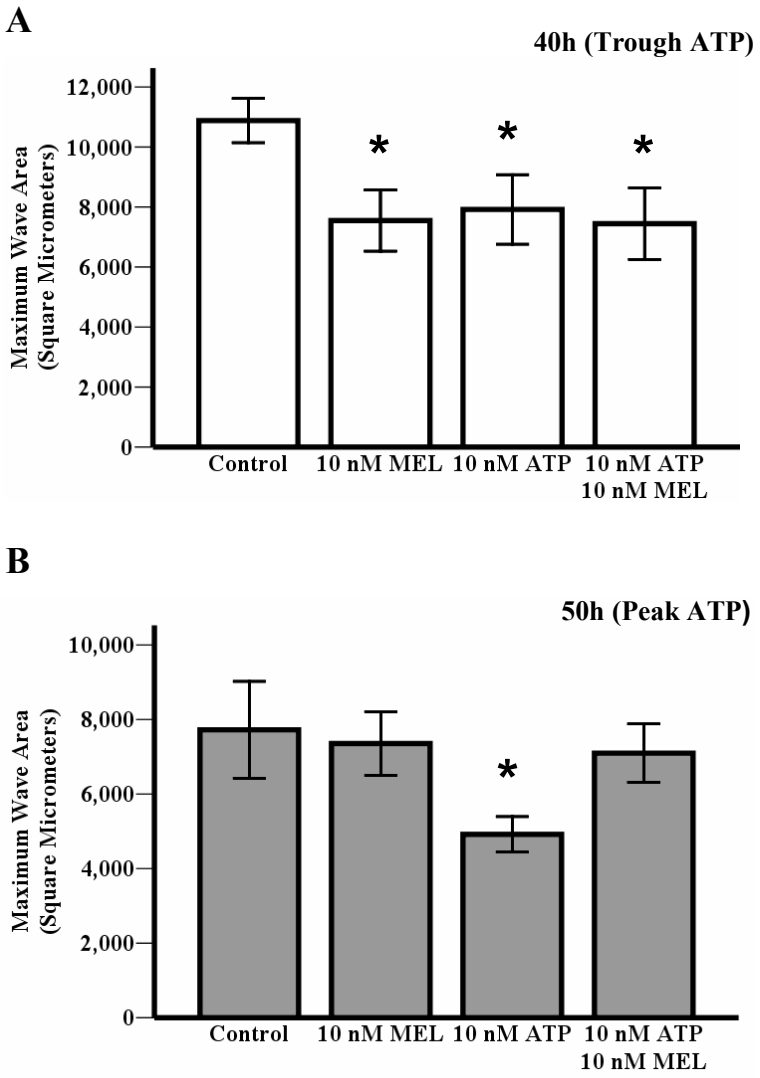

Figure 7. In SCN2.2 cells, melatonin reduced the spread of calcium waves only at the 40h trough in extracellular ATP accumulation; while exogenous ATP applied to the culture medium reduced calcium wave spread at both the trough and peak in the extracellular ATP rhythm. ***A***, Calcium wave spread at the trough (40h) of the endogenous extracellular ATP rhythm. Melatonin (10 nM) significantly reduced (*$p<0.05$, n=20) the average calcium wave area by 31% compared to the average control wave area following vehicle application. Exogenously applied ATP (10 nM) significantly reduced (*$p<0.05$, n=19) the average calcium wave area by 27%, compared to control waves. A combination of both 10 nM ATP and 10 nM melatonin also produced a significant reduction (*$p<0.05$, n=20) in wave area. ***B***, Melatonin (10nM, n=19) had no effect on calcium wave spread at the peak (50h) of the endogenous extracellular ATP rhythm. However, exogenously applied ATP (10 nM) significantly reduced (*$p<0.05$, n=20) the calcium wave area by 36%, compared to control waves. A combination of ATP and melatonin at this time point had no effect on calcium wave propagation.

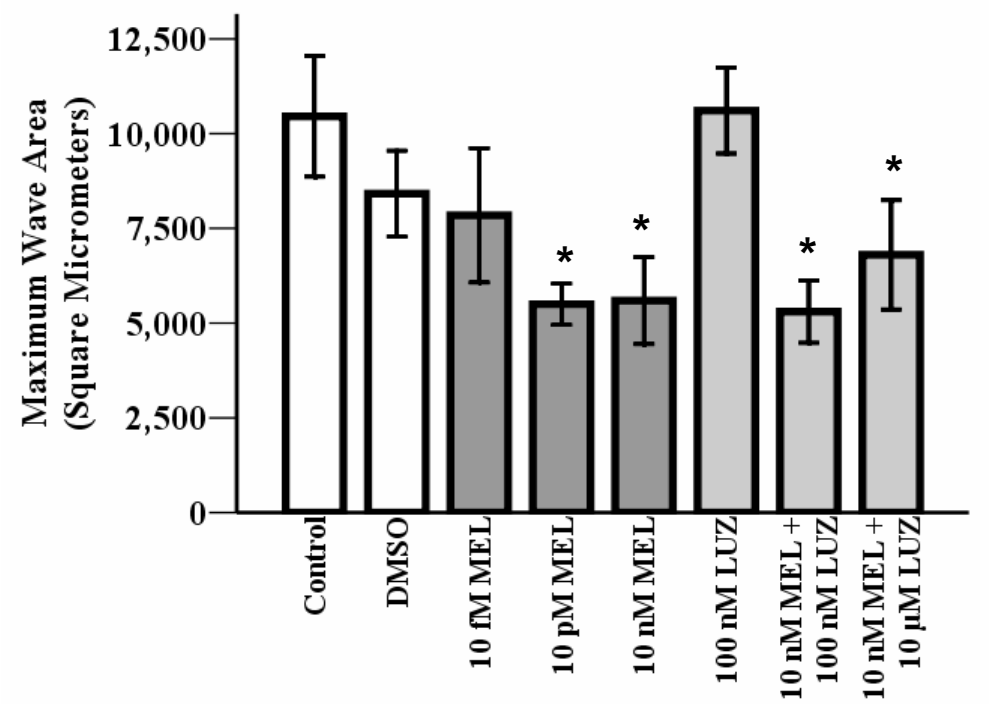

Figure 8. Melatonin dose-dependence at endogenous trough in extracellular ATP. Both 10 pM and 10 nM melatonin significantly reduced (*$p<0.05$, n=10) the average area of calcium wave spread compared to controls (n=10). A combination of melatonin (10 nM) and luzindole (100 nM or 10 µM), a competitive melatonin receptor antagonist, significantly reduced (*$p<0.05$, n=10) the average area of calcium wave spread. Error bars represent 95% confidence intervals.

ATP signaling led me to ask, and test, the following question. Does melatonin affect the levels of extracellular ATP accumulation in SCN2.2 cultures? Melatonin treatment (10 nM) caused a 61% reduction in the level of extracellular ATP at the high ATP time point (Fig. 9*A*), the same time point where it disrupted the effects of exogenously applied ATP. The average area of calcium waves elicited in these cultures was enhanced by approximately 33% compared to wave areas in melatonin treated cultures at the low ATP time point (Fig. 9*B*). Therefore, melatonin treatment produced a reduction in the level of extracellular ATP and lead to a significant enhancement in the propagation of calcium waves in SCN2.2 cell cultures. In contrast, melatonin treatment had no effect on the spread of calcium waves in cultures containing low ATP, where it, in fact, increased ATP levels. Therefore, melatonin can switch polarity in cellular physiological responses, at least with regard to the way it modulates calcium signaling in SCN2.2 cultures, and depending on the state of the endogenous metabolic rhythm, specifically cycles of extracelluar ATP accumulation.

ATP and 2-DG uptake

SCN2.2 cells exhibit circadian rhythmicity in many properties including their extracellular ATP content (Womac et al., 2006) and 2-DG uptake (Earnest et al., 1999b). Specifically, SCN2.2 cell cultures express a circadian rhythm in 2-deoxyglucose cellular uptake and confer this rhythmicity to cocultured fibroblasts. This metabolic rhythmicity likely functions to modulate cellular physiology of the SCN during an appropriate time of day and night. A future goal of our lab is to determine if these two biological rhythms are in phase with each other. In a preliminary study, I determined whether these two rhythms (2-DG uptake and ATP accumulation) possess similar phase relationships. That is, is glucose uptake high when the level of endogenous extracellular ATP is high? To answer this question, the rhythm in 2-DG uptake, monitored as intracellular accumulation of the fluorescent glucose analog 2-NBDG, in SCN2.2 cells was examined at the same trough and peak time points previously demonstrated for the SR protocol. Glucose uptake was 11 % higher when endogenous extracellular ATP levels were at

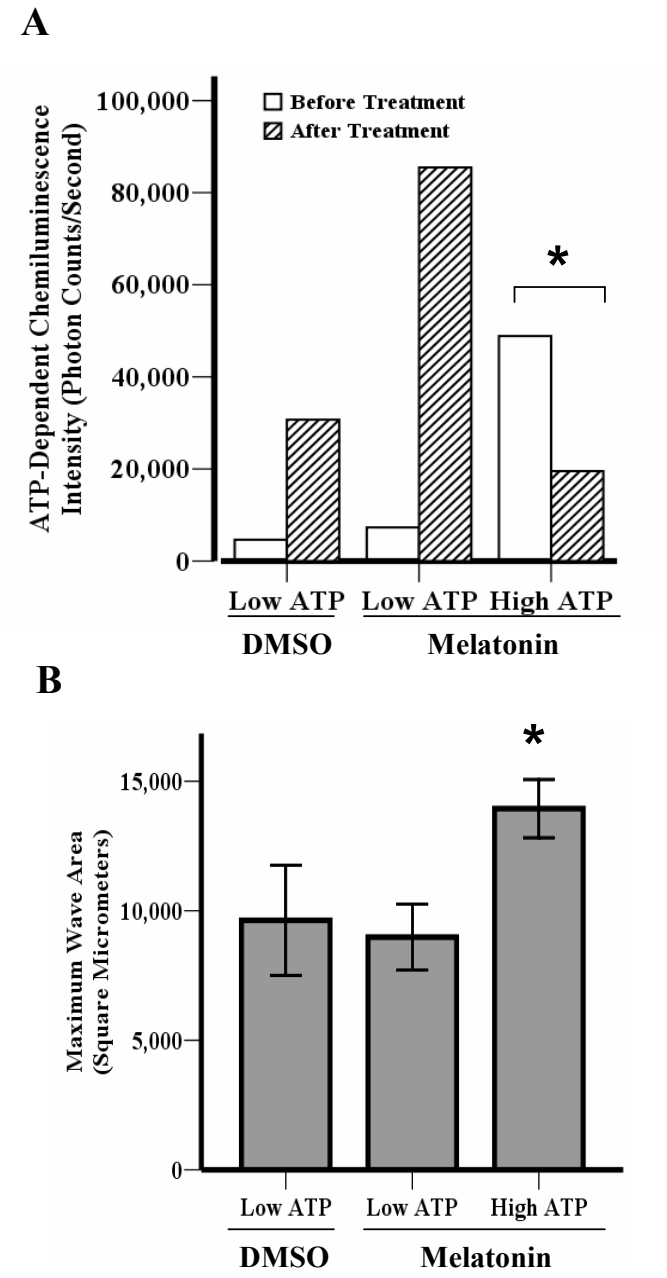

Figure 9. Extracellular ATP affects melatonin-induced changes in wave spread. ***A***, Endogenous levels of extracellular ATP before and after SCN2.2 cell cultures were treated with a physiolocial concentration of melatonin (10 nM) or DMSO (control) for 1h. ATP levels from three representative wells are shown, as determined by luciferin-luciferase chemiluminescence assay. Melatonin treatment caused a 61% reduction in the level of extracellular ATP in the well labeled "High ATP." ***B***, Calcium wave areas for waves elicited in the three corresponding wells shown above in ***A***. The average wave area for the well labeled "High ATP" was significantly increased (*$p<0.05$, $n=10$) by approximately 33% compared to the average control ($n=10$) wave area. Melatonin treatment had no effect on the spread of calcium waves in cultures containing low ATP. Error bars represent 95% confidence intervals.

their peak at the 50h time point, as compared to the 40h trough in ATP (Fig. 10). Therefore, the oscillations in 2-DG uptake and extracellular ATP accumulation appear to correspond in SCN2.2 cell cultures. However, only one protocol was performed that consisted of a 30 minute staining (i.e., 2-NBDG treatment) procedure at two time points. Therefore, at this time, we have only established a starting point for an effective protocol. In the future, the protocol may be modified to: 1) have a shorter staining period to avoid disrupting cellular metabolism, and 2) decrease the non-labeled glucose in the medium instead of completely removing the non-labeled glucose from the medium. Nonetheless, the rhythms in glucose uptake and extracellular ATP accumulation may indicate the presence of a fundamental metabolic rhythm in SCN2.2 cultures, which will need to be examined more thoroughly in the future.

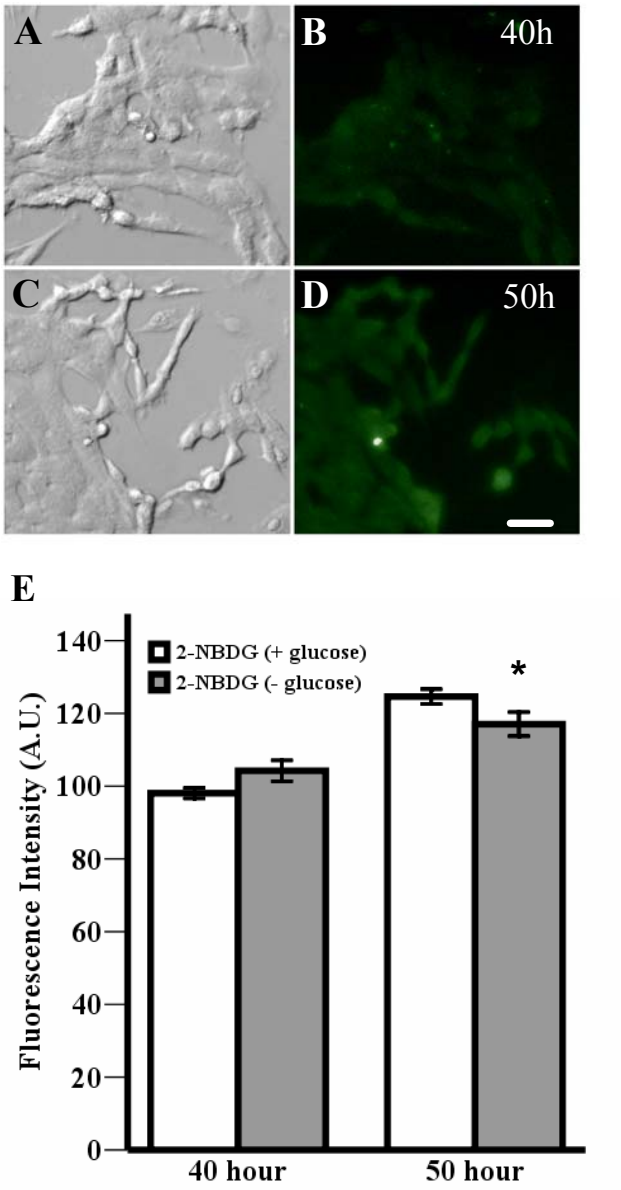

Figure 10. 2-Deoxyglucose (2DG) uptake in SCN2.2 cells was higher during the peak in extracellular ATP accumulation. ***A***,***C***, Representative DIC images of SCN2.2 cultures at low cell density. ***B***,***D***, Representative images showing fluorescence intensity of 2-NBDG uptake in SCN2.2 cells (corresponding to cells in ***A*** and ***C***) at 40h and 50h time points. Bar equals 20 µm (applies to all panels). ***E***, The average 2-NBDG uptake in SCN2.2 cells without glucose in the culture medium (- glucose) was significantly increased (*$p<0.05$, n=12), by 11%, at the 50h peak in the ATP rhythm compared to the 40h trough (n=12). The average 2-NBDG uptake in SCN2.2 cells with glucose in their medium (+ glucose) was significantly greater (*$p<0.05$, n=12), by 22%, at the 50h peak in ATP accumulation compared to the 40h trough (n=12). Error bars represent 95% confidence intervals.

CHAPTER IV
CONCLUSIONS

The neurohormone melatonin modulated cell-to-cell signaling, both dye coupling (gap junctional communication) and calcium waves (ATP-dependent gliotransmission) in SCN2.2 cells, a cell line derived from the rat SCN. The cellular physiology regulating how these cells communicate through gap junctions and ATP-dependent gliotransmission and how that communication might be regulated by melatonin will be discussed in the context of mammalian biological clock function.

Melatonin regulates gap junctional communication

Evidence of gap junctional communication in the suprachiasmatic nucleus of rats has been well established (Batter et al., 1992; Welsh and Reppert, 1996; Blomstrand et al., 1999). In vitro (Batter et al., 1992) and in vivo (Yamamoto et al., 1990a,b) studies demonstrate that gap junction expression is different, among astrocytes from one brain region and between astrocytes derived from different brain regions. These brain-region specific differences in coupling have been suggested to promote functional specialization within the central nervous system. For instance, approximately 30% of hypothalamic astrocytes in the rat brain form functional junctions with their neighbors, whereas striatal astrocytes are less extensively coupled (Batter et al., 1992). Gap junctional coupling strengths are ranked as follows: hippocampus = hypothalamus > cerebral cortex = brain stem (Blomstrand et al., 1999). Although gap junctional coupling is higher in the hypothalamus compared to other brain regions, surprisingly, global gap junctional communication (dye coupling) was low in SCN2.2 cultures (Fig. 2*B*). Multiple approaches were used to assess gap junctional communication, such as extensive scrape loading experiments and single cell injections with lucifer yellow, as well as FRAP analyses. Each of these approaches confirmed the limited nature of dye coupling in these cultures. This apparent contradiction may represent an artifactual restriction in gap junctional coupling between SCN2.2 cells, perhaps due to

consequences of their immortalization or their specific cell culture conditions. Alternatively, coupling among astrocytes within the SCN may not be typical of coupling seen in the hypothalamus in general. In fact, quantitative analyses of gap junctional coupling among rat SCN astrocytes in vivo have not been conducted. Still, low levels of dye coupling in these SCN2.2 cultures does not abrogate the importance of gap junctional communication in the SCN, which may be critical for neuronal cell-cell communication in restricted populations of glial cells, and thus for general SCN cellular function. Colwell (2000) has suggested that gap junctions may provide a mechanism to functionally couple distinct SCN cell populations. Individual SCN neurons express independently phased circadian rhythms (Welsh and Reppert, 1996) and thus are considered single cell circadian oscillators. Considerable interest in the cellular mechanisms underlying coupling of independent oscillators has driven recent research in the SCN (Hastings and Herzog, 2004) and clocks fields (Bell-Pedersen et al., 2005). Consequently, gap junctions may provide a signaling pathway for individual cells to form a linked cellular ensemble and functional timing system in the SCN.

Melatonin has been shown to modulate gap junctional connectivity in chick diencephalic glial cell cultures (Peters et al., 2005a). In agreement with these studies, I have demonstrated here that nanomolar concentrations of melatonin, a physiological level present in the circulatory systems of birds and mammals during the night (Cassone and Menaker, 1983; Cassone et al., 1986; Pang and Ralph, 1975), reduced dye coupling (gap junctional communication) in SCN2.2 cells. These data showing melatonin-mediated reductions in SCN cell dye coupling make sense because: 1) melatonin levels are high at night in all animal species previously examined, regardless of whether they are diurnally or nocturnally active (Simonneaux and Ribeleyga, 2003), and 2) SCN neurons are electrically silent and minimally dye coupled during the night (Colwell, 2000). Thus, a causal relationship may exist whereby melatonin levels rise during the hours of darkness to reduce gap junctional communication in the SCN and create a "nighttime" mode of communication within the mammalian brain. Thus, the current

studies of SCN2.2 astrocytic coupling suggest that nighttime reductions in coupling may be a fundamental feature of both SCN neuronal and glial cell circadian physiology.

The SCN allows the mammalian body to anticipate and prepare for night- and day-specific demands. The SCN's ability to communicate in two ways depending on whether it is daytime or nighttime may provide functionally different information to peripheral targets. Consider the following scenario in the context of astrocytic communication within the rat SCN. The current data, and that of Peters et al. (2005a), suggest that gap junctional coupling is more important to SCN astrocytes during the day. Perhaps this is because SCN neurons at this time exhibit high levels of synchronous neural activity (Colwell, 2000). If SCN neurons are synchronized, then it may be beneficial for nearby astrocytes, which are supplying these neurons with nutrients and buffering transmitter and ionic level in the extracellular fluid, to also be synchronized via gap junctional connectivity. Furthermore, gap junctional communication between astrocytes, and between astrocytes and neurons, may contribute to the generation of this enhanced synchronous activity in the first place. The tripartite synapse hypothesis suggests that a synaptic astrocyte listens to neuronal signaling with appropriate receptors and responds by modulating neurotransmission at that synapse and at those of other synapses in the surrounding area (Volterra et al., 2002a). Thus, melatonin and its reduction of astrocytic gap junctional coupling may be dynamically involved in determining the coupling state of SCN cells, thereby influencing the physiological state of the master biological clock.

Melatonin regulates gliotransmission

One premise of the tripartite synapse hypothesis is that purinergic signaling between synaptic partners, and the astrocytes that surround them, is critical to the modulaton of mammalian neural intregration. Here, I have demonstrated that melatonin also influences ATP-dependent gliotransmission in SCN hypothalamic astrocytes. ATP is known to mediate glial calcium waves in astrocytes derived from various brain regions such as the cortex (Guthrie et al., 1999) and cerebellum (Kirischuk et al., 1995).

Purinergic cell-cell signaling, in the form of calcium waves, occurred in SCN2.2 cells, and these calcium waves were ATP-dependent, as the addition of apyrase, an ATP diphosphohydrolase, to the cells completely abolished calcium wave propagation (Burkeen and Zoran, 2006). Purinergic signaling is critical to neural function because ATP released from astrocytes accumulates as extracellular adenosine, which activates receptors in the hypothalamus to regulate sleep behavior in mammals (Scammell et al., 2001), and regulates synaptic transmission and neural integration in the hippocampus (Pascual et al., 2005). Melatonin, which is high at night, has been shown to modulate ATP-mediated gliotransmission in diurnal mammals (Peters et al., 2005a). I have demonstrated here that melatonin affects the nature of ATP-dependent gliotransmission in SCN2.2 cells. Moreover, we predict that melatonin's effect on astrocytes is an MT_1 mediated modulation since luzindole, primarily an MT_2 antagonist (Dubocovich et al., 1998), did not block the effect of melatonin. However, more pharmacology will be needed to test this idea. In contrast to the effect seen in a diurnal chicken, melatonin suppressed gliotransmission (e.g., calcium waves) in these cells derived from the SCN of the nocturnal rat. Although, the previous studies with diurnal animals (e.g., birds) did not incorporate time elements into their studies, brain physiology, especially in the SCN of the diencephalon, is quite different during the daytime versus nighttime. In our studies, melatonin effects on gliotransmission were highly variable when timing of cultures was not carefully controlled. On the other hand, a clear and significant reduction of calcium waves occurred at specific time points in cycling (as determined by rhythms of ATP accumulation) SCN cultures. If the previous work with diurnal mammals holds true even when the cycling of the SCN is controlled for, then melatonin may affect the diurnal SCN of an animal with higher energy expenditure during the day differently than it affects the nocturnal SCN of an animal with higher energy expenditure at night. This differential modulation by melatonin may be necessary to properly regulate an animal's physiology, since melatonin levels peak at night in both diurnal and nocturnal animals.

Melatonin influences the relationship between gap junctional communication and gliotransmitter signaling

Adding to the complexity of melatonin's role in SCN physiology, melatonin appears to not only modulate gap junctional communication and gliotransmitter signaling separately, but it also has a dynamic effect on the relationship between gap junctional and chemical communication. For example, melatonin imposes a functional switch in the nature of intercellular communication among chick diencephalic astrocytes. Melatonin reduces Cx43 expression, yet enhances intercellular Ca^{2+} wave propagation (Peters et al., 2005a). Thus, ATP-mediated gliotransmission, in this study of chick astrocytes, was greater when gap junctional coupling was low. This data supports the idea of a "nighttime" versus "daytime" neural integration system within the hypothalamus. This data also raises a question as to whether or not gap junctions are needed for the spread of waves. For example, perhaps gap junctions are not as important to intercellular communication at night since melatonin acts to reduce gap junctional coupling.

I demonstrated that CBX, a gap junctional blocker, virtually abolished both dye coupling and Ca^{2+} wave propagation, while increasing resting intracellular calcium levels in SCN2.2 cultures. This increase in intracellular resting calcium may provide a clue into the mechanism of calcium wave suppression. At high doses of melatonin (μM), which caused a significant elevation in resting calcium levels in chick astrocytes, calcium waves were also abolished (M. Zoran, personal communication). Yet, caution must be used when interpreting the effects of CBX because this chemical does not exert its effect by working directly on gap junctions. Carbenoxolone works through some other unknown mechanism that may alter membrane physiology and many membrane-associated proteins. For example, CBX disrupts astrocytic gap junctional communication, yet can still exert its effect when astrocytic gap junctions are absent (Cx43 knockout mice) or nonfunctional (Rouach et al., 2003). In the future, development of pharmacological agents that target gap junctions more directly will be

useful in uncovering the underlying mechanisms involved in the daily rhythms of gap junctional and chemical communication in the SCN.

Extracellular ATP accumulation regulates gliotransmission

The present studies indicated that another important component of gliotransmitter signaling (calcium wave propagation) is the daily rhythm in the accumulation of extracellular ATP in the SCN. However, a discussion of extracellular ATP accumulation must be preceded by an understanding of the cell culture protocol that must be employed to synchronize cycling cell cultures, a procedure that reveals the rhythm in ATP accumulation in the first place. Since individual SCN cells act as single cell circadian oscillators (Hastings and Herzog, 2004), cell culture manipulations are needed to coordinate individual cellular oscillations into ensemble rhythms. Our lab developed a protocol known as the SR protocol to monitor and quantify the circadian release of ATP in SCN2.2 cultures. Cells were manipulated and processed in a specific, thoroughly tested, and carefully controlled protocol (see Material and Methods). This approach involved the use of an entraining stimulus to coordinate individual cellular oscillations allowing SCN2.2 cells to acquire sustained and reproducible synchronization of ATP accumulation in vitro. Most notably, the timing of media exchanges must be precise (e.g., exactly 24h) and exposure to low Ca^{2+} medium must be avoided to circumvent randomness of ATP accumulation in cultures. Consequently, I found that extracellular ATP accumulation is correlated with significant rhythms in gliotransmission (calcium waves) in SCN2.2 cells and that this relationship may be more than correlative. When endogenous levels of extracellular ATP were higher (50 hour time point), calcium waves were smaller (Fig. 6). Yet, exogenously applied ATP (i.e., higher ATP levels) reduced the spread of calcium waves at both the endogenous trough (40 hour time point) and peak (50 hour time point) in extracellular ATP accumulation. Therefore, exogenous ATP reduced the spread of calcium waves regardless of the time of application during the endogenous 24 hour ATP accumulation cycle.

How might ATP be regulating calcium waves? The current and conventional wisdom suggests that vesicular release of ATP mediates calcium-dependent purinergic signaling among astrocytes (Pangrsic et al., 2007). In this model of ATP-dependent gliotransmission, released and extracellularly accumulated ATP binds to P2Y receptors on neighboring cells. This P2Y receptor activation leads to activation of an IP_3–dependent signaling mechanism and the elevation of resting calcium levels via release from intracellular stores (Charles, 2005). Elevation of calcium levels elicits a calcium-dependent release of ATP, which in turn evokes Ca^{2+} responses in nearby astrocytes. Thus, the astrocytic calcium wave is propagated. My results suggest another component is involved in this pathway - an underlying rhythm in purinergic responsiveness. That is, clock outputs may be determining a state of responsiveness to ATP. I have demonstrated here that ATP and calcium waves are both rhythmic and inversely correlated in cycling SCN2.2 cultures (Fig. 11). Although a direct regulation of purinergic receptor gene expression by the clock is possible, or even likely since many ATP- and metabolism-associated genes are know to be rhythmic in both the rat SCN and in SCN2.2 cultures (Menger et al., 2005; Menger et al., 2007), a equally plausible explanation is that accumulating levels of extracellular ATP lead to the desensitization of purinergic receptors. This would create a state of reduced purinergic responsiveness at the peak of the ATP accumulation rhythm, when ATP-dependent calcium waves are reduced in magnitude. Further studies will be required to differentiate among multiple explanations for these results.

The role that ATP accumulation plays in SCN function remains unknown. However, in the central nervous system, work has demonstrated that ATP in extracellular fluids modulates neuronal activities including neurotransmission, endocrine/exocrine secretion, immune reactions, and inflammatory reactions (Dubyak and el-Moatassim, 1993; Bruns, 1990). Clock controlled release of ATP from astrocytes may feedback to cellular components of the SCN to further modulate the cellular release of ATP, or alter the state of the SCN cells' responsiveness to ATP.

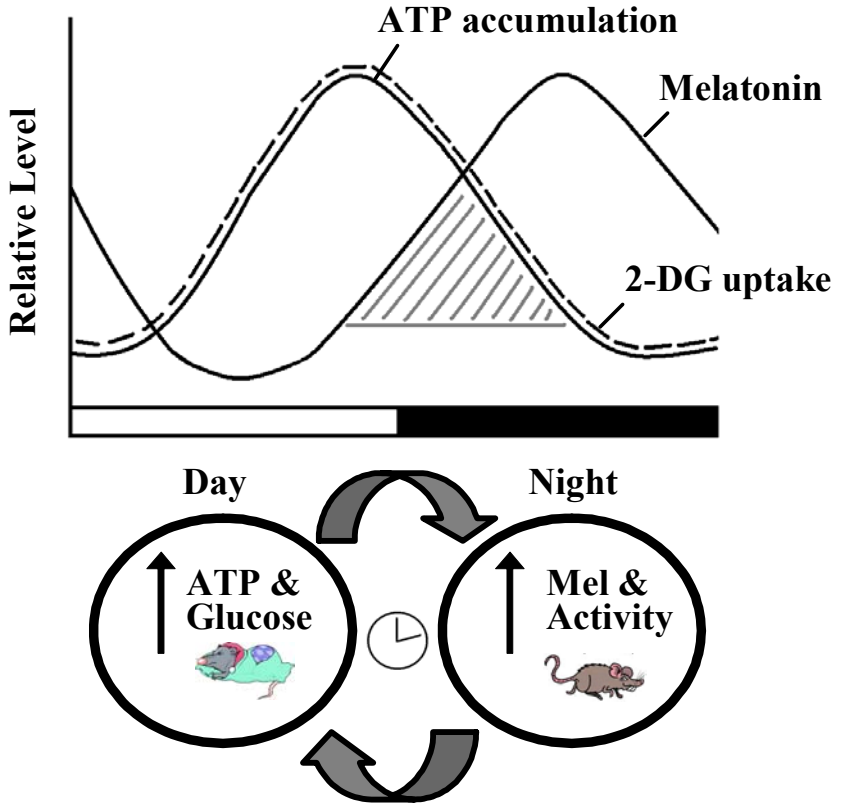

Figure 11. Proposed temporal relationship between melatonin signaling and ATP signaling in the rat SCN to produce precisely timed shifts in intercellular communication among SCN cells. The endogenous rhythms in melatonin production from the pineal gland and extracellular ATP accumulation within the SCN may combine to create windows of sensitivity (grey lines) when intercellular communication (e.g., calcium waves propagation and gap junctional communication) within the SCN can be functionally shifted to accommodate daytime versus nighttime physiological needs. For example, in the hours prior to their daily nocturnal (Night, horizontal black bar) activity, rats experience clock-controlled changes in their body chemistry to prepare them for this upcoming active time period. During this time, glucose utilization (production and uptake) is peaking, extracellular ATP accumulation in the SCN is peaking, and melatonin levels are on the rise. We propose that this period of time is critical to shifting the way cells in the SCN communicate with each other. In this example, the SCN cells will then shift from a daytime state of cell-cell signaling (Day, horizontal white bar) to a nighttime mode of communication, which includes reduced calcium wave signaling (gliotransmitter signaling) due to the combined influence of low extracellular ATP levels and high circulating melatonin levels.

Melatonin and ATP signaling pathways interact to regulate gliotransmission

The present study demonstrates that melatonin and ATP interacted in the regulation of gliotransmission (calcium waves). Additionally, these interactions were specific to endogenous SCN rhythmicity. For example, melatonin affected the spread of calcium waves only at a trough in the endogenous rhythm in extracellular ATP accumulation (Fig. 7). Interestingly, melatonin and ATP did not have an additive effect on the modulation of calcium waves, but melatonin blocked the effect of supplemental ATP only at the endogenous peak in the rhythm of extracellular ATP accumulation. Thus, a complex interaction exists between melatonin and ATP signaling pathways, with this relationship linked to the endogenous cycling of SCN cellular physiology.

Interestingly, my data suggest that part of this relationship may not be simply correlative, but causative, because melatonin altered ATP accumulation in cycling SCN2.2 cultures. In cultures containing high ATP, melatonin treatment reduced the level of extracellular ATP and led to a significant enhancement in the propagation of calcium waves. In contrast, melatonin treatment in cultures containing low ATP caused an elevation in those ATP levels. Therefore, melatonin can alter the amplitude of the endogenous rhythm in ATP accumulation and thereby switch the polarity of calcium signaling in SCN2.2 cultures. It remains to be determined if other effects of melatonin on SCN cells, like neuronal activities, might also be linked to rhythms of ATP accumulation. Like many circadian rhythms, the rhythms in ATP accumulation within the SCN and melatonin production from the pineal gland may come together to create unique physiological states at certain times of day. Fascinatingly, a melatonin-mediated phase shift of circadian rhythms occurs at two windows of sensitivity that correspond to the hours around the day-night (dusk) and night-day (dawn) transitions (Hunt et al., 2001). In rats, the hours just before the day-night transition correspond to the hours just prior to their period of highest activity. Thus, it makes sense that melatonin would be able to modulate ATP accumulation precisely as the rhythm in extracellular ATP is peaking for a given 24 hr period and as the animal is altering its body chemistry in preparation to wake up and become active. The significance of ATP and melatonin

signaling interactions is unclear. Perhaps these interactions are a result of coordinated circadian rhythms that possess a specific phase relationship to modulate intercellular communication in the SCN at certain times of day.

Melatonin action, ATP accumulation, and glucose uptake in SCN2.2 cells

Another circadian rhythm to consider in the context of melatonin and ATP signaling interactions is glucose uptake. SCN2.2 cells are capable of endogenously generating circadian rhythms in 2-deoxyglucose (2-DG) uptake (Earnest, et al., 1999b). The daily rhythms in plasma glucose concentration and glucose uptake both follow the same daily pattern of rising before the onset of activity (la Fleur, 2003). Glucose demands change during a 24h light/dark cycle and glucose metabolism is modulated by the SCN in a rhythmic fashion, independent of the SCN's strong influence on food intake. The CNS relies on blood glucose as its most important energy source. In preliminary experiments, I have demonstrated here that 2-DG uptake in SCN2.2 cells coincided with peak times of ATP accumulation (Fig. 10). That is, the uptake of 2-NBDG, a fluorescent glucose analog, was higher at the 50h peak in the endogenous extracellular ATP rhythm, as compared to the 40h trough. Thus, ATP appears to be accumulated in the extracellular medium of SCN2.2 cultures during the same time that these cultures experienced high glucose uptake. Perhaps the accumulation of extracellular ATP is a result of enhanced glucose utilization.

Many questions are raised by these studies of SCN2.2 cell-cell communication and only a few are answered. One big question that remains is what is the mechanism underlying, and the function of, rhythmic ATP accumulation in SCN cultures. Recent studies in our lab suggest that rhythms in ATP accumulation are not specific to neural astrocytes, but rather may be common to several cell types (Womac et al., 2006). Even so, the fact that melatonin, purine gliotransmission, gap junctional signaling, and calcium signaling all exhibit complex interactions that in one aspect or another are linked to the circadian rhythm in ATP accumulation suggests that SCN physiology is critically influenced by this rhythmic process. Several studies have demonstrated a link

between ATP activation of P2X receptors and calcium-dependent cellular processes (Arcuino et al., 2002; Locovei et al., 2006; Suadicani et al., 2006). This is interesting since regulation of intracellular calcium by membrane channels has been implicated in the regulation of SCN circadian rhythmicity (Nahm et al., 2005). It is likely that an intersection of research in astrocyte-neuron signaling, purinergic regulation of cellular physiology, and coupling mechanisms in the SCN will be required to fully illuminate the broad spectrum of implications and the ultimate significance to biological clock function in the research presented here.

In summary, clock-controlled ATP release and accumulation in SCN cell cultures modulates ATP-dependent gliotransmission in the form of interastrocytic calcium waves. Melatonin signaling and ATP accumulation interact within these SCN cultures to influence each other and the processes they regulate in a fashion dependent on the clock's control of the ATP accumulation rhythm. It will be fundamental to any ultimate understanding of how the SCN clock functions to determine the mechanism by which these processes modulate intercellular communication within the suprachiasmatic nucleus and how that modulation directly affects SCN outputs. In other words, how do rhythms in the regulation of cell-cell communication within the SCN, produce coherent daily oscillations that drive rhythms in behaviors and homeostatic functions of organismal physiology.

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APPENDIX

Experiment protocols

Figure 2 (scrape loading)

1. Obtain 1-60 mm dish containing confluent SCN2.2 cells.
2. Expand the dish to 4 glass coverslip bottom dishes (2 chamber wells per dish).
3. Label the dishes #1 (control), #2 (CBX), #3 (Mel), & #4 (CBX & Mel).
4. Place cells in 37ºC incubator containing 5% CO_2 until the cell cultures reach confluence.
5. Remove cells from incubator and wash twice with CMF (calcium magnesium free) solution containing Dulbecco's PBS (1X) and 0.2% glucose.
6. Add 1,950 µl SCN media consisting of MEM with 10% FBS, 0.4% glucose, and 2 mM L-glutamine to each well.
7. Add 50 µl H_20 (cell culture grade) to each well in dish #1 (control).
   Add 50 µl of 4 mM CBX (100 µM CBX final concentration) to each well in dish #2. Next, pipette solutions in and out to mix. Use new pipette tips for each well.
8. Incubate dishes #1 & #2 for 30 min. at 37ºC.
9. While waiting, begin next incubation:
   a. Remove dishes #3 and #4 from incubator.
   b. Wash each well twice with CMF.
   c. Add 2 ml SCN media to each well.
   d. Add 0.2 µl of 0.1 mM Melatonin in DMSO (10 nM Mel final concentration) to each well in dish #3. Pipette solution in and out to mix. Note: first remove 0.2 µl of media solution from each well before adding melatonin to keep concentrations correct.
   e. Add 50 µl of 4 mM CBX (100 µM CBX final concentration) to each well in dish #4. Pipette to mix.
      Add 0.2 µl of 0.1 mM Melatonin in DMSO (10 nM Mel final concentration) to each well in dish #4. Pipette to mix.
      Note: Before adding CBX or Mel, remove an amount of media from the well equal to the amount of treatment solution being added to keep concentrations correct.
   f. Incubate dishes #3 & #4 for 30 minutes at 37ºC.
10. Remove dishes #1 & #2 from incubator.
    Wash each well twice with Dulbecco's PBS (1X).
11. Add 1,950 µl of dye solution (0.05% Lucifer yellow/ 0.0125 mM Rhodamine dextran in Dulbecco's PBS) to each well in dishes #1 & #2.
    Add 50 µl H_20 (cell culture grade) to each well in dish #1. Pipette to mix.
    Add 50 µl 4 mM CBX (100 µM CBX final concentration) to each well in dish #2. Pipette to mix.

12. Obtain a sterile stainless steel surgical scalpel blade or a pulled glass pipette tip. Perform scrapes in all wells of dishes #1 and #2 by dragging tips gently across the surface of the cells. Change tips between dishes. Next, wash all wells with Dulbecco's PBS (1X) three times to remove background fluorescence.
13. Add 1,950 µl SCN media (without phenol red) to each well in dishes #1 & #2.
    Add 50 µl H_20 (cell culture grade) to each well in dish #1. Pipette to mix.
    Add 50 µl 4 mM CBX (100 µM CBX final concentration) to each well in dish #2. Pipette to mix.
14. Image using Olympus IX70 inverted microscope.
15. Remove dishes #3 & #4 from incubator.
    a. Wash each well twice with Dulbecco's PBS.
    b. Add 2 ml dye solution (0.05% Lucifer yellow/ 0.0125 mM Rhodamine dextran in Dulbecco's PBS) to each well in dishes #3 & #4.
    c. Remove 0.2 µl of dye solution from each well in dish #3.
       Add 0.2 µl of 0.1 mM Melatonin (10 nM Mel final concentration) to each well in dish #3. Pipette to mix.
    d. Remove 50.2 µl of dye solution from each well in dish #4.
       Add 50 µl of 4 mM CBX (100 µM CBX final concentration) to each well in dish #4. Pipette to mix.
       Add 0.2 µl of 0.1 mM Melatonin (10 nM Mel final concentration) to each well in dish #4. Pipette to mix.
    e. Perform scrapes in all wells of dishes #3 & #4 as in step #12 above.
    f. Wash each well three times with Dulbecco's PBS (1X).
    g. Add 2 ml SCN media (without phenol red) to each well.
    h. Remove 0.2 µl of the media from each well in dish #3.
       Add 0.2 µl of 0.1 mM Melatonin (10 nM Mel final concentration) to each well in dish #3. Pipette to mix.
    i. Remove 50.2 µl of media from each well in dish #4.
       Add 50 µl of 4 mM CBX (100 µM CBX final concentration) to each well in dish #4. Pipette to mix.
       Add 0.2 µl of 0.1 mM Melatonin to each well in dish #4. Pipette to mix.
    j. Image using Olympus IX70 inverted microscope.

Figures 3 & 4 (calcium waves)

1. Obtain 1-60 mm dish containing confluent SCN2.2 cells.
2. Expand the dish to 4 glass coverslip bottom dishes (2 chamber wells per dish).
3. Label the dishes #1 (control), #2 (CBX), #3 (Mel), & #4 (CBX & Mel).
4. Place cells in 37ºC incubator containing 5% CO_2 until the cell cultures reach confluence.
5. Remove all wells from incubator. Wash each well twice with CMF. Add 2 ml fresh SCN media to each well. Place wells back in incubator.
6. Prepare Fura-2 AM (calcium indicator) solution:

- Add 15 ml SCN media to one epitube.
- Add 120 µl of 1 mM Fura-2 AM in Pluronic F-127 *20% solution in DMSO to same epitube.
- Vortex and cover in foil to protect from light.
- Final concentration of calcium indicator solution equals 8 µM Fura-2 AM.

7. Remove dish #1 (control) from incubator. Discard media.
   Add 1.5 ml of calcium indicator solution to each well.
   Incubate for 1 hour at 37ºC.
   Wash each well three times with CMF.
   Add 1.5 ml SCN media (without phenol red) to each well.
   Image calcium waves using Olympus IX70 inverted microscope.
8. Remove dish #2 (CBX) from incubator. Discard media.
   Add 1.5 ml calcium indicator solution to each well.
   Remove 37.5 µl of this solution from each well and discard.
   Add 37.5 µl of 4 mM CBX (100 µM CBX final concentration) to each well. Pipette to mix. Next, incubate the dish for 1 hour at 37ºC.
   Wash each well three times with CMF.
   Add 1.5 ml SCN media (without phenol red) to each well.
   Remove 37.5 µl of media from each well and discard.
   Add 37.5 µl of 4 mM CBX (100 µM CBX final concentration) to each well. Pipette to mix.
   Image calcium waves using Olympus IX70 inverted microscope.
9. Remove dish #3 (Mel) from incubator. Discard media.
   Add 1.5 ml calcium indicator solution to each well.
   Remove 0.15 µl of this solution from each well and discard.
   Add 0.15 µl of 0.1 mM Melatonin in DMSO (10 nM Mel final concentration) to each well. Pipette to mix.
   Incubate the dish for 1 hour at 37ºC.
   Wash each well three times with CMF.
   Add 1.5 ml SCN media (without phenol red) to each well.
   Remove 0.15 µl of the media from each well and discard.
   Add 0.15µl of 0.1 mM Melatonin in DMSO (10 nM Mel final concentration) to each well. Pipette to mix.
   Image calcium waves using Olympus IX70 inverted microscope.
10. Remove dish #4 (CBX & Mel) from incubator. Discard media.
    Add 1.5 ml calcium indicator solution to each well.
    Remove 37.65 µl of this solution from each well and discard.
    Add 37.5 µl of 4 mM CBX (100 µM CBX final concentration) to each well. Pipette to mix.
    Add 0.15 µl of 0.1 mM Melatonin in DMSO (10 nM Mel final concentration) to each well. Pipette to mix.
    Incubate the dish for 1 hour at 37ºC.
    Wash each well three times with CMF.

Add 1.5 ml SCN media (without phenol red) to each well.
Remove 37.65 µl of media from each well and discard.
Add 37.5 µl of 4 mM CBX (100 µM CBX final concentration) to each well. Pipette to mix.
Add 0.15 µl of 0.1 mM Melatonin in DMSO (10 nM Mel final concentration) to each well. Pipette to mix.
Image calcium waves using Olympus IX70 inverted microscope.

Figure 5 (calcium waves – SFCF protocol)

Note: This protocol contains time points to target low and high levels of endogenously accumulated extracellular ATP.

1. Obtain 1-60 mm dish containing confluent SCN2.2 cells.
2. Expand the dish to 4 glass coverslip bottom dishes (2 chamber wells per dish).
   During expansion, suspend the pellet of cells in SCN media containing 5% FBS (instead of the usual 10% FBS). The SCN media added to each well should also contain 5% FBS.
   *Note that this step serves as the first serum reduction event.
   Place the cells in 37ºC incubator containing 5% CO_2 until the cell cultures reach 50% confluence. This takes 24 hours.
3. After 24 hours, remove all wells from incubator.
   Wash each well twice with CMF.
   Add 2 ml neurobasal media (contains neurobasal medium along with B-27 supplement, L-glutamine, and glucose) to each well.
   *Note that this step serves as the final serum reduction event.
   Label the wells #1 - #8 and place back in 37ºC incubator.
4. At 34 hours prior to imaging, remove wells #1 - #4 from incubator.
   Add 2 µl of 15 mM forskolin in 100% DMSO (final concentration of 15 µM FSK in 0.1% DMSO) to each well. Pipette to mix.
   Note: first remove 2 µl of media from well and discard before adding the FSK.
   Incubate wells #1 - #4 at 37ºC for 2 hours.
   This serves as the FSK pulse for the 32 hour time point.
5. After 2 hours, remove wells #1 - #4 from incubator and wash each well twice with CMF. Add 2 ml neurobasal media to each well. Place wells in 37ºC incubator. This step serves as time 0 for the 32 hour time point.
6. At 22 hours prior to imaging, remove wells #5 - #8 from incubator.
   Wash each well twice with CMF and add 2 ml neurobasal media to each well.
   Add 2 µl of 15 mM FSK in 100% DMSO (final concentration of 15 µM FSK in 0.1% DMSO) to each well. Remember to first remove 2 µl of media and discard before adding the FSK to each well. Pipette to mix.
   Incubate wells #5 - #8 at 37ºC for 2 hours.
   This serves as the FSK pulse for the 20 hour time point.

7. After 2 hours, remove wells #5 - #8 from incubator and wash each well twice with CMF. Add 2 ml neurobasal media to each well. Place wells in 37ºC incubator. This step serves as time 0 for the 20 hour time point.
8. Note that the 32 hour and 20 hour time points are created by counting backwards from the actual time that calcium waves are imaged. This allows calcium wave imaging to be performed simultaneously for both time points.
9. At 2 hours prior to imaging, begin Fura-2 AM incubations. See protocol for Fig. 3 & 4 step #6 for calcium indicator solution preparation.
10. Remove wells #1 & #2 from incubator and wash each well twice with CMF. Add 1.5 ml Fura-2 AM calcium indicator solution to each well. Incubate wells at 37ºC for 1 hour.
11. Remove wells #1 & #2 from incubator. Wash each well three times with neurobasal media. Add 2 ml neurobasal media to each well.
12. Place wells #1 & #2 in 37ºC for 1 hour for recovery period.
13. After 1 hour, collect a 1 ml media sample from each well and place in -20ºC freezer.
14. Image calcium waves for wells #1 & #2 using an Olympus IX70 inverted microscope.
15. Repeat steps # 9 - #14 for wells #3 - #8. Stagger the incubations for each dish by 30 minutes to so that calcium waves can be imaged while the cells in each dish are still freshly loaded with Fura-2 AM.

Figure 6 (calcium waves – SR protocol)

Note: The SFCF protocol (see above protocol) was used in the experiment for the 20 hour & 32 hour time points, where as the SR protocol was used in the experiment for the 40 hour & 50 hour time points.

1. Obtain 1-60 mm dish containing confluent SCN2.2 cells.
2. Expand the dish to 4 glass coverslip bottom dishes (2 chamber wells per dish). During expansion, suspend the pellet of cells in SCN media containing 5% FBS (instead of the usual 10% FBS). The SCN media added to each well should also contain 5% FBS. Label the wells #1 - #8. *Note that this step serves as the first serum reduction event as well as the plating event (time 0). From this step, at time 0, cells can be carried out to 40 hours or 50 hours depending on the desired time point. Calcium waves are imaged at 40 hours or 50 hours from time 0.
   Place the cells in 37ºC incubator containing 5% CO_2 for exactly 24 hours.
3. After 24 hours, remove all wells from incubator and discard media.
   Wash each well twice with neurobasal media. Add 2 ml neurobasal media to each well. *Note that this step serves as the final serum reduction event.
   Place all wells in 37ºC incubator.
4. Two hours prior to imaging (i.e. desired time point), begin Fura-2 AM incubations. Remove wells #1 - #4 from incubator. Wash each well two times with neurobasal media. Add 2 ml of 8 μM Fura-2 AM calcium indicator solution

to each well. See protocol for Fig. 3 & 4 step #6 for calcium indicator solution preparation. Incubate wells #1 - #4 for 1 hour at 37ºC.

5. Remove wells #1 - #4 from incubator. Wash each well five times with neurobasal media. Add 2 ml neurobasal media to each well. Place wells #1 - #4 in 37ºC incubator for 1 hour to recover.
6. Remove wells #1 - #4 from incubator and collect a 500 µl media sample from each well and freeze sample at -20ºC.
7. Image calcium waves for wells #1 - #4 using an Olympus IX70 inverted microscope.
8. Repeat steps #4 - #7 for wells #5 - #8. Note that the Fura-2 AM incubations are staggered to use cells that have been freshly loaded with calcium indicator.

Figure 7 (2-NBDG uptake)

1. Obtain 1-60 mm dish containing confluent SCN2.2 cells.
2. Expand the dish to 4 glass coverslip bottom dishes (2 chamber wells per dish). During expansion, suspend the pellet of cells in SCN media containing 5% FBS (instead of the usual 10% FBS). The SCN media added to each well should also contain 5% FBS. Label the wells #1 - #8. *Note that this step serves as the first serum reduction event as well as the plating event (time 0). From this step, at time 0, cells can be carried out to 40 hours or 50 hours depending on the desired time point. Place all wells in 37ºC incubator containing 5% CO_2 for exactly 24 hours.
9. After 24 hours, remove all wells from incubator and discard media.
   Wash each well twice with neurobasal media. Add 2 ml neurobasal media to each well. *Note that this step serves as the final serum reduction event.
   Place all wells in 37ºC incubator.
10. Thirty minutes prior to desired time point (e.g., 40 hours),
    Remove wells #1 - #4 from incubator. Wash each well twice with neurobasal media. Immediately continue to next step.
11. Next, perform treatment specific washes. Wash wells #1 & #3 twice with neurobasal media. Then, wash wells #2 & #4 twice with neurobasal media containing no glucose.
12. Begin 2-NBDG incubations:
    Add 2 ml of 0.1 mM 2-NBDG in neurobasal media to wells #1 & #3.
    Add 2 ml of 0.1 mM 2-NBDG in neurobasal media containing no glucose to wells #2 & #4.
13. Incubate wells #1 - #4 for 1 hour at 37ºC (dark).
14. Remove wells #1 - #4 from incubator and wash each well five times with neurobasal media. Pop off sides of chamber wells and prepare a glass wet mount slide for each glass coverslip bottom dish.
15. Immediately image cells with Olympus IX70 inverted microscope.
16. Repeat steps # 10 - #15 for next desired time point (e.g., 50 hours).

Figure 8 (calcium waves)

The protocol followed for the experiment in Figure 8 was identical to the SR protocol for Figure 6. However, melatonin (10 nM) and ATP (10 nM) treatments were added during the Fura-2 AM loading period and/or recovery period.

Figure 9 (calcium waves & melatonin dose-dependence)

The protocol followed for the experiment in Figure 9 was identical to the SR protocol for Figure 6. However, melatonin (10 fM; 10 pM; 10 nM) and luzindole (100 nM; 10 μM) treatments were added during the one hour recovery period.

Figure 10 (calcium waves)

Note: This protocol contains time points to target low and high levels of endogenous extracellular ATP.

1. Obtain 1-60 mm dish containing confluent SCN2.2 cells.
2. Expand the dish to 4 glass coverslip bottom dishes (2 chamber wells per dish). During expansion, suspend the pellet of cells in SCN media containing 5% FBS (instead of the usual 10% FBS). The SCN media added to each well should also contain 5% FBS.
   *Note that this step serves as the first serum reduction event.
   Place the cells in 37ºC incubator containing 5% CO_2 until the cell cultures reach 50% confluence. This usually takes 24 hours.

16. At 34 hours prior to imaging, remove all wells from incubator.
    Wash each well twice with CMF.
    Add 2 ml neurobasal media (contains neurobasal medium along with B-27 supplement, L-glutamine, and glucose) to each well.
    *Note that this step serves as the final serum reduction event.
    Label the wells #1 - #8 and place back in 37ºC incubator.
17. Remove wells #1 - #4 from incubator.
    Add 2 μl of 15 mM forskolin in 100% DMSO (final concentration of 15 μM FSK in 0.1% DMSO) to each well. Pipette to mix.
    Note: first remove 2 μl of media from well and discard before adding the FSK.
    Incubate wells #1 - #4 at 37ºC for 2 hours.
    This serves as the FSK pulse for the 32 hour time point.
18. After 2 hours, remove wells #1 - #4 from incubator and wash each well twice with CMF. Add 2 ml neurobasal media to each well. Place wells in 37ºC incubator. This step serves as time 0 for the 32 hour time point.
19. At 22 hours prior to imaging, remove wells #5 - #8 from incubator.
    Wash each well twice with CMF and add 2 ml neurobasal media to each well.

Add 2 µl of 15 mM FSK in 100% DMSO (final concentration of 15 µM FSK in 0.1% DMSO) to each well. Remember to first remove 2 µl of media and discard before adding the FSK to each well. Pipette to mix.
Incubate wells #5 - #8 at 37ºC for 2 hours.
This serves as the FSK pulse for the 20 hour time point.

20. After 2 hours, remove wells #5 - #8 from incubator and wash each well twice with CMF. Add 2 ml neurobasal media to each well. Place wells in 37ºC incubator. This step serves as time 0 for the 20 hour time point.
21. Note that the 32 hour and 20 hour time points are created by counting backwards from the actual time that calcium waves are imaged. This allows calcium wave imaging to be performed simultaneously for both time points.
22. At 2 hours prior to imaging, begin Fura-2 AM incubations. See protocol for Fig. 3 & 4 step #6 for calcium indicator solution preparation.
23. Remove wells #1 & #2 from incubator and wash each well twice with CMF. Add 1.5 ml Fura-2 AM calcium indicator solution to each well. Incubate wells at 37ºC for 1 hour.
24. Remove wells #1 & #2 from incubator. Wash each well three times with neurobasal media. Add 2 ml neurobasal media to each well.
    Now, begin treatment of DMSO (control) or melatonin.
    Add 2 µl of 100% DMSO (0.1% DMSO final concentration) to well #1. Pipette to mix. Again, first remove 2 µl of media before adding the DMSO.
    Add 2 µl of 10 µM melatonin in 100% DMSO (10 nM Mel in 0.1 % DMSO final concentration) to well #2. Pipette to mix. Remember to remove 2 µl of media and discard media before adding the melatonin to keep concentrations correct.
25. Place wells #1 & #2 in 37ºC for 1 hour for treatment and recovery period.
26. After 1 hour, collect a 1 ml media sample from each well and place in -20ºC freezer.
27. Image calcium waves for wells #1 & #2 using an Olympus IX70 inverted microscope.
28. Repeat steps # 9 - #14 for wells #3 - #8. Stagger the incubations for each dish by 30 minutes to so that calcium waves can be imaged while the cells in each dish are still freshly loaded with Fura-2 AM. The DMSO treatments are for the odd numbered wells and the melatonin treatments are for the even numbered wells. Therefore, each dish contains a control and melatonin well to be imaged immediately following each other.

VITA

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