ASTROCYTES AND THE CIRCADIAN CLOCK: ROLES FOR CALCIUM, LIGHT, AND MELATONIN

A Dissertation

by

JENNIFER LYNN PETERS

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

DOCTOR OF PHILOSOPHY

May 2005

Major Subject: Zoology

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Approved as to style and content by:

Vincent M. Cassone (Chair of Committee)	Deborah Bell-Pedersen (Member)
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ABSTRACT

Astrocytes and the Circadian Clock: Roles for Calcium, Light and Melatonin.

(May 2005)

Jennifer Lynn Peters, B.S., Loras College

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Melatonin is rhythmically synthesized and released by the pineal gland and, in some species, retina during the night and regulates many physiological and behavioral processes in birds and mammals. Chick diencephalic astrocytes express two melatonin receptor subtypes *in vitro*, and melatonin plays a role in regulating metabolic activity. We examined the role of glial cells in circadian function and asked if melatonin modulated glial functions within the retina and the brain. Calcium waves were potentiated by physiological concentrations of melatonin. Melatonin increased resting calcium levels and reduced gap junctional coupling among astrocytes at these same concentrations. Both mouse and chick diencephalic and telencephalic astrocytes express melatonin receptor protein. Nanomolar melatonin modulated astrocytic calcium waves of the mouse and chick diencephalon but not waves of the telencephalon. Mammalian intercellular calcium waves spread farther than avian calcium waves, and the nature of the spread of the waves differed between telencephalic and diencephalic mammalian astrocytes. These differences in propagation were abolished by melatonin.

Using northern analysis, we identified *period2*, *period3*, *cryptochrome1*, *cryptochrome2*, *clock*, melanopsin and peropsin within chick diencephalic astrocytes.

The clock genes *cry1* and*, per2* were expressed rhythmically in a LD cycle, but metabolic activity was not rhythmic. When cells were placed in constant darkness and rhythmically administrated melatonin, a robust rhythm in glucose uptake was induced without a coordinated clock gene rhythm, suggesting rhythmic clock gene expression and metabolic activity are separable processes. Melatonin affected visual function as assessed by electroretinogram. Circadian rhythms of a- and b-wave implicit times and amplitudes were observed. Melatonin (1 mg/kg and 100 ng/kg) decreased a- and b-wave amplitudes greater during the night than during the day and it increased a- and b-wave implicit times while 1 ng/kg melatonin had little to no effect over the saline controls. These data indicate that melatonin modulates glial intercellular communication, affects metabolic activity in astrocytes, and may play a role in regulating a day and night functional shift in the retina, at least partially through Müller glial cells. Thus, melatonin can regulate glia function and thereby, affect outputs of the vertebrate biological clock.

ACKNOWLEDGEMENTS

I would first like to thank my advisor, Dr. Vincent Cassone, whose enthusiasm for research and constant quest for "the data" have taught me how research science should be accomplished and inspired me to question and learn. I am indebted to Dr. Mark Zoran, who not only performed many of these experiments with me, but also spent many, many hours discussing "the data" and teaching me the joy of glial cells and neurobiology. Without his advice and help, I may never have finished. I would like to thank Dr. Dave Earnest for letting more borrow lab space from time to time and for helping me with the perils of molecular biology. I would like to acknowledge the other members of my committee: Drs. Deborah Bell-Pedersen and Stuart Dryer for their advice and input concerning my research and what it all means. I am grateful to my professors at Loras College, especially Drs. Gerald Eagleson and David Czarnecki, who first introduced me to research and its possibilities.

My labmates throughout the years, Beth Cantwell, Steven Karaganis, Hillary Olin, Michael Bailey, Jennifer McGoogan, Arjun Natesan, Akihito Adachi, Paul Bartell, and Barb Earnest, have provided much knowledge, support, and lab camaraderie. They along with friends at TAMU made the journey much more enjoyable.

I would like to thank my parents, Barb and Fred High, for allowing me dig, dissect, splash, and bring home my "science projects" and for their constant encouragement and belief in me. I am thankful to my brother, Ryan, who was my first co-researcher in exploring science and has continued to support his sister. Finally, I would like to thank my husband, Greg Peters, for always knowing I could do it.

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CHAPTER 1

INTRODUCTION

CIRCADIAN RHYTHMS

Circadian rhythms are internally driven daily cycles that occur at all organizational levels of the organism from behavioral and physiological levels to metabolic and molecular levels (Roenneberg and Merrow, 1999; Dunlap, 1999; Cassone, 2000; Hastings and Maywood, 2000). These endogenous timing properties allow the organism to adapt to changes in the environment and more importantly to anticipate environmental changes. This anticipatory behavior is advantageous to the organism because it can activate genes and processes prior to the time cue and therefore, helps the organism succeed in that environment (Pittendrigh, 1981a). Light is the primary time cue, or zeitgeber, and serves as the signal to several input pathways (Pittendrigh, 1961). These inputs lead to one or more oscillators or pacemakers that keep the 24-hour period. The pacemakers then confer rhythmicity to the rest of the organism via outputs to synchronize all of the tissues or cells.

The Pacemaker

The pacemaker is the main timekeeper in the organism. It generates rhythms of approximately 24 hours in duration, and these rhythmicities persist under constant conditions (Pittendrigh, 1961). Some organisms have multiple pacemakers. For

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This dissertation follows the style and format of the Journal of Comparative Neurology.

example, in the avian circadian system, the pineal gland, the retina, and the suprachiasmatic nucleus (SCN), are all pacemakers, although their relative importance in conferring rhythmicity varies somewhat among species (Cassone and Menaker, 1984; Cassone, 1990b). Each organism's endogenous circadian rhythm may be slightly longer or shorter than 24 hours, and therefore, needs to be synchronized each day by the zeitgeber. This resynchronization is known as entrainment (Pittendrigh, 1961). If an organism is placed in constant conditions, and is not receiving daily entraining signals by the zeitgeber, its endogenous or free running period which will persist (Aschoff, 1981). The free running period of a diurnal animal, like a house sparrow (*Passer domesticus*), is generally longer than 24 hours in constant darkness (DD) and less than 24 hours in constant light (LL). Nocturnal animals, such as mice, generally have a free running period of less than 24 hours in DD and longer than 24 hours in LL. This phenomenon is known as Aschoff's rule (Pittendrigh, 1981a). There are frequent exceptions to this rule. One example is Japanese quail (*Coturnix coturnix japonica*), a diurnal species, where the free running period is shorter in DD and longer in LL (Underwood et al. 1997).

Circadian rhythms of the pacemaker are temperature compensated. For most biological processes, as the temperature increases, there is also an increase in the activity. This is expressed as Q_{10}, the value of a rate change of a process in response to 10° temperature steps. For a normal enzymatic biochemical reaction, this rate change is typically 2 to 3 fold, such that the Q_{10} is 2.0 to 3.0. The Q_{10} of circadian rhythms is between 0.9 and 1.2 (Pittendrigh, 1961), meaning the rate of oscillation does not change

over a variety of temperatures. Temperature compensation occurs within the normal

range of temperatures that the animal is exposed, such that daily changes in temperature

do not alter its circadian rhythm (Pittendrigh, 1954).

The Input

As mentioned above, the pacemaker is entrained through a daily time cue via the

input pathways. There are two proposed models as to how this entrainment occurs, the

parametric entrainment model and the non-parametric entrainment model (Pittendrigh,

1981b). The description of the parametric model states that there is a change in the

value of the parameter, such as a change in light intensity or quality, which alters the

period to synchronize. The clock continually or gradually adjusts throughout the day, by

either slowing down or speeding up, in order to achieve a 24 hour period. The

description of the non-parametric model states that it doesn't matter how the parameter

changes, what matters is the time at which the change occurs. The circadian clock then

either abruptly advances forward or backward to compensate for a slow or fast clock and

thereby, be exactly on time. Studies have supported both models and it appears a

combination of the two models is correct (Pittendrigh, 1981b).

Light can entrain an organism's clock and serve as a time cue. Under many

experimental conditions, the photoperiod of 12 hours of light and 12 hours of dark

(12:12) is used, where the time at which lights come on, or dawn, is designated

Zeitgeber Time 0 (ZT 0) and the time of the light to dark transition, or dusk, is

designated Zeitgeber Time 12 (ZT 12). This photoperiod can be modified and under

most environmental conditions, there are not exactly 12 hours of light and 12 hours of dark each day. In many experiments performed under constant conditions, the perceived times at which dawn and dusk would normally occur for the organism in a 12:12 LD cycle, are defined as Circadian Time 0 (CT 0) and Circadian Time 12 (CT 12), respectively. When an organism is exposed to an environmental stimulus such as light for a short period under constant conditions, they can experience a shift in their phase on the subsequent day. This phase shift in response to the stimulus occurs as either a jump forward (phase advance) or jump back (phase delay) in time, and is in accordance with the non-parametric entrainment model (Pittendrigh, 1981b).

The Output

The input pathways translate the zeitgebers, or time cues, into biological signals sent to the pacemaker. The pacemaker then renders signals to the rest of the organism via output pathways in order to generate circadian rhythmicities. The output pathways can affect an organism on many different levels. On the most basic level, they generate rhythms in gene expression and protein levels in a cell (Dunlap, 1999; Hirota and Fukada, 2004). They can regulate homeostatic processes, such as body temperature, heart rate, and the sleep/wake cycle (Warren et al., 1994; Moore, 1997; Czeisler et al. 1999). They influence rhythmicity in activity patterns, such as feeding, locomotion, and reproductive behavior (Farner, 1980; Gillette and Tischkau, 1999; Lincoln et al., 2003). They can affect photoreception and visual system function (McGoogan and Cassone, 1999; Kavakli and Sancar, 2002). The outputs can generate rhythms in neurotrophin and

neurohormone release (Reiter, 1991; Cassone, 1998; Earnest et al., 1999). Some output

pathways may even feedback into the oscillator and affect further rhythms in the

organism (Lakin-Thomas and Brody, 2004; Cassone, 1998). Many of these outputs will

be discussed in greater detail below.

CIRCADIAN ORGANIZATION

The Avian Circadian System

In the avian circadian system, there are three oscillatory or pacemaker

components, the pineal gland, the retina, and the mammalian homologue to the SCN, the

visual suprachiasmatic nucleus (vSCN; Cassone, 1990b). The relative importance of

each of these pacemakers in conferring rhythmicity varies somewhat among different

species (Cassone and Menaker, 1984). Each of these components contains

photoreceptive structures, and through these structures, light can then influence the

circadian system (Menaker, 1982).

The Pineal Gland

The pineal gland is located in the epithalamus of the diencephalon of the brain,

and its photoreceptive cells receive direct light cues. Oscillations of the pineal gland

affect behavior. Gaston and Menaker (1968) removed the pineal gland of the house

sparrow and found there was no effect on its locomotor activity while the bird was

housed in an LD cycle, but when the pinealectomized bird was housed under constant

darkness its activity became arrhythmic. This suggested that although the pineal gland

plays a critical role in generating rhythmicity, there may be other components of the circadian system which allowed the bird to retained rhythmicity in LD without its pineal. When the pineal gland is transferred from an entrained LD bird to a pinealectomized bird that is free running in DD, it confers the rhythm and phase of the donor bird to the recipient bird (Zimmerman and Menaker, 1979). Additionally, while pinealectomy causes disruption or abolition of locomotor activity in sparrows (Gaston and Menaker, 1968), it has little or no effect on pigeons and chickens (Ebihara et al., 1984; Nyce and Binkley, 1977). Oscillations of the pineal gland drive rhythmic synthesis and release of the hormone melatonin (Klein et al., 1997), and these rhythms persist *in vitro* with pineal glands continuing to secrete higher levels of melatonin during the night than during the day (Takahashi et al., 1980).

In the pineal gland the photoreceptive cells are the pinealocytes (Menaker 1982). Pinealocytes have cilia that extend from processes on the cell membranes similar to the cilia found on photoreceptor cells of the retina (Zimmerman and Tso, 1975), and they express S-antigen, a specific protein found in retinal photoreceptors (Collin et al., 1986). Deguchi (1981) reported that pinealocytes contain a rhodopsin-like photopigment, and more recently other opsin molecules have been identified in the pineal. The photopigment iodopsin is expressed in the pineal gland (Araki et al., 1992), and its pattern and level of expression are different than those of other pineal opsins (Yamao et al., 1999). Pinopsin is a blue light-sensitive pigment, and while similar to retinal opsins, is found only in the pineal gland (Okano et al., 1994). The mRNA levels of *pinopsin* are increased in response to light, and although they are not rhythmic in a circadian fashion,

it has been suggested that pinopsin might regulate acute effects of light (Takanaka et al., 1998). A more recently discovered opsin is melanopsin. Melanopsin expression in the pineal exhibits a daily rhythm with higher levels during the night than during the day and this opsin is thought to play a role in photic regulation and entrainment (Chaurasia et al., 2005). Two opsin-based molecules, peropsin and RGR opsin, are rhythmically expressed in the pineal and are thought to be photoisomerases responsible for the regeneration of the photosensory opsins (Bailey and Cassone, 2004).

The Retina

The retina, like the pineal gland, also receives light input directly to its photoreceptive cells. The retina is important to circadian functions as bilateral enucleation (removal of both eyes) disrupts locomotor and body temperature rhythms in pigeons, chickens, and Japanese quail (Nyce and Binkley, 1977; Ebihara et al., 1984; Underwood et al., 1984). The retina can generate rhythms in melatonin content within the eye that persist even in pinealectomized birds, indicating the source of the hormone and its rhythm are located within the retina (Hamm and Menaker, 1980). The mRNA of two retinal opsins of the photoreceptive layer, peropsin and RGR-opsin, are regulated in a circadian fashion, peaking in the late subjective day (Bailey and Cassone, 2004). Melanopsin is found in the retina and similar to its expression in the pineal gland, the mRNA of melanopsin expresses a daily, but not circadian, rhythm (Bailey et al., 2004). Retinal explants in culture show daily rhythms in transcription of iodopsin, the red light-sensitive cone photopigment, which persist for several days under constant conditions

(Zhang et al., 2003). Cryptochrome (*cry*), a flavin-based blue light photopigment, is expressed in the retina, and in chicks, *cry1* is regulated in a circadian fashion (Bailey et al., 2004). Disc shedding of the outer segments of the photoreceptors of the retina is rhythmically regulated (Young, 1978). Additionally, it has been shown that the cGMP-gated channels of retinal photoreceptors have a higher affinity for cGMP at night, and that this is driven by rhythms in two kinases, Erk and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). Erk is more active at night, and CaMKII is more active during the day (Ko et al., 2001).

The retina is not only an oscillatory component in avian species, but obviously a critical part of the visual system. The retina is the sensory layer in the eye that perceives light and plays a direct role in vision. When a photon of light is absorbed by the opsin molecule of the rod or cone photoreceptor, it can elicit a response and initiate the phototransduction cascade. For rod photoreceptors, this is induced by a conformational change in the structure of the rhodopsin molecule from the 11-cis-retinal to the all-trans-retinal form. The change to metarhodopsin II allows it to interact and activate the G-protein, transducin. Transducin, then, activates phosphodiesterase, which breaks down to cGMP. cGMP-binding to cGMP channels is reduced and the channels close causing a decrease in Na^+ influx, and thus a hyperpolarization of the cell. (For review, Bear et al., 2001). In a diurnal animal, such as a bird, the cone photoreceptors are more active during the day when the animal is awake and interacts with its environment (Schaeffel et al., 1991). Cones can distinguish different colors as there are several types of cones, each with its own specific opsin and oil droplet combination for different wavelengths of

visible light (Dowling, 1987). Cone photoreceptor transduction is faster than that of

rods. At night, the rod photoreceptors are more active. They are more sensitive than

cones, and they can sense the slightest changes in light intensity. This day-night shift

from cones to rods is known as the Purkinje shift (Schaeffel et al., 1991; McGoogan and

Cassone, 1999).

The photoreceptive cells, the bipolar cells, and the retinal ganglion cells are the

neuronal components of the retina directly involved in photoreception and visual

processing. The horizontal and amacrine neuronal cells are responsible for lateral

processing in the retina. The axons from the ganglion cells comprise the optic nerve of

the eye (Bear et al., 2001). These projections continue via the optic tracts to the brain

and form the four visual pathways, the retinohypothalamic tract, the accessory optic

pathway, the tectofugal pathway, and the thalamofugal pathway (Benowitz and Karten,

1976; Brecha and Karten, 1981; Hodos et al., 1984; Cassone and Moore, 1987). The

retinohypothalamic tract (RHT) projects from the retina to the SCN (Cassone and

Moore, 1987), whose afferents reach the paraventricular nucleus (PVN) which projects

to the intermedial spinal cord cells (IML) to the superior cervical ganglia (SCG). The

superior cervical ganglia (SCG) release norepinephrine (NE) to the pineal gland.

Disruption of the pathway affects or abolishes melatonin production in the pineal gland

(Cassone et al., 1986c). The accessory optic pathway projects from the retina to the

ectomammillary root to the ocular motor complex to the cerebellum and is responsible

for controlling movement (Brecha and Karten, 1981). The tectofugal pathway projects

from the retina to the contralateral optic tectum to the nucleus rotundus and to the

ectostriatum (Benowitz and Karten, 1976). It detects brightness, color, contrast and movement. Lastly, the thalamofugal pathway projects from the retina to the thalamus to the caudal part of the telencephalon or visual Wülst, and it is involved in fine acuity and binocular vision (Hodos et al., 1984). The visual structures along all of these pathways have been found to bind the hormone melatonin (Brooks and Cassone, 1992).

These visual pathways are all affected by the retina and one method used to examine retinal visual function is the electroretinogram (ERG). ERG measures the visual evoked potential of the retina in response to a flash of light. The a-wave represents the response of the photoreceptive layer. The b-wave represents the response of the rest of the retina, mainly the Müller glial cell uptake of potassium (K^+) from the extracellular space (Rager, 1979; Miller and Dowling, 1970). There is a circadian rhythm in the electroretinogram (ERG) in several species (Brandenburg et al., 1983; Nozaki et al., 1983; Dearry and Barlow, 1987; Lu et al, 1995; McGoogan and Cassone, 1999; Miranda-Anaya et al., 2000). Using chickens, McGoogan and Cassone (1999) demonstrated that the amplitude of b-wave is higher during the day than during the night and the implicit time, or time from the onset of the stimulus until the time of the peak of the wave, is shorter during the day than during the night. In chickens, an injection of hormone melatonin given during the day can decrease amplitude of b-wave to levels normally observed during the night (Lu et al., 1995). Continuously administered exogenous melatonin abolishes rhythmicity of a- and b-wave implicit time and b-wave amplitude of chickens in DD (McGoogan and Cassone, 1999). Dopamine is another neurohormone that is secreted from the amacrine cells of the retina in 180° anti-phase of

melatonin release in pigeons (Adachi et al., 1998). It increases the amplitude of the

ERG b-wave in the iguana (Miranda-Anaya et al., 2002), and appears to act on the

photoreceptors in the retina of *Xenopus* (Cahill and Besharse, 1993).

Melatonin

The pineal gland and the retina both produce the indoleamine hormone

melatonin. One of the important outputs of this circadian system, and potentially an

input component, is melatonin. Melatonin is rhythmically synthesized in the

photoreceptors of the retina and the pinealocytes in the pineal gland (Klein, et al., 1997).

Its synthesis involves four enzymes, some of which are under regulation by the circadian

clock. The biosynthetic pathway is as follows: tryptophan is converted to 5-

Hydroxytryptophan by tryptophan hydroxylase, which is then decarboxylated to form 5-

Hydroxytryptamine (5-HT), or serotonin, by aromatic 1-amino acid decarboxylase;

serotonin is converted to N-acetylserotonin by arylalkylamine N-acetyltransferase (AA-

NAT); N-acetylserotonin is converted to melatonin by hydroxyindole-O-

methyltransferase (HIOMT). The melatonin biosynthetic enzymes tryptophan

hydroxylase (Thomas and Iuvone, 1991), AA-NAT (Besharse and Iuvone, 1983;

Bernard et al., 1997; Klein et al., 1997), and HIOMT (Bernard et al., 1999), are

expressed rhythmically in the pineal and the retina.

Melatonin levels are rhythmic, such that levels are high during the night and low

during the day (Adachi et al., 1995; Hamm and Menaker, 1980; Underwood, et al, 1990;

Klein, et al., 1997). In chickens, this rhythmicity is retained in constant darkness

(Hamm and Menaker, 1980). Pineal cells in culture also generate a circadian rhythm of melatonin release (Natesan et al., 2002). Timed daily administration of melatonin can restore rhythmicity to pinealectomized birds (Gwinner and Benzinger, 1978; Lu and Cassone, 1993a), while continuous melatonin can abolish ERG rhythms (McGoogan and Cassone, 1999) and activity rhythms (Turek et al., 1976) in DD. 2-[^{125}I]iodomelatonin (IMEL) studies identified sites of melatonin binding in the chick retina and brain visual system structures, such as the vSCN (Dubocovich and Takahashi, 1987; Rivkees et al, 1989; Cassone et al, 1995). This IMEL binding is rhythmic, such that binding is greater during the day than during the night with peak binding at ZT 10 and CT 10 (Brooks and Cassone, 1992). The metabolic activity of the brain is affected by melatonin. Birds injected with exogenous melatonin have decreased glucose uptake in the vSCN during the day when uptake levels normally peak (Cantwell and Cassone, 2002). *In vitro* studies with diencephalic astrocytes, demonstrated that administration of melatonin induces rhythms in 2-Deoxyglucose (2-DG) uptake and in the release of the glycolytic byproducts, lactate and pyruvate (Adachi et al., 2002).

As in birds, melatonin is rhythmically synthesized in the pineal and retina of mammals, along with its rate limiting enzyme, AA-NAT (Klein and Weller, 1970; Lewy et al., 1980; Ebihara et al., 1986; Reiter, 1991; Roseboom et al., 1996). This rhythmic synthesis is regulated by the master pacemaker, the SCN. The mammalian SCN binds melatonin with high affinity as demonstrated in IMEL binding studies (Cassone, 1998). IMEL binding was also found in the median eminence, par tuberalis of the anterior pituitary, and several nuclei in the thalamus (Weaver et al., 1989). Melatonin affects the

SCN *in vivo* and *in vitro* in a time and dose dependent fashion, such that the greatest effects are seen at late subjective day and early subjective night during the light to dark transition (Shibata et al., 1989; McArthur et al., 1991; Margraf and Lynch, 1993). In a study of several hypothalamic areas, melatonin decreased 2DG uptake in a dose-dependent fashion within the SCN area alone. This inhibition was seen primarily in the late subjective day (Cassone et al., 1987). Application of melatonin to *in vitro* SCN slices causes a phase advance in the neuronal firing rate (Starkey et al., 1995). Melatonin can entrain locomotor activity in rats that are free running in DD (Redman et al., 1983). However, unlike pinealectomized birds, mammals with lesions of the master pacemaker cannot re-entrain with melatonin alone (Cassone et al., 1986a). This demonstrates how melatonin can differentially affect birds and mammals as melatonin alone does not restore rhythmicity to mammals whose master pacemaker is abolished, while it does restore rhythmicity to birds with a lesioned master pacemaker.

Melatonin Receptor Subtypes

The differential effects of melatonin could be due to the melatonin receptor subtype expressed by the species. Melatonin receptors are members of the guanine nucleotide-binding protein (G-protein)-coupled 7-pass receptor family that act to inhibit adenylate cyclase (Reppert, 1997). Mammals express the melatonin receptors subtypes, MT1 (Mel$_{1a}$) and MT2 (Mel$_{1b}$; Reppert et al., 1996), while birds express an addition subtype, Mel$_{1c}$. In mammals, there is differential expression of the two subtypes, MT1 and MT2. Mice housed under a LD cycle express mRNA of the MT1 melatonin

receptor that peaks at the beginning of the dark period (ZT14), while in DD expression occurs around mid-subjective night (CT18). This suggests MT1 receptor rhythms in the SCN are regulated by light and the biological clock (Masana et al., 2000). Using specific melatonin receptor antagonists, Hunt et al. (2001) demonstrated that melatonin's modulation of phase advances occurs via the MT2 receptor signaling. In situ hybridization of the melatonin receptor subtype expression demonstrated MT2 melatonin receptor mRNA expression in human cerebellar Bergmann glia and astrocytes (Al-Ghoul et al., 1998). In the retina, ganglion cells and the amacrine cells that secrete dopamine, express the MT1 receptor subtype (Fujieda et al., 2000).

The three melatonin receptors, Mel_{1a}, Mel_{1b}, and Mel_{1c}, are all found in chickens (Reppert et al., 1995; Liu et al., 1995). Studies of the mRNA expression for the receptors, Mel_{1a} and Mel_{1c}, determined that the Mel_{1a} receptor appears to bind to mainly neuronal cell bodies, while the Mel_{1c} receptor binds to non-neuronal components of the retina and brain (Reppert et al., 1995). These non-neuronal components are glial astrocytes. All astrocytes of the diencephalon express the mRNA of the Mel_{1c} receptor, while only 25% express the Mel_{1a} receptor (Adachi et al., 2002). Several groups have demonstrated the expression of Mel_{1a}, Mel_{1b}, and Mel_{1c} mRNA in the inner segments of the photoreceptor layer, the vitread portion of the inner nuclear layer, and the ganglion cell layer of the retina (Reppert et al., 1995; Natesan and Cassone, 2002). The mRNA of the receptors is rhythmic, such that Mel_{1a} and Mel_{1c} mRNA levels are higher during midday in LD (Natesan and Cassone, 2002), and the rhythm of Mel_{1a} RNA, persists in DD. The Mel_{1b} receptor mRNA is constitutively expressed in the photoreceptor layer,

the ganglion cell layer, and throughout the inner nuclear layer. Antisera developed for

the Mel_{1a} and Mel_{1c} receptors showed similar results in the retina. The Mel_{1a} receptor

protein is found in the neuronal amacrine, bipolar and ganglion cells of the retina and

Mel_{1c} receptor protein binds not to neurons, but rather to surrounding glial cells

(McGoogan, 2000).

The Suprachiasmatic Nucleus

The third component of the avian circadian system is a homolog to the

mammalian SCN. In mammals, the SCN, which is located close to the 3rd ventricle in

the hypothalamus of the brain, is the sole master pacemaker (Moore, 1983). It is

composed of small compactly organized cells that can be divided into at least two

groups. Endogenously rhythmic neurons are primarily found in ventromedial,

dorsomedial and rostral portions of the nucleus having a "shell-like" appearance. The

light-responsive cells that receive direct retinohypothalamic input from the retina are

largely located in a central "core" region of the SCN and the majority of these cells are

nonrhythmic (Lee et al., 2003). A lesion to the SCN that abolishes circadian rhythmicity

can be repaired by transplanting fetal SCN tissue (Lehman et al., 1987). Lesions to the

SCN also disrupt other rhythms in the animal, such as heart rhythms, which are driven

by the SCN through the sympathetic nervous system (Warren et al., 1994).

Transplanting immortalized SCN cells to a SCN-lesioned rat restores rhythmic

locomotor activity (Earnest et al., 1999). Single isolated SCN neurons express a

circadian rhythm in firing rate (Honma et al., 1998; van Esseveldt et al., 2000) and

immortalized SCN cells generate robust metabolic rhythms (Earnest et al., 1999).

Studies with 2-DG demonstrated that glucose levels in the SCN are high during the day

and low during the night (Schwartz and Gainer, 1977; Schwartz et al., 1983).

Norepinephrine (NE) is released via the sympathetic nervous system due to SCN

oscillations and it targets the pineal gland to drive the production of the hormone

melatonin (Klein et al., 1970). The SCN is the master pacemaker and the pineal gland

its "slave" as pinealectomized rats are still rhythmic in locomotor activity in LD and in

DD (Aschoff et al., 1982). However, the "slave" oscillator may regulate photic

sensitivity as the circadian rhythms of pinealectomized rats placed into constant light

dissociate into ultradian bouts of activity, while sham controls remained in their

circadian activity rhythm (Cassone, 1992).

The avian SCN is more complex with two areas of the brain apparent candidates

for the homolog of the mammalian SCN. The first is the medial SCN (mSCN) located

dorsal to the optic chiasm in the preoptic recess of the third ventricle (Norgren and

Silver, 1990). The second is the visual suprachiasmatic nucleus (vSCN) also located

dorsal to the optic chiasm, but lateral and caudal to the mSCN (Cassone and Moore,

1987). It is termed the vSCN because it receives light input via the retinohypothalamic

tract (RHT), via the signals from the pineal gland, and possibly directly via deep brain

photoreceptors (Foster et al., 1994). The vSCN contains peptides that are analogous to

those found in the mammalian SCN, such as glutamic acid decarboxylase, 5-

hydroxytryptamine, neuropeptide Y, neurotensin, vasoactive intestinal polypeptide, and

arginine vasopressin (Cassone and Moore, 1987). The vSCN expresses rhythmic

metabolic activity in the sparrow and chicken (Cassone, 1988; Lu and Cassone, 1993a; Lu and Cassone 1993b; Cantwell and Cassone, 2002) with glucose uptake higher during the subjective day than during the subjective night. Lesions to this area abolish locomotor activity (Ebihara and Kawamura, 1981; Takahashi and Menaker, 1982) and rhythms of NE turnover in the pineal gland (Cassone et al., 1990).

The mSCN does not display rhythmicities in glucose uptake or bind melatonin. However, it does contain genes that are rhythmic components of the clock in quail (discussed in greater detail later; Yoshimura et al., 2000). Lesions to the mSCN of pigeons disrupted circadian rhythms in locomotor activity under constant dim light (Yoshimura et al., 2000). Track tracing studies in pigeons found retinorecipient staining in the mSCN as well as the vSCN (Shimizu et al., 1994) and the ring dove mSCN receives retinal input as well (Norgren and Silver, 1990). Perhaps, both regions contribute to the rhythms of the SCN and are somehow coupled together as Cantwell and Cassone (personal communication) have found an astrocytic bridge and a neuronal link between the mSCN and the vSCN.

The multiple avian pacemakers are thought to be explained by a looping model. The neuroendocrine loop model proposes that these are damped oscillators, which interact with each through output pathways, such as the RHT, the sympathetic nervous system, and the bloodstream (Cassone and Menaker, 1984; McGoogan and Cassone, 1999).   This interaction is inhibitory, such that during the day the SCN inhibits the pineal gland by secretion of NE, while during the night the pineal gland inhibits the SCN by release of melatonin. Abolition of one of these pacemakers may not destroy the

circadian rhythm of the organism, although the rhythm may dampen over time. There are

other complementary models of this system. Gwinner et al. (1997) describes an internal

resonance model, whereby pacemaker components synchronize and amplify each other

through resonance. The output of the overall pacemaker depends on the amplitude of the

individual components. Reduction of the amplitude of any of the components then

reduces the amplitude of the overall pacemaker output rhythms.   There is also a model

which includes a separate metabolic oscillator. This model is discussed in a later chapter

(CHAPTER VI).

Glial Cells

As we have stated previously, glial astrocytes from the diencephalon express the

Mel_{1a} and Mel_{1c}, receptor subtypes, and in the retina glial cells are responsible for the b-

wave component of the ERG. The name glia comes from the Greek word "glue",

suggesting the role of glia in holding the brain together. In fact in the human brain glia

are thought to outnumber neurons 10 to 1. There are two main types of neuroglia,

macroglia and microglia. Microglia have a macrophage-like role in the nervous system.

Macroglia include oligodendrocytes, Schwann cells, Bergmann glia, Müller glia, and

astrocytes. There are many roles for these macroglia cells (For review, Volterra et al.,

2002). They provide support, like connective tissue. They facilitate neural

integration/signaling between neurons. They provide insulation of neurons via an

encapsulating myelin sheath. They act as buffers or scavengers maintaining an ion

gradient by taking up K^+, glutamate or other ions from the extracellular space. They are

important as producers of energy for the brain by uptake and conversion of glutamate to glutamine that is released back to neurons. They also serve as energy stores by converting glucose to glycogen for later use by themselves and neurons. Astrocytic endfeet help maintain, and possibly regulate, the blood-brain barrier. They produce trophic factors, such as cytokines, that promote survival of neurons. They are important in the development of the nervous system, functioning like a monorail to move neurons from ventricular zones during development. As a fuel source for these activities, astrocytes via glucose transporters uptake glucose, that is readily used to make ATP.

Astrocytes are found throughout the central nervous system and their relatively short projections form many contacts with neighboring neuronal synapses and other glia cells (Volterra et al., 2002). The Müller glial cells span the entire retina from the pigmented epithelium to the inner limiting membrane where they play an important role in all vertebrate species (Newman and Reichenbach, 1996). Similar to astrocytes, they mediate metabolic activity and provide nutrition to the neuronal cells, which is especially important in an avascular bird eye. In fact, glycogen stores within the retina are restricted to the Müller cells (Newman and Reichenbach, 1996). Müller cells also mediate ionic processes through a variety of ion channels, such as K^+, Na^+, and Ca^{2+}. Following the release of K^+ into the extracellular space by light-stimulated photoreceptive cells, the Müller cells rapidly depolarize to modulate this K^+ concentration through spatial buffering (Newman et al., 1984). This has also been shown to cause an increase in the intracellular concentration of Ca^{2+} within the glial cell (Keirstead and Miller, 1995), which has further implications discussed later. Moreover,

Müller cells, like astrocytes, express receptors for catecholamines, amino acids, growth factors, and hormones.

Recent studies have shown a new role for glia in which they modulate neuronal signaling. Glial synapses are closely associated with presynaptic and postsynaptic nerve terminals forming a tripartite synapse (Araque et al., 1999). Calcium homeostasis is highly regulated in both neurons and glial cells. The concentration gradient with greater amounts on calcium outside of the cell than inside, favors calcium entry into the cell (Verkhratsky et al., 1998). This transient increase in intracellular calcium plays an important role in many glial cell functions from gene expression and metabolic activity to electrical processing and neurotransmitter release (Finkbeiner, 1993). Calcium ions can enter the cell through voltage-gated ion channels, ligand-gated channels, and the Na^+/Ca^{2+} exchanger (Verkhratsky et al., 1998). Increases in intracellular calcium can also occur when calcium is released from internal stores following activation of (1,4,5)-trisphosphate (IP_3)- gated calcium release channels on the endoplasmic reticulum (Verkhratsky et al., 1998).

Changes in intracellular calcium affect glial function as well as their responses to neuronal activity via signaling transduction. In mixed cultures of astrocytes and neurons, electrically-stimulated elevations in calcium in a single astrocyte will initiate calcium increases in other astrocytes and neurons in the culture (Nedergaard, 1994). Elevations in Ca^{2+} concentrations in astrocytes can cause them to release glutamate to modulate adjacent neurons (Parpura et al., 1994; Parpura and Haydon, 2000). A wave of calcium through a culture of glial cells elevates intracellular calcium levels and increases

electrical activity in hippocampal neurons grown with them (Hassinger et al., 1995).

Prostaglandin E_2 (PGE_2) is a second messenger that when applied to astrocyte cultures

can induce Ca^{2+}-dependent glutamate release (Bezzi et al., 1998). In neurons co-cultured

with astrocytes, a glutamate-dependent slow inward current (SIC) was caused in the

neurons following mechanical stimulation of the astrocytes, and using pharmacological

manipulations it was determined that this effect was due to extracellular glutamate acting

on N-methyl-D-aspartate (NMDA) and non-NMDA glutamate receptors (Araque et al.,

1998). In retinal eyecup preparations, mechanical stimulation of astrocytes and Müller

cells modulates the light-induced excitation of ganglion cells (Newman and Zahs, 1998).

Intercellular Calcium Waves

A population of astrocytes can communicate as elevations of internal Ca^{2+} in a

single cell propagate to neighboring cells as an intercellular Ca^{2+} wave (Nedergaard,

1994). This was first shown as bath application of glutamate to a hippocampal astrocyte

culture caused increases in calcium which spread throughout the cells (Cornell-Bell et

al., 1990). Further studies indicated that glutamate, 5-HT, and ATP could induce long-

distance communication between glial cells, seen as waves of fluorescence moving from

cell to cell (Cornell-Bell et al., 1990; van den Pol et al., 1992). Focal application of

PGE_2 elicits intercellular calcium waves evoking a SIC in adjacent neurons (Sanzgiri et

al., 1999). Additionally, mechanical stimulation of a single astrocyte elicited an increase

in intracellular calcium that spread as a wave to surrounding cells (Charles et al., 1991).

There are two methods by which the waves are thought to propagate, by gap junctions and by extracellular signaling. Astrocyte gap junction channels, primarily composed of the protein connexin43 (Cx43), allow for cell-to-cell communication as small molecules like inositol (1,4,5)-trisphosphate (IP_3) pass through the channel. Blockade of functional gap junctions prevented the intercellular waves from propagating to neighboring cells (Finkbeiner, 1992). Charles et al. (1992) demonstrated that the calcium waves occur via gap junctions. They transfected cells with little or no intercellular waves with Cx43 cDNA, and increased the number and distance of the calcium waves. The second messenger IP_3 can bind to the endoplasmic reticulum (ER) and cause the release of calcium from its stores, thus elevating the intracellular concentration of calcium. Exposure to thapsigargin, an inhibitor of ER calcium release, blocks the communication of the waves suggesting that IP_3 mediates propagation of the waves (Charles et al., 1993). In co-cultures of neurons and astrocytes, following neuronal upregulation of gap-junctional communication and the expression of Cx43, there was an increase in the propagation of intercellular calcium waves (Rouach et al., 2000).

Several extracellular signals have been investigated as to their role in intercellular calcium wave propagation. Isolated groups of astrocytes that were separated from each other could still communicate using intercellular waves across gaps of less than 120 microns, suggesting an extracellular diffusible signal was involved (Hassinger et al., 1996). Several groups have demonstrated that intercellular signaling in astrocytes is mediated by the release of ATP and purinergic receptor stimulation

(Cotrina et al., 1998; Guthrie et al. 1999). As mentioned above, release of glutamate has been shown to mediate glial-neuronal signaling (Parpura et al., 1994), and application of nitric oxide (NO), acetylcholine, and histamine also induces intercellular calcium waves in cultures of astrocytes (Shelton and McCarthy, 2000; Willmott et al., 2000).

The method of propagation may depend on the location or type of glia cell, or it may involve a combination of the two methods (Charles, 1998). In the retina, where both astrocytes and Müller glial cells are present, there seems to be two types of calcium wave propagation (Newman, 2001). Mechanical stimulation evoked the release of ATP in the retinal eyecup preparation. The waves that propagate between the astrocytes are mediated by diffusion of an internal messenger as purinergic antagonists did not greatly reduce the calcium waves. However, waves that propagated from astrocytes to Müller cells and from Müller cells to other Müller cells were significantly reduced by purinergic antagonists suggesting mediation by the release of ATP. ATP acting on purinergic receptors (P2Y) produced a transient Ca^{2+} release by IP_3 in hippocampal astrocytes (Koizumi et al., 2002). It has recently been shown that ATP can be released via hemichannels, or connexins, of individual astrocytes and thereby, induce intercellular calcium waves (Stout et al., 2002). Additionally, using Cx43 knockout mice, Suadicani et al. (2003) demonstrated that downregulation of Cx43 not only affected gap junctional communication, but also decreased expression of the adenine-sensitive P2Y receptor altering the mode of intercellular communication.

While there has been much study of intercellular calcium waves, no clear physiological role has been established. Given the glial cells ability to trigger the release

of neurotransmitters, activate ion channels, and modulate neuronal signaling, it is possible that the waves act in development of the nervous system or in coordinating temporal and spatial cues throughout the brain. This could have wide implications for many fields of study, including circadian rhythms.

Glia and Circadian Rhythms

The idea that astrocytes may play a role in circadian rhythms production and regulation was first suggested by Morin et al. (1989) when they found that the intergeniculate leaflet (IGL) and the SCN in the hamster and the rat had a high number of cells that were immunopositive for glial fibrillary acidic protein (GFAP). There are daily fluctuations in GFAP expression in the mammalian SCN (Lavialle & Serviere, 1993). Plasticity in GFAP expression can occur, as even rats allowed to grow in DD after birth whose GFAP immunoreactivity is reduced compared to controls, will experience an increase in expression when later transferred to an LD cycle (T Ikeda et al., 2003). In enucleated animals, the area of the SCN that receives the retinal projections had decreased GFAP expression, while areas that still expressed GFAP continued to be rhythmic (Lavialle et al., 2001). Changes in GFAP activity in the hamster SCN could be induced by increased expression of serotonin receptors in a phase-dependent manner, such that high levels during mid-day decreased GFAP content (Glass and Chen, 1999). Glial cells in the SCN of the Syrian hamster express transforming growth factor-alpha (TGF-alpha), which was recently suggested to be a clock output signal regulating the activity/rest rhythm (Li et al, 2002).

The RHT synapses that terminate in the SCN release the neurotransmitter, glutamate, and its content there fluctuates on a circadian basis. Astrocytes develop along with the RHT neurons and have been demonstrated to control the extracellular glutamate concentration found in the SCN (Lavialle and Serviere, 1995). The release of glutamate and serotonin at the RHT synapses cause an increase in the intracellular Ca^{2+} concentrations in the SCN neurons and astrocytes, which can lead to intercellular calcium waves (van den Pol et al., 1992). Injection of fluorocitrate into an intact rat SCN leads to inhibition of glial metabolism and induces behavioral arrhythmicity for 1 week (Prosser et al., 1994).

Astrocytes, but not neurons, in the SCN of a rat, are coupled to each other by connexin43-positive gap junctions (Welsh and Reppert, 1996), suggesting a possible role in neuronal coupling. Additionally, it is these gap-junctionally connected astrocytes that surround the glutamate-immunoreactive axons that are in synaptic contact with SCN dendrites and isolate neurons from each other (van den Pol et al., 1992). Disruption of glial activity by blockade of gap junctions using high concentrations of octanol flattened rhythmicity of neuronal activity, and low concentrations of the blocker caused a small phase delay (Prosser et al., 1994). In the chick pineal gland, astrocytes coupled via the Cx43 gap junction protein help synchronize the heterogeneous population of cells to produce the circadian rhythm of pineal activity (Berthoud et al., 2000). As mentioned previously, rhythmic administration of melatonin to chick diencephalic astrocyte cultures induces rhythms in metabolic activity in these cells (Adachi et al., 2002).

MOLECULAR BASIS OF RHYTHMS

Clock Genes

Other components of cells are rhythmic as well, including a large number of genes. Genes that regulate circadian rhythms in an organism have been termed "clock genes". The first animal mutation for circadian rhythms was found by Konopka and Benzer in 1971 while screening mutant flies for abnormal circadian behavior. The *period* (*per*) mutant was arrhythmic in its ecolsion rhythms, and thus the study of molecular circadian rhythms began (Kyriacou and Hall, 1980). On this molecular level, several model systems have been extensively studied. These include the *Drosophila* and mammalian species as well as cyanobacteria and *Neurospora crassa*, and in more recent years, work has been published on the avian species. In the molecular core of clock components, there appears to be a central theme as to the importance in the role of negative and positive elements (Dunlap, 1999). The positive elements are generally transcription factors that activate transcription of clock genes. These genes are translated to clock proteins, which are the negative elements as they feedback to in some way block the action of the positive elements, and thusly inhibit their own transcription. The positive elements are *clock* (*clk*) and *cycle* (*cyc*) in flies, and *clk* and *brain muscle ARNT-like protein* (*Bmal*) in mammals. The negative elements are *period* (*per*) and *timeless* (*tim*) in flies, and *per* and *cryptochrome* (*cry*) in mammals. Additionally, some studies (Sherman et al., 2000) have suggested interlocking loops, in which the negative elements play a positive role by in some way activating/co-activating the transcription of the positive elements. For example as discussed in several papers and reviews (Sherman

et al., 2000; Hastings and Maywood, 2000; Whitmore et al., 2000), in mammals, the positive elements CLK and BMAL form a dimer, which binds to the E-box on the negative elements, *mCry1* and *mCry2,* and *mPer1,2,3* to activate their transcription. PER and CRY proteins dimerize in the cytoplasm. This dimerization is affected by the phosphorylation of PER by the casein kinase I. The PER/CRY dimer enters the nucleus where CRY negatively interacts with CLK/BMAL to inhibit further transcription of *cry* and *per* genes. A similar interlocking loop has been proposed in flies, although with different partners (Glossop et al., 1999). There is a lag time between peak of the mRNA and protein of *per* and *Bmal* that is around 4 to 6 hours, and is important in the generation of a 24-hr rhythm.

Following transcription, kinases and other common components play important roles. Casein kinase I and mitogen-activated protein kinase (MAPK) phosphorylate clock proteins and modulate their stability, or function (Vielhaber et al., 2000; Sanada et al., 2002). In mammals, phosphorylation levels of MAPK are regulated by the circadian clock (Obrietan et al., 1998). The positive elements and some of the negative elements have PAS (Per- ARNT- Sim protein) domains, which helps them to form heterodimers with their "partner". Many of these clock genes contain an E-box in their promoter region (Dunlap, 1999). Transcription factors, such as CLK and BMAL bind to this promoter region to initiate transcription. Positive elements of the circadian loop also activate clock-controlled genes via the circadian clock regulatory elements (CCRE). These clock controlled genes affect a wide variety of processes in an organism. The processes, often called outputs, include temperature rhythms, behavior rhythms,

metabolic processes, development, neuropeptide release, and many others. Some of these processes may be directly activated as is the case in mammals. CLK /BMAL directly bind to the E-box of the gene arginine vasopressin (AVP), a neuropeptide rhythmically expressed in the brain (Hastings and Maywood, 2000). They activate its transcription whereas *per*, *tim*, and *cry* negatively affect this positive AVP regulation. The melatonin synthesis rate limiting enzyme, AA-NAT, which is rhythmically expressed in the retina and the pineal gland, also has an E-box in its promoter region (Chen and Baler, 2000). This suggests a role as to how the clock cells work on output genes and how cells in peripheral tissue may be affected.

Avian orthologs of the mammalian clock genes exist in several species of birds, including sparrows, quail, and chickens (Yoshimura et al., 2000; Bailey et al., 2002; Abraham et al., 2003; Bailey et al., 2003; Bailey et al., 2004). They have been identified in all three of the avian pacemakers, the retina, the pineal, and the SCN. Expression of genes in quail demonstrated that *qPer2* and *qPer3* were rhythmically expressed in both the pineal and the retina, while *qClock* mRNA was constitutively expressed throughout the day (Yoshimura et al., 2000). In cultures of chick pineal cells, analysis showed that CLK and BMAL formed heterodimers, which activated E-box element-dependent transcription (Okano et al., 2001). In the chick pineal gland, active MAPK levels are rhythmic with peaks during the subjective night (Hayashi et al., 2001). Analysis of cryptochrome2 (*Cry2*) in chick revealed rhythmic mRNA expression in the photoreceptors of the retina and in visual and circadian system structures, including the pineal gland, with levels highest in the late subjective night (Bailey et al., 2002).

Cryptochrome is not only a clock gene, but also a flavin-based, blue-light absorbing phytochrome found in many organisms (Van Gelder, 2002). The role of light is important in resetting, or entraining, the clock mechanism on a daily basis as mentioned previously. Light input pathways may act via photoreceptive elements, such as rhodopsin and cryptochrome (Yoshizawa, 1984; Thresher et al., 1998; Stanewsky et al., 1998; Emery et al., 1998). The role of cryptochrome varies depending on the organism. In plants, *cry* mediates blue light-dependent development as well as high-intensity blue light signals for circadian period length control (Lin et al., 1998; Somers et al., 1998). *Cry2* is induced by light in chickens (Bailey et al., 2002), but neither *cry1* nor *cry2* is light induced in mammals (Griffin et al., 1999). Instead, light plays a more direct role in activating the transcription of *mPer1* and *mPer2*, but not *mPer3* (For review see Hirota and Fukada, 2004). In mammals, daily entrainment with light is thought to occur via the retinal photoreception as bilaterally enucleated animals cannot phase shift locomotor activity following light exposure (Yamazaki et al., 1999). Recently, though, a role for melanopsin, a non-visual opsin found in the retinal ganglion cells that compose the RHT to the SCN (Provencio et al., 2000; Hannibal et al., 2002), was discovered after blind mice normally reset their clocks (Foster and Hankins, 2002). As mentioned earlier, several other visual and non-visual opsins have been discovered in the retina, pineal, and SCN oscillatory components (Natesan et al., 2002; Van Gelder, 2003; Rollag et al., 2003; Bailey and Cassone, 2004) and studies are ongoing as to their role in the entrainment of the circadian clock.

OBJECTIVES AND SIGNIFICANCE

Melatonin receptors are expressed on glial cells in the brain and the retina. Although several studies have demonstrated melatonin's antioxidant effects in protecting astrocytes against oxidative stress (Martin et al., 2002), but little aside from the Adachi et al. (2002) study is known about other effects of melatonin on glial cells. As the role of glia is further explored, especially in their communication to each other and neurons, their role in circadian rhythms becomes of interest. The following series of experiments were conducted to discover how melatonin modulates glial cells and their communication, and thus, affecting neurons, and having a global effect on the brain communication and homeostasis. Examination of the effects of melatonin on calcium waves in mammalian and avian astrocytes will allow us to discover similarities and differences that may exist among various species. Additionally, the presence of clock genes in the chick glial cells and how these genes are regulated can give important insights into the outputs of the circadian clock and a better overall view of circadian clock function within the hypothalamus.

CHAPTER II

MELATONIN MODULATES INTERCELLULAR COMMUNICATION AMONG

CULTURED CHICK ASTROCYTES[*]

INTRODUCTION

The pineal neurohormone, melatonin, is important for the stability and

expression of circadian rhythms in many vertebrates, especially in diurnal animals with

visually biased neurobehavioral repertoires (Cassone, 1998). Melatonin production is a

direct output of the circadian pacemaker such that melatonin is synthesized primarily

during the night in animals maintained in light:dark cycles (LD) and persists during the

subjective night in animals maintained in constant darkness (DD; Cassone, 1998). In

birds, the location of the pacemaker generating melatonin rhythms resides within the

gland itself, while in mammals the pacemaker resides within the hypothalamic

suprachiasmatic nucleus (SCN), which then regulates pineal rhythms via the sympathetic

nervous system (Cassone and Menaker, 1984; Miller et al., 1996; Klein et al., 1997;

Gwinner and Brandstatter, 2001). In turn, melatonin signaling affects the diencephalon,

including hypothalamic areas such as the SCN to modulate functional aspects of the

biological clock (Cassone, 1990a). Glial cells within the diencephalon are abundant and

express melatonin receptors. In chick diencephalic astrocytes, $Mel_{1c}R$ and $Mel_{1a}R$

mRNA are expressed in 100% and 25% of cells respectively, while none express the

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Mel_{1b} receptor (Adachi et al., 2002). Melatonin, signaling through these G_i-coupled receptors, modulates glucose uptake and the release of glycolytic byproducts from astrocytic cultures (Adachi et al., 2002) and has broad impact on the metabolism of many brain regions *in vivo* (Lu and Cassone 1993a; Cantwell and Cassone, 2002).

The role of glial cells as modulators of brain activity has recently received much interest (Volterra et al., 2002). The neurotransmitter glutamate induces calcium waves in astrocytes (Cornell-Bell et al., 1990) and, in turn, changes in astrocytic calcium stimulate glutamate release from glia, thereby evoking glutamergic currents in neighboring neurons and modulating the activity of adjacent synapses (Parpura et al., 1994; Parpura and Haydon, 2000). Calcium waves in retinal glial cells alter light-evoked spiking activity of retinal ganglion cells, demonstrating that glial calcium waves are capable of modulating information processing within the CNS (Newman and Zahs, 1998). Although intercellular calcium waves propagate among neuroglial cells in many brain regions and affect neuroglial processes, a larger functional context for this signaling phenomenon has not been elucidated. In addition, astrocytes are known to communicate via gap junctions, which conduct ionic and small signaling molecules from cell to cell. The functional significance of these astrocytic processes to neuronal integration is not known.

Using chick diencephalic astrocytes in culture, we tested the hypothesis that glial cell calcium homeostasis, intercellular calcium waves, and gap junctional communication are modulatory targets of melatonin. We conducted ratiometric Ca^{+2} imaging studies and demonstrated that melatonin potentiates the propagation of calcium

waves at physiological doses. In addition, we asked whether gap junctional communication among astrocytes is similarly affected and, interestingly, found an opposite response. This is glial coupling was suppressed by melatonin. This switch in interastrocytic communication implicates glial communication pathways as a functional link between circadian behavioral states and the wide spectrum of glial brain functions.

MATERIALS AND METHODS

Cell Culture

Glial cells were prepared from chick embryonic (E17) eggs (Hyline Hatcheries, Bryan, TX). Embryos were extracted and the brains were removed. Diencephalic tissue was placed in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO) and dissociated using titration with 1% trypsin. Cells were filtered through nylon mesh (180 mm pore diameter) and centrifuged for 5 minutes at 1000X g. The supernatant was removed and the pellet was re-suspended in DMEM with 10% fetal bovine serum (FBS) and 1% PSN antibiotic (penicillin, streptomycin, neomycin; Gibco/Invitrogen, Grand Island, NY). The suspension was filtered again through nylon mesh (77 mm pore diameter) and seeded into 75 cm^2 tissue culture flasks. Cultures were maintained in darkness at 37°C with 95% air and 5% CO_2 in a humidified Napco CO_2 incubator. Culture media was changed every other day until cell cultures reached confluence (approximately 10 days).

In all experiments, cells were incubated in melatonin (Sigma) presented in a solution of culture media containing 1% DMSO. Incubations varied from 30 to 60

minutes, depending upon the experiment. All parallel controls contained a 1% DMSO vehicle. Although DMSO has been reported to affect intercellular coupling (Haller and Luft, 1998) and connexin protein expression (Kojima et al., 1997), our results demonstrate significant effects of melatonin on calcium signaling and junctional coupling when compared to this DMSO vehicle control.

Ratiometric Calcium Imaging

Confluent cultures were re-plated onto coverglass chambers (Lab-Tek) and allowed to grow to confluence (5-6 days). Cells were loaded with 5 μM Fura-2 AM (diluted from a 50 μM Fura-2 AM stock in a 20% Pluronic F-127 in DMSO; Molecular Probes, Eugene, OR) in culture medium for 1 hour at 37º C prior to imaging. In experiments using forskolin, cells were treated with forskolin (10 μM; Calbiochem, La Jolla, CA) during Fura-2 loading. Following extensive washes, cultures were treated with melatonin (Sigma) or a 1% DMSO vehicle for 30 minutes prior to imaging. Ratiometric calcium imaging was conducted at room temperature with an Olympus IX70 inverted microscope connected to an Orca ER CCD camera (Hamamatsu). The 40x 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). The number of cells in the ROI was manually counted from raw fluorescence images, where cell boundaries were obvious at 40x magnification, using SimplePCI 4.0 imaging software (Compix, Inc., Cranberry Township, PA) and a ROI consisted of approximately 33 to 50 cells with an overall average of 41.6 cells per ROI. Resting calcium levels were first obtained from a

ROI and then a single glial cell was stimulated once using gentle mechanical stimulation with a micromanipulated, glass micropipette to elicit an immediate calcium wave. In 97% of the ROIs, only a single stimulation was required to initiate the calcium elevation and wave. The number of cells involved in the wave was counted using the difference in the background-corrected ratio (Fluorescent emission at 340 wavelength / Fluorescent emission at 380 wavelength) calculated using SimplePCI 4.0 imaging software. A minimal criterion for inclusion of cells in a wave was that defined as a change in intracellular calcium greater than 5% of the average change of all cells observed in the ROI. The average change in calcium for ROIs ranged from 200-300 nM. Therefore, the minimal level of calcium change required for inclusion in a calcium wave ranged from 10-15 nM. The estimated calcium concentrations were calculated using conventional methods (Grynkiewicz et al., 1985). The percentage of cells involved in a wave was calculated based on the total number of cells in the ROI. Images were collected for 40 seconds, sufficient time to detect any calcium changes in cell within the ROI. In these astrocyte cultures, calcium transients were initiated in cells within the ROI over a range of 1 to 18 seconds from the onset of stimulation (n = 316 cells from ten ROI sampled). Therefore, we were confident that a sampling time of 40 seconds was sufficient to detect all cells within the ROI involved in a calcium wave. Since a 40x objective was used in these experiments, calculations of cells involved in calcium waves are undoubtedly underestimates of the extent of the wave, since they often propagated beyond the field of view (Figure 1A).
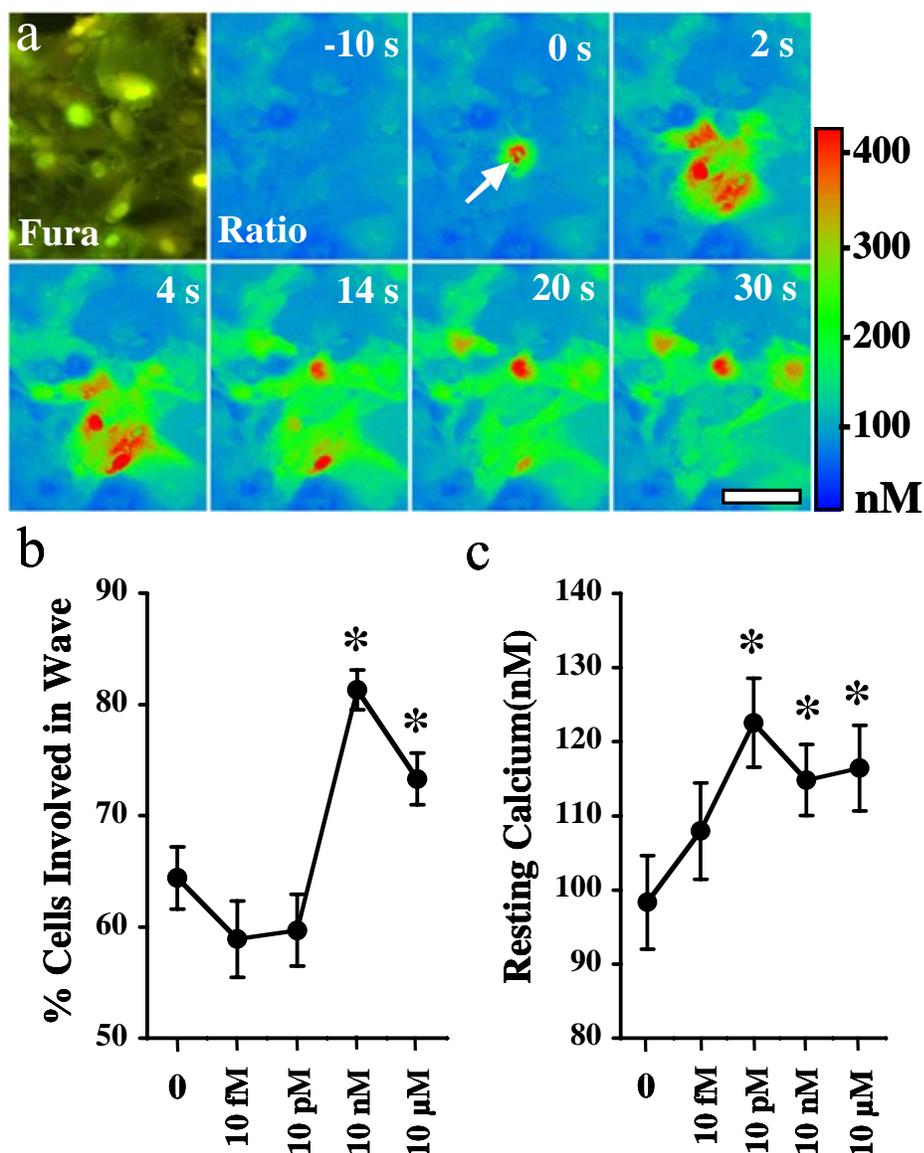
Fig. 1. Mechanically-induced calcium waves among chick astrocytes are modulated by melatonin. **A**. A confluent culture of chick diencephalic astrocytes loaded with Fura-2 AM. Ratiometric image showing a single cell, activated by mechanical stimulation (arrow), initiated a Ca^{+2} wave that spread through surrounding glial cells during the first 30 seconds of wave propagation. Initial resting calcium levels (-10 s) in these forskolin-activated cells ranged from 60-100 nM. Bar equals 20 µm. **B**. Dose response curves for melatonin show increased spread of Ca^{+2} waves at 10 nM and 10 µM concentrations (*, p<0.005, n=58) as compared to control cultures without melatonin exposure (n=68). Waves at 10 fM (n=40) and 10 pM (n=45) concentrations of melatonin were similar to those of control astrocytes. **C**. Resting calcium levels of individual cells were significantly increased over control levels at all concentrations greater than 10 pM melatonin (*, p<0.0001, n ranged from 600 to 2100 cells per dose), with a peak elevation at the pM dose.

Receptor and Second Messenger Pharmacology

In studies using melatonin blockers, Fura-2-loaded astrocyte cultures were incubated at 37º C in the melatonin receptor antagonist, N-acetyltryptamine (1 μM or 100 μM; Tocris, Ellisville, MO), while they were simultaneously treated with melatonin or the 1% DMSO vehicle. In experiments investigating the PKA pathway, the cAMP analogs, Rp-cAMP, a PKA inhibitor, or Sp-cAMP, an activator of PKA (100 μM; Calbiochem), were bath applied for 1 hour at 37º C. Pertussis toxin (PTX; 1 μg/ml; List Biological Labs, Campbell, CA) was added to the culture media to treat the cells for at least 4 hours at 37º C prior to imaging. Additional treatment of forskolin and/or melatonin was given in the continued in the presence of PTX. In similar experiments, the purinergic antagonists, suramin (100 μM; Calbiochem, La Jolla, CA) and pyridoxal phosphate-6-azophenyl-2', 4'-disulfonic acid tetrasodium (PPADS; 50-100 μM; Tocris), the inositol 1,4,5-triphosphate (IP_3) receptor antagonist, 2-Aminoethoxydiphenylborate (2-APB; 100 μM; Tocris), the nitric-oxide synthase inhibitor, N^G-Nitro-L-arginine methyl ester hydrochloride (L-NAME; 1.2 mM; Tocris), and the prostaglandin synthesis inhibitors, acetylsalicylic acid (ASA; 10 μM; Sigma) and indomethacin (50 μM; Sigma) were bath applied to FSK-treated, Fura-2-loaded astrocytic cultures.

Scrape Loading Dye Transfer

For studies of intercellular dye-coupling, a scrape loading dye transfer technique was used (Trosko et al., 2000). Neurobiotin (323 Da; 0.5 mg/ml; Vector Laboratories, Burglingame, CA) and rhodamine-dextran (10,000 Da; 0.125 mg/ml; Molecular Probes)

were bath applied to control or forskolin-activated glial cell cultures treated with 1%

DMSO or melatonin (10 pM, 10 nM, or 10 µM). In experiments using pertussis toxin,

cells were treated with PTX (1 µg/ml) for 7 hours at 37º C prior to addition of forskolin.

Confluent cells on glass-bottom dishes were then damaged with a fresh, sterile scalpel

blade and incubated in the dark for 10 minutes at room temperature. Following cell

loading and diffusion, wells were washed with media, flushed with 4%

paraformaldehyde fixative for 10 minutes, and washed three times in phosphate-buffered

saline (PBS). They were reacted with avidin-FITC (1:1000) overnight at 4ºC, washed

with PBS, and incubated in FITC-tagged, anti-avidin antibody (1:1000) overnight at 4ºC.

They were then washed with PBS and viewed on an inverted fluorescence microscope

using FITC and RITC optical configurations. Dye coupling coefficient was calculated as

the ratio of non-damaged astrocyte cell fluorescence intensity to the damaged astrocyte

cell fluorescence intensity minus the background.

Immunocytochemistry

Cell cultures were plated on glass-bottom dishes for 5-7 days. Control or

forskolin-activated glial cell cultures treated with 1% DMSO or melatonin (10 pM, 10

nM, or 10 µM) for 15 minutes. Wells were fixed with 4% paraformaldehyde fixative for

10 minutes, and were then washed three times in PBS for 10 minutes each. They were

reacted with mouse anti-connexin43 (Cx43; Chemicon, Temecula, CA; 1:1000) in PBS

with 1% Triton-X and 5% goat serum (GT) overnight at 4ºC, washed with PBS as above,

and incubated in FITC-tagged, goat anti-mouse antibody (1:1000) in PBS-GT overnight

at 4ºC. Slides were then rinsed with PBS and viewed on an inverted fluorescence

microscope using FITC optical configurations. The mean intensity of fluorescence and

distribution of fluorescent clustering (extent of punctate staining) was measured for ten

regions of interest in all experimental and control glial cultures. The number of discrete

areas expressing Cx43 (fluorescent puncta) was discriminated by image analysis

(SimplePCI 4.0), where fluorescent spots detectable above a set threshold of intensity

were counted. The intensity threshold criterion was set identically for all regions

measured and cultures examined. In additional immunocytochemical studies, cultures

were confirmed to be 98% glial with anti-glial fibrillary acidic protein (GFAP; Incstar,

Stillwater, MN), consistent with previous descriptions (Adachi et al., 2002).

Statistics

In all experiments, multiple ROIs were sampled from multiple glial cultures.

Multiple culture wells used in any experiment were derived from the passage of a single

culture. Thus, experimental number (n) throughout the paper equals the total number of

ROIs studied across multiple cultures. Ratiometric calcium imaging data and dye

coupling ratios were analyzed using Microsoft Excel and treatment groups statistically

compared using t-tests or one-way ANOVA. Student-Newman-Keuls posthoc test was

used when significant differences were found. In all cases $p < 0.05$ level was used to

determine minimum significance.

RESULTS

Effects of Melatonin on Calcium Waves

Chick diencephalic astrocytes possessed resting cytosolic calcium concentration of 80-100 nM, as estimated by ratiometric image analysis with the calcium indicator Fura 2. Mechanical stimulation of these astrocytes induced elevations in intracellular calcium that spread through out 40-50 cells over a time course of 20 to 30 s (Fig. 1A). Calcium transients associated with waves involved changes in calcium concentration ranging from 12 to 300 nM. We tested a wide range of concentrations of melatonin (from 10 fM to 10 μM) for their influence on astrocytic intracellular calcium levels and intercellular calcium waves. Melatonin caused an increase in the spread of astrocytic calcium waves at physiological concentrations (10 nM), where $81.3 \pm 1.8\%$ of cells were involved in the wave (Fig. 1B) as compared to $64.4 \pm 2.2\%$ in the absence of melatonin. Treatment of glial cell cultures with lower concentrations of melatonin (10 fM and 10 pM) did not alter calcium waves, but did significantly increase resting calcium levels (Fig. 1C). In general, effective doses of melatonin caused 10-20% increases in resting calcium concentration. Thus, melatonin significantly altered glial cell calcium handling, both resting levels and evoked waves.

Melatonin signaling mechanisms involve regulation of adenylate cyclase activity and control of intercellular cAMP levels (Masana and Dubocovich, 2001). To investigate whether the level of intracellular cAMP affected glial calcium waves, cultures were treated with forskolin (10 μM FSK) or control vehicle prior to calcium imaging. Resting calcium levels were elevated slightly ($p<0.02$) from 91.4 ± 8.3 nM in

 controls to 107.9 ± 23.2 nM in cells treated with FSK. Calcium waves in astrocytes

activated with FSK prior to stimulation involved, on average, $45.4 \pm 2.2\%$ of the cells

within a region of interest. In contrast, a smaller percentage of glial cells were involved

($31.3 \pm 4.1\%$) in waves stimulated in the absence of FSK pretreatment (Fig. 2).

Melatonin (10 nM) again caused facilitation of calcium waves in the presence of FSK

treatment (Fig. 2). However, the effects of melatonin on calcium waves were present

even in the absence of FSK treatment. The facilitation of calcium waves by 10 nM

melatonin minus FSK was 23.1% (n=23, $p<0.05$, data not shown), compared to 31.1%

facilitation following activation plus FSK (Fig. 2). Therefore, forskolin-induced

elevation of cAMP potentiated the general spread of the calcium waves, but the

modulatory effects of melatonin were independent of FSK pretreatment.

Calcium waves among chick astrocytes treated with melatonin (10 nM),

following exposure to cAMP analogs, were indistinguishable from control waves treated

with melatonin alone. Both inhibition of cAMP-dependent protein kinase (PKA)

activity with Rp-cAMP (100 μM) and activation of PKA activity with Sp-cAMP (100

μM) caused no differences in the propagation of the waves ($53.3 \pm 3.2\%$ and $55.3 \pm$

2.9% respectively). Additionally, waves in cAMP analog-treated cultures were not

different from those of parallel control cultures ($46.7 \pm 4.6\%$). Thus, taken together with

FSK results, the potentiation of calcium waves by melatonin was not dependent on

intracellular cAMP levels or cAMP-dependent protein kinase activity. Regulation of

cellular events by melatonin is mediated by GTP-binding proteins coupled to specific

melatonin receptors (Reppert, 1997). The effects of melatonin on calcium waves
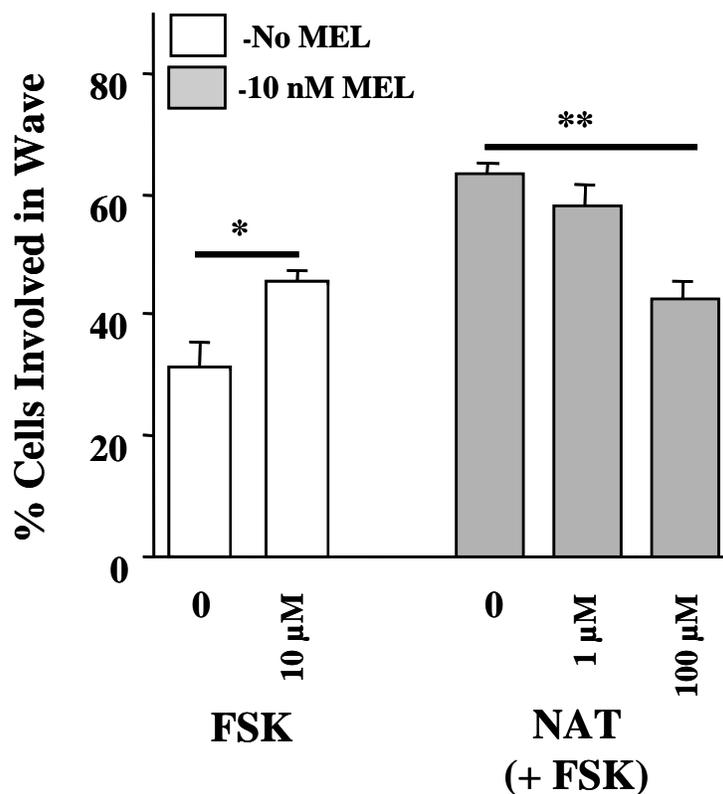
Fig. 2. Melatonin-induced enhancement of calcium signaling in forskolin-activated astrocytes is antagonized by receptor blockade. Application of forskolin (FSK) prior to stimulation significantly increased (*, p<0.003, n=56) the percentage of cells involved in glial Ca^{+2} waves, as compared to control cultures without FSK (n=38). Bath application of a non-specific melatonin receptor blocker, N-acetyltryptamine (NAT; 100 μM), caused a significant amelioration of the 10 nM melatonin-induced enhancement (**, p<0.0001, n=35), while 1 μM NAT had little effect (n=25).
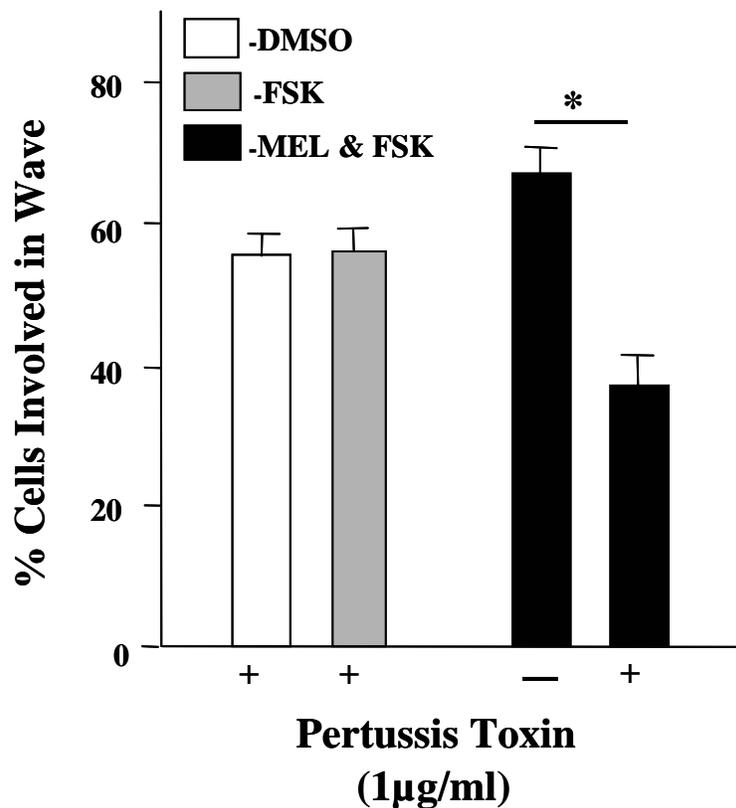
Fig. 3. Effects of melatonin are mediated through G_i protein signaling. The percentage of cells involved in the calcium waves was not different following application of pertussis toxin (1 μg/ml) to forskolin-treated and non-treated glial cells (n=25 per group). The 10 nM melatonin-mediated enhancement of Ca^{+2} waves was significantly reduced by PTX treatment (*, p< 0.005, n=15-25).

were reduced by a melatonin receptor antagonist. Application of N-acetyltryptamine (100 µM), a non-specific melatonin receptor partial agonist/antagonist (Sugden et al., 1997), caused a significant reversal of the nanomolar melatonin-induced facilitation of intercellular calcium waves (Fig 2). The effect was concentration dependent in that 1 µM NAT had little or no effect (Fig 2). Pertussis toxin (PTX; 1 µg/ml) blocked the effects of melatonin on calcium wave propagation (Fig. 3). Exposure to nanomolar melatonin following PTX-mediated inhibition produced a significant reduction in the spread of calcium waves as compared to similar cultures lacking PTX (Fig. 3). Control glial cultures, lacking adenylate cyclase activation, treated with PTX exhibited calcium waves that involved equal numbers of cells as those of cultures pretreated with FSK along with PTX treatment. These results indicate that melatonin-induced facilitation of intercellular communication via calcium waves was a PTX-sensitive event.

Mechanism of Calcium Wave Propagation

We tested the hypothesis that blockade of IP_3 receptors, with the antagonist 2-APB, would disrupt chick glial calcium waves. Pretreatment with the 100 µM 2-APB dramatically reduced the propagation of calcium waves and subsequent treatment with melatonin did not rescue wave propagation (Fig. 4). Thus, IP_3 signal transduction pathways mediate intracellular signaling events responsible for calcium transient generation. However, the intercellular signaling mechanism underlying wave propagation remained to be determined.
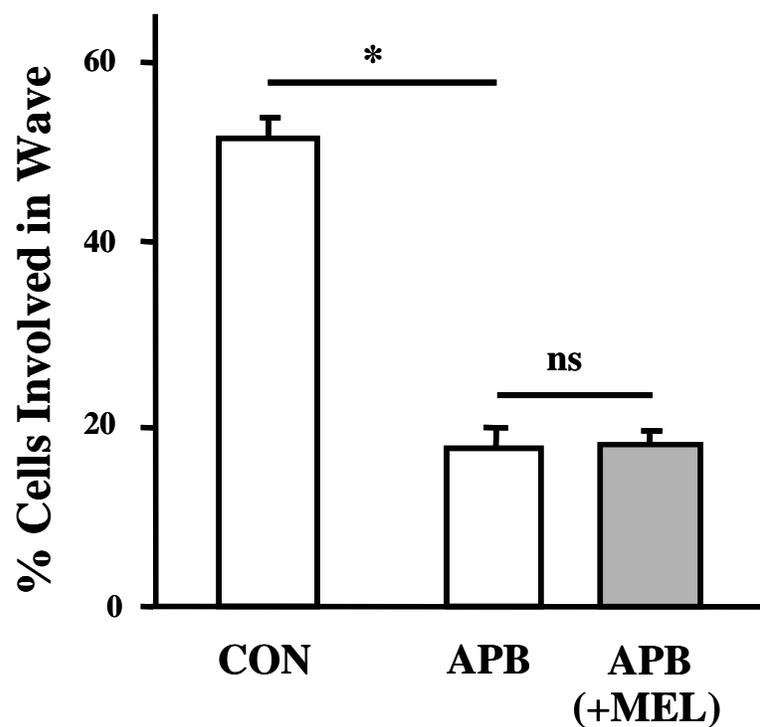
Fig. 4. Calcium waves are mediated via inositol (1,4,5)-trisphosphate (IP$_3$). 2-ABP (100 µM), an IP$_3$ receptor antagonist, significantly reduced the percentage of cells involved in calcium waves as compared to forskolin-treated controls (*, $p<0.0001$, n=15). Addition of 10 nM melatonin was did not overcome this ABP-induced suppression (ns, not significant; $p>0.05$, n=15).

We investigated the potential role of several intercellular signals in calcium wave propagation. Suramin (100 µM), a general purinergic receptor blocker, and PPADS (50-100 µM), a P2Y/P2X selective antagonist, significantly reduced the percentage of cells involved in calcium waves as compared to waves in control cultures (Table 1). Application of nanomolar melatonin was unable to overcome the reduction caused by suramin. Studies in mammalian astrocytic cultures have demonstrated that ATP is a potential mediator of intercellular calcium waves (Guthrie et al., 1999; Fam et al., 2000). Nonetheless, focal application of ATP (1-100 µM) by pressure application from fine-tipped pipettes (Picospritzer II, General Valve) failed to generate calcium waves comparable to those elicited by mechanical stimulation. N^G-Nitro-L-arginine methyl ester hydrochloride (L-NAME; 1.2 mM), acetylsalicylic acid (ASA; 10 µM) and indomethacin (INDO; 50 µM), inhibitors of nitric oxide synthase (L-NAME), and cyclooxygenase (ASA and INDO), also failed to appreciably diminish the spread of calcium waves through these cultures (Table 1). Thus, the search for an extracellular mediator (i.e., signaling molecule) responsible for the intercellular propagation of chicken diencephalic calcium waves was not successful, when limited to those signals tested in Table 1.

Effects of Melatonin on Gap Junctional Communication

Since a mechanism of melatonin-mediated modulation of extracellular signaling pathways was not sufficient to explain the effects of melatonin on calcium waves, we tested whether melatonin modulated gap junctional coupling in a fashion consistent with

TABLE 1. Effects of drug treatments on percentage of astrocytes involved in the calcium wave

	Suramin (100 µM)	PPADS (50-100 µM)	L-NAME (1.2 mM)	ASA (10 µM)	INDO (50 µM)
Control	83.2 ± 2.31	76.0 ± 2.77	52.2 ± 2.75	51.5 ± 2.65	51.5 ± 2.65
Drug	60.3 ± 6.0^a	55.8 ± 6.0^a	45.8 ± 4.2	56.5 ± 4.77	53.2 ± 2.37
Drug + Mel*	60.0 ± 2.45^a	70.8 ± 3.0	57.6 ± 3.0^b	58.3 ± 3.22	43.4 ± 3.7^b

Data are means ($\pm$ sem).
* concentration administered is 10 nM melatonin
[a] $p<0.01$ compared with corresponding control
[b] $p<0.03$ compared with corresponding drug treatment
n = 15-25 ROI per group
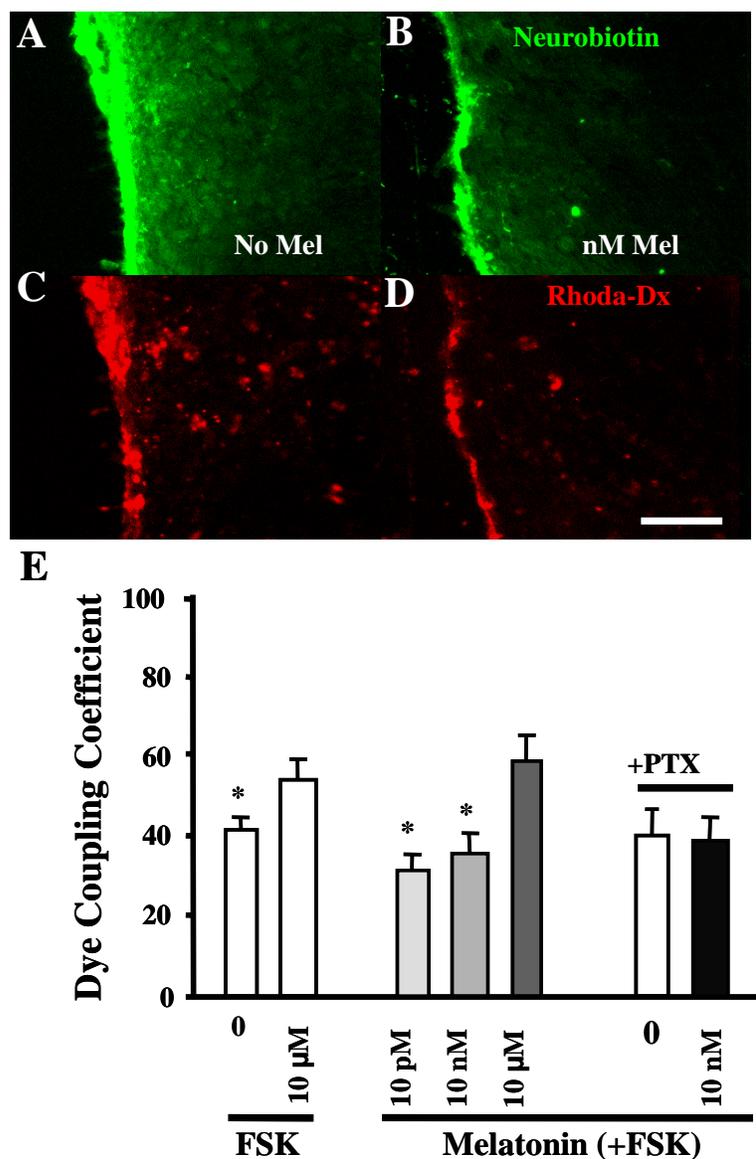
Fig. 5. Dye coupling is altered by melatonin in astrocytic cultures. **A-B**. Using a neurobiotin/scrape loading technique to examine dye coupling, neurobiotin spread readily among FSK-stimulated astrocytes in the absence of melatonin (**A**, No Mel); whereas, spread of the dye between cells was reduced following treatment with 10 nM melatonin (**B**, nM Mel). Rhodamine-dextran fluorescence (**C-D**, Rhoda-Dx) indicates the cells that survived scrape damage and incorporated dye. These loading controls are shown below their corresponding neurobiotin fluorescence image. The site of scrape, represented by a lack of cells, is to the left in each panel. Bar equals 80 µm. **E**. Forskolin (10 µM; n=18) significantly enhanced dye coupling compared to vehicle-treated controls (n=22). In the presence of FSK, melatonin reduced coupling (10 pM and 10 nM; n=12). Dye coupling in glial cells treated with 10 µM melatonin (n=12) was not different from FSK-stimulated controls (n=9). Pertussis toxin (PTX; 1 µg/ml) treated cultures were not different from untreated controls and did not exhibit reduced coupling in the presence of 10 nM melatonin (n=10). In all cases, * equals $p < 0.03$.

its effects on calcium wave propagation. As determined by a neurobiotin/scrape loading

technique (Fig. 5A - D), 10 pM and 10 nM melatonin significantly reduced gap

junctional coupling, while coupling in the presence of higher concentrations (10 μM),

was not different than that of FSK controls (Fig. 5E). In these experiments, the loading

control, rhodamine dextran, remained restricted to the scrape-damaged cells (Fig. 5C and

D). As in calcium wave studies, the modulation of junctional coupling by melatonin was

affected by the activation of adenylate cyclase as cells treated with FSK exhibited a

pronounced enhancement in dye coupling (Fig. 5E) and cells not pretreated with FSK

exhibited statistically insignificant changes in dye coupling, although the trend was

similar (data not shown). Additionally, just as PTX blocked melatonin-mediated

facilitation of calcium waves, PTX treatment abolished the ability of melatonin (10 nM)

to affect a reduction in dye coupling between glial cells (Fig. 5E).

Forskolin not only enhanced dye coupling between astrocytes, but also

significantly increased the expression of gap junctional protein levels, which were

determined by connexin43 immunocytochemistry (ICC; Fig. 6A and B). Forskolin

treatment (10 μM) caused a small, but statistically significant increase (12%) in

immunofluorescence intensity from 59.3 ± 2.4 in control cultures to 67.4 ± 1.9 in treated

cultures. More importantly, FSK-treated cells, following exposure to melatonin,

exhibited marked changes in both the level of Cx43 expression and distribution, as

measured by ICC (Fig. 6C and D). Melatonin applied to astrocytes in the absence of

FSK prestimulation caused no detectable change in Cx43 immunoreactivity (data not

shown). However, physiological levels of melatonin (10 pM and 10 nM) caused a
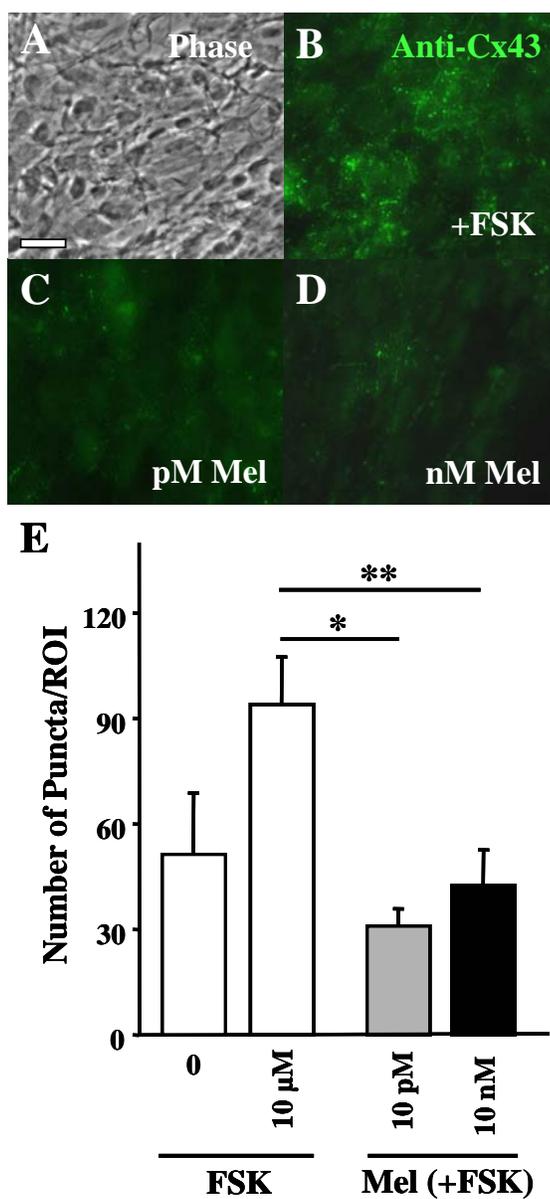
Fig. 6. Forskolin and melatonin enhance connexin-43 immunoreactivity and distribution. (**A-D**) Micrographs of glial cell cultures immunocytologically stained with an antibody to connexin-43. Control cells treated with 10 μM FSK (**A**, phase contrast; **B**, anti-Cx43 immunofluorescence) exhibited intense staining. Both pM (**C**) and nM (**D**) concentrations of melatonin caused a reduction in anti-Cx43 staining ($p<0.001$) in FSK-treated cultures, as compared to FSK-stimulated controls. Scale bar equals 40 μm. (**E**) Image analysis of fluorescent puncta (intensely fluorescing spots per ROI) was used to assess the distribution (clustering) of immunostained proteins. The number of puncta per ROI was significantly decreased by pM (*, $p<0.001$) and nM (**, $p<0.01$) melatonin. Histograms equal mean ± sem. Ten ROIs were imaged per group and all ROIs were 100 μm^2.

significant reduction in anti-Cx43 staining (fluorescent intensity) compared to control (FSK-treated) cultures. These changes in fluorescence intensity, following acute melatonin exposure were accompanied by alterations in the distribution or clustering, of immunostained gap junctional proteins (Fig. 6E). The number of detectable fluorescent puncta was significantly decreased by both 10 pM and 10 nM melatonin, suggesting that melatonin alters the expression of the connexin proteins and their localization in glial cell membranes. Therefore, melatonin elicited changes in gap junctional communication between chick astrocytes, both dye coupling and Cx43 expression that were precisely opposite to the facilitatory effects of this neurohormone on intercellular calcium waves.

DISCUSSION

Glial cells are abundant in many brain regions, including the diencephalon, and regulate a wide range of activities. Here, using chick diencephalic glial cell cultures, we demonstrate that the pineal neurohormone melatonin modulated the spread of calcium waves, resting calcium levels, and gap junctional connectivity. The facilitatory effect of melatonin on calcium waves was observed at physiological (nM) concentrations, levels of neurohormone that are present in the circulatory systems of birds and mammals during the night (Pang and Ralph, 1975; Cassone and Menaker, 1983; Cassone et al., 1986b). Surprisingly, picomolar and nanomolar concentrations of melatonin caused elevations in intracellular calcium and marked reductions in gap junctional coupling and connexin43 expression. These results demonstrated that melatonin imposes a functional switch in the nature of intercellular communication among diencephalic astrocytes.

Chick diencephalic astrocytes express the RNA of both Mel_{1a} and Mel_{1c} receptors (Adachi et al., 2002) and the melatonin receptor blocker, N-acetyltryptamine, reduced the melatonin-mediated enhancement of calcium wave propagation. Melatonin action on these chick astrocytes is likely mediated by multiple melatonin receptors. Unfortunately, other antagonists are not available in the avian system. Luzindole, a receptor antagonist in mammalian cells with highest affinity for the Mel_{1b} receptor subtype, cannot be utilized as the Mel_{1b} receptor is not expressed in these cells (Adachi et al., 2002). Until better antagonists are available, the relative roles of specific receptors in these cellular events will remain undefined.

The effects of melatonin on calcium signaling in these astrocyte cultures were augmented by activation of adenylate cyclase with forskolin and were disrupted by pertussis toxin. However, melatonin-induced facilitation of calcium waves was not dependent on cAMP elevation by FSK. Melatonin when applied to chick astrocytic cultures, inhibits forskolin stimulated 2-deoxyglucose (2DG) uptake and this inhibition by melatonin is pertussis toxin sensitive (Adachi et al., 2002). Taken together, these studies suggest that elevation of cAMP enhances melatonin's effects on calcium waves and metabolism by priming cellular mechanisms or synchronizing cellular events.

Gap junctions allow for biochemical exchange of small molecules between cells, including the second messenger IP_3. The cellular mechanisms underlying calcium waves often involve the activation of calcium release from intracellular stores and, in many systems including glial cell cultures, IP_3 mediates this process (Charles, 1998). The propagation of calcium waves in our chick glial cultures was virtually abolished by

disruption of IP_3 signaling. This suggested the possibility that gap junctional communication might mediate the spread of calcium waves in theses glial cell cultures. However, as indicated above, melatonin conferred a dose-dependent reduction on dye-coupling and connexin43 expression among astrocytes that is precisely opposite that on calcium waves. In the presence of picomolar and nanomolar melatonin, gap junctional coupling was decreased as resting levels of calcium were elevated. Whether or not elevated intracellular calcium, induced by melatonin, was mechanistically-linked to gap junction gating in these astrocytes remains to be determined, but calcium elevation leads to changes in junctional coupling in many systems (Peracchia, 2004). Thus, if gap junctional coupling mediates propagation of calcium waves in these cultures, mechanisms must exist to facilitate this aspect of their function at the same time that their general permeability is reduced.

Although gap junctions provide a conduit for intercellular exchange, including IP_3, other mechanisms of cell-cell communication might underlie the effect of melatonin on intercellular calcium waves. Thus, we investigated several paracrine signaling molecules (i.e., ATP, nitric oxide and prostaglandin) as potential mediators of IP_3-dependent calcium waves among glial cells as demonstrated by other laboratories (Charles, 1998). For example, ATP induces changes in astrocytic intracellular calcium, is released during calcium wave propagation, and, when blocked by purinoceptor antagonists, fails to activate intercellular waves (Guthrie et al., 1999; Fam et al., 2000). We found that in our cultures, although blockade of purinergic receptors and inhibition of synthases required for prostaglandin and nitric oxide signaling altered calcium waves

to a detectable extent, it is unlikely that any one of these signaling molecules is the primary messenger governing calcium waves in chick astrocytes. Therefore, the question of whether these waves are regulated by gap junctional signaling, extracellular signaling, or combined signaling pathways remains unclear.

The effects of melatonin on dye coupling were dependent on forskolin-stimulation of cAMP formation, an action that facilitates gap junction clustering at astrocytic contacts (Paulson et al., 2000). Furthermore, exposure to pertussis toxin blocked the reduction in dye coupling induced by melatonin suggesting a $G_{\alpha i}$-dependent coupling of MelR activation to the inhibition of gap junctional gating. Following pertussis toxin exposure, melatonin markedly suppressed intercellular calcium waves, indicating that gap junctions may not play an important role in melatonin-induced potentiation of glial calcium wave propagation. Studies investigating pertussis toxin (PTX)-sensitive mechanisms have demonstrated a role for the coupling of G_i proteins to melatonin receptors in mammals (Carlson et al., 1989) and chickens (Adachi et al., 2002). In chick astrocytic cultures, PTX exposure removed G_i-mediated adenylate cyclase inhibition and caused the conversion of calcium wave facilitation to suppression following exposure to nanomolar melatonin.

Current models of melatonin receptor-mediated signal transduction, based largely on the mammalian $Mel_{1a}R$, suggest a $G_{\alpha i}$-mediated inhibition of cAMP formation and protein kinase A activity, and a $G_{\beta\gamma}$-mediated potentiation of phospholipase C activity and IP_3 stimulation of calcium release from intracellular stores (Masana and Dubocovich, 2001). Here, manipulation of PKA activation had no effect on calcium

wave propagation and FSK stimulation was not required for calcium wave modulation by melatonin. Consequently, we predict that potentiation of calcium waves by melatonin may be mediated by a $G_{\beta\gamma}$-coupled stimulation of a PLC/IP$_3$ cascade, rather than a cAMP-dependent mechanism. It is possible, however, that other G protein-coupled mechanisms, for example G_q, contribute to this neuroglial modulation. Thus, the current studies suggest a complex interaction between melatonin receptors, G protein activation, and IP$_3$ second messenger cascades in this neurohormone regulation of intercellular calcium waves.

Functional Switch in Astrocytic Coupling

During mid-night, the brains of birds and mammals experience their highest exposure to circulating melatonin (1-100 nM; Pang and Ralph, 1975; Cassone and Menaker, 1983; Cassone et al., 1986b). We hypothesize a model for melatonin's mechanism of action on glial cells at this time and neurohormone titer (nM; Fig. 7). This model predicts that activation of melatonin receptors on chick glial cells elicits dichotomous alternations of intercellular communication. First, melatonin, upon activation of glial receptors, induces an elevation in resting calcium levels. Second, $G_{\alpha i}$-mediated inhibition of adenylate cyclase imparts a decline in cAMP-dependent signaling, and consequently, a reduction in gap junction distribution and coupling. Rising calcium levels could also contribute to changes in gap junctional gating. Third, and finally, melatonin enhances the propagation of intercellular calcium waves via an IP$_3$-dependent mechanism, mediated by $G_{\beta\gamma}$-activation of phospholipase C.
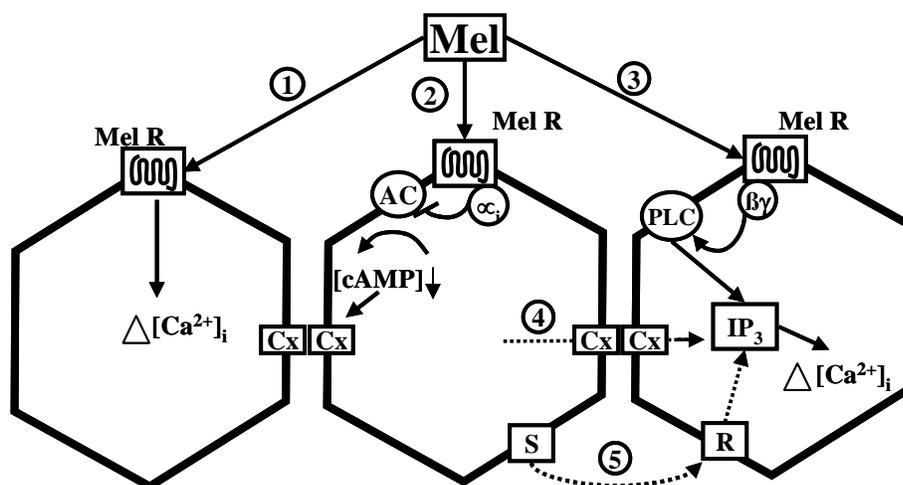
Fig. 7. Predicted model for melatonin regulation of astrocytic communication. Melatonin (Mel) binds to seven-transmembrane receptors, Mel R, on the surface of contacting astrocytes (hexagons) and alters intercellular communication through multiple signal transduction mechanisms. ① Melatonin causes a significant elevation in resting calcium concentration. ② $G_{\alpha i}$-activation inhibits adenylate cyclase, and thereby decreases cAMP signaling and gap-junctional coupling between glial cells. ③ $G_{\beta\gamma}$-activation leads to phospholipase C induction and IP_3-dependent release of calcium from intercellular stores. In this model, IP_3-signaling mediates an undefined mechanism of wave propagation (dotted lines), either by gap junctional passage of an intercellular signal, such as IP_3 itself ④, or a paracrine mechanism of calcium wave propagation (S→R) that in turn activate IP_3 signaling ⑤. At nanomolar concentrations of melatonin that facilitate intercellular calcium waves, gap junctional communication is suppressed. Therefore, this model suggests a functional switch in astrocytic communication mediated by melatonin receptor activation.

Recent studies of biological clocks in vertebrate animals have focused on the search for neural coupling pathways that govern circadian rhythmicity of hypothalamic outputs, including activity patterns, sleep/wake cycles, temperature and metabolic rhythms, feeding patterns, and cycles of neuroendocrine secretion. SCN neuronal oscillations are cell autonomous (Pennartz et al., 2002); yet, SCN astrocytes, but not neurons, are coupled by gap junctions (Welsh and Reppert, 1996). SCN transplantation restores rhythmicity of outputs without extensive new synaptic connectivity (LeSauter and Silver, 1998) and SCN cultures confer rhythmicity on arrhythmic follower cells in the absence of cell-cell contacts (Allen et al., 2001). Therefore, mechanisms of SCN interneuronal communication, other than conventional synaptic pathways, must exist. The spread of calcium waves through astrocytes might represent a potential signaling pathway for intercellular communication between hypothalamic neurons. In addition, melatonin-mediated modulation of interastrocytic calcium signaling may represent a neurohormonal mechanism for regulation of wide-spread brain coupling, or "volume transmission" (Agnati et al., 1992). Such global influences are likely the kind of broad modulatory effects that might be disrupted in long-distance travelers and shift workers, dysfunctions that are ameliorated by clock-resetting or, in some cases, by the administration of melatonin (Skene et al., 1999).

Astrocyte Coupling and Brain Metabolism

Coupling between brain activity and energy utilization is largely regulated by astrocytes. Astrocytic endfeet interact with both synaptic sites of neuronal

communication and capillary endothelial sites of energy substrate uptake. Neural activity likely induces glucose uptake and stimulates glycolysis in astrocytes (Magistretti and Pellerin, 1999). Melatonin acutely inhibits 2DG uptake and the release of lactate and pyruvate in chick astrocytes (Adachi et al., 2002), globally affects avian brain metabolism (Lu and Cassone 1993a; Lu and Cassone 1993b; Cantwell and Cassone, 2002), and modulates chick astrocytic morphology (Cassone, unpublished data). Thus, this neurohormone is uniquely positioned to link environmentally entrained physiologic oscillations to the changing energetic demands of a diurnal animal. It has been suggested that mechanisms that serve to link cellular metabolic state with circadian oscillations might constitute evolutionarily ancient connections (Rutter et al., 2002). In the case of diencephalic structures, the same suprachiasmatic nuclei that regulate the rhythmic production of pineal melatonin are themselves targets of this hormone's metabolic and calcium homeostatic regulation.

Intercellular calcium waves occur between astrocytes and endothelial cells and likely play a role in astrocyte-endothelial interactions at the blood brain barrier (Leybaert et al., 1998). This is interesting since the restoration of brain energy has been hypothesized as the function of animal sleep, such that sleep is homeostatically regulated and physiologically linked to brain energy consumption (Benington and Heller, 1995). Regardless of the ultimate function of sleep, a switch in glial cell connectivity induced by melatonin suggests a critical connection between glial physiology, brain metabolism, and mechanisms of biological rhythms, including sleep/wake cycles. Melatonin, which causes sleepiness in diurnal organisms and facilitates recovery from jet lag in humans

(Skene et al., 1999), may initiate an astrocytic pseudostasis of energy deficiency, thereby shifting the brain into an altered, energy-conserving state. The switch in the nature of astrocytic communication reported here represents the first demonstration of a pervasive, functional consequence of melatonin on the intercellular signals that propagate among neuroglial cells in the brains of diverse animal species.

CHAPTER III

MODULATION OF INTERCELLULAR CALCIUM SIGNALING BY

MELATONIN, IN AVIAN AND MAMMALIAN ASTROCYTES, IS BRAIN

REGION SPECIFIC

INTRODUCTION

Neurons of the suprachiasmatic nucleus (SCN) in mammals, and a homologous

hypothalamic structure in birds, are considered the master pacemakers of vertebrate

biological rhythms (Ebihara and Kawamura, 1981; Takahashi and Menaker, 1982;

Moore, 1983; Klein et al., 1997). Rhythmic SCN outputs activate hypothalamic-to-

autonomic signaling pathways that culminate in sympathetic modulation (via the

superior cervical ganglia) of the pineal gland (Klein et al., 1997; Moore & Silver 1998).

This modulatory pathway regulates the rhythmic production and release of melatonin

from the pineal, such that circulating blood levels of the neurohormone are low during

the day and high during the night in both birds and mammals (Pang and Ralph, 1975;

Cassone and Menaker, 1983; Cassone et al., 1986b). Day-night rhythms of melatonin

release are entrained to environmental light-dark cycles by sensory inputs to the SCN

from the neural retina via the retinohypothalamic tract (RHT; Rusak, 1979; Reppert et

al., 1981; Cassone et al., 1990).

Melatonin directly influences circadian rhythms. Rhythmic melatonin

administration entrains locomotor activity, inhibits SCN metabolic activity *in vivo*,

inhibits SCN electrical activity *in vitro* and phase shifts the SCN clock *in vitro* (Redman

et al., 1983; Cassone et al., 1988; Shibata et al., 1989; Gillette & McArthur, 1996). Not surprisingly, two of the three G-protein-coupled melatonin receptors that have been identified, the Mel_{1a} (MT1) and Mel_{1b} (MT2) subtypes, are highly expressed in the mammalian SCN (Weaver et al., 1989; Reppert et al., 1996; von Gall et al., 2002; Dubocovich et al., 2003). In birds, melatonin receptor expression is high in many brain regions, including the hypothalamus and visual system structures (Rivkees et al., 1989; Weaver et al., 1989; Brooks & Cassone, 1992; Cassone et al., 1995). Thus, the same hypothalamic structures that drive or entrain rhythmic melatonin production and release, are direct targets of the neurohormone's modulatory action.

Identification of the avian melatonin receptor subtypes suggest that the Mel_{1a} receptor is found mainly on neurons of the brain, while the Mel_{1c} receptor is found on non-neuronal cells, primarily astrocytes (Reppert et al., 1995). In the chick, all astrocytes of the diencephalon express the mRNA of the Mel_{1c} receptor, while only 25% express the Mel_{1a} receptor (Adachi et al., 2002). None of these astrocytes appear to express the Mel_{1b} receptor. Melatonin modulates glucose uptake and the release of glycolytic byproducts from astrocytic cultures (Adachi et al., 2002) and affects changes in brain metabolism *in vivo* (Lu and Cassone, 1993a; Cantwell and Cassone, 2002). Melatonin also modulates resting levels of astrocytic calcium and facilitates the spread of intercellular calcium waves among astrocytes (CHAPTER II).

Because melatonin receptors are expressed in many brain regions (Rivkees et al., 1989; Weaver et al., 1989; Reppert et al., 1995), including telencephalic structures, we have tested whether melatonin affects differential responses in mouse and chicken

astrocytes isolated from both the diencephalon and telencephalon. We hypothesized that the effects of melatonin on vertebrate brain physiology, in this case calcium signaling, would be greatest in diencephalic astrocytes.

MATERIALS AND METHODS

Chick Astrocyte Cell Culture

Glial cells were prepared from chick embryonic (E17) eggs (Hyline Hatcheries, Bryan, TX). Embryos were extracted, the brains were dissected, and meninges were removed. Telencephalic tissue and diencephalic tissue, where the SCN of the hypothalamus is located, were placed in separate petri dishes containing minimal essential medium with Earle's salts (MEM; Gibco/Invitrogen, Grand Island, NY) and 1% PSN antibiotic (penicillin, streptomycin, neomycin; Gibco/Invitrogen). Tissue was dissociated using gentle trituration with MEM containing 3U/ml dispase (Gibco/Invitrogen) while stirring at a low speed on a stir plate. Dissociated tissue in suspension was extracted after 10 minutes and placed into a 15 ml conical tube. Additional dissociation media was added to the non-dissociated tissue along with 80 ul of 8000 U/ml Dnase I solution (Sigma, St. Louis, MO) and allowed to stir for 10 more minutes. Another extraction of dissociated tissue was performed. Dissociated tissue from the 15 ml conical tubes was pooled into a 50 ml conical tube and centrifuged at 1000X g for 10 minutes at 4° C. Media was aspirated from the cell pellets, and cells were re-suspended in MEM with 10% fetal bovine serum (FBS) and 1% PSN antibiotic, and seeded into 75 cm^2 tissue culture flasks. Cultures were maintained in darkness at

37°C with 95% air and 5% CO_2 in a humidified Napco CO_2 incubator. Culture media was changed after 18-24 hours and then every three days until cell cultures reached confluence (approximately 10 – 14 days).

Mammalian Astrocyte Culture

One day old C57Bl/6J mice were killed by decapitation and primary astrocytes were isolated from cortex (telencephalon) or hypothalamus (diencephalon) essentially as previously described (Aschner et al., 1992; Erikson and Aschner, 2002). Tissue was rapidly dissected and meninges were removed under a stereomicroscope (Olympus) in isolation medium consisting of Modified Eagle's Medium containing Earle's salts (Invitrogen) and penicillin-streptomycin-neomycin (PSN) at twice the concentration present in normal growth medium (0.001 mg/ml for penicillin and streptomycine and 0.002 mg/ml for neomycin, respectively). Tissue extractions were performed in isolation medium containing 1.5 U/ml dispase (Sigma). The number of extractions was reduced by half to accommodate the lower yield of tissue from mice as compared to rats. Astroglial cultures were maintained at 37 C, 5% CO2 in Minimal Essential Medium (MEM) supplemented with Earle's salts, 10% FBS, and PSN, and grown 18 days to maturity prior to experiments. In our laboratory, this method routinely yields cultures consisting of 95-98% astrocytes, based upon immunofluorescence staining for glial fibrillary acidic protein. All studies utilizing primary cultured astrocytes from mice was carried out under approved animal use protocol number 2001-209 at Texas A&M University.

Calcium Imaging

Confluent astrocyte cultures were sub-cultured onto coverglass chamber slides (Lab-Tek) and allowed to grow to confluence (6-7 days). Cells were loaded with 4 µM Fluo-4 (diluted from a 2 mM Fluo-4 stock in DMSO; Molecular Probes, Eugene, OR) in imaging medium (MEM with HEPES and L-glutamine and without phenol red) for 45 minutes at 37º C. Cells were then washed twice and treated with melatonin (Sigma) or DMSO vehicle (0.01%) for 30 minutes prior to imaging. Calcium imaging was conducted at room temperature with an Olympus IX70 inverted microscope and 10X PlanAprochromat air objective. Images were acquired using an Orca-ER CCD camera (Hamamatsu) and an entire field of cells (approximately 1000 mm^2) was designated as a region of interest (ROI) for analysis. Resting calcium levels were first obtained from a ROI and then a single glial cell was stimulated using gentle mechanical stimulation with a micromanipulated, glass micropipette to elicit a calcium wave. The area of the spread of the wave was calculated using SimplePCI 4.0 imaging software (Compix, Inc., Cranberry Township, PA). Images were collected every 0.5 seconds for 1.5 minutes in order to permit recording of the initiation, rate of spread, and maximal extent of the spread of the calcium wave through each ROI. Even using the 10x objective, the wave occasionally spread beyond the field of view, indicating that the calculated areas of cells involved in the wave are likely somewhat underestimate the full extent of wave propagation (Figure 8A).
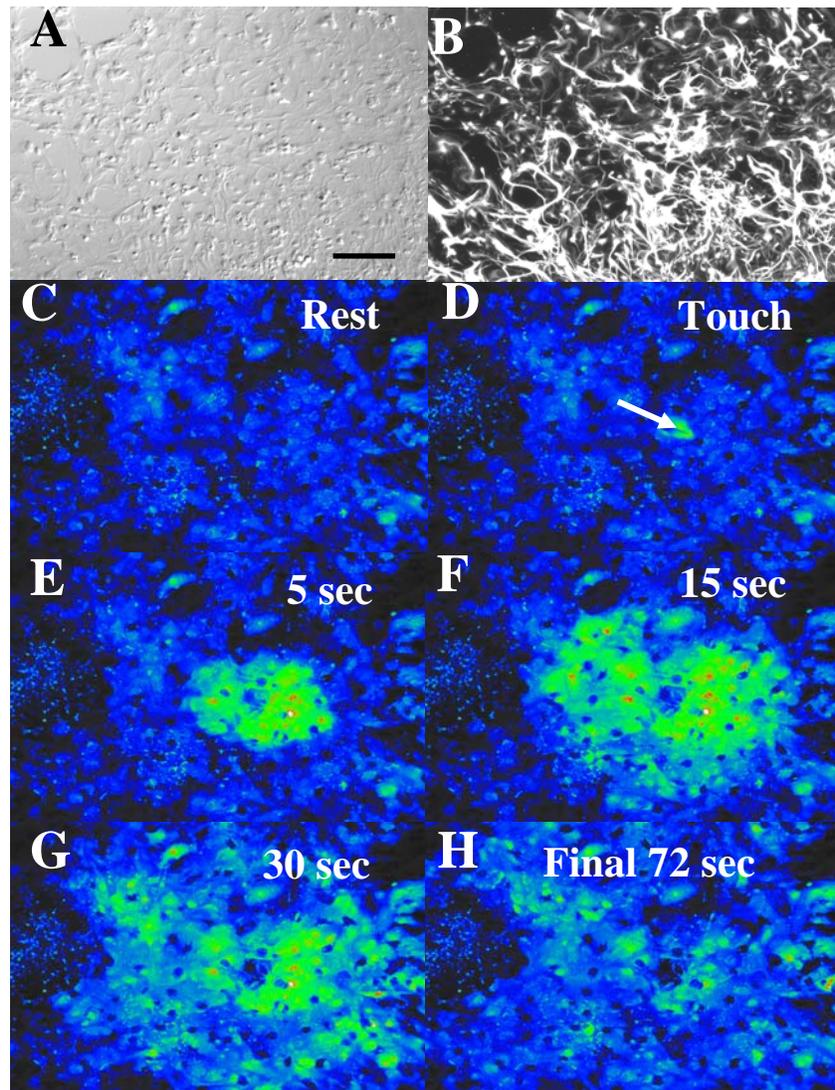
Fig. 8. Ca^{2+} wave propagation through a confluent mammalian astrocyte culture. **A-B**. DIC image of a region of interest of diencephalic mammalian astrocytes (**A**). Astrocyte cultures were 98% glial as determined by anti-GFAP staining (**B**). Bar equals 200 μm. **C-H.** Pseudo-colored image an intercellular calcium wave. A region of telencephalic mouse astrocytes, loaded with Fluo-4 AM, shows selected frames from a calcium wave sequence. After resting levels of calcium were obtained, a single cell was activated by mechanical stimulation (arrow in D) and this 'touch' stimulation initiated a Ca^{+2} wave that spread to surrounding glial cells

Immunocytochemistry

Cell cultures were plated on glass-bottom dishes for 5-7 days. Wells were fixed with 4% paraformaldehyde fixative for 10 minutes, and were then washed three times in PBS for 10 minutes each. Next they were washed with PBS with 1% Triton-X (PBT) for 10 minutes and blocked with 5% goat serum in PBT (PBS-GT) for 10 minutes. They were reacted with glial fibrillary acidic protein antibody (GFAP; Incstar, Stillwater, MN; 1:1000) or melatonin receptor antibody (chick Mel_{1a} or Mel_{1c}; 1:1000 and mammalian MT1 (Mel_{1a}) 1:1000 or MT2 (Mel_{1b}); 1:250; Lifespan Biosciences, Seattle, WA) in PBT-GT overnight at 4ºC. Controls were incubated overnight with block (PBS-GT) instead of primary antibody. The cells were then put through a series of 10 minutes washes with block, PBT, and PBS. The cells were incubated in FITC-tagged, goat anti-rabbit antibody (1:1000) in PBS-GT overnight at 4ºC. Slides were then rinsed with PBS and viewed on an inverted fluorescence microscope using FITC optical configurations. Images were obtained of the cells using the Orca ER CCD camera. The cultures were confirmed to be 98% glial with GFAP, consistent with previous descriptions (Adachi et al., 2002). Images were obtained of the cells using the Orca ER CCD camera. The cultures were confirmed to be 98% glial with GFAP, consistent with previous descriptions (Adachi et al., 2002).

Western Blot Analysis

Protein was extracted from confluent cell cultures with trizol reagent using Invitrogen trizol protocol, quantified with Total Protein Assay (Sigma), and 30ug was

loaded and run on Tris-HCl (4-20% gradient; Bio-Rad) gels. Protein was transferred to

nitrocellulose membrane. Membrane was blocked with blocking buffer (10% dry milk

in PBS) for 30 minutes and incubated with melatonin receptor antibody (mammalian

MT1 (Mel$_{1a}$) 1:1000 or MT2 (Mel$_{1b}$); 1:250; Lifespan Biosciences) in blocking buffer

overnight at 4ºC. Membrane was washed three times with PBS for 10 minutes each

wash and then incubated with biotinylated goat anti-rabbit antibody (1:500) in blocking

buffer for 1 hour at room temperature. It was then washed three times with PBS for 10

minutes each wash and then incubated with Avitin-Biotin Complex (ABC kit; Vector

labs) for 1 hour at room temperature. The membrane was washed three times with PBS

for 10 minutes each wash and then incubated with DAB until there was a color contrast

(about 10-15 minutes).

Statistical Analysis

In all experiments, multiple ROIs were sampled from individual wells. Multiple

wells used in an experiment were derived from the passage of a single culture. n

throughout the paper equals the total number of ROIs studied across culture wells.

Calcium imaging data was analyzed using Microsoft Excel and treatment groups

statistically compared using t-tests or one-way ANOVA. Student-Newman-Keuls

posthoc test was used when significant differences were found. In all cases $p < 0.05$ level

was used to determine minimum significance.

RESULTS

Following dissociation of mouse and avian brain tissues, telencephalic and diencephalic astrocyte-enriched cultures of each were selected based on growth conditions and brought to confluence (Fig. 8A). Cultured cells displayed almost ubiquitous expression of glial fibrillary acidic protein (GFAP), demonstrating that the cultures were composed of >95% astrocytes (Fig. 8B). Astrocyte cultures, whether telencephalic or diencephalic, elicited intercellular calcium waves when a single cell was stimulate by the touch of a micropipette. Calcium waves spread from the stimulated cell to neighboring cells and propagated among large numbers of glial cells in the confluent culture (Fig. 8C-H).

The spread of the calcium waves varied dramatically between avian and mammalian glial cultures. In mouse telencephalic cultures, waves spread 3.4-fold farther than the mouse diencephalic waves (466.1 ± 13.7 mm^2 versus 135.8 ± 26.7 mm^2; Fig. 9A). Calcium waves in the telencephalic cultures initially spread at a regular rate. This spread was radial in nature, with the leading edge of the wave advancing concentrically (Fig. 9B). In diencephalic cultures, wave spread was irregular and characterized by an uneven rate of wave advance (Fig. 9C, black triangles). Additionally, the direction of the wave spread meandered throughout the ROI, often making erratic turns that left large areas of the culture uninvolved in the wave (Fig. 9C, arrow).

In chick astrocyte cultures, the area of the spread of the calcium wave was similar in both the telencephalic and diencephalic groups (58.0 ± 4.5 mm^2 and 59.6 ± 4.5
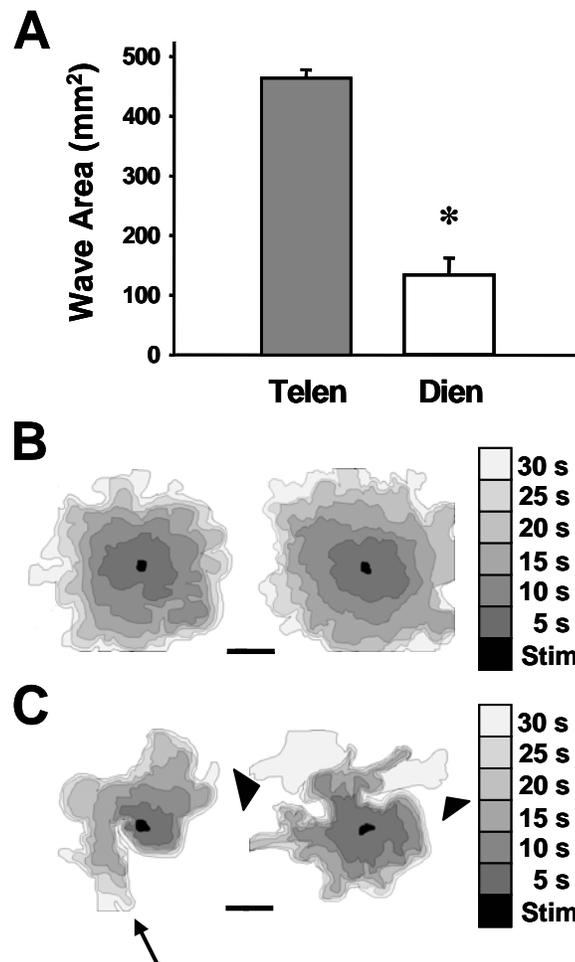
Fig. 9. Differential spread of calcium waves in mammalian astrocyte cultures. **A**. Following stimulation of mammalian astrocytes, Ca^{+2} waves had spread farther in telencephalic (Telen; *, $p<0.0001$, n=10) than in diencephalic cultures (Dien; n=10) after 90 seconds of propagation. **B-C.** The spread of Ca^{+2} waves in the telencephalic cultures (**B**) was radial and more synchronous than in the diencephalic cultures (**C**), where waves spread irregularly. Two representative waves from each group indicate the area of wave spread in 5-second intervals (shades of gray) from touch stimulation site (black area). Calcium waves among diencephalic astrocytes made hairpin turns (arrow) and unequal advances of the wave front (arrowheads). These features were not seen in telencephalic cultures. Bar equals 200 μm.
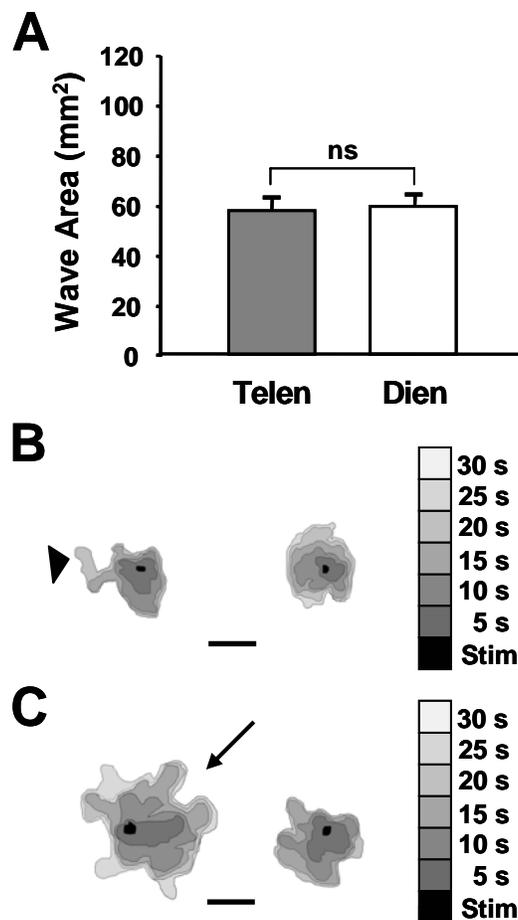
Fig. 10. Calcium wave spread among avian astrocytes was homogeneous. **A**. In chick astrocyte cultures, the spread of the Ca^{+2} waves after 90 seconds was the same in telencephalic (Telen; n=12) and diencephalic (Dien; n=27) cultures. However the spread of the waves was dramatically reduced from those seen in the mammalian astrocytes. Note the scale of the y-axis. **B-C.** The nature of the spread of the wave was similar in both the chick telencephalic (**B**) and diencephalic (**C**) cultures. Two representative waves from each group indicate the area of wave spread in 5-second intervals (shades of gray) from touch stimulation site (black area). Calcium waves among the astrocyte cultures made hairpin turns (arrow) and had unequal advances of the wave front (arrowhead). Bar equals 200 μm.

mm^2 respectively; Fig. 10A). The telencephalic (Fig. 10B) and the diencephalic (Fig. 10C) waves spread from the point of stimulation in a highly variable fashion. That is, the rate of spread was uneven, with the wave advancing into some areas of the ROI rapidly, but more slowly into others.

Differences in Calcium Waves Among Astrocytes of Mammals and Birds

Calcium waves in mouse astrocytes involved many more cells (Fig. 11A) and spread more rapidly (Fig. 11B) than waves in chick astrocytes. The total estimated number of cells involved in the wave, following an increase in intracellular calcium in a single stimulated cell, was greatest in the telencephalic mouse cultures with approximately 1500 cells involved compared to 700 cells in the diencephalic mouse cultures (Fig. 11A). Thus, mouse telencephalic waves involved greater than 2 times the number of astrocytes, compared to diencephalic waves. In addition, mouse astrocytic calcium waves involved 4 to 8 times the number of cells involved in chick astrocytic waves. Chick telencephalic and diencephalic cultures had essentially the same number of cells involved, 180 and 160, respectively (Fig. 11A). Calcium waves in mouse astrocyte cultures spread faster than those of chick astrocytes (Fig. 11B). The rate of the spread was approximately 33 cells/second (telencephalic) and 19 cells/second (diencephalic) for mouse astrocytes as compared to 8 cells/second in both telencephalic and diencephalic chick astrocytes.
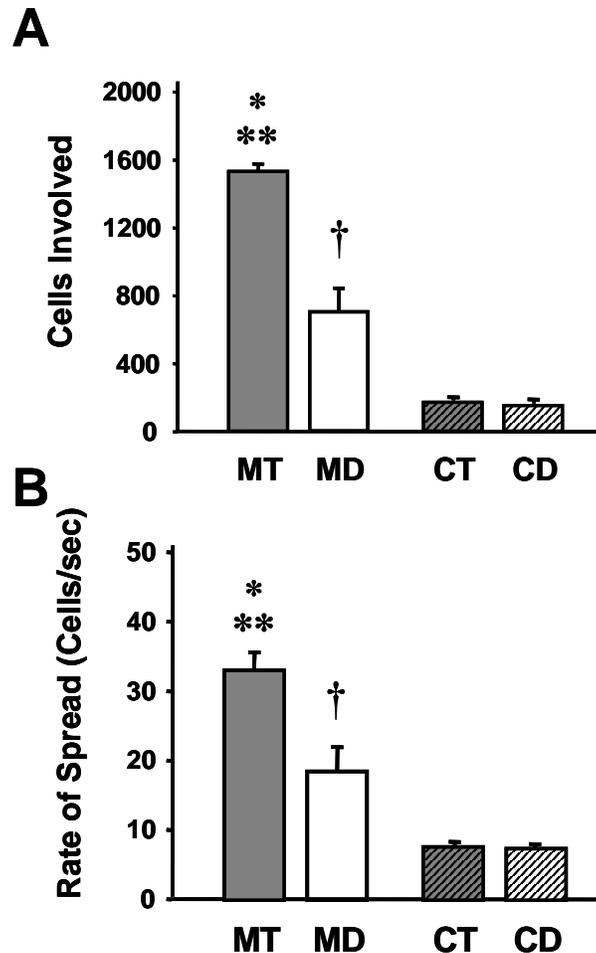
Fig. 11. Ca^{2+} wave propagation differs between mammalian and avian astrocytes. **A**. The estimated number of cells involved in calcium waves was greater in telencephalic mouse cultures than in the diencephalic mouse cultures (MT vs MD; *, p<0.001, n=10). There were also more cells involved in the mouse telencephalic (MT vs CT and CD; **, p<0.0001, n=10-27) and mouse diencephalic (MD vs CT and CD; †, p<0.003, n=10-27) cultures than in either of the chick astrocyte groups. Chick telencephalic and diencephalic cultures had essentially the same number of cells involved in Ca^{+2} waves (n=13-27). **B**. Similar to the number of cells involved in Ca^{+2} waves, the rate of the spread of the wave was faster in the mouse telencephalic and diencephalic astrocytes than either of the chick astrocyte cultures (MT vs CT and CD; **, p<0.0001, n=10-27; and MD vs CT and CD; †, p<0.01, n=10-27). Mouse telencephalic waves spread faster than mouse diencephalic waves (MT vs MD; *, p<0.003, n=10), whereas the rate of spread was similar between the chick telencephalic and diencephalic waves (n=13-27).

Effects of Melatonin on Calcium Waves

When 10 nM melatonin was administered to the cultures for 30 minutes prior to touch stimulation, the area of the spread of the wave (at 30 seconds after touch) increased 32% over control waves among diencephalic mouse astrocytes (252.7 ± 21.0 mm^2 versus 172.7 ± 34.2 mm^2; Fig. 12A). However, there was no effect of the melatonin on the telencephalic mouse astrocytes. The area of the calcium wave of the controls (277.8 ± 22.5 mm^2) was not different from that of melatonin-treated cultures (284.8 ± 20.5 mm^2; Fig. 12A). Melatonin (10 nM) increased the spread of the calcium wave 23% in chick diencephalic cultures (77.4 ± 5.5 mm^2 versus 59.7 ± 4.5 mm^2 in control cultures; Fig. 12B). The telencephalic chick astrocytes were not affected by melatonin as the area of the spread of the wave in hormone-treated cultures was similar to that of controls (60.4 ± 3.6 mm^2 and 58.0 ± 4.5 mm^2, respectively; Fig. 12B).

Melatonin also significantly increased the rate of wave spread among mammalian diencephalic astroglia (27 cells/second) as compared to untreated controls (19 cells/second), but had no effect on the rate of propagation of telencephalic astrocytes (31 cells/second versus 33 cells/second in control cultures; Figure 13A). A similar facilitation of wave propagation rate was induced by melatonin in chick diencephalic cultures (10 cells/second versus 8 cells/second in control cultures; Figure 13B). Again, melatonin had little effect on the rate of wave spread among chick telencephalic astrocytes (9 cells/second versus 8 cells/second in control cultures).

The effects of 10 nM melatonin on the mouse diencephalic astrocytes, not only involved increases in the area and rate of wave spread, but elicited other wave
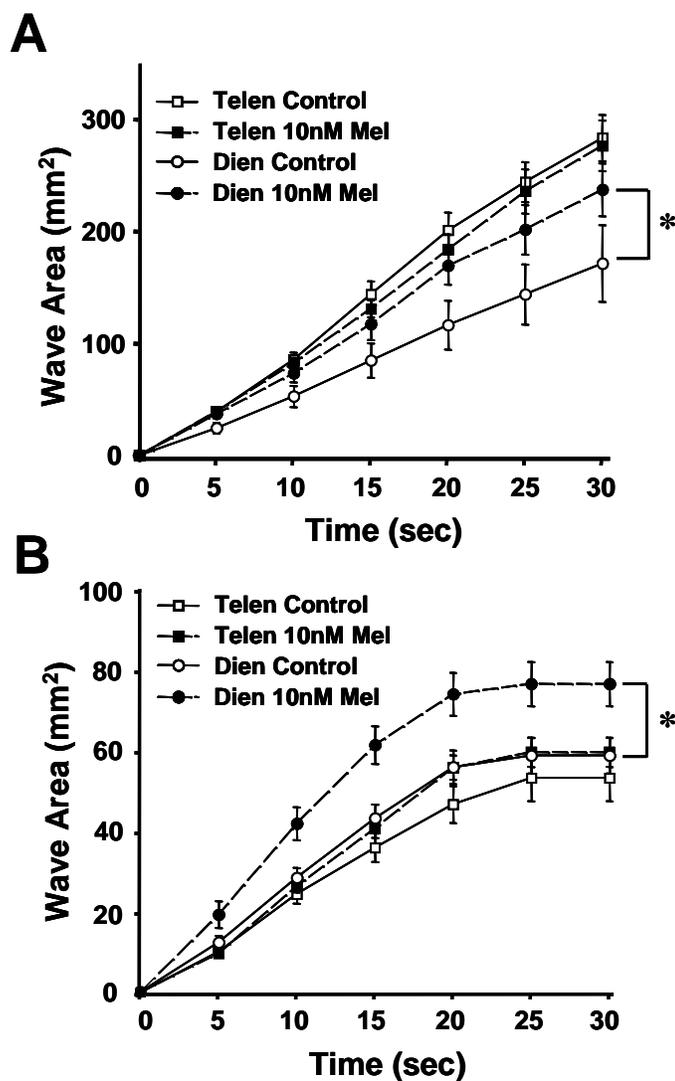
Fig. 12. Melatonin enhances intercellular calcium waves in diencephalic, but not telencephalic, glial cultures. **A.** Following application of 10 nM melatonin, the area of the Ca^{+2} wave spread after 30 seconds was increased over control (DMSO-treated) cultures in mouse diencephalic astrocytes (*, $p<0.03$, n=10), while there was little effect of melatonin on calcium waves among telencephalic glial cells (n=10). **B.** Nanomolar melatonin also increased the area of the Ca^{+2} wave spread after 30 seconds in chick diencephalic astrocytes (*, $p<0.01$, n=27), as compared to DMSO-treated controls, but had no effect on waves of telencephalic cultures (n=10).
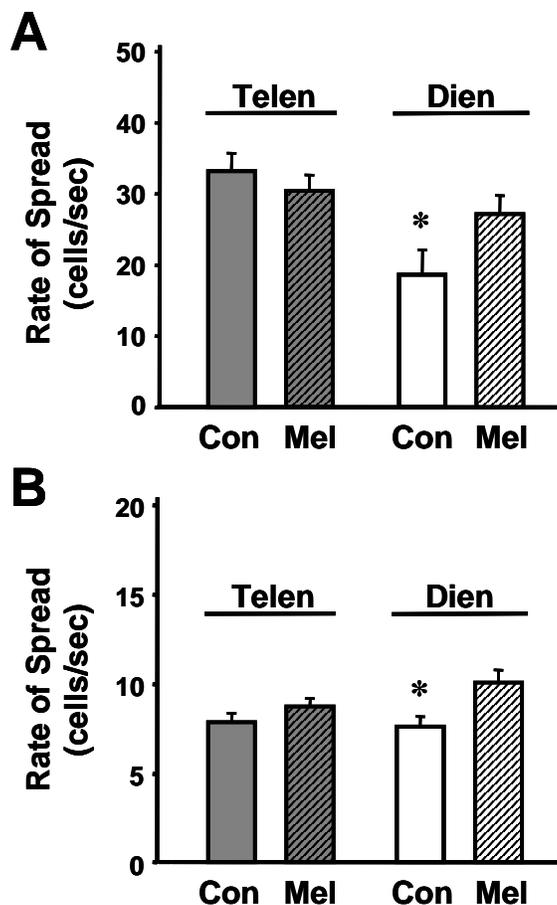
Fig. 13. Melatonin increased the rate of calcium wave spread among diencephalic, but not telencephalic, glial cultures. **A.** Mouse astrocyte cultures. Following application of 10 nM melatonin, the rate of the wave spread in these cultures was faster than the rate of wave spread in control (DMSO-treated) cultures in mouse diencephalic astrocytes (*, $p<0.04$, n=10). There was no effect of melatonin on the rate of Ca^{+2} wave spread among telencephalic glial cells (n=10). **B.** Chick astrocyte cultures. Nanomolar melatonin also increased the rate of the Ca^{+2} wave spread among chick diencephalic astrocytes (*, $p<0.005$, n=27), as compared to DMSO-treated controls, but had no effect on the rate of Ca^{+2} wave spread in the telencephalic cultures (n=10).
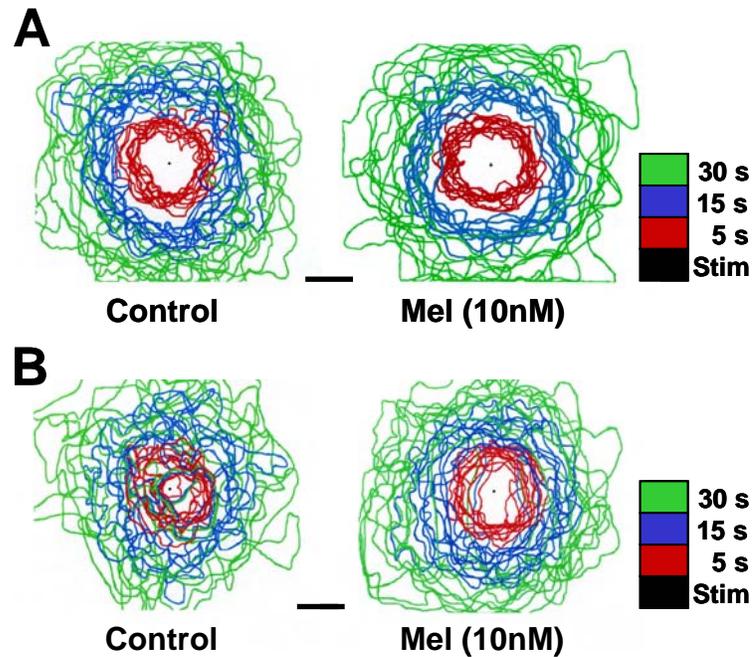
Fig. 14. Melatonin affects the dynamic nature of Ca^{+2} waves spreading among mammalian astrocytes. **A**. Mouse telencephalic astrocytes. Ca^{+2} waves were propagated radially at consistent rates, as indicated by the virtual lack of overlap of lines indicating the leading edge of the wave at 3 time points (colored lines). Application of 10 nM melatonin had no effect on the spread of the Ca^{+2} waves in mouse telencephalic cultures. **B.** Mouse diencephalic astrocytes. Ca^{+2} waves were propagated with greater randomness of direction and rate among diencephalic astroglia. Note the extensive overlap of lines depicting the leading edge of the wave at all time points. Application of 10 nM melatonin increased the spread of the Ca^{+2} waves. Diencephalic Ca^{+2} wave propagation was more telencephalic-like following melatonin receptor activation, such that the spread of the Ca^{+2} waves through confluent cultures became more radial and concentric. The leading edge of the Ca^{+2} wave was outlined at 5 (Red), 15 (Blue), and 30 (Green) seconds. Black dots indicate the site of mechanical stimulation. Each plot shows the overlay of 10 representative waves from each astrocyte group. Bar equals 200 μm.

characteristics similar to those observed in telencephalic astrocytes. Telencephalic waves were propagated in a radial and concentric manner, as shown in Figure 14A. The leading edge of the wave at 5, 15, and 30 second time points, rarely overlapped in telencephalic astrocytes demonstrating that they spread in all directions at similar rates. The spread of diencephalic waves in control cultures was more varied with inconsistent rates and directions of propagation (Fig. 14B). Following melatonin treatment, diencephalic waves were indistinguishable from the telencephalic waves (Fig. 14B), suggesting that melatonin induced a change in the underlying mechanisms of wave propagation.

It should be noted that species-specific differences are not likely due to differences in cell cultures conditions. We employed several cell culture approaches for the avian astrocytes including conventional chick astrocytic protocols, as well as approaches that more closely matched our mammalian culture conditions. In each case, control and treated cultures produced similar results (data not shown).

Melatonin Receptor Expression in Mouse and Chick Astrocytes

We performed immunocytochemistry on confluent cell cultures of chick and mouse glia using antibodies against the known melatonin receptor subtypes for each species. The chick diencephalic and telencephalic astrocytes expressed the Mel_{1c} (Fig. 15A & B) and the Mel_{1a} receptor proteins (data not shown). Likewise, both the mouse diencephalic and telencephalic astrocytes expressed MT2 (Mel_{1b}; Fig. 15C & D) and the
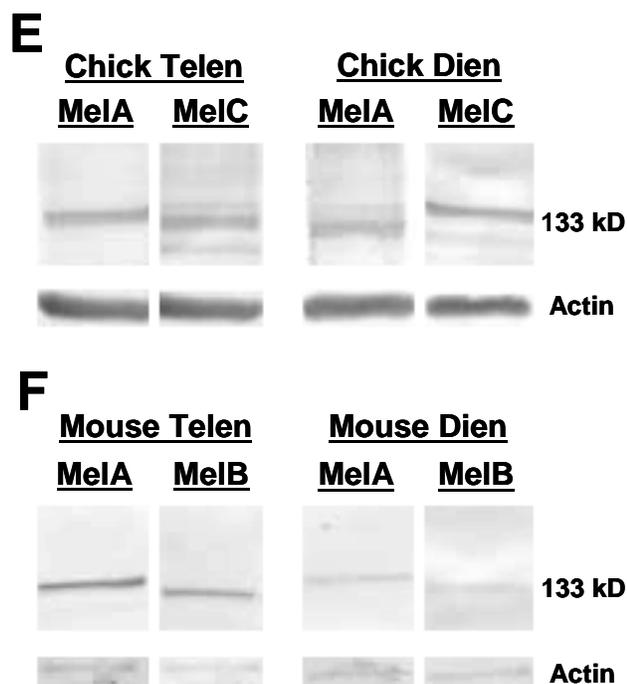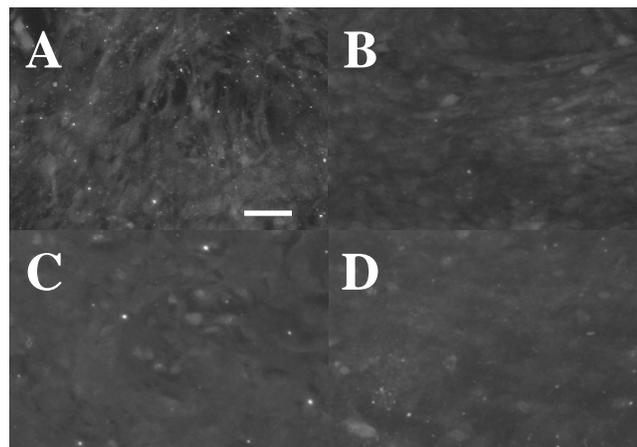
Fig. 15. Melatonin receptor subtypes are expressed in diencephalic and telencephalic astrocytes. **A-D.** Immunocytochemistry of known receptor subtypes. Chick diencephalic (**A**) and telencephalic (**B**) astrocyte cultures expressed the Mel_{1c} receptor subtypes. The Mel_{1b} (MT2) receptor subtype was present on diencephalic (**C**) and telencephalic (**D**) mouse glia. Bar equals 100 μm. **E-F.** Western blot analysis. **E.** Western blot analysis from cell culture extracts demonstrated the expression of both the Mel_{1a} and Mel_{1c} receptor proteins in chick diencephalic and chick telencephalic astrocytes. **F.** Protein extracts of mouse diencephalic and telencephalic glia cells expressed both the Mel_{1a} (MT1) and Mel_{1b} (MT2) receptor subtypes.

MT1 (Mel$_{1a}$; data not shown) receptor subtypes. Staining above background for the melatonin receptor proteins was detected in all of the glial cells examined (data not shown). Immunocytochemical approaches were not sensitive enough to detect differences in receptor expression, presumably due to low antigen-antibody affinity. Therefore, we conducted western blot analyses to test for the presence of the receptor subtypes in protein extracts derived from cell cultures of diencephalic and telencephalic astrocytes from both mouse and chick. Both Mel$_{1a}$ and Mel$_{1c}$ antibodies labeled proteins in whole cell extracts from chick diencephalic and telencephalic cultures (Fig. 15E). Similarly, antibodies to mammalian melatonin receptors, Mel$_{1a}$ and Mel$_{1b}$, detected proteins in extracts from mouse diencephalic and telencephalic astrocytes (Fig. 15F).

DISCUSSION

Astrocytes have many functions, ranging from buffering ions and neurotransmitters to regulating the transport of metabolites between the blood vessels and neurons. Astrocytes are closely apposed to neurons and synapses of the central nervous system are typically ensheathed by glial process (Schikorski and Stevens, 1997; Ventura and Harris, 1999). Recently, a role for astrocytes in the regulation of neural communication and synaptic function has been discovered (Haydon, 2001; Volterra et al., 2002; Auld and Robitaille, 2003; Braet et al., 2004). In the light of this emerging integrative function of astrocytes, it is important to determine whether astrocyte-astrocyte and astrocyte-neuron interactions are different among diverse brain regions and how modulators of neural function affect these interactions. For example, in the

hypothalamus, astrocyte-neuron signaling is thought to regulate gonadotropin-releasing hormone (GnRH) secretion and production (Dhandapani et al., 2003). Hypothalamic astrocytes modulate synaptic plasticity associated with GnRH neurons, perhaps involving dynamic glial ensheathment, the extension and retraction of glial processes (Witkin et al., 1995; Witkin et al., 1997). Glial cells modulate synaptic activity of hippocampal neurons by releasing glutamate in a calcium-dependent manner (Parpura and Haydon, 2000) and glial glutamate release synchronizes neural activity in hippocampal slices (Angulo et al., 2004).

Astrocytes are abundant in the hypothalamus (Morin et al., 1989). Diencephalic structures, including the hypothalamus, express several types of G-protein-coupled melatonin receptors (Weaver et al., 1989; Brooks & Cassone, 1992; Cassone et al., 1995; Reppert et al., 1995; Reppert et al., 1996; von Gall et al., 2002) and calcium signaling and gap junctional communication among diencephalic astrocytes is modulated by melatonin (CHAPTER II). Using western blot analyses, we have demonstrated that Mel_{1a} (MT1) and Mel_{1b} (MT2) proteins are present in both hypothalamic and cortical astrocytes of mice, while Mel_{1a} (MT1) and Mel_{1c} receptor proteins are present in chick diencephalic and telencephalic astrocytes. Melatonin caused robust enhancement in the spread of intercellular calcium waves among diencephalic astrocytes of both birds and mammals, but had no effect on calcium waves among telencephalic astrocytes in either species. Thus, besides its canonical roles in the regulation of vertebrate visual and circadian pathways, melatonin regulates calcium signal communication among hypothalamic glial cells.

Why does melatonin affect calcium signaling only in diencephalic astrocytes, when telencephalic glial cells also expressed melatonin receptors? Calcium waves propagating among mouse telencephalic astrocytes were robust and pervasive, involving many more cells on average than control diencephalic cultures. Following melatonin-induced facilitation, mouse diencephalic calcium waves were indistinguishable from those of telencephalic cultures, including the number of cells involved, the rate of wave spread, and the dynamic manner of propagation (i.e., radial rather than multidirectional). It is possible that melatonin had little effect on calcium signaling in mouse cortical astrocytes because these cultures already possessed maximal propagation dynamics prior to melatonin receptor activation; however, it is more likely that region-specific astrocytes have intrinsically difference cellular responses to melatonin receptor activation. In fact, the extent of astroglial calcium wave propagation varies across brain regions, with cortical and hippocampal waves often spreading twice that of those in astrocytes from hypothalamus and brain stem (Blomstrand et al., 1999). Interestingly, serotonin affects calcium signaling in a manner that is completely opposite that of melatonin. Serotonin decreases the spread of calcium waves in cortical and hippocampal astrocytes, but has no effect on hypothalamic astroglial waves and inhibits waves among brain stem astrocytes (Blomstrand et al., 1999).

Astrocytes respond to increases in brain activity, via neuronal glutamate release, by increasing consumption of glucose and production of lactate (Pellerin and Magistretti, 1994). Neurons then preferentially utilized this glia-supplied lactate to meet their energy demands, a process called the astrocyte-neuron lactate shuttle (Kasischke et al., 2004).

Melatonin modulates glucose uptake in astrocytes and their production and release of pyruvate and lactate (Adachi et al., 2002). Sodium-dependent glucose uptake by glial cells is also influenced by intercellular calcium waves propagating among mouse cortical astrocytes (Bernardinelli et al., 2004) and astrocytic calcium signals influence endothelial and smooth muscle cells of brain vasculature, affecting blood flow (Braet et al., 2004). Astrocytes, therefore, function as a cellular network for metabolic coupling in the brain and this coupling can be regulated by intercellular calcium signal communication. Melatonin-mediated regulation of glial calcium signaling or glucose uptake, we predict, would have a significant homeostatic impact on melatonin-sensitive brain regions, particularly the hypothalamus, but also astrocyte-neuron networks in telencephalic structures.

Although calcium waves among diencephalic, but not telencephalic, astrocytes were facilitated by melatonin in both mouse and chick, fundamental differences were observed between these chick and mouse intercellular waves. Calcium waves among chick astrocytes involved many fewer cells than did those in mouse cultures. Why do intercellular calcium waves spread better among mouse astrocytes than among chick astrocytes? These species differences are not likely due to cell cultures conditions as several were compared, including conventional chick astrocytic protocols and approaches that more closely match mammalian culture conditions. More likely, these differences reflect species-specific heterogeneity of astrocytes (e.g., receptor phenotypes, signaling mechanisms) in the glial cultures.

The mechanisms underlying mammalian calcium waves have been studied in much greater detail than avian calcium waves. Purinergic extracellular signaling via ATP mediates calcium waves among mammalian astrocytes (Guthrie et al. 1999), as well as other diffusible signals such as nitric oxide and prostaglandins (Charles, 1998). In addition, cytoplasmic signaling via gap junctions likely mediates calcium waves among some glia (Charles, 1998; Scemes, 2000; Bennett et al., 2003; Braet et al., 2004). It is likely that both avian and mammalian astrocytes employ combinations of signaling mechanisms for wave propagation. In fact, downregulation of connexin43 alters expression of the P2Y receptor in spinal cord astrocytes, suggesting a complex interaction between gap junctional and extracellular modes of glial communication (Suadicani et al., 2003). The irregular, multidirectional and restricted spread of chick calcium waves indicates a greater role for gap junctions in these avian astrocytes. While in mouse astrocytes, the radial, pulsating and pervasive spread of calcium waves suggests a diffusible and less constrained paracrine mechanism. Rat astrocyte cultures derived from the hypothalamus possess greater dye coupling and levels of connexin43 expression than cortical glial cell cultures (Blomstrand et al., 1999). In chick astrocytes, melatonin induces a fundamental shift in cell-cell communication, facilitating calcium waves, while suppressing gap junctional coupling and connexin43 expression (CHAPTER II). Interestingly, a potential role for connexins in both gap junctional and paracrine (i.e., functioning as hemichannels) signaling is emerging (Goodenough and Paul, 2003; Bennett et al., 2003). Thus, we hypothesize that disparate mechanisms of astrocytic signaling account for the differences in propagation of intercellular calcium

waves between birds and mammals. In support of this interpretation, disruption of extracellular signaling pathways known to mediate calcium waves among mammalian astrocytes, namely ATP, nitric oxide, and prostaglandins (Charles, 1998; Guthrie et al., 1999; Fam et al., 2000), have little effect on waves among chick diencephalic astrocytes (CHAPTER II).

Nocturnal Versus Diurnal Activity Patterns

Rodents are generally nocturnal animals (i.e., more active at night), whereas most birds are diurnal (i.e., more active in the day). One might expect pineal melatonin levels to be phase-linked with activity patterns across species; that is, melatonin should be highest in all species when they sleep. On the contrary, melatonin levels peak in the circulation of both diurnal birds and nocturnal rodents at night (Pang and Ralph, 1975; Cassone and Menaker, 1983; Cassone et al., 1986b). Therefore, the link between melatonin rhythms and activity rhythms must be found in the nature of the cellular and molecular mechanisms of melatonin action. In part, this will be answered as more becomes known about the signaling pathways involved in melatonin transduction in diverse species. As mentioned above, mammals and birds, express Mel_{1a} (MT1) and Mel_{1b} (MT2) receptors; however, birds, but not mammals, express the Mel_{1c} receptor, particularly on astrocytes (Weaver et al., 1989; Cassone et al., 1995; Reppert et al., 1996; von Gall et al., 2002; Adachi et al., 2002). Nevertheless, with regard to intercellular calcium signaling, melatonin facilitated both avian and mammalian astrocytic waves.

Why is the effect of melatonin on calcium waves the same in nocturnal mice and diurnal chicks? In fact, although the melatonin-induced response was facilitatory in both species, the astrocytic responses were not identical. Melatonin facilitated avian diencephalic calcium signaling such that waves became less telencephalic in their propagation. In fact, chick diencephalic astrocytes exhibit a fundamental shift in intercellular coupling following activation of their melatonin receptors (CHAPTER II). This switch in mode of coupling involves a facilitation of paracrine signaling (i.e., intercellular calcium waves as reported here) and a suppression of cytoplasmic signaling (i.e., gap junctional coupling). In comparison, melatonin facilitated calcium waves in mouse diencephalic astrocytes such that their spread attained more telencephalic-like features, including a radial and concentric wave, rather than the meandering one present prior to melatonin receptor activation. Like chick astrocytes, melatonin may induce a shift in the mode of mammalian astroglial cell coupling that is detectable as alterations in the nature of wave transmission. Until more is known about the transduction pathways mediating melatonin receptor and calcium signaling in nocturnal and diurnal species, and how these cellular pathways mediate circadian behavior, the role of melatonin and glial calcium waves in these processes will remain unclear.

Glial Calcium Signaling and Brain Function

The similarity and disparity of calcium wave propagation between mouse and chick astrocytes are only meaningful if such waves impact brain function and animal behavior. Recently, a role for glia in modulating neuronal signaling in the brain and

retina has been determined and has given rise to the tripartite synapse hypothesis (Araque et al., 1999; Volterra et al., 2002; Nedergaard et al., 2003; Newman, 2003). This idea implicates astrocytes in neural integration at synaptic contacts. Specifically, glial calcium signaling regulates neurotransmitter release from glia cells in cell culture (Parpura et al., 1994; Araque et al., 1999), modulates retinal activity (Newman and Zahs, 1998), and likely mediates glutamate release from astrocytes, which in turn synchronizes neuronal activity in hippocampal slices (Angulo et al., 2004).

Viewed in the context of the tripartite synapse hypothesis, a melatonin-mediated change in hypothalamic calcium waves would impart fundamental and far-reaching changes in an animal's physiology. Hypothalamic nuclei mediate vast regulatory influences over autonomic physiology, endocrine signaling, and behavioral states of vertebrate animals (Aston-Jones et al., 2001; Saper et al., 2001), including blood pressure, body temperature, sleep/wake cycles, food intake, and energy metabolism. It is, in fact, neurons of hypothalamic nuclei (i.e., the SCN) in mammals and birds that set the pace of biological rhythms (Ebihara and Kawamura, 1981; Takahashi and Menaker, 1982; Moore, 1983; Klein et al., 1997), including pineal production and release of melatonin (Cassone and Menaker, 1983; Klein et al., 1997; Moore & Silver 1998). Since high affinity melatonin receptors are expressed in neural cells of the hypothalamus, a regulatory feedback loop has been suggested involving the hypothalamus and the pineal gland (Cassone and Menaker, 1984; Cassone et al., 1986a; Cagampang et al, 1994). Melatonin alters diencephalic glial physiology in many ways, both in terms of metabolic activity (Adachi et al., 2002) and in cell-to-cell communication (CHAPTER II). The

modulation of glial calcium waves reported here, defines a feedback mechanism by which melatonin could profoundly alter the synchrony of neuronal activities in the hypothalamus. Thus, the daily rise and fall of melatonin levels in the brain would profoundly impact the function of the same neural structures that drive the ebb and flow of this neurohormone's production.

CHAPTER IV

**DIFFERENTIAL REGULATION OF CIRCADIAN RHYTHMS OF
METABOLIC ACTIVITY AND CLOCK GENE EXPRESSION IN CHICK
DIENCEPHALIC ASTROCYTES**

INTRODUCTION

The avian circadian system comprises multiple oscillators and photosensitive
structures (Cassone and Menaker, 1984; Cassone, 1990a). These include the retina
(Ebihara et al., 1984; Underwood et al., 1984), the avian homolog to the suprachiasmatic
nucleus (SCN; Ebihara and Kawamura, 1981; Takahashi and Menaker, 1982; Cassone et
al., 1990), and the pineal gland (Gaston and Menaker, 1968; Zimmerman and Menaker,
1979). The pineal gland and, in some species, the retina influence the system by
secreting the neurohormone melatonin during the night (Klein et al., 1997), such that
levels are higher during the night than during the day (Hamm and Menaker, 1980;
Underwood et al., 1990; Adachi et al., 1995). However, the molecular mechanisms by
which melatonin influences physiology and circadian behavior are not completely
known.

In the course of cloning and isolating the multiple melatonin receptors, the Mel_{1a}
or MT1 receptor, the Mel_{1b} or MT2 receptor, and the Mel_{1c} receptors, it was found that
the Mel_{1c} receptors resided primarily on non-neuronal elements within the nervous
system (Reppert et al., 1995). The most abundant of these were glial astrocytes. Using

receptor binding studies and non-radioactive *in situ* hybridization of the 3 receptor sub-

types on chick diencephalic astrocytes, we (Adachi et al., 2002) demonstrated that 25%

of the cells expressed the Mel_{1a} receptor mRNA, none expressed the Mel_{1b} receptor

mRNA, and all expressed the Mel_{1c} receptor mRNA. Additionally, when melatonin was

rhythmically administered to these cultures, there were rhythms in glucose utilization

and in the release of the glycolytic end products, pyruvate and lactate (Adachi et al.,

2002).

Molecular components responsible for the generation of circadian rhythms have

been identified and extensively studied in several model systems (Dunlap, 1999). In

mammals, a network of "clock genes" forms an interlocking feedback loop (Sherman et

al., 2000). "Positive elements", *clock* (*clk*) and *brain muscle ARNT-like protein* (*bmal1*

and 2), are translated, dimerize, enter the nucleus and activate the transcription of

"negative elements", *period* (*per*), and *cryptochrome* (*cry*), which then are translated,

dimerize, and feedback to inhibit their own transcription by inhibiting the action of

clk/bmal dimers.

Transcriptional profiling and DNA microarrays have been employed to identify

rhythmic molecular components of the chick pineal gland (Bailey et al., 2003) and retina

(Bailey et al., 2004). The mRNA encoding several enzymes involved in the melatonin

biosynthetic pathway and all of the known clock genes and their orthologs were

identified, many of which were rhythmic. N-acetyltransferase (AA-NAT), for example,

the rate-limiting step in pineal melatonin synthesis converting serotonin to N-

acetylserotonin, was rhythmic with higher expression during the night than during the

day as previously shown (Bernard et al, 1997). Clock genes were expressed

rhythmically as well with phases that are consistent with the literature (e.g. Hirota and

Fukada, 2004) and confirmed by northern blot analysis. Similarly, retinal melatonin

biosynthesis and clock gene rhythms were identified and confirmed by northern analysis

(Bailey et al., 2004).

To determine whether the rhythmic regulation of metabolic and/or ionic

homeostasis involved the rhythmic regulation of clock genes, we asked if cultured

diencephalic astrocytes express these clock genes. This preliminary study showed that

astrocytes express *period2*, *period3*, *cryptochrome1*, *cryptochrome2*, and *clock*, as well

as two opsins that have recently been implicated in circadian clock function, melanopsin

(*Opn4*) and peropsin (*Rrh)* (Peters et al., 2003). These data will be discussed in greater

detail in the results. Since these cells expressed several opsins and cryptochromes, the

possibility that astrocytes were directly light sensitive was an intriguing consideration.

We therefore ask here whether rhythmic light and/or melatonin cycles affect metabolic

activity and clock gene expression in cultured astrocytes.

MATERIALS AND METHODS

Cell Culture

Glial cells were prepared from chick embryonic (E17) eggs (Hyline Hatcheries,

Bryan, TX). Embryos were extracted and the brains were removed. Diencephalic tissue

was placed in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO)

and dissociated using trituration with 1% trypsin. Cells were filtered through nylon

mesh (180 μm pore diameter) and centrifuged for 5 minutes at 1000X g. The

supernatant was removed and the pellet was re-suspended in DMEM with 10% fetal

bovine serum (FBS) and 1% PSN antibiotic (penicillin, streptomycin, neomycin;

Gibco/Invitrogen, Grand Island, NY). The suspension was filtered again through nylon

mesh (77 μm pore diameter) and seeded into 75 cm^2 tissue culture flasks. Cultures were

maintained in darkness at 37°C with 95% air and 5% CO_2 in a humidified Napco CO_2

incubator. Culture media was changed every other day until cell cultures reached

confluence (approximately 10 days).

2-Deoxy[^{14}C]Glucose Uptake in Astrocyte Cultures

Confluent cultures were split, re-plated new 75 cm^2 tissue culture flasks and

allowed to grow to almost confluence (3-4 days). Flasks (n = 4 per treatment) were then

placed into treatment cycles of light, melatonin, or a combination of light and melatonin

(Table 2, Days 1-7). The flasks treated with light were given either a light:dark cycle

(LD; 12:12-hr) or an opposing dark:light cycle (DL; 12:12-hr). The flasks treated with

melatonin were placed into constant darkness (DD) given a cycle of melatonin media (5

nM) during the subjective night and normal media during the subjective day (MN) or an

opposing cycle (in DD) of melatonin media (5 nM) during the subjective day and normal

media during the subjective night (MD). The flasks that were treated with both light and

melatonin (LD & MN) were placed into a LD cycle and given melatonin media (5 nM)

during the night or dark phase, and normal media during the day or light phase.

Following seven days of the treatment cycle, the sampling began. Samples were taken

TABLE 2. Experimental design for cycling treatment groups of cell culture flasks

Sampling begins

Cycle		Days 1-7		Day 8		Day 9		Day 10	
		1-12 hrs	12-24 hrs	1-12 hrs	12-24 hrs	1-12 hrs	12-24 hrs	1-12 hrs	12-24 hrs
1	LD	(white)	(black)	(white)	(black)	(white)	(black)	Sampling done	(white)
2	DL	(black)	(white)	(black)	(white)	(black)	(white)	Sampling done	(white)
3	LD to DD	(white)	(black)	(white)	(black)	(black)	(black)	(black)	(black)
4	MN	(hatched)	MEL	(hatched)	MEL	(hatched)	MEL	Sampling done	(white)
5	MD	MEL	(hatched)	MEL	(hatched)	MEL	(hatched)	Sampling done	(white)
6	MN to No mel	(hatched)	MEL	(hatched)	MEL	(gray)	(gray)	(gray)	(gray)
7	LD & MN	(white)	MEL	(white)	MEL	(gray)	(gray)	(gray)	(gray)

every 4 hours for 48 or 72 hours. During this sampling period (Table 2, Day 8, Day 9, and Day 10, in some cases), the flasks either remained in their treatment cycle or were placed into constant conditions after one day of sampling. There were seven treatment/sampling groups that were used: 1) in light:dark (LD; 12:12-hr); 2) in dark:light (DL; 12:12-hr); 3) in LD for one day and then transferred to DD for 2 days; 4) in constant darkness (DD) with melatonin media (5 nM) given during the subjective night and normal media given during the subjective day (MN); 5) in DD with melatonin media (5 nM) given during the subjective day and normal media given during the subjective night (MD); 5); 6) in DD with melatonin media (5 nM) given during the subjective night for one day and then transferred to no melatonin for 2 days (MN to No mel); and 7) in LD with melatonin media (5 nM) given during the night for one day and then transferred to DD and no melatonin for 2 days (LD & MN).

On the day of sampling one hour prior to the timepoint at which the sample was taken, the cells were incubated with ^{14}C-labeled 2-deoxyglucose (2-DG; 0.2 µCi/ml; American Radiolabeled Chemicals, St. Louis, MO). Following incubation, media was removed and saved for scintillation count. Cells were rinsed twice with Dulbecco's PBS (Gibco/Invitrogen), and harvested with a cell scrapper and Trizol reagent (Invitrogen) to extract total cellular RNA and soluble protein. Aliquots of cell lysate (200 µl) were placed in scintillant in duplicate and counted on a Beckman scintillation counter. Uptake of 2-DG was normalized to protein content, following protein extraction and quantification using the bicinchoninic acid method (Pierce, Rockford, IL).

Northern Analysis

Total RNA was isolated from cell lysates using Trizol reagent as described by the manufacturer. RNA samples (10 μg each lane) was fractionated on a 1.5% agarose/0.66 M formaldehyde gel, and probed for *gPer2, gPer3, gCry1, gCry2, gClk, gOpn4,* or *gRrh.* Probes were labeled with [α-^{32}P] dATP by random priming (DECA Prime II kit, Ambion, Austin, TX) and hybridized overnight at 42˚C. Blots were washed in saline sodium citrate (SSC; 2X) twice for 5 minutes, and then 0.2X SSC with 0.1% SDS twice for 15 minutes each. Blots were exposed to x-ray film (Biomax MS, Kodak, Rochester, NY) for 2-3 days. Film was analyzed using the JAVA image analysis program (SPSS Inc., Chicago, IL). Data were normalized for variation in RNA loading and transfer efficiency by comparison to the 18S ribosomal band.

Statistical Analysis

Changes in 2-DG uptake were analyzed using one-way analysis of variance (ANOVA). Student-Newman-Keuls post-hoc test was performed when significant differences were found. In all cases $p<0.05$ was used to determine minimum significance.

RESULTS

These studies were conducted with confluent cultures of chick diencephalic astrocytes (Fig. 16A). Using the sequences from the transcriptional profiling and DNA microarray studies (Bailey et al., 2003; Bailey et al., 2004), we found that the
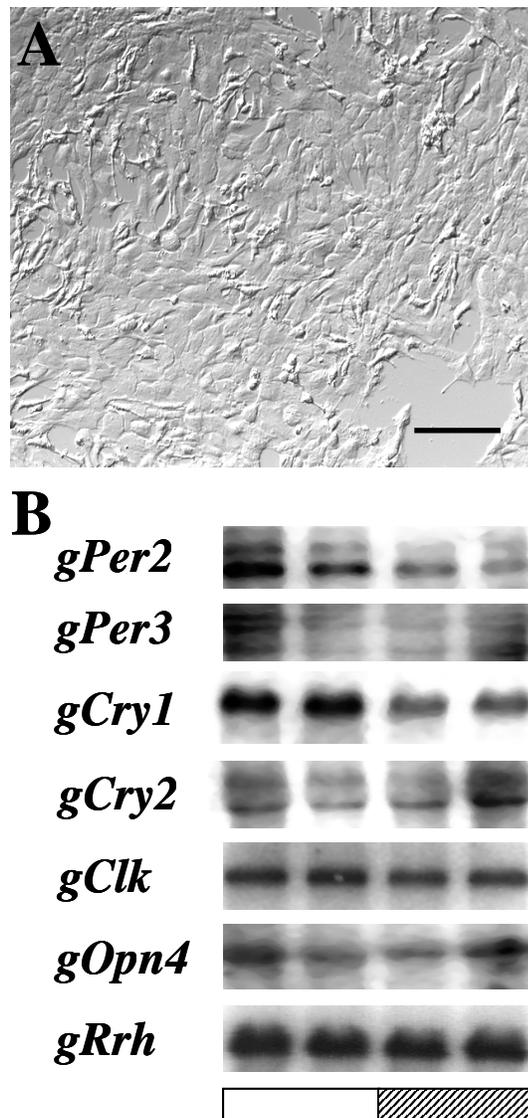
Fig. 16. Chick diencephalic astrocytes rhythmically express known clock genes and opsins. **A.** A DIC image of a region of confluent diencephalic chick astrocytes. Bar equals 200 μm. **B.** Using Northern analysis, preliminary studies of four times of day demonstrated that chick glial cells expressed mRNA of *period2 (per2)*, *period3 (per3)*, *cryptochrome1 (cry1)*, *cryptochrome2 (cry2)*, and *clock (clk)*, melanopsin (*gOpn4*), and peropsin (*gRrh*). The clock genes *gPer2* and *gCry1* were expressed rhythmically with higher levels of mRNA during the early and late day.

astrocytes expressed many of the clock genes (Fig. 16B). They express *period2*, *period3*, *cryptochrome1*, *cryptochrome2*, and *clock*, as well as melanopsin (*gOpn4*), and peropsin (*gRrh*). Several of the genes, such as *gPer2* and *gCry1,* were expressed rhythmically with higher levels of mRNA during the early and late day.

Under constant conditions, cultures administered melatonin in opposing cycles, melatonin at night (MN) or melatonin during the day (MD), had opposing rhythms in 2DG uptake. For both cycling groups, peak utilization coincided with the time at which melatonin was not present, at Circadian Time 9 (CT 9; Fig. 17A, upper & lower panels). Utilization of glucose was lowest at early subjective day (CT 1), following the melatonin to no melatonin transition. In contrast, cultures were given opposing light cycles, LD or DL, and there was no systematic relationship between the light:dark cycle and glucose utilization (Fig. 17B, upper & lower panels).

Cells placed in the LD to DD treatment cycle (Fig. 17C, upper panel), again did not exhibit any rhythmicity in glucose uptake due to the light cycle. Cultures were treated with a cycle of MN and then transferred no melatonin media (Fig. 17C, lower panel), and this treatment did induce oscillations that persisted perhaps for 1 cycle, but not very robustly. Combining light with melatonin administration (LD & MN), also synchronized the cells, but the effect was not additive, and the rhythms did not persist longer under constant conditions (data not shown).

Northern analysis was used to examine clock gene expression in these exact same cultures. In a LD cycle, expression of *gPer2* was rhythmic with peak expression during the day at Zeitgeber Time 5 (ZT 5; Fig. 18A), and expression of *gCry1* was also
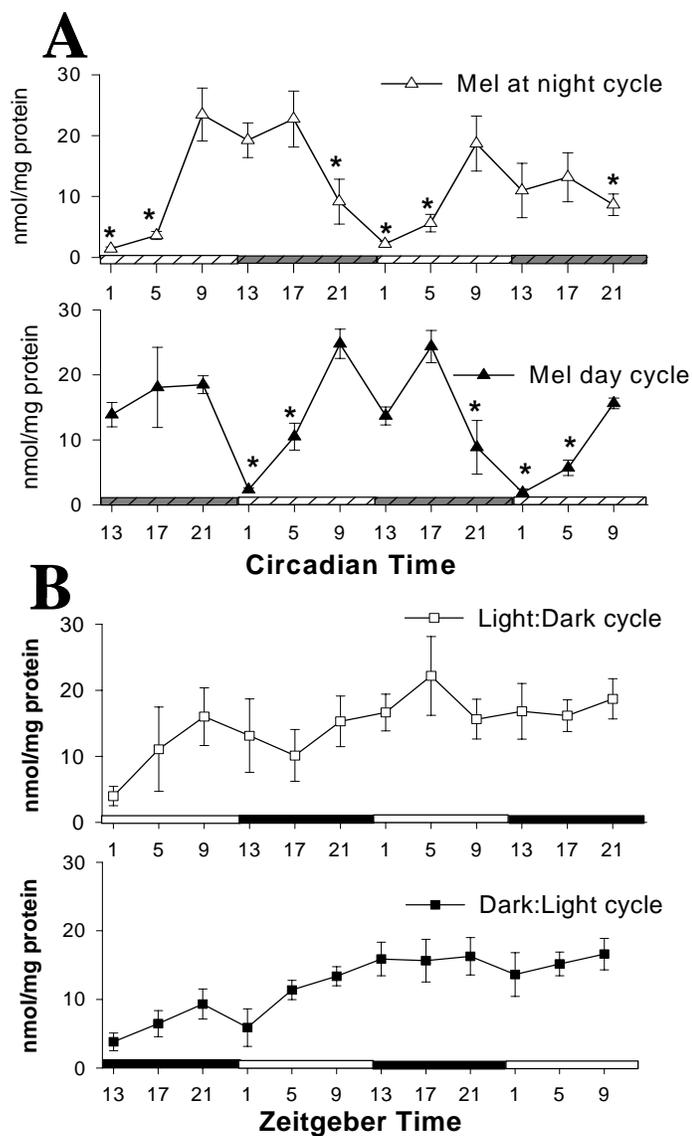
Fig. 17. Melatonin cycles, but not light cycles, induce metabolic rhythms in chick astrocytes. **A.** Under constant conditions, opposing cycles of rhythmic melatonin (nM) administration, either during the night (upper panel) or during the day (lower panel), caused rhythmic uptake of glucose in the cultures, such that 2DG uptake was higher during the time which melatonin was not present, CT 9 (n=4). **B.** Glial cultures placed in either light:dark (upper panel) or dark:light (lower panel) cycles demonstrated no rhythms in glucose utilization (n=4).
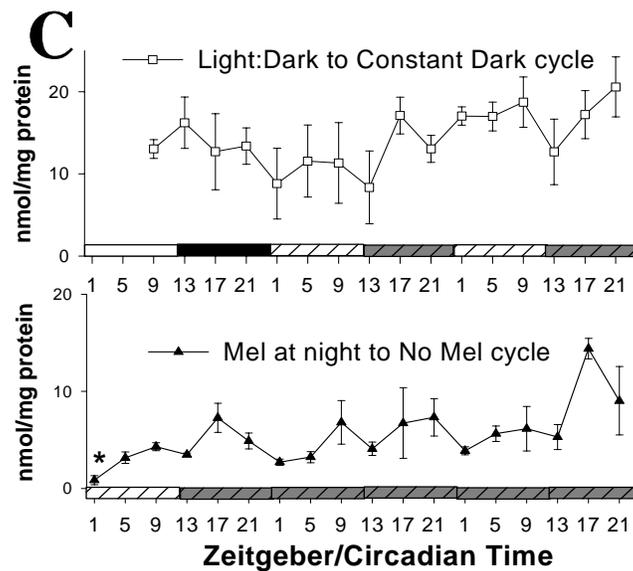
Fig. 17. (cont.) **C.** Cells placed in light:dark cycles and then transferred to constant darkness showed no rhythms in glucose uptake (upper panel; n=4). However under constant conditions, astroglia given rhythmic melatonin administration and then to no melatonin, displayed rhythmic glucose utilization for perhaps one additional cycle, although not as robustly (lower panel; n=4). In all cases, * equals p<0.001.
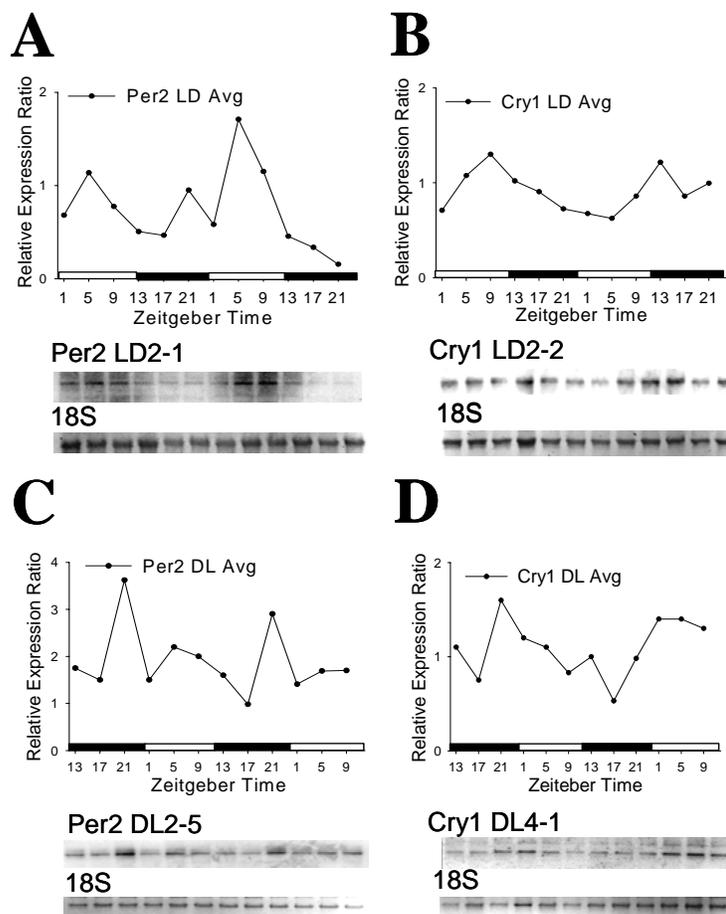
Fig. 18. Glial astrocyte cultures express rhythmic oscillations in the clock genes, *gPer2* and *gCry1*. **A.** Chick astrocyte cultures placed in a light:dark (LD) cycle express rhythms in *gPer2* mRNA with peaks during mid-day. **B.** Rhythms of *gCry1* mRNA peak during the late day (ZT 9) in LD. **C.** When astrocyte cultures are placed into a dark:light (DL) cycle, cultures express rhythms in *gPer2* mRNA that peak during the late night (ZT 21) **D.** The mRNA of *gCry1* of astroglial cultures is rhythmic in DL, peaking in late night to early morning. (n=2-3).

rhythmic with peak expression during late day at ZT 9 (Fig. 18B). In DL cycle entrained

rhythmic expression of *gPer2* and *gCry1* mRNA as well, but with a different phase

relationship, such that *gPer2* peaked during late night at ZT 21 (Fig. 18C) and *gCry1*

peaked during late night to early morning (Fig. 18D).

The expression of *gPer2* mRNA in cultures that were placed in the MN cycle

showed no rhythmicity (Fig. 19A). Astroglia under these conditions also failed to

exhibit rhythms in *gCry1* mRNA (Fig. 19B). There was no rhythmicity of *gPer2* in

cultures administered the MD cycle (Fig. 19C). The expression of *gCry1* mRNA was

rhythmic with peak expression during late subjective night (CT 21; Fig. 19D).

Cultures that were placed in LD to DD cycles showed rhythmic expression of

gPer2, which peaked during the day, and perhaps persisted for one cycle in DD (Fig.

20A). When placed into these conditions, astrocyte cultures did not exhibit any

rhythmicity of *gCry1* mRNA under constant conditions (Fig. 20B). The LD & MN

treatment cycle, where after one day of sampling the cultures were transferred to

constant conditions (no light or melatonin), had a greater effect of the rhythmicity of the

clock genes. In these cultures, *gPer2* was rhythmic for 1 to 2 cycles in DD with peak

expression during the day (Fig. 20C). Expression of *gCry1* mRNA also exhibited

rhythmicity that persisted for at least 1 cycle in DD (Fig. 20D). Peak expression was at

ZT/CT 5.
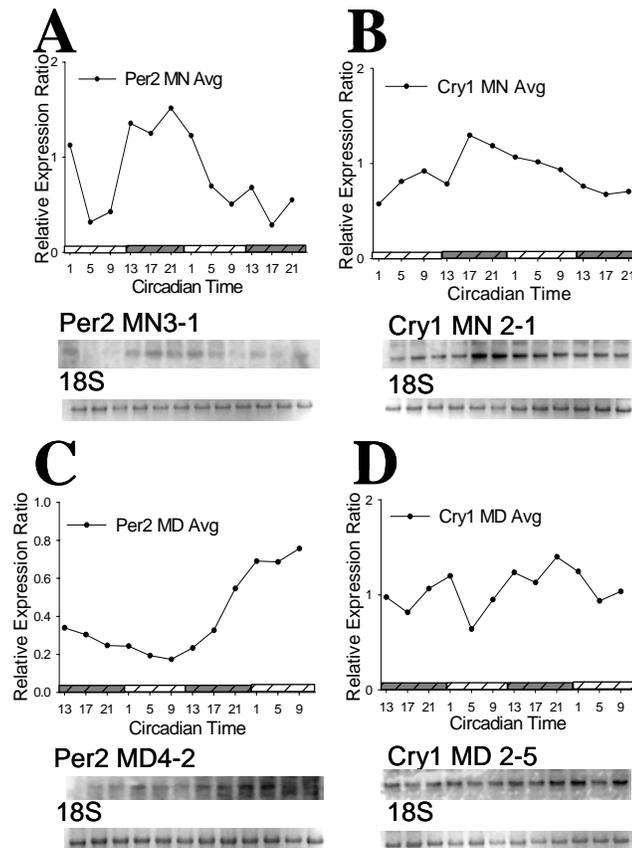
Fig. 19. Most clock gene rhythms are not synchronized by melatonin administration. **A-D.** Under constant conditions, melatonin was rhythmically administrated such that melatonin was present either during the night (MN) or during the day (MD). The mRNA of *gPer2* (**A**) and *gCry1* (**B**) in cultures of glial cells was not synchronized by MN cycles. **C**. The MD cycles also failed to elicit any rhythms of *gPer2* in astrocyte cultures. **D**. However, *gCry1* mRNA was rhythmic with peak expression during late subjective night. (n=2-3).
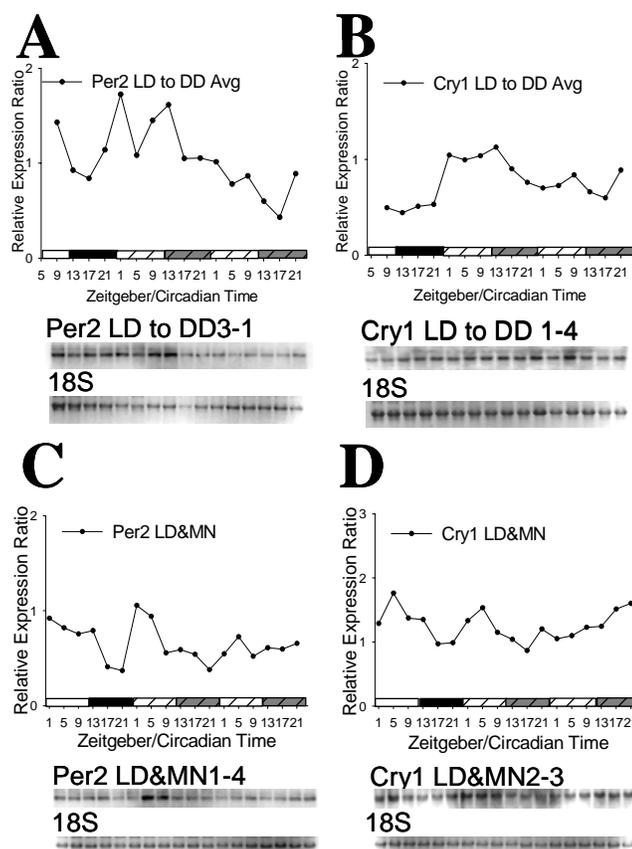
Fig. 20. Glial cultures synchronized by light and melatonin express *gPer2* and *gCry1* mRNA rhythms that persist for one cycle under constant conditions. **A-B.** Astrocyte cultures were placed in LD cycles and then transferred to constant darkness (DD). Cell cultures expressed rhythms in *gPer2* (**A**) that perhaps persisted for one cycle in DD with peaks in the late subjective day, while *gCry1* (**B**) mRNA did not exhibit any rhythmicity under constant conditions. **C-D.** Chick astrocytes were placed in LD cycles with rhythmic melatonin administered during the night (MN) and then transferred to DD with no melatonin. The mRNA of *gPer2* (**C**) and *gCry1* (**D**) in astroglial cultures was rhythmic and oscillations persisted for one to two cycles in constant conditions, peaking during early to mid-subjective day. (n=2-3).

DISCUSSION

Astrocytes in the central nervous system perform many important functions, ranging from providing support and growth factors to neurons to handling neurotransmitters and buffering extracellular ions (Volterra et al., 2002). One of the most crucial of roles is that of mediator of energy consumption in the brain (Magistretti, 2000). Astrocytes are metabolically coupled to neurons (Tsacopoulos and Magistretti, 1996), providing metabolically inexpensive pyruvate and lactate to electrically active neurons. This metabolic coupling and perhaps the release of more specific humoral signals likely results in modulation of neuronal activity as well (Nedergaard, 1994; Parpura et al., 1994; Newman and Zahs, 1998). This modulation may be focused on neurons that are adjacent to astrocytes as the astrocytes communicate with each other via intercellular calcium waves (Cornell-Bell et al., 1990; Sanzgiri et al., 1999). These calcium waves, often studied as waves of fluorescence moving form cell to cell, are induced by glutamate, serotonin, and ATP (Cornell-Bell et al., 1990; van den Pol et al., 1992). Alternatively, astrocytes may modulate neuronal circuits through the release of paracrine signals, including adenosine, nitric oxide, and several growth factors and leutrienes (Koyama et al., 1997; Sanzgiri et al., 1999; Vesce et al., 1999; Willmott et al., 2000).

There is a growing body of evidence suggesting astrocytes affect neurons in the mammalian SCN involved in circadian rhythm generation and possibly mediate at least one pathway by which the SCN influences downstream processes. First, pharmacological inhibition of glial metabolism abolishes or disrupts circadian rhythms

in intact rat SCN (Prosser et al., 1994). Secondly, transforming growth factor-alpha (TGF-α), released from SCN glia, has been suggested to be a clock output signal regulating rhythms of activity and rest (Li et al., 2002). Finally, astrocytes derived from the cortex of transgenic mice expressing luciferase conjugated to the *per1* and *per2* promoters express damped circadian rhythms of bioluminescence (Prolo et al., 2005).

In the present study, rhythmic administration of the pineal hormone melatonin clearly synchronizes a robust rhythm in glucose uptake in chick astrocytes (Fig. 17A), corroborating and extending our previous observations (Adachi et al., 2002) in that timed melatonin administration can entrain and/or drive 2DG uptake rhythms in 180° antiphase, depending upon the time at which melatonin is present. There is a hint in the data that the effect of melatonin on 2DG uptake involves entrainment of a damped oscillatory capacity in the cells, since a small, but significant peak in 2DG uptake is expressed following transfer of the cells from the melatonin cycle to continuous media without the hormone (Fig. 17C, lower panel). In stark contrast, even though these cells express the mRNA for both melanopsin and peropsin (Fig. 16B), LD cycles or DL cycles have no systematic effect on astrocyte 2DG uptake. No systematic circadian variation in 2DG uptakes is apparent following transfer from LD to DD, although there is significant variation (Fig. 17C, upper panel).

Of course, the presence of both opsins and clock genes in these cells suggested to us that they might be photosensitive, even though the 2DG uptake data clearly showed no effect of LD cycles (Fig. 17). Therefore, using the exact same cell extracts from which we obtained the 2DG values, we performed northern blot analyses of *gPer2* and

gCry1 expression. The northern blots indicated very robust expression rhythms that coincided with the phases of the LD cycles in which the cells were housed (Fig. 18). The DL cycles also affected the phase as well, but did not completely re-entrain the cells as melatonin did for the glucose utilization. Therefore, the data do support the view that chick astrocytes are photosensitive, although more experiments are needed. When the cultures were placed in LD cycles and transferred to DD, the expression rhythms of *gPer2* and *gCry1* did not persist, at least not robustly, under constant conditions (Fig. 20A & 20B). However, the clock gene expression patterns in cells that received melatonin cycles were considerably less rhythmic (Fig. 19), strongly suggesting that light has an effect on clock gene expression in cultured chick astrocytes. Combining light and melatonin cycles elicited rhythms in both clock genes that persisted for one to two cycles (Fig. 20C & 20D). This suggests that these hypothalamic astrocytes have some damped oscillatory capacity.

A recent study by Welsh et al. (2004) demonstrated that individual fibroblast cells were capable of generating robust rhythms in clock gene expression for several weeks. While it appeared that the rhythms of the fibroblast cultures were damping over time, this damping was actually due to a loss in synchrony among the individual cells. The SCN could serve to synchronize these cells to the light: dark cycle via humoral signals (LeSauter & Silver, 1998). SCN explants co-cultured with cortical astrocytes are able to sustain clock gene rhythms in the glia for several days longer than when the glia cells are cultured alone (Prolo et al., 2005). Whether or not our hypothalamic astrocytes

are damping due to a loss of synchrony among individual cells or if it is due to a loss in the rhythm, remains to be investigated.

In any case, the data presented herein clearly show that whereas melatonin cycles induce and likely entrain rhythms of glucose utilization in cultured astrocytes, no such relationship can be found between glucose utilization and light:dark cycles. However, light:dark cycles are able to entrain rhythms in clock gene expression. Thus, these two output processes in these important cell-types are differentially regulated and call into question the notion that downstream rhythms are the result of peripheral clock gene expression. In this case, we have a physiologically relevant rhythm, but it is not accompanied by rhythmic clock gene expression.

Whether or not astrocytes are affected by melatonin or are photoreceptive *in vivo* is still under investigation. *In situ* hybridization of the Mel$_{1c}$ receptor demonstrated a wide distribution of the receptor in areas without neuronal cell bodies (Reppert et al., 1995). Areas of the brain, such as the vSCN, exhibit 2DG uptake that is higher during the day than during the night (Cassone, 1998) when melatonin is present (Klein et al., 1997). Additionally, this uptake can be inhibited by administration of exogenous melatonin (Cantwell and Cassone, 2002) and pinealectomized sparrows that are arrhythmic in metabolic activity and behavior can be synchronized with daily administration of melatonin (Lu and Cassone, 1993a). In the pineal gland, peropsin and melanopsin have been localized to interstitial areas where astrocytes are found (Bailey and Cassone, 2004; accepted). Furthermore, throughout the rest of the brain there were

low levels of opsin expression above that of sense controls, suggesting this expression may be due to astrocytes (Bailey and Cassone, 2004; accepted).

Other studies have suggested differential regulation of metabolic activity and clock gene expression. Co-cultures of SCN2.2 cells and NIH3T3 fibroblasts demonstrated that the SCN cells, which retain their circadian rhythmicity *in vitro* (Earnest et al., 1999), can induce clock gene rhythms and 2DG uptake in the fibroblasts (Allen et al., 2001). However, while the rhythm in 2DG uptake in the fibroblasts lags the uptake in the SCN cells by 4 hours, the expression of *per1* and *per2* in the fibroblasts lag the SCN by 12 hours. Here we have suggested a way that clock gene expression and glucose uptake could be differentially regulated. The first could be regulated by light, and the second by melatonin release from the pineal gland.

CHAPTER V

MELATONIN REGULATES CIRCADIAN ELECTRORETINOGRAM

RHYTHMS IN A DOSE- AND TIME-DEPENDENT FASHION[*]

INTRODUCTION

In addition to its critical role in vision, the retina is an important component of the circadian system of many avian species (Ebihara et al., 1984; Underwood et al., 1984), due in part to its circadian synthesis and secretion of the indoleamine melatonin. Melatonin is rhythmically synthesized by retinal photoreceptors, such that its levels are high during the night and low during the day, and this rhythm persists in constant darkness (DD; Hamm and Menaker, 1980; Underwood et al., 1990; Adachi et al., 1995; Bernard et al., 1997) The melatonin biosynthetic enzymes tryptophan hydroxylase (Thomas and Iuvone, 1991), serotonin N-acetyltransferase (NAT; Besharse and Iuvone, 1983; Bernard et al., 1997; Klein et al., 1997), and hydroxyindole-O-methyltransferase (HIOMT; Bernard et al., 1999), are expressed rhythmically in the retina at the protein and mRNA levels.

The retina and central visual system structures are sites of direct melatonin action. Using the melatonin agonist 2-[^{125}I]-Iodomelatonin (IMEL) several authors have identified sites of high affinity IMEL binding in the chick retina and brain visual system structures (Dubocovich and Takahashi, 1987; Rivkees et al., 1989; Brooks and Cassone,

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1992; Cassone et al., 1995), while genes encoding three melatonin receptors, Mel_{1A}, Mel_{1B}, and Mel_{1C}, are expressed in chick retina and visual system structures (Liu et al., 1995; Reppert et al., 1995; Natesan and Cassone, 2002). The expression of melatonin receptor mRNA is differentially distributed. The Mel_{1A} receptor is expressed by neuronal components while Mel_{1C} receptor is expressed by non-neuronal parts of the retina and brain (Reppert et al., 1995). The Mel_{1B} receptor is predominantly expressed in all cell layers of the retina with reduced expression in the brain (Natesan and Cassone, 2002).

The circadian system influences visual function within the retina and brain at least partially via the rhythmic production of melatonin by the retina and pineal gland. Retinal visual function has been accurately assessed using electroretinographic techniques (ERG; Rager, 1979; Schaeffel et al., 1991; Lu et al., 1995; McGoogan and Cassone, 1999; McGoogan et al., 2000; Wu et al., 2000). In chickens and pigeons, the amplitude of the ERG b-wave, which represents the photic response of the inner retina, primarily due to Müller glial cell uptake of K^+ from the extracellular space (Miller and Dowling, 1970; Rager, 1979), is higher during the day than during the night (Lu et al., 1995; McGoogan and Cassone, 1999; McGoogan et al., 2000; Wu et al., 2000). The implicit times, a measure of response latency, of both a-wave, which represents the hyperpolarizing response of the photoreceptor layer (Rager, 1979), and b-wave are shorter during the day than during the night (McGoogan and Cassone, 1999; McGoogan et al., 2000). This rhythmic change in the amplitude and implicit time persists in constant darkness (DD), indicating it is under control of a circadian timing system as

well as the light: dark cycle (LD; McGoogan and Cassone, 1999; McGoogan et al., 2000; Wu et al., 2000). Conversely, in both chickens and pigeons, light sensitivity of a-wave is higher during the night than during the day (McGoogan and Cassone, 1999; McGoogan et al., 2000; Wu et al., 2000). Injection of melatonin in the afternoon (Zeitgeber Time 10) reduces chick ERG b-wave amplitude to nighttime levels (Lu et al., 1995). Constant melatonin administration abolishes rhythmicity of a-wave and b-wave implicit time and b-wave amplitude in DD (McGoogan and Cassone, 1999).

In the present study, we assess the dose response characteristics of melatonin on chick ERG, and ask whether the time of day at which melatonin is administered differentially affects retinal function. We conclude that physiological levels of melatonin have a greater effect during the night than during the day.

MATERIALS AND METHODS

Animals

Male White Leghorn chicks (n=168) were obtained at one day post hatch (Hy-line Hatchery, Bryan, Texas). They were maintained in heated brooders on a 12:12-h light: dark (LD) cycle until 3 weeks of age (lights on from 6:00 a.m. to 6:00 p.m., CST). Food (Purina All in One) and water were available at all times. The animal use described in this study was in strict accordance with Ethical Law guidelines and has been approved by the University Laboratory Animal Care Committee (Protocols, #98-00006 and #9-156B).

ERG Recordings

At 3 weeks of age, chicks were anesthetized with intramuscular injection of a ketamine/xylazine (90mg/kg: 10mg/kg) drug cocktail, and placed into a stereotaxic instrument (Stoelting Instruments, Wood Dale, IL). A 3-mm active gold electrode (Astro-Med, West Warwick, RI) was placed on the inferior corneal surface. A ground needle electrode was placed in the breast muscle and a reference electrode in the comb. A Grass PS33T strobe lamp was positioned 20 cm in front of the right eye. A series of 6 consecutive 10 μs white light stimuli (1.2×10^{16} $Q/cm^2/s$) separated by 30 seconds was given.

ERGs were recorded 6 times in a 24-hour period, or every four hours beginning at Zeitgeber Time 1 (ZT 1; 1 hour after lights on), ZT 5, ZT 9, ZT 13, ZT 17, and ZT 21. For recordings in constant darkness (DD), the chicks were moved to another room one day prior to the experimental work and kept in constant darkness throughout the remainder of the experiment. The times of recording were designated Circadian Time 1 (CT 1), CT 5, CT 9, CT 13, CT 17, and CT 21. Each LD bird was allowed to dark-adapt for 20 minutes prior to stimulation. The DD birds had been kept in constant darkness and it was, therefore, not necessary for them to dark-adapt. Latencies, a- and b-wave amplitudes, and a- and b-wave implicit times were obtained.

Melatonin, 1 mg/kg, 100 ng/kg, or 1 ng/kg in 1% DMSO (in 0.75% avian physiological saline), or a saline control was then injected in the breast muscle of each bird, and 45 minutes post-injection, another series of ERG recordings was obtained.

Each bird served as its own control by preinjection ERG and was only used once to prevent the effects of residual melatonin from affecting the subsequent data. Electrical signals were amplified, digitized using a MacLab/4d interface, and then recorded using MacLab Scope software (MacLab, Milford, MA). Four birds were tested at 6 times of day in LD and in DD. Averages of 6 responses for all parameters of each bird were calculated and plotted against time of day using Sigmaplot 5.0 (SPSS Inc, Chicago, IL). Light stimulus intensity was determined using a Biospherical Instrument (San Diego, CA) QSL-100 radiometer.

Statistical Analysis

Latency was defined as the interval from the beginning of the stimulus to the initial retinal response. A- and b-wave implicit time was defined as the interval from the stimulus to the peak voltage of each response. The a-wave amplitude was designated the peak voltage of the initial downward deflection. B-wave was the subsequent upward deflection. The b-wave amplitude was the change in voltage from the peak of a-wave to the peak of b-wave (Fig. 21A). The responses for all parameters were averaged for each bird at each time point. Each plot therefore represents the average of the average responses from 4-birds/time point (± SEM).

The effect of the saline control for each of the parameters was determined and the difference between preinjection and post-injection was calculated. The difference between the preinjection and post-injection value for each parameter of each dose was calculated as well. The fold difference over the baseline, or saline control, was
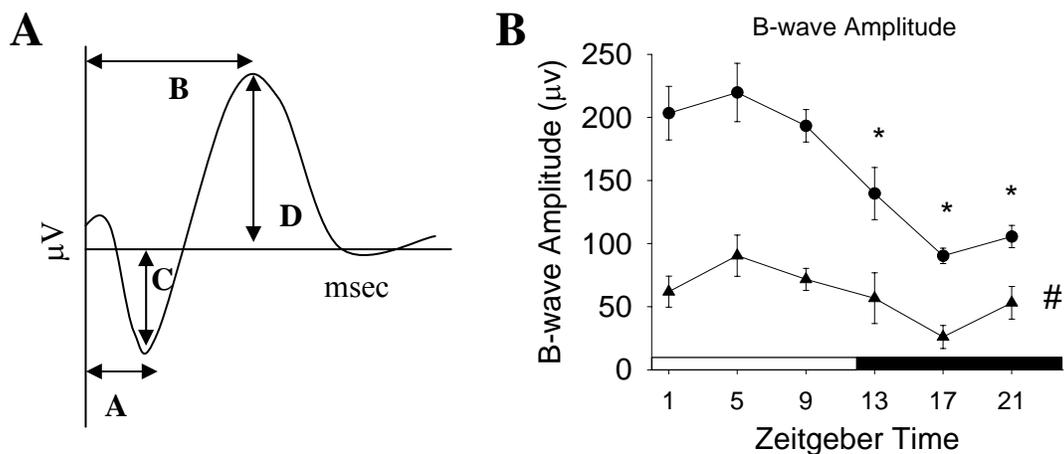
Fig. 21. Electroretinogram (ERG). **A**. Electroretinogram (ERG). (A) A-wave implicit time was defined as the interval from the stimulus to the peak voltage of the downward deflection; (B) B-wave implicit time was defined as the interval from the stimulus to the peak voltage of the upward deflection; (C) A-wave amplitude was defined as the peak voltage of the downward deflection; (D) B-wave amplitude was defined as the voltage change from the peak of a-wave to the peak of the subsequent upward deflection or b-wave. **B.** Recorded values of the data of the effects of melatonin on b-wave amplitude. Prior to injection of melatonin, there was an ERG rhythm of b-wave amplitude (circles) such that amplitude was higher during the day than during the night. The amplitude of the rhythm for b-wave was significantly decreased by 1 mg/kg melatonin injection (triangles). Values are the b-wave amplitudes prior to division of baseline, or saline control, effects.
* Relative to ZT 5 ($p < 0.05$). # Entire data set relative to preinjection ($p < 0.05$).

determined by dividing the dose difference by the saline control difference. Statistical

analysis was performed for all parameters using SAS statistical analysis software (SAS

Institute, Cary, NC). One-way ANOVA was used to determine significance on time of

day data and on melatonin dosage differences data. A 2-way ANOVA was used to test

the interaction of melatonin and time of day. Duncan's parametric post-hoc test was

used when significant differences were found. In all cases, $p < 0.05$ level was used to

determine minimum significance.

RESULTS

ERG Rhythms in LD and DD

As previously shown, there is a rhythm in b-wave amplitude in LD (McGoogan

and Cassone, 1999; McGoogan et al., 2000) and an injection of melatonin significantly

decreased the amplitude (Lu et al., 1995; Fig. 21B). This decrease occurred at all times

of the day (data shown are those prior to calculation of fold difference over baseline,

saline control). There was a rhythm of a- and b-wave implicit time observed in LD and

in DD, such that the interval from stimulus to the peak of each was shorter during the

day than during the night (Fig. 22A, B).   Latency was not rhythmic in LD or in DD

(data not shown).

A-wave and b-wave amplitudes also were rhythmic in LD and in DD. The

amplitude of each was greater during the day than during the night (Fig. 22C, D). The b-

wave amplitude rhythm was more robust than was the rhythm of a-wave amplitude. In
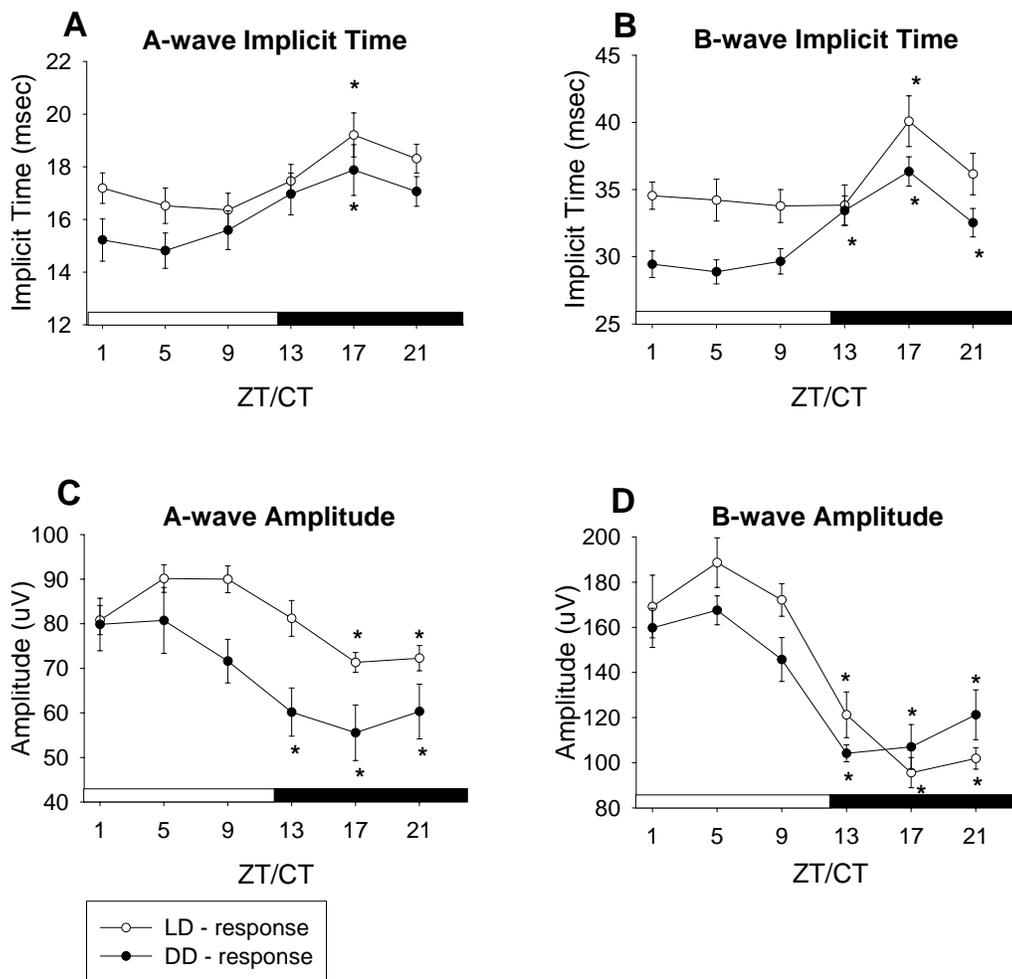
Fig. 22. Rhythmic parameters of the ERG in LD and in DD. **A & B.** The implicit times of a- and b-wave were rhythmic in LD and in DD. During mid- to late afternoon, (ZT/CT 5 to ZT/CT 9), a-wave implicit time was shorter than it was at mid-night (ZT/CT 17). B-wave implicit time was similar with shorter times during the day (ZT/CT5) than during the night (ZT/CT 17). **C & D.** Circadian rhythms of a- and b-wave amplitude were observed in both LD and DD. During the day (ZT/CT 5), a-wave amplitude was greatest and then it gradually decreased to lower nighttime amplitudes (ZT/CT 17). In the same way, b-wave amplitude was also higher at ZT/CT 5 than it was at ZT/CT 17.
* Relative to ZT/CT 5 (p<0.05).
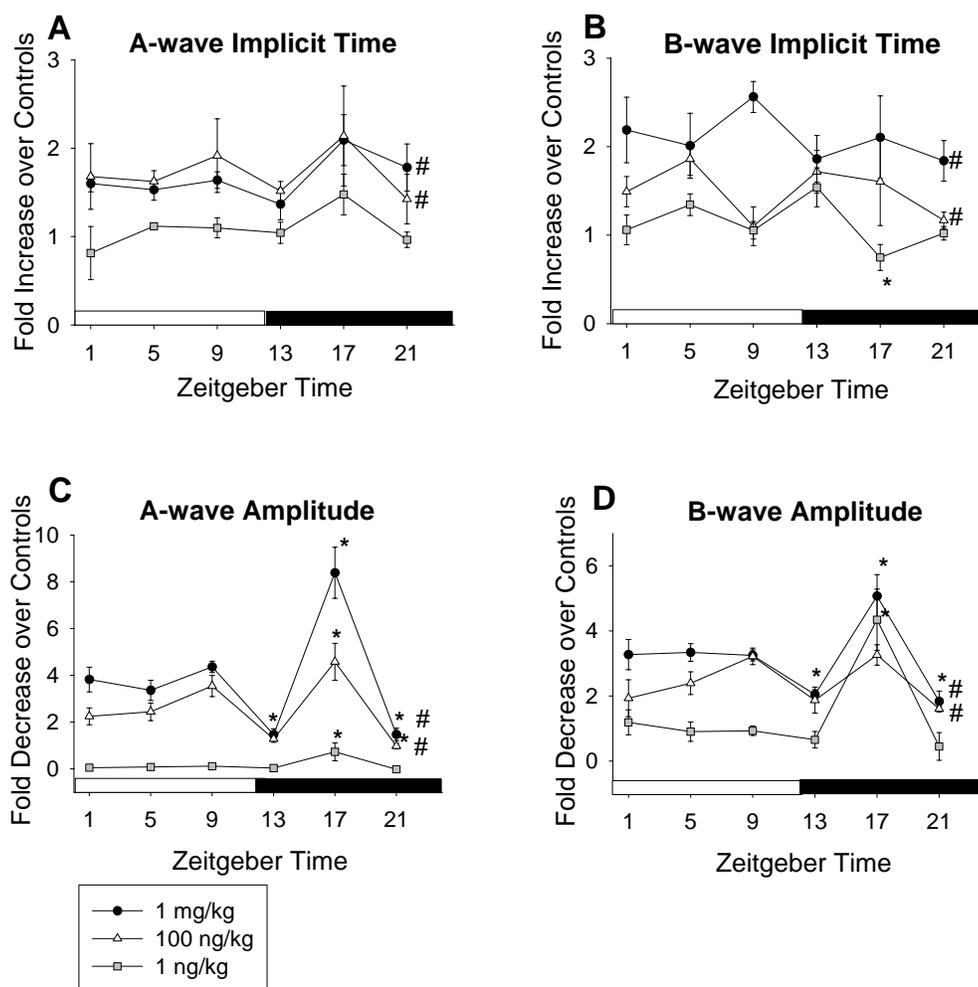
Fig. 23. Effects of melatonin on the ERG in LD. **A.** 1 mg/kg and 100 ng/kg lengthened the a-wave implicit time over that observed for the saline controls while the 1 ng/kg melatonin had little or no effect. **B.** Similarly, the effects of 1 mg/kg melatonin and 100 ng/kg melatonin for b-wave implicit time were significantly greater than those observed for 1 ng/kg melatonin as they increased the implicit times throughout the day. The 1 ng/kg dose, actually shortened the implicit time at ZT 17 as compared to the saline controls. **C.** The fold decrease in the a-wave amplitude due to 1 mg/kg and 100 ng/kg melatonin, although not 1 ng/kg melatonin, was 1.5 to 8-fold over that of the saline controls. This effect was greatest during the night at ZT 17. **D.** B-wave amplitude also had a significant fold decrease over saline controls following administration of 1 mg/kg and 100 ng/kg melatonin while 1 ng/kg melatonin had only a slight effect. The amplitude decrease due to 1 mg/kg and 1 ng/kg melatonin was greatest at mid-night (ZT 17).
* Relative to ZT 5 ($p < 0.05$). # Relative to 1 ng/kg melatonin ($p < 0.05$).

DD, the rhythm of both a- and b-wave amplitude persisted (Fig. 22C, D), such that amplitudes were higher during subjective day than in subjective night.

Effects of Melatonin on ERG Rhythms in LD and DD

In LD, melatonin administration at 1 mg/kg and 100 ng/kg increased the a-wave implicit time 1.5 to 2-fold over that of the saline controls (Fig. 23A), irrespective of the time of day. Administration of 1 ng/kg melatonin had little effect over that of saline controls. A similar effect was observed for b-wave implicit time (Fig. 23B). 1 mg/kg melatonin and 100 ng/kg melatonin had a significantly greater effect over saline controls than did 1 ng/kg melatonin. The 1 ng/kg melatonin injection shortened the b-wave implicit time at ZT 17 as compared to daytime values (ZT 5).

A- and b-wave amplitudes were decreased by melatonin. This administration of melatonin during the day decreased the amplitude to night time levels as shown in one example in Figure 21B of the effects of melatonin on the ERG, prior to division of baseline values (saline control values). The 1 mg/kg and 100 ng/kg doses of melatonin had a 1.5 to 8-fold decrease on the a-wave amplitude over that of the saline controls (Fig. 23C). This decrease was significantly greater than that of 1 ng/kg melatonin, which had little to no effect on the amplitude over that of the controls. The effect of all 3 doses was greater during the night at ZT 17 than during the day at ZT 5. B-wave amplitude was similarly affected. 1 mg/kg melatonin and 100 ng/kg melatonin had a significantly greater effect at decreasing the amplitude than did 1 ng/kg melatonin, as compared to the saline controls (Fig. 23D). 1 ng/kg melatonin had only a slight effect

over the saline controls. The effect of 1 mg/kg and 1 ng/kg melatonin was greater during

the night (ZT 17) than during the day (ZT 5).

Since 100 ng/kg melatonin had as great of an effect on the ERG as did the 1

mg/kg melatonin dose, we used the 100 ng/kg and 1 ng/kg doses for the subsequent

experiments in DD. As in LD, 100 ng/kg melatonin lengthened the a- and b-wave

implicit time in DD. This increase in implicit time was seen at all times of day, except

ZT/CT 17. The effect of 100 ng/kg melatonin on a-wave implicit time was a half-fold

greater than the effects of the saline control (Fig. 24A) and was significantly greater than

that of 1 ng/kg melatonin, which had no effect over that of the saline control. A similar

effect was observed for the b-wave implicit time (Fig. 24B). 100 ng/kg melatonin had a

2-fold increase over that of saline controls, and this effect was significantly greater than

that of 1 ng/kg melatonin. Both doses of melatonin shortened the b-wave implicit time

at CT 17 as compared to daytime levels (CT 5).

The 100 ng/kg dose of melatonin decreased the amplitude of a- and b-wave at all

times in DD. This reduction in a-wave amplitude was 2-fold greater than the effect of the

saline control (Fig. 24C) and similar at all times of day. 100 ng/kg melatonin's effect

was not significantly greater than that of 1 ng/kg melatonin. However, the effects of 100

ng/kg melatonin were significantly greater than 1 ng/kg melatonin for b-wave amplitude

(Fig. 24D). 100 ng/kg melatonin had a 2-4 fold decrease over the saline control.   This

effect was greater during the night (CT 17) than during the day (CT 5). The 1 ng/kg

dose of melatonin had a slight effect over the saline controls.
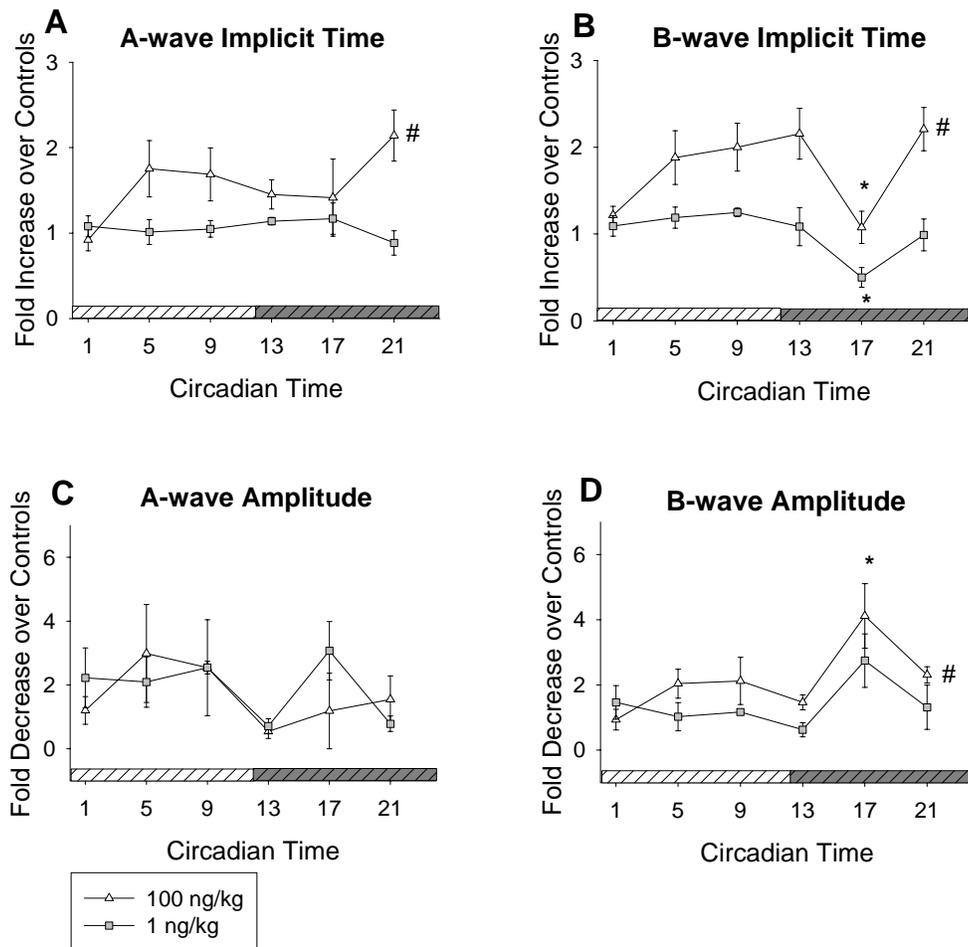
Fig. 24. Effects of melatonin on the ERG in DD. **A & B.** The 100 ng/kg dose of melatonin lengthened the implicit times of a- and b-wave over the effects observed by the saline control. However, the 1 ng/kg dose had no effect over that of the saline controls. While the increase in a-wave implicit time was seen throughout most of the day (**A**), the implicit time of b-wave was actually decreased or shortened by both doses of melatonin as compared to the saline controls at CT 17 (**B**). **C.** The 100 ng/kg dose of melatonin decreased a-wave amplitude over the saline controls, although there was not a time of day when this effect was more significant, and these effects were not significantly greater than those of 1 ng/kg melatonin. **D.** B-wave amplitude also had a significant fold decrease over saline controls following administration of 100 ng/kg melatonin while 1 ng/kg melatonin had only a slight effect. The amplitude decrease due to 100 ng/kg melatonin was greatest at during mid-subjective night (CT 17).
* Relative to CT 5 ($p<0.05$). # Relative to 1 ng/kg melatonin ($p<0.05$).

DISCUSSION

Here we observed diurnal and circadian rhythms of ERG a- and b-wave amplitudes and implicit times in LD and in DD. Consistent with our previous studies (Lu et al., 1995; McGoogan and Cassone, 1999; McGoogan et al., 2000), amplitudes were higher during the day in LD and subjective day in DD than during the night and subjective night, respectively. Conversely, implicit times were longer during the night in LD and subjective night in DD than during the day and subjective day respectively. Intramuscular injections of melatonin at doses of 1 mg/kg and 100 ng/kg increased implicit times and decreased amplitudes of a- and b-wave in LD, and 100 ng/kg melatonin had similar effects in DD.

The effect of melatonin on a- and b-wave amplitude in LD and on b-wave amplitude in DD was greater during the night at ZT/CT 17 than during the day at ZT/CT 5. In a diurnal bird where there are a greater number of cones, the cone photoreceptors are active during the day and rods predominantly are active during the night (Schaeffel et al., 1991). The greater effect of melatonin on a-wave amplitude during the night suggests a greater effect of melatonin on the rod photoreceptors. This effect is also seen on b-wave amplitude, which suggests a large effect of melatonin is on the Müller glial (Rager, 1979; Miller and Dowling, 1970).

Müller glial cells are known to play an important role within the retina of all vertebrate species (Newman and Reichenbach, 1996). These glial cells span the entire retina from the pigmented epithelium to the inner limiting membrane. While we do not have data from the Müller cells themselves, the rhythmic administration of melatonin to

astrocytes, which share similarities to Müller cells (Hollander et al., 1991), in culture has been shown to play an important role in the regulation of their metabolic activities (Adachi et al., 2002). Glucose uptake is greater during the day than during the night, and the release of the glycolytic by-products, lactate and pyruvate, is higher during times of day when melatonin is not present. Moreover, the Mel_{1c} melatonin receptor is expressed on all of these glial astrocytes (Adachi et al., 2002). Müller glial cells are also known to mediate metabolic processes within the retina, and they are very sensitive to changes in pH and to oxygen levels.

Müller cells also mediate ionic processes, as following the release of K^+ into the extracellular space by light-stimulated retinal neurons, Müller cells rapidly depolarize to modulate this extracellular K^+ concentration through spatial buffering (Newman et al., 1984). This depolarization has been shown to cause an increase in the intracellular concentration of Ca^{2+} within the glial cell as well (Keirstead and Miller, 1995). Melatonin may alter the ionic state of the Müller cell via nonspecific cation channels (NSC) and second messenger systems, thereby decreasing K^+ uptake and the amplitude of the b-wave, as shown here. Physiological concentrations of melatonin have been shown to increase the spread of intercellular calcium waves and decrease gap junctional communication amongst chick diencephalic astrocytes *in vitro* (CHAPTER II).

Another possible explanation of the effects of melatonin on the ERG b-wave amplitude is that it is a reflection of the ON-bipolar cells. While the Müller glial cell serves as the direct producer of the b-wave (Dowling, 1987), the Müller cell potential changes are not directly light induced. The light-induced increase in extracellular

potassium concentration in both the inner (proximal) and outer (distal) retina are likely

the result of the ON-bipolar cell depolarization (Stockton and Slaughter, 1989). The

currents across the Müller cell membrane are a result of this depolarization. The ON-

bipolar cells communicate with the amacrine cells where dopamine is produced. A

reduction in dopamine levels by melatonin could alter inputs to the ON-bipolar cells,

thus affecting the b-wave of the ERG. This is additionally supported by our DD data, in

that the effects of melatonin at different times of day are abolished for a-wave amplitude

and somewhat reduced for b-wave amplitude (Fig. 24C, D), indicating an important role

for light and dopamine.

Amacrine cells in the INL, which express melatonin receptors, secrete dopamine

180° anti-phase from melatonin, such that levels are high during the day and low during

the night (Cahill et al., 1991; Adachi et al., 1998). Retinal dopamine synthesis and

release are stimulated by light (Adachi et al., 1998) and appear to act on photoreceptors

(Cahill and Besharse, 1993). Dopamine prohibits the photoreceptors from secreting

melatonin (Cahill and Besharse, 1991), increases the amplitude of the ERG b-wave

(Miranda-Anaya et al., 2002), and phase shifts the retinal' clock (Adachi et al., 1998;

Steenhard and Besharse, 2001). Conversely, at night, when the photoreceptors secrete

melatonin, dopamine release is suppressed. The inverse relationship between dopamine

and melatonin in the duck retina was recently demonstrated in that 1) exposure to light

during the night increased dopamine levels and decreased melatonin content, 2)

intraocular administration of melatonin decreased retinal dopamine content, and 3)

intraocular administration of a dopamine receptor agonist decreased NAT activity and

melatonin content in the retina (Zawilska et al., 2003). A similar relationship has been shown in chicks (Nowak et al., 1992) and this loop in which melatonin and dopamine negatively feed back on their respective layers within the retina in a circadian fashion may drive the rhythmic oscillations we observe in the ERG. The retinal rhythmicities generated in the eye would in turn act locally to regulate visual function (Thomas and Iuvone, 1991; Cahill and Besharse, 1993).

The sensitivity of a- and b-wave amplitude to melatonin during the night may be associated with one or more circadian processes. Several groups (Reppert et al., 1995; Natesan and Cassone, 2002) have demonstrated the expression of Mel_{1a}, Mel_{1b}, and Mel_{1c} mRNA in the inner segments of the photoreceptor layer, the vitread portion of the inner nuclear layer (INL), and the ganglion cell layer (GCL). Further, there is a rhythm of the mRNA encoding these receptors, such that Mel_{1a} and Mel_{1c} mRNA levels are higher during midday in LD (Natesan and Cassone, 2002). The rhythm of Mel_{1a} RNA, at least, persists in DD, while Mel_{1b} mRNA is constitutively expressed. If we assume a delay in protein expression and activated protein of the receptor, this would be consistent with our data as melatonin has a greater effect on decreasing the amplitude during the night than during the day. Alternatively, the constitutively expressed Mel_{1b} receptor may mediate this process. The avian system is very sensitive to melatonin, especially at night, as demonstrated by this study as exogenous melatonin was able to affect the ERG at night when levels of melatonin are already high, perhaps indicating that the melatonin receptors may not be saturated at night. This also suggests that these effects are not due

to a shift of the circadian clock, but rather to a rhythmic sensitivity of the system to melatonin.

The increased effects of melatonin during the night point to a restorative role for melatonin during the night while the diurnal bird is not active. Melatonin has been found to have antioxidant effects, albeit at very high concentrations (Tan et al., 1993; Allegra et al., 2003). Light can cause photodamage through the generation of reactive oxygen species (ROS). In cell culture experiments, melatonin rescues retinal neurons from reactive oxygen species injury (Lee et al., 2001). It is able to scavenge hydroxyl radicals and peroxyl radicals, thus preventing oxidative injury. This would be most beneficial if it occurred on a regular, circadian basis. At night when the retina is most susceptible to damage due to less shielding by the retinal pigmented epithelium, melatonin may prevent ROS damage by removing toxins that could still be present.

The Purkinje shift model of avian retinal function describes the change from a cone active retina during the day, when the implicit times are shorter, to a rod active retina at night, when the implicit times are longer as rods take longer to respond (Schaeffel et al., 1991). Melatonin is thought to at least partially mediate this shift (McGoogan and Cassone, 1999). As stated above, there are several melatonin receptors located on the photoreceptive cells and other retinal cells. The different receptors may mediate different functions within the same cell. One might regulate changes in the ionic state of the cell through second messenger systems and alter the amplitude of the ERG via Muller glial cells, and another could regulate the implicit time of the ERG through effects on the photoreceptors via the Purkinje shift.

In conclusion, we have shown that disparate concentrations of melatonin, ranging from 1 mg/kg to 100 ng/kg, have similar effects on the function of the chick retina. Melatonin given during the day reduces the b-wave amplitude to night-time levels. The effects of melatonin on b-wave implicit time and a- and b-wave amplitude changes depend on the time of day at which it is administered.

CHAPTER VI

CONCLUSIONS

The aim of this research was to examine the role of neural glial cells in the vertebrate circadian clock and to learn to what extent, if any, the neurohormone melatonin has in mediating glial functions within the retina and the brain. Melatonin is an important output of the circadian clock and receptors for it are found in the retina and visual system structures throughout the brain, including the SCN (Dubocovich and Takahashi, 1987; Rivkees et al., 1989; Cassone et al., 1995; Klein et al., 1997; Cassone, 1998). Some of the cells that have these receptors are not neuronal in nature, but are glial cells (Reppert et al., 1995). In these experiments, we studied melatonin responses in astrocytic glial cells from the telencephalon and diencephalon of both birds and mammals. In addition, we investigated the roles of melatonin and light in the regulation of glia metabolism and clock gene expression. Finally, we examined retinal responses to melatonin via electroretinography.

First, chick diencephalic astrocyte communication via intercellular waves and gap junctions is modulated by melatonin. Physiological concentrations of melatonin increase the percent of astrocytes involved in an intercellular calcium wave. These intercellular calcium waves of glia are mediated via an IP_3-dependent mechanism. Melatonin also increases intracellular resting calcium concentrations. The same nanomolar concentrations of melatonin that increase the spread of the calcium wave, decrease gap junctional coupling. Cultures treated with picomolar and nanomolar

melatonin have decreased immunochemical staining of the gap junctional protein connexin43. These opposing effects of melatonin indicate that the presence of melatonin during the night brings about a functional switch in the nature of intercellular communication among diencephalic astrocytes.

Second, melatonin modulates both avian and mammalian diencephalic astrocytic intercellular calcium waves. However, it has no effect on telencephalic glial calcium waves. Intercellular calcium waves of mouse astrocytes spread 2 to 5 fold farther than those of chick glia. Physiological concentrations of melatonin increase the area and the rate of the spread of intercellular calcium waves among chick and mouse diencephalic astrocytes, but have no effect on telencephalic astrocyte communication. Interestingly, both chick and mouse diencephalic and telencephalic astrocytes express multiple melatonin receptors. Mouse telencephalic waves spread the farthest of the four types of cells tested. The spread of the wave was concentric with all wave fronts advancing at approximately the same rate. On the other hand, mouse diencephalic calcium waves meandered throughout the culture from the point of stimulation at various rates of propagation. Nanomolar concentrations of melatonin make these diencephalic waves more telencephalic-like. This suggests that melatonin receptors in diverse areas of the brain may differentially influence glial communication and outputs of the circadian clock.

Thirdly, metabolic activity and clock gene expression in chick glial cells are differentially regulated. Diencephalic astrocytes express melanopsin, peropsin, and several of the clock genes, including *clk, cry1, cry2, per2, per3*, and others. Melatonin

affects metabolic activity such that under constant conditions glucose uptake is higher when melatonin is not present. Thus, rhythmic administration of the hormone generates robust rhythms in metabolic activity. However, melatonin has little or no effect on clock gene expression. In contrast, light does not affect metabolic activity as LD cycles cannot induce rhythmic utilization of glucose. Light cycles do affect clock gene expression, as light synchronizes the expression of both, *cry1* and *per2*. This effect may persist in DD following previous synchronization by LD cycles. Rhythmic expression of *cry1* and *per2* mRNA does persist for 1 to 2 cycles in constant conditions following cycles of light and melatonin. This suggests that endogenous oscillatory elements exist in these astrocytes, which may normally be coordinated by signals or messages released by neighboring neurons. These data strongly suggest that both rhythmic clock gene expression and metabolic function are differentially regulated, the first by light and the second by melatonin.

Lastly, melatonin modulates a day – night shift in the retina of the chick. There is a daily and circadian rhythm of the a- and b-wave implicit times and amplitudes of the electroretinogram, one way to assess retinal visual function. Physiological concentrations of melatonin affect these parameters of the ERG, such that amplitudes are decreased and implicit times are increased. This reduction of a- and b-wave amplitudes in LD and b-wave amplitude in DD is greater during the night (or subjective night) than during the day (or subjective day). The b-wave amplitude is due mainly to response of Muller glial cells (Miller and Dowling, 1970). These data indicate that melatonin plays

a role in regulating a day and night functional shift in the retina via regulation of a retinal clock.

GLIA AND THE ROLE OF CALCIUM

There are astrocytes and/or other glial cells found in all three of the avian pacemakers, the retina, the pineal gland, and the vSCN as well as the mammalian pacemaker, the SCN (Cassone and Menaker, 1984; Morin et al., 1989). In the central nervous system, astrocytes perform many important functions from providing support and growth factors to neurons, to handling neurotransmitters and buffering extracellular ions (Volterra et al., 2002). Glial cells are able to react to a large number of neuroactive substances by modulation of intracellular cascades that almost always involve a change in intracellular calcium concentration (Verkhratsky et al., 1998). For example, activation of glutamate receptors on glia increases the influx of Ca^{2+} into the cell through ligand-gated Ca^{2+} channels. These and other modulatory changes can have marked affects on ionic buffering of neuroglia since regulation of calcium homeostasis is vital to cell health and brain function, and neural astrocytes possess a mechanism to closely regulate cytosolic calcium. Removal of Ca^{2+} from the glial cell occurs via plasmalemmal Ca^{2+} pumps and an electrochemically driven Na^+/Ca^{2+} exchanger. While little is known about astrocyte Ca^{2+} pumps, it appears they are Ca^{2+}- ATPases that sense increased intracellular calcium and utilize energy to decrease it (Kirischuk et al., 1995). The Na^+/Ca^{2+} exchanger function is controlled by the extracellular Na^+. When the Na^+ driving force decreases, the rate of Ca^{2+} efflux decreases, and consequently, the Ca^{2+}

concentration in the cell increases (Verkhratsky et al., 1998). Neuronal activity can alter Na^+ and Ca^{2+} concentrations and thus, the activity of the exchanger. Therefore, Ca^{2+} ions are exchanged not only to maintain proper ionic gradients of glial cells, but also to control the ionic environment of neighboring neuronal cells.

Glutamate is an important excitatory neurotransmitter in the central nervous system that plays many roles in normal brain function. Glial cells, particularly astrocytes, play a critical role in transmitter scavenging. Glutamate accumulates in the synaptic cleft following synaptic transmission. Therefore, it needs to be removed quickly, so as not to be toxic due to over stimulation of the glutamate receptors (Volterra et al., 2002). Additionally, stimulation of excitatory neurons with glutamate leads to an increased energy demand in the neurons. This energy demand is met by astrocytes, which sense the neuronal activity via glutamate and respond by increasing glucose uptake and production of lactate (Pellerin and Magistretti, 1994). Lactate is then released by the glia into the extracellular pool where neurons preferentially utilize it to meet their energy needs in a process known as the astrocyte-neuron lactate shuttle (Kasischke et al., 2004). In the astrocyte, increases in intracellular Ca^{2+} promote glycogen breakdown that can be transferred to neurons to provide energy (Giaume and Venance, 1998).

Glutamate re-uptake in astrocytes is accomplished by glutamate transporters and is driven by an electrochemical gradient for Na^+. The increase in the intracellular concentration of Na^+ is sensed in the cell as an additional signal that glutamate was released at the synapse. Na^+ homeostasis is restored to the glial cell by activation of the

Na^+/ K^+ ATPase, which causes increased energy demands that are met by glucose utilization (Pellerin and Magistretti, 1997). Bernardinelli et al. (2004) demonstrated that intercellular Na^+ waves mediated by glutamate, induced glucose uptake amongst cortical astrocytes and that the Na^+ waves were driven by propagated changes in intracellular Ca^{2+}. Astrocytes also require energy (ATP) to convert glutamate to glutamine, which when released is taken up by neurons, where the glutamine can be converted back to glutamate. As part of the tripartite synapse, astrocytes are able to modify neural communication and synaptic function in the retina and the brain (Nedergaard, 1994; Parpura et al., 1994; Newman and Zahs, 1998; Araque et al., 1999; Haydon, 2001; Volterra et al., 2002). This can occur by release of glutamate in a Ca^{2+}-dependent manner (Parpura and Haydon, 1994) and neural activity in hippocampal slices can be synchronized by glutamate release from glia (Angulo et al., 2004). Alternatively, astrocytes may release paracrine signals, including adenosine, nitric oxide, and several growth factors and leutrienes (Koyama et al., 1997; Sanzgiri et al., 1999; Vesce et al., 1999; Willmott et al., 2000).

Elevated intracellular Ca^{2+}, which leads to release of glutamate and other neurotransmitters (e.g., serotonin and ATP), often generates intercellular Ca^{2+} waves (Cornell-Bell et al., 1990; van den Pol et al., 1992). These interastrocytic waves are thought to transfer information within the CNS by modulation of networks of glia and neurons. In fact, this type of lateral information transfer could represent a wide-spread form of brain coupling or volume transmission. In our studies (CHAPTERS II & III), we observed an increase in astrocytic intracellular Ca^{2+} following mechanical stimulation, of

the cell, which spread as an intercellular Ca^{2+} wave in the both diencephalon and telencephalon of two different species, mice and chicks. The Ca^{2+} waves propagating among mouse telencephalic astrocytes were robust and pervasive, involving many more cells on average than mouse diencephalic cultures. In fact, regional differences in the extent of astroglial calcium wave propagation exist, with cortical and hippocampal waves often spreading twice that of those in astrocytes from hypothalamus and brain stem (Blomstrand et al., 1999). Additionally, fundamental differences were observed between these chick and mouse intercellular waves. Calcium waves among chick astrocytes involved many fewer cells than did those in mouse cultures. We propose that these differences reflect species-specific heterogeneity of astrocytes (e.g., receptor phenotypes, signaling mechanisms) in the glial cultures.

Differences in signaling mechanisms likely determine the disparate way in which Ca^{2+} waves are propagated. The irregular, multidirectional and restricted spread of chick calcium waves indicates a greater role for gap junctions in these avian astrocytes. In mouse astrocytes, the radial, pulsating and pervasive spread of calcium waves suggests the involvement of a diffusible signal and a less constrained paracrine mechanism. Disruption of extracellular signaling pathways known to mediate calcium waves among mammalian astrocytes, namely ATP, nitric oxide, and prostaglandins (Charles, 1998; Guthrie et al., 1999; Fam et al., 2000), had little effect on waves among chick diencephalic astrocytes (CHAPTER II). The presence or sensitivity of various receptors may also account for differences observed. Application of glutamate to mammalian

astrocytes induces waves of calcium (Cornell-Bell et al., 1990); however, glutamate had no effect on chick diencephalic astrocytes (data not shown).

Following the influx of calcium into the cell, most of the ions are bound to Ca^{2+}-binding proteins that lead to several signal transduction pathways. One of these proteins is calmodulin (CaM). It activates CaM-dependent protein kinases affecting the pathway responsible for gene expression. Finkbeiner and Greenberg (1996) demonstrated that cytoplasmic Ca^{2+} activates Ras proteins inducing phosphorylation cascades, which lead to the modification of gene expression. Synthesis of immediate early genes can occur if cytoplasmic Ca^{2+} enters the nucleus of the cells (Verkhratsky et al., 1998). Thus, calcium homeostasis, involving Ca^{2+} influx as well as intercellular calcium waves, is important for cells in the brain. We have found differences in Ca^{2+} handling between species and brain regions. These differences in calcium homeostasis could have effects on the role of the endocrine system or circadian clock function, and may be critical for the function of an animal's nervous system.

GLIA AND THE ROLE OF LIGHT

Light affects chick glial cells of the both hypothalamus and retina. This influences the circadian pacemaker through both retinal visual function and clock genes as seen in these studies. Light acts as a time cue to entrain an organism to its environment (Pittendrigh, 1961). Dopamine release, stimulated by light, suppresses melatonin synthesis in the retina (Adachi et al., 1998; Zawilska et al., 2003) and increases b-wave amplitude of the ERG (Miranda-Anaya et al., 2002). Calcium may

have a role in transmitting this input signal to the core clock. The light signal to the

SCN is transmitted by release of glutamate (Ebling, 1996) and pituitary adenylate

cyclase-activating polypeptide (PACAP; Hannibal, 2002) at the end of the RHT

terminals. As mentioned previously, glutamate can induce an increase in intracellular

Ca^{2+} in cells, which activates signal transduction pathways (i.e. CaM-dependent kinase,

Ras, etc.) and phosphorylation events leading to alterations in gene expression.

Tischkau et al. (2003) demonstrated that following the phosphorylation of cAMP-

response-element-binding protein (CREB), it binds to a cAMP-response element (CRE)

on the 5' upstream end of *Per1* and initiates transcription of this clock gene. Light-

induced phase shifts in locomotor activity rhythms were blocked by disruption of this

binding (Yokota et al., 2001).

L-type voltage-gated Ca^{2+} channels undergo daily modulations that effect

intracellular Ca^{2+} homeostasis (Pennartz et al., 2002) and in fact, intracellular

concentrations of Ca^{2+} in SCN neurons are higher during the day than during the night

(Colwell, 2000). In SCN neurons there is a circadian rhythm in cytosolic Ca^{2+}

concentrations that is independent of the neuronal firing rhythm as neither TTX nor an

L-type Ca^{2+} channel blocker, which both affected neuronal activity rhythms, had any

affect on the intracellular Ca^{2+} rhythms (M Ikeda et al., 2003). The rhythmic increase in

intracellular Ca^{2+} is due to release of Ca^{2+} from ryanodine-sensitive stores (Ding et al.,

1998; M Ikeda et al., 2003). This suggests that the regulator of the rhythm is one of the

core components of the clock, and that these Ca^{2+} rhythms could serve to generate

rhythms in other downstream outputs, such as transcription and translation, clock-controlled cellular processes, integrative neural functions, and animal behaviors.

The light-induced release of glutamate from the RHT increases the energy demands of neurons and glia in the SCN and thereby increases glucose utilization. Glucose utilization is rhythmic in mammals and birds with greater uptake during the day than during the night (Cassone, 1988; Cassone et al., 1988; Schwartz, 1990; Cantwell & Cassone, 2002). Data from our lab (Cantwell & Cassone, 2002) indicates an important role for light, since the rhythmic amplitude of the glucose uptake is lower in the vSCN under constant darkness compared to that of the amplitude in LD. Additionally, changes in astrocyte GFAP expression in the SCN are related to light and rhythmic glutamate release from the RHT (Glass et al., 1993). In LD, the photic signals induce GFAP rhythms that are sustained by the circadian rhythm of glutamate release in DD, but are abolished in LL where glutamate release is constant (Lavialle et al., 2001).

Individual SCN neurons in culture express circadian rhythmicity, but are not synchronized (Welsh et al., 1995) and the inhibition of glial metabolism disrupts the circadian rhythm of neuronal firing in the SCN (Prosser et al., 1994). This indicates to an important role for astrocytes in coordinating rhythmicity between populations of cells within the SCN. Rat astrocytes have recently been shown to express rhythms in two of the clock genes, *mPer1* and *mPer2* (Prolo et al., 2004). These cells were able to entrain to daily time cues and be phase shifted. Rhythms damped out after one week *in vitro*, but when co-cultured with adult SCN explants, the rhythms were sustained for several days longer suggesting a diffusible signal from the SCN coordinates rhythmicity. Welsh

et al. (2004) demonstrated rhythms in fibroblast cultures that damped over time in constant conditions. This damping was due to a loss in synchrony among the individual cells. The SCN could serve to synchronize these cells to the light: dark cycle via humoral signals (LeSauter & Silver, 1998) and this idea is supported by the fact that SCN cultures confer rhythmicity on arrhythmic follower fibroblast cells in the absence of cell-cell contacts (Allen et al., 2001).

It is unclear whether or not the chick astrocytes in our studies are photoreceptive. They do express mRNA for the two opsins, melanopsin and peropsin, and light is able to synchronize rhythms in both *cry1,* and *per2* (CHAPTER IV). One opsin detected in the pineal gland of birds, peropsin, has been localized to interstitial areas corresponding to the astrocyte cells (Bailey and Cassone, 2004). It has been proposed that astrocytes coupled via the Cx43 gap junction protein help synchronize the heterogeneous population of cells found in the pineal to produce the circadian rhythm of pineal activity (Berthoud et al., 2000). It is interesting to speculate that this synchronization occurs via light activation of signaling pathway that lead to changes in calcium homeostasis. *In vivo*, this signal could come in the form of glutamate release from neighboring neurons or some other neurotransmitter, such as nitric oxide. The damping of the clock gene rhythm of our *in vitro* cultures in DD may be due to a loss of synchrony among individual cells or to a loss in the rhythm altogether. Thus light's ability to influence the astrocytes could be yet another way to regulate and synchronize circadian rhythmicity.

GLIA AND THE ROLE OF MELATONIN

Not only are glial cells present in the avian circadian pacemakers, but melatonin also plays a role in each of these oscillators. The pineal gland secretes the neurohormone during the night (Gaston and Menaker, 1968). Studies using chicken have found that birds express three melatonin receptors: Mel_{1a}, Mel_{1b}, and Mel_{1c}, (Reppert et al., 1995; Lui et al., 1995).The SCN contains melatonin receptors and binding of melatonin in this region is rhythmic (Rivkees et al., 1989; Brooks and Cassone, 1992). In culture, all diencephalic astrocytes express the Mel_{1c} melatonin receptor and 25% of them express the Mel_{1a} melatonin receptor (Adachi et al., 2002). The retina, like the pineal gland, produces melatonin during the night (Hamm and Menaker, 1980; Adachi et al., 1995; Bernard et al., 1997). There are melatonin receptors in the retina on the ganglion cells, the inner nuclear layer, and the photoreceptive layer (Dubocovich and Takahashi, 1987; Natesan and Cassone, 2002). Melatonin release from the photoreceptors during the night inhibits dopamine release from the amacrine cells in the inner nuclear layer (Nowak et al., 1992; Adachi et al., 1998). The cell bodies of Müller glial cells are located in the vitread portion of the inner nuclear layer where melatonin receptors are observed (McGoogan, 2000; Natesan and Cassone, 2002).

Mammals also have astrocytes throughout the brain and the retina. The master circadian pacemaker in mammals is the SCN, and it directly affects melatonin output via neural pathways to the pineal gland, its slave oscillator (Klein et al., 1997; Moore & Silver, 1998). Mammals express the melatonin receptor subtypes, MT1 (Mel_{1a}) and MT2 (Mel_{1b}), but not the Mel_{1c} receptor (Reppert et al., 1996; Dubocovich et al., 2003).

Melatonin receptors are found in several regions of the brain, with high affinity receptors located in the SCN of the hypothalamus (Weaver et al., 1989; von Gall et al., 2002). Melatonin receptor expression has been localized to astrocytes and cerebellar Bergmann glia in humans (Al-Ghoul et al., 1998). Melatonin levels peak in the circulation of birds and mammals at night (Pang and Ralph, 1975; Cassone and Menaker, 1983; Cassone et al., 1986b).

In the retina, melatonin alters the amplitude of the b-wave of the ERG (McGoogan and Cassone, 1999; CHAPTER V), which is mainly attributed to uptake of extracellular K^+ by Müller glial cells following K^+ efflux from light-stimulated neurons. This uptake also causes an increase in intracellular Ca^{2+} concentrations in the glial cell (Keirstead and Miller, 1995). Melatonin could act through its receptors and second messenger systems and alter the ionic homeostatic state of the cell, thereby decreasing K^+ uptake and the amplitude of the b-wave. Melatonin's influence on the Müller glial cells points toward an important role for these cells in modulating the retinal clock. As mentioned earlier, melatonin also affects Ca^{2+} homeostasis in diencephalic astrocytes. Melatonin increases the resting Ca^{2+} concentration in these cells (CHAPTER II), and since these changes in intracellular Ca^{2+} levels are vast, this could have wide-spread affects on the entire brain in both metabolic and integrative forms.

Melatonin alters Ca^{2+} waves propagating among mouse and chick diencephalic astrocytes. In chick astrocytes, melatonin induces a fundamental shift in cell-cell communication, facilitating Ca^{2+} waves, while suppressing gap junctional coupling and connexin43 expression (CHAPTER II). Melatonin also facilitated Ca^{2+} waves among

diencephalic mouse astroglia, but had no effect on telencephalic astrocytes of either mice or chicks (CHAPTER III). It was surprising that melatonin had similar effects on both species, but upon closer examination the astrocytic responses were not identical. Melatonin facilitated avian diencephalic Ca^{2+} signaling such that waves became less telencephalic in their propagation. In fact, following activation of their melatonin receptors, chick diencephalic astrocytes exhibit a fundamental shift in intercellular coupling (CHAPTER II). This switch the in mode of coupling involves a facilitation of paracrine signaling (i.e., intercellular Ca^{2+} waves) and a suppression of cytoplasmic signaling (i.e., gap junctional coupling). By comparison, in mouse diencephalic cultures, melatonin facilitated Ca^{2+} waves such that their spread attained more telencephalic-like features, including a radial and concentric wave, rather than the meandering one present prior to melatonin receptor activation (CHAPTER III). Like chick astrocytes, melatonin may induce a shift in the mode of mammalian astroglial cell coupling that is detectable as alterations in the nature of wave transmission. Until more is known about the transduction pathways mediating melatonin receptor and Ca^{2+} signaling in nocturnal and diurnal species, and how these cellular pathways mediate circadian behavior, the role of melatonin and glial Ca^{2+} waves in these processes will remain unclear. Interestingly, the neurotransmitter serotonin affects Ca^{2+} signaling in a manner that is completely opposite that of melatonin. Serotonin decreases the spread of Ca^{2+} waves in cortical and hippocampal astrocytes, but has no effect on hypothalamic astroglial waves (Blomstrand et al., 1999).

Astrocytes are responsible for regulating brain energy utilization, and melatonin globally shapes avian brain metabolism (Lu and Cassone 1993; Cantwell and Cassone, 2002). As mentioned previously, glucose uptake levels are higher during the day in both nocturnal and diurnal animals (Cassone, 1988; Cassone et al., 1988; Schwartz, 1990; Cantwell & Cassone, 2002). This suggests that there must be a "sign" change in second messenger cascades and/or the mechanism of cellular coupling within these species, such that nocturnal mammals receive a signal that inhibits behavior at the same time of day that the behavior of a diurnal bird is activated. This difference in temporal activity patterns is not due to melatonin. Melatonin has similar effects on glucose uptake in both species at CT 10 with decreases in 2DG levels within the SCN of rats (Cassone et al., 1988) and the vSCN of chicks (Cantwell & Cassone, 2002). While there is glucose uptake in other regions of the brain and melatonin has receptors in other brain regions (CHAPTER III), it is interesting that this effect occurs specifically in the SCN. A melatonin-mediated change in hypothalamic activity could affect a large number of functions within the organism involving the homeostatic processes regulated there, such as blood pressure, body temperature, sleep/wake cycles, food intake, and energy metabolism.

Glucose utilization in chick diencephalic astrocytes is greater during the day than during the night, and the release of the glycolytic by-products, lactate and pyruvate, is higher during times of day when melatonin is not present (Adachi et al., 2002). In our studies, melatonin was able to generate opposing rhythms in 2DG uptake in astrocytes (CHAPTER IV). *In vivo*, the mediation of metabolic activity by melatonin via

diencephalic astrocytes could affect the neighboring SCN neurons. Inhibition of glial

metabolism can cause arrhythmicity in an intact rat SCN (Prosser et al., 1994). Glial

SCN cells release transforming growth factor-alpha (TGF-α), which has been suggested

to be a clock output signal regulating rhythms of activity and rest (Li et al., 2002). The

changing energy demands of an organism can be altered by melatonin as it asserts itself

to entrain physiological rhythms through the glial astrocytes.

In our studies, we found that melatonin had little or no effect on clock gene

expression in chick diencephalic astrocytes (CHAPTER IV). Yasuo et al. (2002) also

found no effect of exogenous melatonin on clock gene expression in Japanese quail.

However, melatonin is involved in phase shifting the circadian clock (for reviews,

Cassone, 1990b; Dubocovich et al., 1996; Lewy, 1999). This suggests that melatonin

could affect other cells that impose a downstream modulation of clock gene expression.

Thus, the effect would be indirect. We found that melatonin modulated ionic

homeostasis, cell to cell communication, and metabolic activity of glia cells (CHAPTER

II, III, & IV), and perhaps, through one of these processes clock gene expression could

be altered.

Growing data support the existence of multiple oscillators within cells, including

one that drives metabolic activity. Co-cultures of SCN2.2 cells and NIH3T3 fibroblasts

demonstrated that the SCN cells, which retain their circadian rhythmicity *in vitro*

(Earnest et al., 1999), can induce clock gene rhythms and 2DG uptake in the fibroblasts

(Allen et al., 2001). Even though the rhythm in 2DG uptake in the fibroblasts lags the

uptake in the SCN cells by 4 hours, the expression of *per1* and *per2* in the fibroblasts lag

the SCN by 12 hours suggesting clock gene rhythms and metabolic activity may be differentially regulated. Additionally, inhibition of *Clock* with antisense morpholinos in both the SCN2.2 cells and co-cultured NIH/3T3 fibroblasts caused *Per2* expression to become arrhythmic while 2-DG uptake continued to oscillate, albeit with an increase in the peak-to-peak interval (Allen et al., 2004). Mendoza et al. (2005) demonstrated that decreasing the caloric-intake of mice in LD altered clock gene expression in the SCN as well as the phase of outputs rhythms of AVP mRNA, locomotor behavior, and pineal melatonin as compared to normal-caloric fed mice. Thus, linking repetitive metabolic energy cues to entrainment of the circadian rhythms of the SCN to light.

In birds, pinealectomy and administration of exogenous melatonin have no effect on clock gene expression (Yasuo et al., 2002). One way this could be explained is if there are two oscillatory elements, one that is sensitive to melatonin but does not directly influence clock gene expression, and the other that is insensitive to melatonin but affects transcription of clock genes. Our data corroborates this and suggests that light drives clock gene expression of one oscillator while melatonin is involved in a separate metabolic oscillator. Interestingly, Bernardinelli et al. (2004) demonstrated that there are not only intercellular waves of Ca^{2+} in cortical astrocytes, but also intercellular waves of Na^+ that exist in parallel with the Ca^{2+} waves. These Na^+ waves affect metabolic processes in the astrocytes such that the glutamate-mediated Na^+ waves bring about a wave in glucose uptake in these same cells and Ca^{2+} waves may drive the sodium waves. As astrocytes modulate neuronal signaling and function, it is possible that these Na^+ waves are one way to globally influence metabolic activity as the wave spreads

throughout a region of astrocytes, thereby affects neighboring neurons. As melatonin modulates the spread of the Ca^{2+} wave, if the calcium wave does indeed "drive" the Na^+ waves, then melatonin modulates glucose uptake mediated by the Na^+ waves as well.

We hypothesize a model based on the findings of these studies (Fig. 25). First, melatonin affects astrocytic glucose utilization and, in turn, the astrocytes then mediate metabolic activity in neighboring neurons. Second, melatonin affects calcium homeostasis, which could generate ionic rhythms that could influence neuronal behavior. Additionally, melatonin enhances intercellular calcium wave propagation and reduces gap junctional coupling, thus lending to wide-spread changes in brain cell communication. Melatonin does not directly affect clock gene rhythms, but rather light affects glial rhythmicity, suggesting clock gene rhythms are regulated in a different manner than metabolic activity and calcium homeostasis in astrocytes. Although more studies are needed to fully understand the pathways involved in these processes and how they influence downstream outputs and overt behaviors.

Astrocytes function as a cellular network for metabolic coupling in the brain and this coupling can be regulated by intercellular calcium signal communication. Melatonin-mediated regulation of glial calcium signaling or glucose uptake would have a significant homeostatic impact on melatonin-sensitive brain regions, particularly the hypothalamus. While these cells do express clock genes, they are not self-sustained oscillators. Their main role in the circadian system may be to provide a permission environment to sustain rhythms in surrounding neuronal cells through the regulation of
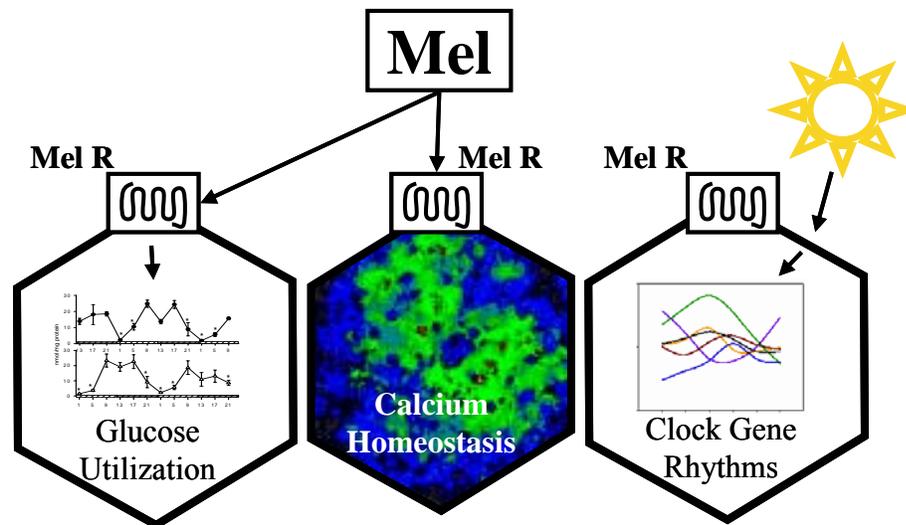
Fig. 25. Model of the effects of melatonin and light on astrocytes. Melatonin (Mel) binds to seven-transmembrane receptors, Mel R, on the surface of astrocytes (hexagons). Melatonin affects glucose utilization, such that glucose uptake is greater during the day in the absence of melatonin than during the night when the hormone is present. Melatonin affects calcium homeostasis as it increases resting calcium concentrations and enhances intercellular calcium wave propagation. Melatonin does not directly affect clock gene rhythms. However, light induces rhythms in clock gene expression.

ionic and metabolic states, and melatonin would provide daily entrainment cues to these cells, thus modifying glial coordination of rhythms within the hypothalamus.

FUTURE STUDIES

There are several directions that future studies could take, based on the present work. The majority of these studies have been performed in cultured astrocytes. Whether or not astrocytes are affected by melatonin or are photoreceptive *in vivo* is still under investigation. The ability to examine melatonin's effects on intercellular calcium waves in an intact brain would be informative. There is wide distribution of the Mel_{1c} receptor in areas without neuronal cell bodies (Reppert et al., 1995) and cultures of astrocytes express the melatonin receptors (Adachi et al., 2002). However, one can never be sure what other hormonal influences affect cellular functions, such as calcium waves and metabolic activity, in a live animal.

Creating a "bird in a dish" by co-culturing pinealocytes and diencephalic astrocytes would allow us to examine if the pineal cells can drive rhythms in output cells located within the SCN. For example, the release of melatonin by pineal cells could generate rhythms in metabolic activity in astrocytes, while the release of other neurotransmitters may drive rhythms in clock gene expression in the astrocytes. It may be possible to block melatonin release or manipulate clock gene expression using siRNA techniques. Blocking the release of melatonin from the pinealocytes would allow one to examine if metabolic activity was disrupted as well, and if so, could this activity be restored with rhythmic administration of exogenous melatonin. Manipulating different

clock genes in the pinealocytes would allow us to study the astrocytic output cells to better understand clock gene expression in these cells as well.

Experiments with mammalian SCN2.2 and astrocyte co-cultures could also be performed for many of the same reasons as the "bird in a dish" experiment. However, the lab already has knockout mice for the *per1* and *per2* genes, which will allow easier manipulation of the genes. Using these knockouts, one could examine whether melatonin still affects metabolic activity or if SCN2.2 cells drive oscillations in glucose uptake and clock gene expression in the astrocytes from knockout mice. Allen et al. (2001) demonstrated that in co-cultures of SCN2.2 cells and NIH3T3 fibroblasts the SCN cells induced clock gene rhythms and 2DG uptake in the fibroblasts. In preliminary data from co-cultures of SCN2.2 cells and diencephalic astrocytes, we observed rhythms of glucose uptake in both SCN2.2 cells and astrocytes, with peak uptake of the astrocytes lagging that of the SCN2.2 cells by 4 hours. However, many more experiments are needed to fully understand what occurs in these co-cultures.

Finally, our studies of glial cell calcium waves and their modulation by melatonin will only be of significant importance to neuroscientists in general when we demonstrate that such wave modulation affects brain integration. Therefore, future studies must focus on neuronal physiology and how that physiology is impacted by glia calcium waves and how melatonin's regulation of those waves influences brain function and the generation of rhythmic animal behaviors.

In conclusion, melatonin modulates the spread of calcium waves, resting calcium levels, and gap junctional connectivity among chick glial cells. This modulation occurs

in both mammalian and avian diencephalic astrocytes. Melatonin drives rhythms in metabolic activity, but not clock gene expression. This hormone also plays a role in the regulation of retinal visual function, through, at least to some extent, its effect on Müller glial cells. Glial cells ability to mediate neuronal function in the brain and the retina, and melatonin's ability to modulate glial cells, suggests an important role for glial cells in circadian rhythms.

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APPENDIX

ELSEVIER

28 October 2004-10-29   Our ref: HW/ TL/Nov04/J003

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Dear Dr. Peters

BRAIN RESEARCH, Accepted for publication "Melatonin Modulates Intercellular…"

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        Thank you for your time and prompt attention to this request.

Sincerely,
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October 2003 - present	Member of the Society for Neuroscience

Publications

Peters, J.L., V.M. Cassone, and M.J. Zoran. 2005. Melatonin Modulates Intercellular Calcium Waves Among Brain Astrocytes. Brain Research 1031:10-19.

Peters, J.L., and V.M. Cassone. 2005. Melatonin Regulates Circadian Electroretinogram Rhythms in a Dose- and Time-Dependent Fashion. Journal of Pineal Research 38:209-215.

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