TOWARD THE BEGINNING OF TIME: CIRCADIAN RHYTHMS IN

DEVELOPMENT

A Dissertation

by

JIFFIN KURIACKO PAULOSE

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Chair of Committee,	Vincent Cassone
Committee Members,	David Earnest
	Gladys Ko
	Mark Zoran
Head of Department,	Thomas McKnight

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ABSTRACT

The presence of circadian rhythms during early development has been poorly

examined until very recently in vertebrate species. Previous studies have focused on the

development of specific structures involved in the control of circadian regulation;

however, these structures develop relatively late during development and, in most cases,

only mature after parturition. The experiments in this thesis aim to answer the basic

question, "When do circadian rhythms first appear?" with a comparative approach

utilizing mouse embryonic stem cells in culture and chicken embryos *in ovo* to 1)

establish the first appearance of the full complement of canonical clock genes, 2)

analyze the temporal expression patterns at the earliest stages of development, and 3)

determine whether external cues can induce a functional molecular clockwork in these

tissues. The role of pineal melatonin in regulating circadian rhythms of developing

astrocytes is also investigated. The results of these studies show that, while the putative

molecular clock is not functional in undifferentiated embryonic stem cells, a

physiological rhythm of glucose utilization is present. This metabolic rhythm is

amplified upon non-specific differentiation, at which point the molecular clock also

starts. In contrast, circadian rhythms of chick embryos *in ovo* do not appear

spontaneously, although all components of the molecular machinery are expressed. The

clock in early chick embryos can be driven by – but not entrained to – temperature

cycles, but is not responsive to light cycles. Finally, some clock gene rhythms can be

driven by rhythmic melatonin signals; however, glucose utilization is fully entrained

after several days of rhythmic exposure. Remarkably, pineal melatonin increases mitotic activity in these cells in a dose-dependent fashion, suggesting an auxiliary role of melatonin in embryonic development. While these studies show the earliest observed circadian rhythms in both mammalian and avian model systems, they also show that metabolic rhythms precede those of the molecular mechanism and provide compelling evidence that the ontogeny of circadian time-keeping lies outside of the canonical molecular clockworks.

DEDICATION

This dissertation is dedicated to Alka Paulose, without whom I would not have

had the confidence to pursue my dream. Thank you for believing in me: I cherish you,

always.

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1. INTRODUCTION

1.1 Formal Properties of Circadian Rhythms

Life on Earth has evolved around an environment that is constantly changing. These changes pose direct and indirect challenges to organisms that are competing to survive under such conditions. While some of these changes are unpredictable, others appear periodically: temperature minima and maxima across seasons in temperate zones, tidal volume in coastal areas and, the most universal across the planet, daily changes that occur with the rising and setting of the sun. It is the precision with which the latter phenomenon occurs – and has occurred for billions of years – that provides organisms with the ability to anticipate the challenges that coincide with it. For example, increased humidity in the early morning hours is crucial for the emergence of larvae from their eggs to prevent desiccation. Similarly, the ebb and flow of the tides change the environment from terrestrial to aquatic at certain times of day. These and other environmental fluctuations that coincide with the daily light cycle have imposed a selective pressure on all organisms to evolve mechanisms, collectively referred to as circadian rhythms, to anticipate and respond to daily changes in the environment. The term circadian is literal in its description of rhythms that occur with a period of about (Circa) a day (Diem). Although it is now believed that circadian rhythms evolved multiple times across different taxa, the primary effect remains universal: to maintain a synchrony between molecular, physiological, and behavioral processes and the external environment (Pittendrigh, 1993, Menaker et al., 1997).

Several biological processes coincide with daily occurring phenomena; however some of these occur as a direct response to environmental conditions. While these diurnal rhythms are important, they are fundamentally different from circadian rhythms. Experimentally, a rhythmic process can be defined as circadian if it exhibits 3 properties. Firstly, the rhythm must be maintained under constant conditions, - such as constant dark (DD), and thus be capable of free-running with a period (τ) of close to – but not exactly – 24 hours (Pittendrigh, 1993, Bell-Pedersen et al., 2005). The persistence of circadian rhythms under constant conditions serves as proof that the mechanism underlying the rhythm is generated endogenously, as opposed to reactively in response to an external oscillation.

The intimate relationship between organism and environment has provided the former with a milieu of stimuli, many of which occur at the same time each day. When such a stimulus is strong enough to impose the phase (φ) of its rhythm onto the endogenous mechanism of an organism, thereby creating a stable phase relationship (Ψ) between the two, the stimulus can be referred to as a "zeitgeber", literally time giver. Several environmental cues have been shown to entrain circadian rhythms, including light, temperature, and food availability, with light being the most efficacious (Pittendrigh, 1981). The process of establishing a stable phase relationship between zeitgeber and endogenous clock is referred to as entrainment and is the second formal property of circadian rhythms. Although the period of artificial zeitgebers is usually constant (12:12 light:dark cycles, for example) the variability observed in any organism's τ, in that it is rarely exactly 24 hours, provides an adaptive advantage to re-entrain its

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timekeeping mechanism to naturally occurring zeitgebers that may vary in their periodicities throughout the year.

While our understanding of entrainment, especially at the molecular level, is limited, there are two models describing the mechanism underlying a zeitgeber's ability to synchronize an oscillator: the so-called parametric and non-parametric entrainment models (Pittendrigh, 1981, Daan, 2000). Parametric – or tonic – entrainment posits that the zeitgeber acts on the oscillator continuously, and the oscillator responds by changing its angular velocity towards a new phase. The empirical basis of the parametric model lies in the lengthening and shortening of τ as a result of different intensities of constant light, where entrainment is achieved by sinusoidal oscillations of the light intensity (Daan, 1977). The nonparametric model regards the zeitgeber as a discrete signal; a singular event that, dependent upon the phase at which it occurs, can shift the phase of the oscillator forwards or back. The nonparametric model can be – and almost always is – illustrated by a phase response curve (PRC), the graphical representation of the resulting phase shifts based on the times at which the zeitgeber is administered (Decoursey, 1960, Pittendrigh, 1981).

The third trait shared by all circadian rhythms is that they remain stable – with respect to period length – across a wide range of physiologically permissible temperatures. Most biochemical processes are dependent upon thermodynamic properties such that increases in temperature speed up reactions while decreases have the inverse effect. Mathematically, the temperature coefficient of a chemical/biochemical reaction based on the reaction speed after a change in temperature of 10˚C is designated

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as the Q10 value. While most biological processes exhibit a Q10 of 2.0 to 3.0, circadian

rhythms express Q10 values between 0.6 and 1.2 (Kalmus, 1940, Pittendrigh, 1954,

Zimmerman et al., 1968, Reyes et al., 2008). Of course this compensation has its limits:

period length will remain mostly unchanged under a range of constantly held

temperatures, but amplitude and phase may be affected. Additionally, the incubation

temperature itself must be physiologically tolerable to the preparation or organism to

survive. Although little is understood regarding the mechanism of temperature

compensation, it lends itself to a wide variety of mathematical modeling paradigms in

various plant, animal, and tissue culture systems (Jolma et al., 2010).

1.2 Hierarchical Organization of Circadian Clocks

Despite the myriad differences in how circadian timekeeping mechanisms have

evolved among various organisms – the autoregulatory rhythm in Synechococcus KAI-C

protein vs. transcription-translation feedback loops in vertebrates comes to mind, the

mechanism itself is made up of three universally shared components, in their broadest

sense: input pathways, central pacemakers, and output pathways. Input pathways to the

clock include the organs and/or proteins (and the signal transduction cascades they

activate or repress) that receive external signals, i.e., zeitgebers, and relay those signals

to the pacemaker, thus establishing entrainment. The most obvious input pathway that

comes to mind is light-sensing retina in animals, although other photoreceptive

structures have been found to act directly upon pacemakers such as the pineal gland in

non-mammalian vertebrates. However, it should be noted that input pathways are not

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relegated to photoreception, as food-restriction and temperature also can act as zeitgebers. Neither are input pathways restricted to complex organs; in unicellular cyanobacteria (Synechococcus) and multicellular fungi (Neurospora) protein complexes within each cell are responsible for transducing time of day information and providing it to the central machinery. That machinery, the central pacemaker, is responsible for conferring time of day information, as interpreted by the input pathway, to the rest of the organism, be it within a single cell or across multiple organ systems (Pittendrigh, 1993, Dunlap, 1999, Bell-Pedersen et al., 2005). Regardless of the complexity underlying any given clockworks, the pacemaker by definition must be capable of entrainment, self-sustained rhythmicity, and regulation of downstream processes that make up the output pathways as manifest in cellular physiology and/or overt behavior.

In mammals – as well as birds, to some extent – the central pacemaker resides in the suprachiasmatic nuclei (SCN), a bilateral cluster of 20,000 or so neurons at the base of the hypothalamus just above the optic chiasm. The SCN receives photic input from the retinae via the retinohypothalamic tract (RHT) that originates with intrinsically photo-receptive ganglion cells and terminates in the SCN (Cassone et al., 1988, Hannibal and Fahrenkrug, 2002, Antle and Silver, 2005, Lupi et al., 2006). The SCN itself is comprised of disparate populations of neurons, most broadly grouped into a dorsal "shell" and ventral "core", which differ in their pacemaking capacity, neurotransmitter accoutrement, and downstream targets among others (Aton and Herzog, 2005). As a whole, the SCN in mammals is necessary and sufficient to entrain overt rhythmicity as seen in ablation studies (Stephan and Zucker, 1972) as well as transplantation studies

with SCN tissue (Sherwin et al., 2004) and immortalized SCN cells (Earnest et al., 1999a).

In birds, the SCN homologue is divided between two separate nuclei within the hypothalamus: the medial SCN (mSCN) and visual SCN (vSCN), the former of which is situated in a similar location as in mammals. Both structures are retinorecipient through a similar RHT as in mammals (Cassone and Moore, 1987, Cantwell and Cassone, 2006a), however only the vSCN expresses melatonin receptors and rhythmic melatonin binding (Lu and Cassone, 1993b). The vSCN also exhibits rhythmic metabolism as measured by 14C-2-deoxyglucose (2DG) uptake (Cassone, 1988, Cantwell and Cassone, 2002). Evidence for the mSCN as a pacemaker in birds comes from ablation studies where lesion of the mSCN, but not the vSCN, was sufficient to abolish locomotor activity in pigeons (Yoshimura et al., 2001). Comprehensive chemoarchitecture analysis of both nuclei showed that both areas share similar neurochemical immunoreactivity as the mammalian SCN (Cantwell and Cassone, 2006b). Furthermore, the avian SCN are connected by an "astrocyte bridge" that may facilitate communication between the two structures (Cantwell and Cassone, 2006b). While the specific roles of each structure have yet to be numerated, it is clear that both structures participate in the central time-keeping mechanism of the organism.

In addition to the SCN, the pineal gland also plays a significant role in the establishment and maintenance of circadian rhythms. In birds, the pineal gland is also photoreceptive by way of pineal-specific photoreceptors and photoisomerases (Bailey and Cassone, 2004). In addition, the pineal gland also contains the necessary molecular

machinery to sustain circadian outputs; primarily the neurohormone melatonin. In this way, the pineal gland is also considered a pacemaker in birds and other non-mammalian vertebrates. Evidence of this has been established along similar empirical studies as the mammalian SCN: pinealectomy abolishes locomotor behavior in some species of birds, and transplantation of the pineal gland restores rhythmicity to that of the donor pineal (Gaston and Menaker, 1968). Furthermore, the phase of the rhythm is conferred by melatonin itself, as transplantation of the pineal to the anterior chamber of the eye is sufficient to re-establish rhythmicity before neuronal integration (Zimmerman and Menaker, 1979). These and other studies have been coalesced into the "neuroendocrine loop" model where the pineal gland (and retinae, which also can secrete melatonin and serve as pacemaker in the absence of the pineal in some species) secretes melatonin during the night, inhibiting electrical activity the SCN via melatonin receptor-based signaling. As dawn approaches, pineal melatonin decreases, releasing SCN inhibition. The SCN projects to the pineal via the superior cervical ganglia (SCG) and – during the day – inhibits pineal melatonin production via inhibitory norepinephrine (NE) signaling (Cassone and Menaker, 1984).

1.3 Molecular Basis of Circadian Rhythms

Notwithstanding the systems level coordination of circadian rhythms by the central pacemakers mentioned above, within each cell lies a molecular clockworks, first established in *Drosophila* with the *period* gene mutant by Ron Konopka (Konopka and Benzer, 1971). Since that time, so-called "clock genes" have been demonstrated in all

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model organisms in which circadian rhythms are studied (Bell-Pedersen et al., 2005).

Most of these are driven by a transcriptional-translational feedback loop wherein

transcriptional activation and repression of a subset of genes throughout a 24 hour period

constitute the core oscillator (Dunlap, 1999). In birds and mammals, this feedback loop

is largely the same, and depicted in Figure 1. The "positive limb" of transcriptional

activators consists of the CLOCK and BMAL1 proteins. These basic Helix-Loop-Helix

(bHLH) – PAS (Per, Arnt, Sim) domain-binding proteins heterodimerize and bind E-box

(5'-CACGTG-3') and E'-box (5'-CACGTT-3') motifs to activate transcription in a

number of genes, including those that make up the "negative limb", *periods* (*per*) and

cryptochromes (*cry*). The protein products of these genes, the PERs – PER1 and PER2

in mammals and PER2 and PER3 in birds – and CRYs (CRY1 and CRY2) form a

complex and enter the nucleus where they inhibit CLOCK/BMAL1 transcriptional

activity, thereby inhibiting their own expression. The repressive activity of PER/CRY is

what drives the period of the rhythm, and proteosomal degradation – induced via

phosphorylation by CASEIN KINASE I – lifts the repression of CLOCK/BMAL1

activity to renew the cycle (Bell-Pedersen et al., 2005, Koike et al., 2012, Buhr and

Takahashi, 2013). Additionally, there is a second loop involving the orphan nuclear

receptors RORα and REVERBα that compete for binding onto the retinoic acid-related

orphan receptor response element (RORE) of the *bmal1* promoter region to activate or

repress its transcription, respectively (Guillaumond et al., 2005). Although little data

exists on putative ligands of these orphan receptors, it is known that REVERBa

reversibly
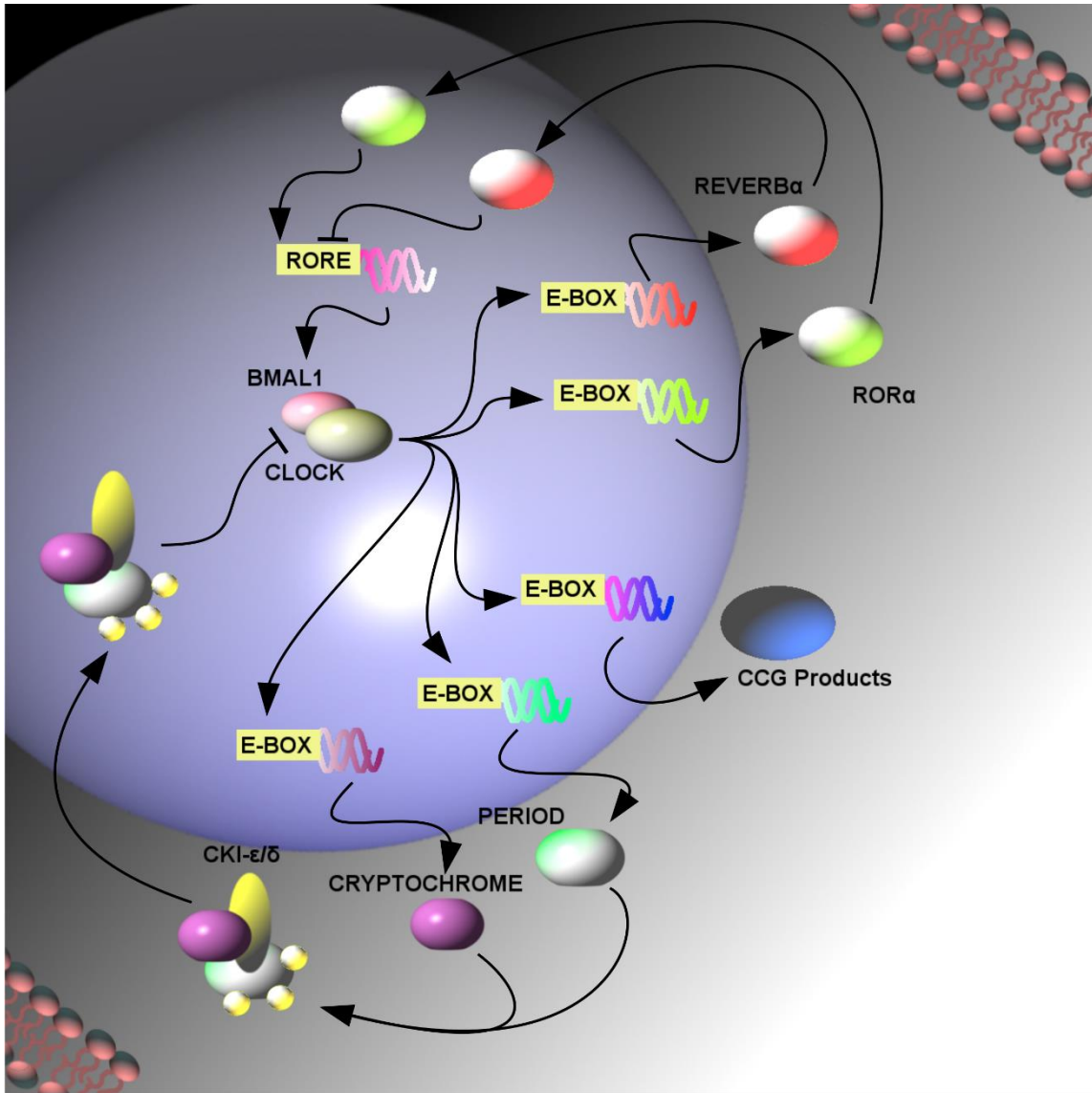
Figure 1. The mammalian/avian molecular clockworks is a transcriptional/translational feedback loop. Positive elements BMAL1 and CLOCK heterodimerize to initiate transcription of genes containing E-box promoter elements, such as the negative clock components PERs and CRYs. The negative elements act to repress BMAL1/CLOCK activity and, thus, their own transcription. In addition, the orphan nuclear receptors RORα and REVERBα, both encoded by E-box containing genes, competitively bind to ROR elements within the promoter region of *bmal1* to, respectively, activate and repress its transcription.

associates with heme and, once bound, acts as a redox sensor (Guillaumond and Teboul, 2008). Furthermore, *Rora* disruption results in decreased robustness of circadian rhythms, suggesting a role in circadian time-keeping maintenance (Teboul et al., 2009).

While the "core clock" consists of only a handful proteins, the regulatory network created by the transcriptional-translational feedback loop encompasses multiple pathways that affect every aspect of cellular activity (Zhang et al., 2009). It comes as no surprise then that the molecular clockworks is present in nearly every cell of the body, and that each can act as a cell-autonomous oscillator, with the aggregate organ and tissue systems being synchronized by the pacemaker (Yoo et al., 2004). Although several peripheral tissue preparations are capable of sustaining rhythmicity *ex vivo*, the rhythms damp out over time in a process thought to be linked to desynchrony among cells (Dibner et al., 2010).

The notion that individual cells can maintain rhythmicity in a variety of metabolic and homeostatic pathways is indicative of the overall importance of circadian rhythms to cellular and organismal physiology. This has been punctuated most recently by the discovery of transcription-independent oscillations within human red blood cells and a single-celled eukaryote (O'Neill and Reddy, 2011, O'Neill et al., 2011)). Both studies show a persistent circadian rhythm in the oxidation state of a highly conserved peroxiredoxin protein. Later, these studies were expanded to include representative model organisms from nearly all taxa, including *Synechecoccus*, *Neurospora*, and *Mus* (Edgar et al., 2012). Edgar, et al. also showed that mutations in the molecular oscillator have little effect on the redox oscillator and vice versa, suggesting an independent role of

the metabolic circadian rhythm that is coupled to, but not reliant upon, the transcriptional machinery.

1.4 Circadian Rhythms in Development

Until recently, the role of circadian rhythms in embryonic development has focused specifically on the pacemaking structures: the SCN in mammals and the pineal in birds. In rodents, the SCN as a structure begins developing relatively late in gestation at approximately embryonic day 14 and gradually continues through parturition (Seron-Ferre et al., 2007, Vallone et al., 2007, Serón-Ferré et al., 2011). Although synaptogenesis is not complete until weeks after birth, circadian variation in 2-deoxyglucose (2DG) uptake – indicative of metabolic activity – is observed as early as E19 (Reppert and Schwartz, 1984). Remarkably, the 2DG uptake rhythm in the SCN precedes that of the core molecular clock which becomes rhythmic between postnatal days 3-10 (Shimomura et al., 2001, Ohta et al., 2002, Sládek et al., 2004). Furthermore, ontogeny of peripheral circadian rhythms has been shown to be tissue specific, ranging from days to weeks after birth (Sumová et al., 2008, Dolatshad et al., 2010, Sumová et al., 2012). SCN ablation of the mother during gestation is not sufficient to disrupt post-natal rhythms, but does cause desynchronization among litter-mates (Reppert and Schwartz, 1986, Reppert et al., 1988, Shibata and Moore, 1988). Similarly, clock gene knockout females – when mated with wild-type males – produce heterozygous offspring with functional rhythms (Jud and Albrecht, 2006, Serón-Ferré et al., 2007, Serón-Ferré et al., 2011). The interpretation of these data liken the developing fetus as analogous to a

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peripheral or slave oscillator to the mother's master pacemaker (Serón-Ferré et al. 2011; Serón-Ferré et al. 2007), capable of entrainment within or outside of the context of the womb.

In contrast, examination of earlier expression of clock gene expression in the developing mouse embryo has not so been comprehensive. A comprehensive screen of clock gene expression by Johnson et al. found transcripts of all "core" clock genes in post-fertilization zygote to blastocyst stages (Johnson et al., 2002). Real-time bioluminescence imaging has also shown *per1*:*luc* expression *in utero* as early as E9 (Saxena et al., 2007), however neither of these were time-series studies. Whole embryos at E10-11 were shown to be not rhythmic in a later study, however the authors conceded that individual cells within the embryos may be rhythmic (Dolatshad et al., 2010).

Similar to the mammalian models, studies on circadian development in birds has focused on the pineal gland and melatonin secretion, as these are the primary pacemaker and entraining signal, respectively. Virtually all *ex vivo* studies utilize late embryonic pineal glands for measurement of melatonin and molecular rhythms, due to the intrinsic ability of isolated pinealocytes to detect light. Previous studies have shown that isolated pineal glands from chick embryos express rhythmic melatonin secretion no earlier than day 16 (Lamosová et al., 1995), and at the same time *gPer2* transcript expression is rhythmic in the SCN (Okabayashi et al., 2003). Interestingly, the early rhythms of *gPer2* require light:dark cycles to entrain, but melatonin secretion can also be entrained by exposure to temperature cycles (Lamosová et al., 1995, Okabayashi et al., 2003). The ontogeny of rhythmic melatonin in birds occurs earlier than in mammals, presumably

due to lack of maternal melatonin in the former (Zeman and Herichová, 2011). However, maternal deposits of melatonin and its synthesis components, AANAT and HIOMT *(*transcripts and activities), are found in the yolk and albumen of Japanese Quail eggs (Olszańska et al., 2007) This would suggest a role for melatonin in early avian development, and it has been postulated that melatonin's potent antioxidant activity is beneficial during embryogenesis (Olszańska et al., 2007). As is the case in mammalian studies, examination for circadian clock presence/activity is severely lacking.

1.5 Objectives

Since the first elucidation of circadian clock function, a great deal of attention has been directed towards understanding circadian rhythms from the physiological level down to the molecular underpinnings. Furthermore, several lines of evidence from different model organisms suggest that the circadian clock is initiated very early in embryogenesis, earlier even than the formation of central pacemaking structures within the vertebrate brain. However, the field still lacks a comprehensive investigation into the earliest stages of development and an answer to the question, "When does the clock start?" This dissertation aims to answer that question through a comparative approach, utilizing primary mouse embryonic stem cells (mESCs) and chick embryos at the onset of differentiation.

The mammalian studies were conducted using standard cell culture techniques. We exploited mESCs tendency towards differentiation to establish different cellular/physiological states for comparison. We utilized new (quantitative, real-time

PCR) and classic (2-deoxyglucose method) technologies to establish a temporal profile of core clock gene transcript abundance as well as glucose utilization and associated transcripts. Similarly, the earliest available tissues from fertilized chick eggs were isolated and assayed for presence of core clock genes. Embryos were then subjected to time-course analysis using either light or temperature cycles to determine if the embryonic clock could be entrained. Here, transcript abundance was quantified using the relatively new NanoString technology. Finally, we investigated the role of pineal melatonin as an entraining signal for embryonic astrocytes using a co-culture system to simulate the transport of melatonin from the pineal gland to peripheral brain cells.

These studies allowed us to approach the following hypotheses: 1) mouse embryonic stem cells express all of the required clock genes, however circadian oscillations of clock gene expression and glucose uptake do not begin until after differentiation, 2) chick embryos similarly express all clock genes and that these clock genes are entrained by temperature cycles, but not light, and 3) pineal melatonin acts as an entraining signal to isolated chick astrocytes *ex vivo*. We show that circadian rhythms, even at the embryonic cell level, are complex and not necessarily coupled to clock gene expression, and that pineal melatonin may play a previously unnoticed role in central nervous system development through regulation of mitosis.

2. CIRCADIAN RHYTHMS IN MOUSE EMBRYONIC STEM CELLS[*]

2.1 Introduction

Previously, investigations into the development of the mammalian circadian system have concentrated primarily on the development of the SCN pacemaker (Seron-Ferre et al., 2007) or, more recently, on the ontogeny of rhythms in peripheral tissues (Sládek et al., 2004, Sládek et al., 2007). These studies showed that the onset of molecular circadian rhythms in the brain occurs just after birth, followed by rhythms expressed by peripheral tissues. However, the postnatal rhythms of clock gene expression and electrical activity in the SCN are preceded by rhythmic glucose uptake 3–4 days before parturition and well before the completion of synaptogenesis within the SCN (Reppert and Schwartz, 1984). Several studies have investigated clock gene expression earlier in mammalian embryogenesis. Saxena et al (2007) investigated embryonic mPer1 expression *in utero* using live fluorescence imaging. Although this was a proof of concept study, the authors clearly showed mPer1::luciferase expression as early as day 7. Earlier, Johnson et al. (2002) showed evidence of zygotic expression of several clock genes in the pre-implantation embryo as well as uterine tissues, suggesting that perhaps clock gene expression plays a role in embryo-uterus interaction during early embryogenesis. It is important to note, however, that these studies were from embryos taken directly from the uterus, placing the expression of embryonic genes in the context

of the uterine environment. Recently, two independent groups have investigated circadian rhythms in embryonic stem cells. Yagita et al. (2010) used ES cells stably transfected with bioluminescent luciferase driven by either an mBmal1 promoter or clock-controlled gene mDbp promoter. The results from this study showed that undifferentiated cells were not rhythmic with respect to mBmal1 or mDbp, but cells that were directed toward a neural fate were rhythmic after synchronization with forskolin. Kowalska et al. (2010) expanded on those experiments and showed that individual undifferentiated ESCs are not rhythmic. Both of these studies provided ample evidence that the canonical molecular clock is not rhythmic in ESCs and concluded that the clock is not functional this early in development. However, given the previous evidence that glucose uptake – a well-established physiological output of the circadian clock - is rhythmic in the SCN before clock genes are rhythmic during development, it is premature to equate lack of canonical clock gene rhythmicity to a lack of a functional timekeeping mechanism. Here we present evidence that rhythmicity in ESCs precedes the development of clock gene rhythms. In addition, we show a dissociation in rhythmicity after further differentiating ESCs towards a neural fate. Primary ESCs were cultured either in the presence or absence of the differentiation inhibitor Leukemia Inhibitory Factor (LIF) to promote spontaneous differentiation. ESCs were also subjected to a directed differentiation towards a neural fate. 2-DG uptake was assayed as a physiologically relevant output of the clock and a comprehensive profile of transcripts from both positive and negative limbs of the molecular clock was analyzed for rhythmicity over 2 days in culture, as well as the ''stabilizing loop'' consisting of

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mReverb-α and *mRor-α*.

2.2 Materials and Methods

Cell Culture

ES cells were derived from blastocysts of SV129 mice for molecular studies and

C57BL/6 mice for the real-time analyses. Females (3 weeks of age, n = 3–5) were

superovulated by intraperitoneal injection of pregnant mare serum gonadotropin (PMSG,

5IU, Sigma, St. Louis, MO) followed by human chorionic gonadotropin (hCG, 2.5IU,

Sigma, St. Louis, MO) prior to mating with males of the same respective genotype). ES

colonies were initially expanded from a 96-well format to 24-well plates, and finally to

6-well plates while being maintained on mitomycin C-inactivated STO feeder cells. For

the experiments, ES cells were passaged nine times into feeder-free cultures, resulting in

pure ESC cultures that were maintained as pluripotent ESCs or allowed to differentiate

by the removal of LIF, which was done one passage prior to experimentation (passage

8). The culture environment consisted of a standard water-jacketed incubator held at

37°C and 5% CO_2 and controlled humidity. Standard ES media (ES-DMEM),

Dulbecco's Modified Eagle Medium (DMEM) supplemented with 15% fetal bovine

serum, sodium bicarbonate (2.2 g/L), MEM Non-Essential Amino Acids (0.1 mM), L-

glutamine (2 mM), β-mercaptoethanol (0.1 mM), penicillin (50 U/ml)/streptomycin (50

µg/ml) antibiotic, and LIF (500–1000 U/ml, Millipore, MA) was used for maintenance

of ES cell pluripotency. Differentiation medium was identical to ES-DMEM but without

LIF. Neural differentiation was directed using a well-established protocol of retinoic

acid incubation (Bain et al. 1995). Briefly, ESCs were incubated without LIF to promote the formation of embryoid bodies (EBs). EBs were gently passaged and maintained as free-floating cultures for four days in DMEM without LIF before incubating for four days in DMEM with 50g of retinoic acid. Induced neural stem cells (NSCs) were dissociated during passage and maintained in DMEM supplemented with N12 as dispersed NSCs. Confirmation of neural differentiation was accomplished by immunocytochemistry (ICC) using anti-nestin antibody (developed by S. Hockfield, Cold Spring Harbor Laboratory and obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242). Cultures were maintained in 100 mm, gelatin-coated culture dishes, fed daily, passaged every 2 days and replated at 1×10^6 per well into 6-well culture plates (BD Bioscience) for experiments. Cells were allowed to grow for 12 hours after final passage before sampling began, at which point time is referred to as 0 hours on each graph.

^{14}C-2-DG Uptake Assay

At the onset of each timepoint, one 6-well plate from each cell type was incubated with ^{14}C-2-deoxyglucose (2-DG, 0.1 mCi/ml; American Radiolabeled Chemicals, St. Louis, MO) for one hour by complete removal and replacement of the medium. The medium was then removed and the cells rinsed twice with Dulbecco's PBS (Invitrogen). Cells were harvested in 1 ml Trizol reagent (Invitrogen) to extract cellular RNA and soluble protein. 200 µl of cell lysate was placed in 5 ml of scintillant and each sample counted in duplicate on a Beckman scintillation counter. Disintegrations per

minute (DPM) were converted to molar quantities as per Sokoloff's method (Sokoloff et al. 1977). 2-DG uptake was normalized to total RNA as measured by spectrophotometry on a Nanospec 1000 (Nanometrics, Milpitas, CA). Total RNA was isolated from the remaining cell lysate and subsequently treated with DNase I (Invitrogen) for quantitative PCR analysis of gene expression.

Real-Time Quantitative Polymerase Chain Reaction

DNase-treated cDNA from total RNA was generated using Superscript II reverse transcriptase (Invitrogen). Relative quantification of clock genes and stem cell marker genes was accomplished using SYBR chemistry-based qPCR on either ABI Prism 7500 Fast or StepOne Plus system (Applied Biosystems Foster, CA). Both machines are capable of generating identical thermal profiles and this was confirmed by testing identical samples on both machines. Table 1 lists all genes profiled and the corresponding primer sequences which were identified through literature search or using Primer Express software (Applied Biosystems). The relative quantification was based on a standard curve of dilutions 1:50, 1:100, 1:250, 1:500, and 1:1000 with triplicate samples diluted 1:100. All transcripts were normalized to corresponding values of *mCyclophilin D*.

Real-Time Bioluminescent Protein Expression

ESCs of C57Bl/6/SV129 hybrid mice expressing the PER2::LUCIFERASE fusion protein (Yoo et al., 2004) were used to monitor the real-time expression of mPER2 throughout differentiation. For these studies, feeder-free ESCs or dESCs (passage 9) were plated onto gelatin-coated 35 mm dishes (n = 10 for both ESCs and dESCs) (BD-

Falcon) in ES media at 1×10^5 cells per plate and allowed to attach and grow for 12–15 hours. At the onset of the experiment, media was replaced with ES media with the following modifications: Sodium bicarbonate concentration reduced to 0.35 g/L and 10 mM HEPES added to compensate for atmospheric conditions. The dishes were sealed with glass coverslips and vacuum grease and placed in a Lumicycle luminometer (Actimetrics, IL). One group of cells was maintained in the undifferentiated state via LIF administration, while another group of cells, passaged from the same initial culture, did not receive LIF and, therefore, underwent differentiation. Bioluminescence recording and detrended plotting of the counts from the cells was accomplished using a Lumicycle photomultiplier detection system and Lumicycle software, respectively (Actimetrics, IL).

Statistics

Time course data were subjected to cosinor analysis based on linear harmonic regression (Circwave Software, Roelof Hut) where each 24-hour period was tested separately. The software assumes a 24 hour period and applies harmonics to the basic sinusoidal function. These attributes allowed us to use the software to determine whether the data were rhythmic for each day of the 2 day sampling as well as providing centers of gravity to determine acrophase for each day. The criteria for determining bona fide circadian rhythms were as follows: a) both days show statistically significant rhythms using Circwave analysis and b) the acrophases of day 1 and day 2 occur within 23–25 hours of each other. Data were also tested using ANOVA for non-rhythmic expression

Table 1. Primers used for qPCR on embryonic stem cells

Gene	Forward Primer Seq. 5'-3'	Reverse Primer Seq. 5'-3'
mCycD	CTCATCTGGACGGGAAAAT	CCAGTCATCCCCTTCTTTCA
mGlut8	GCTCTCAGTGTCCTATTCA	AAATGGGCTGTGACTTGT
mGlut1	CAGGAGGATATTCAGGACTT	CAGTGTGGAGATAGGAGAG
mClock	GGCGTTGTTGATTGGACTAGG	GAATGGAGTCTCCAACACCCA
mBmal1	GGACTTCGCCTCTACCTGTTCA	AACCATGTGCGAGTGCAGGCGC
mRor-a	CCAACCGTGTCCATGGCAGAAC	GCACACAGCTGCACATCACCT
mPer1	GCTGGGCCGGTTTTGTG	CACTTTATGGCGACCCAAC
mPer2	ATTGGGAGGCACAAAGTCAG	ATCAGTAGCCGGTGGATTTG
mCry1	TACAGCAGCCACAAACAACC	TTCTTGTCCCAAGGGATCTG
mReverb-a	CCCTGGACTCCAATAACAACACA	GCCATTGGAGCTGTCACTGTAG

profiles to determine significant changes over time. ANOVA was performed using

Sigmastat software (Systat Software Inc., Point Richmond, CA). Where performed,

amplitudes were calculated by subtracting absolute peak levels from previous or

following trough levels – as previously determined using ANOVA – and dividing by 2.

2.3 Results

Stem Cell Morphology/Identity

      ESCs maintained under LIF displayed morphology consistent with that of

undifferentiated embryonic stem cells when imaged just before sampling (Fig. 2A).

Multiple cells formed isolated colonies in which individual cell borders were indistinct

and colony edges rounded. Flattened cells, indicative of differentiation are minimal

across cultures. Additionally, mOct4 expression was high during the first day of culture

and decreased during the second, suggesting that some of the cells began differentiating

on the second day (Fig. 2A, inset). Acutely differentiated cells, also imaged just before

sampling began, were morphologically distinct from undifferentiated cultures. Individual

cells were easily identifiable and flattened in appearance (Fig. 2B). Furthermore, mOct4

expression was very low across both days of sampling, confirming that the cultures were

differentiated (Fig. 2B, inset). Neural differentiation proceeded as expected with EB

formation (Figure 2C, left), and NSCs showed robust staining of nestin antibody,

confirming neural differentiation after incubation with retinoic acid (Figure 2C, right).

2-DG Uptake

      ESCs cultured in the presence of LIF exhibited two-fold oscillations of 2-DG
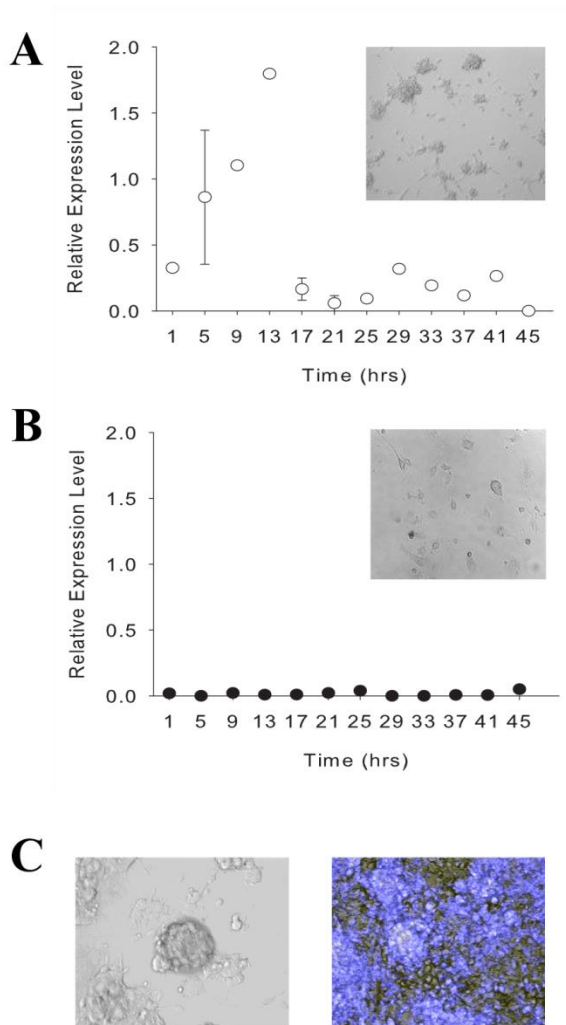
Figure 2. Identification of different stages of ES cell development by morphology and molecular marker. A) ESCs exhibited expression of mOct4 mRNA and grew as tightly clustered colonies of rounded cells (inset). B) Upon differentiation, mOct4 expression was nearly undetectable, and dESCs exhibited a flattened appearance (inset). C) Left, Neural differentiation began with cells forming EBs in culture before retinoic acid administration. Right, NSCs stained positively for anti-nestin antibody, confirming the neural progenitor identity.

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uptake over two cycles, with peaks occurring at 9 and 33 hours of sampling (p<0.001,

Fig. 3A). Acutely differentiated cells were similarly rhythmic with peak uptake

occurring at approximately 9 and 37 hours (p<0.001, Fig. 3B). There was no significant

difference in acrophase between ESCs and dESCs. Furthermore, the amplitude of 2-DG

uptake in dESCs was markedly increased, as was the basal level of uptake, each being

almost 10-fold higher than the corresponding level in undifferentiated ESCs (p<0.001).

2-DG uptake in NSCs varied greatly over time and exhibited significant peaks at 13, 21,

and 33 hours of sampling (p<0.001, Figure 3C).

Glucose Transporter Gene Expression

The rhythms in 2-DG uptake indicate that glucose transporter (*mGlut*) expression

might also be rhythmic. qPCR analysis of 6 different *mGlut* members revealed only two

that were detectable: *mGlut1* and *mGlut8*. Of these two, *mGlut1* was not rhythmic in

either ESCs or dESCs (Fig. 4A). *mGlut8* was rhythmic in both ESCs and dESCs

(p<0.001, Fig. 4B). In both cell types, the expression profile of *mGlut8* was phase

delayed to that of the 2-DG uptake; the rhythms peaked between 17 and 21 hours and

again towards the end of the sampling period, around 45 hours. There was no

statistically significant difference in overall expression levels of *mGlut8* between either

cell types.

Clock Gene Expression

Rhythmic 2-DG uptake and glucose transporter expression in both ESCs and

dESCs suggested that the canonical molecular clockwork may be present in these
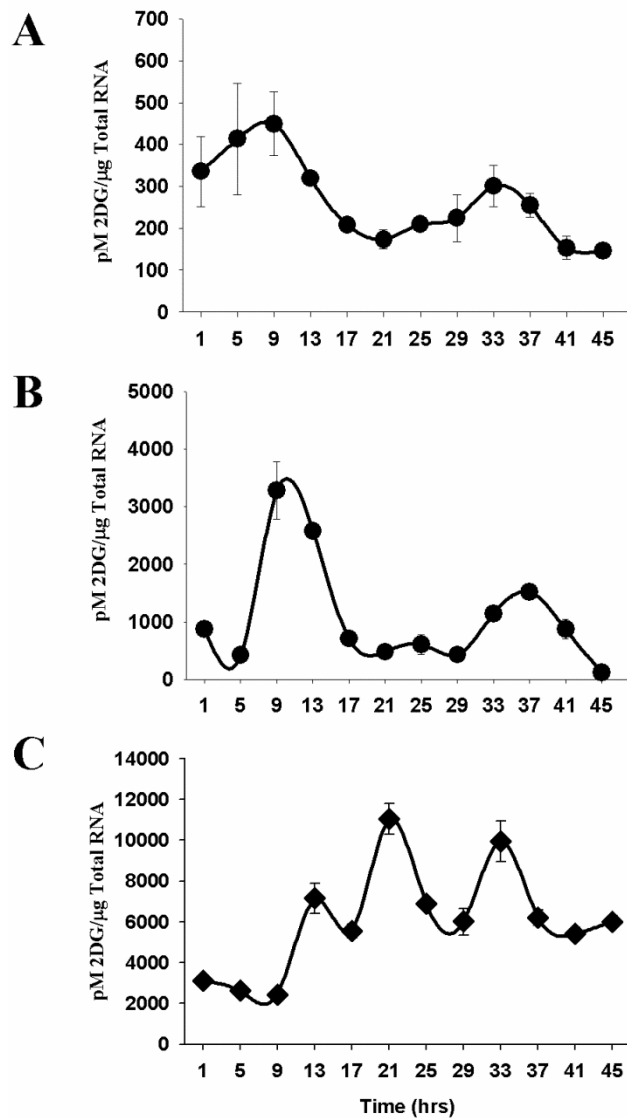
Figure 3. Glucose utilization is rhythmic in ESCs, dESCs, and possibly in NSCs. ESCs (A) displayed peaks in glucose utilization at 9 hours and 33 hours. DESCs (B) peaked in glucose utilization at 9 hours and 37 hours. NSC (C) glucose utilization showed peaks at 13, 21, and 33 hours. Lines connecting data points indicate significant rhythmicity across 24 hour periods as tested by Circwave software.
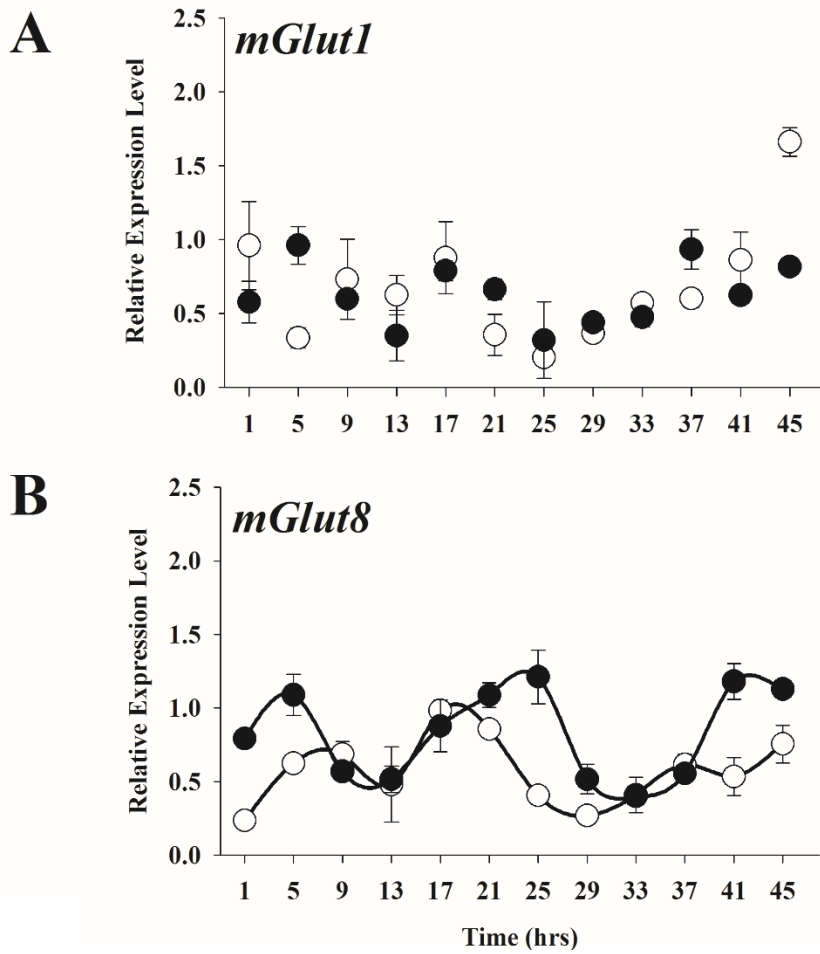
Figure 4. Glucose transporter gene expression reflects glucose utilization rhythms in ESCs (white circles) and dESCs (black circles). *mGlut1* **expression (A) is not rhythmic in either cell type.** *mGlut8* **expression (B) is rhythmic in both, as indicated by lines connecting time points.**

cultures. However, quantitative, real-time PCR against clock gene transcripts revealed

differential expression patterns both within and among the three conditions. The positive

elements, *mClock* and *mBmal1* were not rhythmic in ESCs (white circles, Fig. 5A and B,

respectively). Upon differentiation, however, only *mBmal1* displayed circadian

rhythmicity in dESCs (p<0.05, Fig. 5A, black circles), and low-amplitude rhythmicity in

NSCs, peaking at 9 and 33 hours (p<0.05, Figure 5B, gray circles). *mRor-α* was not

detectable in ESCs, but was rhythmic in dESCs, peaking at approximately 17 hours and

again at approximately 41 hours (p<0.001, Fig. 5C, black circles). In NSCs, *mRor-α*

abundance peaked at approximately 21 hours with a second peak at approximately 37

hours (Fig. 5C, gray circles). Among the negative elements, neither *mPer1* nor *mPer2*

transcripts were rhythmic in ESCs, but the temporal profiles of both were similar (Fig.

6A and B, respectively, white circles). Both *mPer1* and *mPer2* were rhythmic in dESCs,

however, with peaks occurring at 21 hours of sampling in both (p<0.001, Fig. 6A and B,

respectively, black circles. Neither *mCry1* nor *mReverb-α* were rhythmic in ESCs or

dESCs (Fig. 6C and D, respectively), however *mReverb-α* did show an oscillatory

expression pattern, troughing at approximately 17 hours and again at 41 hours. The

expression pattern of mPER2 protein, as visualized by real-time bioluminescence, was

similarly non-rhythmic in ESCs, but highly rhythmic in dESCs (p<0.001, Fig. 7A and B,

respectively). Furthermore, the rhythm in dESCs was reinstated by culture medium

exchange (Fig. 7B, arrow) despite a diminishing baseline of protein expression which

may be attributed to cell death over time.
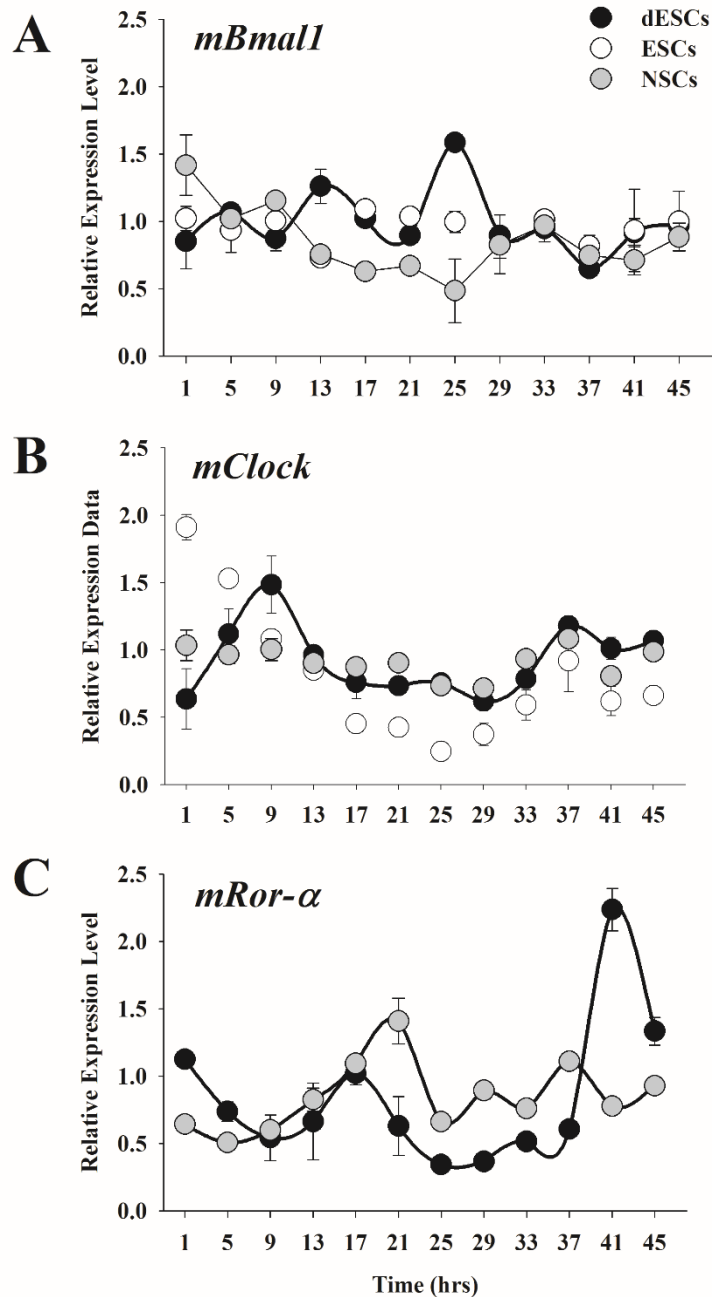
Figure 5. Positive regulators of the putative molecular clock vary in rhythmicity across differentiation. ESCs, dESCs, and NSCs (n=6 per cell type per timepoint) are indicated by white, black, and grey circles, respectively. Lines connecting timepoints indicate rhythmic expression in *mBmal1* (A), *mClock* (B), and *mRor-α* (C) expression levels.
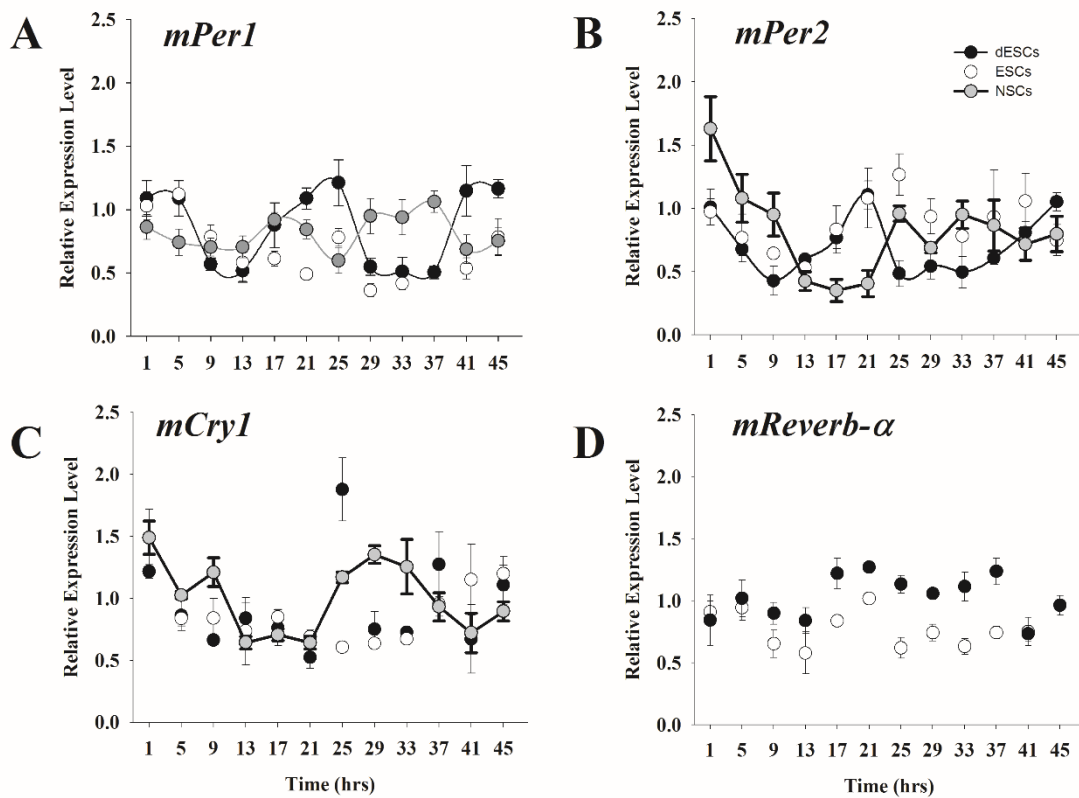
Figure 6. Clock genes comprising the inhibitory arm of feedback loop are rhythmic in dESCs and NSCs, but not ESCs. Relative abundance of A) *mPer1*, **B)** *mPer2*, **C)** *mCry1*, **and D)** *mReverb-α* **mRNAs were measured every 4 hours for 2 days in constant conditions. ESCs (white circles, n=6 per timepoint) displayed no rhythms. dESCs (black circles, n=6 per timepoint) showed rhythmic expression of both** *period* **mRNAs. NSCs (grey circles, n=6 per timepoint) displayed rhythmic expression of both** *period* **mRNAs as well as** *mCry1*, **but was not tested for** *mReverb-α*. **Solid lines between data points indicate statistically significant circadian rhythms.**
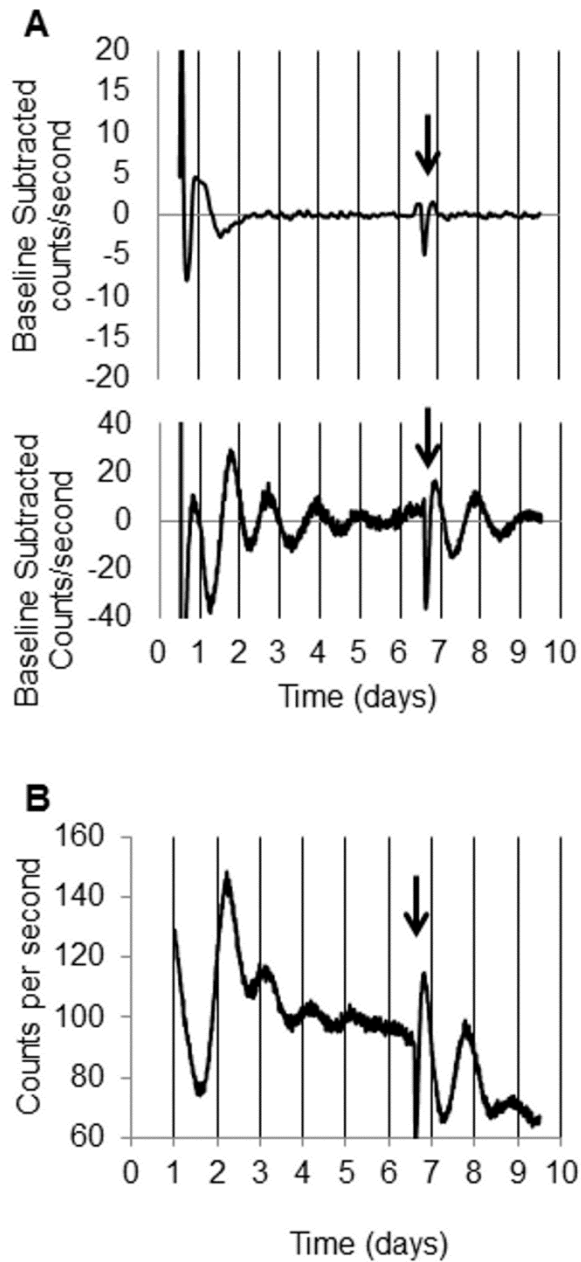
Figure 7. Real-time bioluminescence of mPER2::LUCIFERASE protein shows no rhythmicity in ESCs (A, top) and robust circadian rhythms in dESCs (A, bottom). Panel A show baseline subtracted counts per second. Raw counts of dESCs (B) show damping rhythmicity that is re-established upon culture medium exchange (arrows in A, bottom and B).

2.4 Discussion

Rhythmic glucose utilization precedes the development of the molecular clockworks in embryonic stem cells; however clock gene expression becomes rhythmic upon short-term differentiation. Previous investigations of the ontogeny of circadian rhythms concluded that undifferentiated mouse embryonic stem cells do not contain a functional clock based upon the lack of molecular rhythmicity in the expression of known components of the molecular clock (Bell-Pedersen et al., 2005), both in synchronized cultures as well as at the single cell level (Kowalska et al., 2010, Yagita et al., 2010). These studies, however, limited their analyses to some, but not all, putative clock genes. Here we show – in agreement with those studies as well as in a more comprehensive manner – that undifferentiated cells indeed do not possess a functioning canonical molecular clock, based upon expression of mRNA of most of these genes as well as protein expression of PER2. Undifferentiated cells were not rhythmic with respect to the clock genes tested, which is also in accord with previous studies. Upon non-specific differentiation, however, all of the tested clock genes were expressed rhythmically, with the exception of *mClock*, *mCry1*, and *mReverb*-α. Furthermore, the rhythm of luciferase bioluminescence in mPER2::LUC dESCs confirms the expression data. Although previous studies also looked at differentiated cells, the fate of those cultures was directed towards that of neural tissues. These data show the earliest developmental point at which clock genes exhibit circadian rhythms. This study investigated gene expression rhythms in primary cell cultures without the use of chemical synchronization as well as a physiological output of the clock, glucose uptake,

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which is a measure directly indicative of glucose utilization (Sokoloff et al., 1977). In addition to investigating whether undifferentiated cells exhibit uninduced rhythmicity, undirected differentiation was included in this set of experiments in order to observe any potential reorganization of clock elements in a manner that recapitulates the development of the embryo *in utero*.

Remarkably, rhythmic glucose utilization in undifferentiated stem cells does not necessarily coincide with rhythmic canonical clock gene expression; these processes are developmentally and experimentally separable. Previous studies in juvenile chicks showed that enucleation abolishes 2-DG uptake in the brain while clock genes remained rhythmic (Karaganis et al., 2009). Along with the previously mentioned rhythm of 2-DG uptake in neonatal rats (Reppert and Schwartz, 1984) as well as the recently discovered transcription-independent rhythm of red-ox cycles in human red blood cells (O'Neill and Reddy, 2011) the data presented here provide compelling evidence that metabolic rhythms are not regulated solely by the canonical molecular clockworks.

The glucose utilization rhythms in both ESCs and dESCs were corroborated by rhythmic glucose transporter expression, *mGlut8* in both ESCs and dESCs, indicating that the rhythms are driven by a transcriptional mechanism separate from the rhythmic expression of the canonical clock genes. Previously, Tonack, et al. (2006) showed that ESCs expressed several *mGlut* transcripts throughout embryoid body differentiation, including glut1 and glut8 in undifferentiated cultures. Although neither *mGlut1* nor *mGlut8* have been implicated in circadian rhythms, mGlut1 is necessary for ESC viability (Heilig et al., 2003) and up-regulation of *mGlut8* in embryoid bodies suggests

an increased need for glucose in differentiating cells (Tonack et al., 2006). Interestingly, the amplitude of *mGlut8* in both cultures remained the same, while the average glucose utilization was markedly increased in differentiated cells. Despite rhythmic expression of *mBmal1* and *mCry1*, glucose utilization in NSCs was ultradian, with peaks occurring approximately 8 hours and 20 hours after the initial peak. The intermediate peak may be indicative of a harmonic of the rhythm, as described by Hughes and colleagues (Hughes et al., 2009). If so, it would appear that the circadian rhythm of glucose utilization persists across multiple cell lineages. In this case, it would also appear that the phase of the rhythm is, on the whole, maintained across differentiation.

The rhythmic clock gene expression upon differentiation is quite remarkable for a number of reasons. The relative profiles of those genes that were rhythmic are consistent with the canonical molecular mechanism of circadian transcription, the negative elements *mPer1* and *mPer2* were identical in their phasing, and the positive element mBmal1 was expressed in anti-phase. Also, the cultures were rhythmic in the absence of any chemical synchronization. Previous studies had used dexamethasone (Kowalska et al., 2010, Yagita et al., 2010) or forskolin (Yagita et al., 2010) to synchronize cultures, as is common practice. In this study, the only conceivable synchronizing factors could have been centrifugation during passage or the absence of LIF, however there is no evidence of either phenomenon occurring in ESCs. Despite this, rhythms in 2-DG and clock genes were synchronized differentially between ESCs, dESCs, and NSCs. Secondly, the signal for differentiation away from the pluripotent

state immediately synchronized the cultures in this study, as seen in the bioluminescence data.

In contrast with the data presented here, Yagita et al (2010) showed that an *mBmal1* reporter remained arrhythmic throughout an induced differentiation process and required over two weeks of incubation with retinoic acid to obtain rhythmic bioluminescence, whereas we observed a low-amplitude oscillation after eight days of incubation. We also observed a more robust rhythm in *mCry1* expression, which would suggest, at the very least, that the monitoring of a single clock gene is insufficient to draw conclusions regarding the molecular clockworks. In combination with the data presented here, it would appear that clock gene rhythmicity is a dynamic phenomenon that can change depending on developmental stage, and perhaps within developmental contexts. Indeed, previous studies have shown that the synchrony of clock genes changes throughout development in both the SCN (Sládek et al., 2004) and the liver (Sládek et al., 2007). The nature of this synchronization is unknown but, considering the aggregate nature of the cultures, functional gap junctions between cells may facilitate communication and synchrony (Wong et al., 2008). Finally, the relative phases of the clock genes, once synchronized, align in a manner that would suggest functional molecular clockworks, with the positive and negative elements expressed anti-phase to each other.

Unlike previous studies examining rhythms in fate-specific differentiated cells, the media in this study differed with respect to one ingredient; the differentiation inhibitor LIF. LIF acts as a cytokine binding to a heterodimeric receptor complex of its

own receptor, LIFR, and gp130. The pathway ultimately leads to activation and translocation of STAT3 to the nucleus where it binds and activates various genes, presumably those involved in maintaining pluripotency (Chambers and Smith, 2004). However, there is no known, direct link between the LIF pathway and glucose transporter/uptake. The absence of LIF in differentiated cultures may account for the rhythmic expression of clock genes, but it does not explain the persistence of the 2-DG uptake rhythm in undifferentiated cells.

These data cannot be explained by possible effects of 2-DG on metabolism itself. While high mM concentrations of 2-DG inhibit cellular metabolism *in vitro* (Krieglstein et al., 1982), and high dosages of 2-DG can affect circadian clock light sensitivity *in vivo* (Challet et al., 1999), the concentrations of ^{14}C-2DG employed here (8 µM) and elsewhere *in vitro* (Earnest et al., 1999b), and the dosages employed for metabolic markers *in vivo* have no effect on metabolism (Sokoloff et al., 1977, Swanson and Benington, 1996) or circadian clock function (Schwartz and Gainer, 1977). Further, the 2-DG concentrations employed here are 0.4% of the 2 mM Kondoh et al. (2007) have shown to have no effect on ES cell proliferation: much higher concentrations than 2 mM are required to affect differentiation. Moreover, these data cannot be explained by cell cycle effects on clock gene expression and/or metabolism, since cell-cycle period for these cells is 11–12 hours rather than the 24 hours observed here (Stead et al., 2002). The most parsimonious explanation of the present results is that a circadian clockworks that does not entail the rhythmic expression of clock genes is present in mouse embryonic stem cells before differentiation into germ lines.

Thus, although undifferentiated ESCs do not possess a functioning canonical molecular clock, a circadian rhythm of glucose utilization persists in these cells. This rhythm is coincident with rhythmic expression of one glucose transporter gene, suggesting rhythmic transcriptional control of glucose utilization. Whether this rhythm is initiated intrinsically or in response to glucose availability is unknown. In addition, acute differentiation by withdrawal of LIF increases the amplitude of glucose utilization rhythms and initiates rhythms of the expression of canonical clock genes, suggesting a potential role for the cytokine in initiating the transcriptional-translational feedback loop. Finally, differentiation to a neural state further reorganizes rhythmic clock gene expression, and glucose utilization rhythmicity may persist, albeit with an intermediary harmonic.

3. ONTOGENY OF CIRCADIAN RHYTHMS IN CHICK EMBRYOS

3.1 Introduction

Unlike viviparous organisms, avian development proceeds – for the most part – in the absence of nutritive contribution afforded within the womb. As such, the myriad environmental signals relayed from the mother to the gestating embryo are absent, including those encoding time-of-day information. However, the lack of maternal signaling does not preclude a role of the clock *in ovo*, as mid-gestation chicken embryos have been shown to exhibit rhythms in melatonin production from the pineal gland (Lamosová et al., 1995) and clock gene expression in both the SCN and pineal gland (Okabayashi et al., 2003). The developing avian embryo does not benefit from circadian signals passed through the circulatory system as in mammals, but there is evidence that information pertinent to initiating the rhythm may be imparted maternally. Indeed, follicular development in chickens is regulated in part via clock genes, where the surge in luteinizing hormone influences clock gene expression specifically within the ovary (Tischkau et al., 2011). Similarly, egg laying in quails appears to be under the control of a circadian mechanism (Houdelier et al., 2002). One conclusion from these data is that, as is the case in mammals, avian maternal germline tissues contain a functional clock mechanism. It would not be unreasonable, especially in light of the data presented previously, to hypothesize the presence of a circadian clock in early avian embryogenesis.

Until recently, it was not known whether clock genes are present at all in early embryonic tissues. *In situ* analysis showed that two clock gene mRNAs, *gBmal1* and *gClock* are expressed throughout early chick embryos (Gonçalves et al., 2012). Remarkably, Gonçalves and colleagues showed a decrease in expression of the two clock genes between the first and fourth Hamilton and Hamburger stages of development and suggested that this may be evidence of maternal contribution of clock genes in early embryogenesis, before the transition to zygotic transcription (Gonçalves et al., 2012). Similarly, melatonin and transcripts of *Aanat* and *Hiomt* were found in fertilized, unincubated quail eggs as well as in the yolk (Olszańska et al., 2007). Again, the data suggest that the fertilized egg is supplied with low levels of transcript that quickly disappear, only to increase when zygotic transcription has taken over.

The presence of melatonin and its synthesizing hormones in the egg yolk would imply that the embryo can make use of melatonin at some point during development. Synthesis of melatonin by embryonic pineal glands *in vitro* is detectable in cultures from embryos as early as 10 days post incubation (Möller and Möller, 1990), however rhythmic melatonin secretion in DD is only seen in cultures from embryos at least 18-days post incubation (Akasaka et al., 1995). Akasaka et al, also found that 14-day old pineal cultures were able to entrain to L:D cycles, which suggests the presence of an underlying molecular clockworks.

While these studies are compelling, they are insufficient in demonstrating a functional circadian mechanism in embryogenesis. Here, we extend the previous *in situ* data with RT-PCR analysis of all clock gene transcripts from early chick embryos and,

using a no amplification-based method of RNA quantification, examine the molecular clock function under entraining cycles of incubation temperature.

3.2 Methods

Embryos

Fertilized chick eggs (Hyline Strain W38) were obtained from the University of Kentucky Poultry Science Research Institute for all studies. Eggs were incubated in Sportsman 1502 Egg Incubators with automatic turning (GQF Manufacturing, Savannah, GA) at 37.5°C except during temperature entrainment. For time series experiments, embryos were split into three incubation groups: constant high temperature (39°C, H:H), constant low temperature (36°C, L:L), or transferred every 12 hours between the two, thus establishing a potential temperature zeitgeber (H:L). Zeitgeber time (ZT) was defined in reference to when H:L embryos were transferred to low temperature, i.e. ZT 12 was when embryos were transferred from low to high temperature. After 3 days of incubation, timepoint samples were taken every four hours and consisted of removal of embryos (n = 5 per timepoint per treatment) from the egg, with care taken to eliminate as much yolk material as possible. Embryos were placed directly into 500ul of Trizol reagent in silicon/graphite bead-filled tubes and homogenized in a Thermo FastPrep sample homogenizer. Homogenates were stored at -80°C until RNA isolation.

RNA Isolation

Whole embryos, brain, and pineal glands, as applicable, were isolated and placed directly in homogenizing vials containing 500 μL of Trizol reagent. Samples were

homogenized using a Thermo FastPrep homogenizer and MolBio lysing matrix F tubes (MolBio, OH). RNA was isolated using Directzol RNA purification kits (Zymo Research, Irvine, CA) with in-column DNAse treatment to remove genomic DNA. Total RNA was quantified using spectrophotometry (Nanodrop 1000, Thermo Fisher) to determine concentration and purity (260:280 and 260:230 ratios).

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

For RT-PCR, total RNA was reverse-transcribed using Q-script cDNA synthesis kits (Quanta Biosciences, Gaithersburg, MD) and cDNA amplified with primers for clock genes and melatonin biosynthesis and receptor genes (Table 2). PCR products were visualized on 1% agarose gels by electrophoresis and digitized by Bio-Docit gel documentation system (UVP, Upland, CA).

NanoString Analysis

For quantitative expression analysis, 100ng of total RNA was hybridized to a commercially designed array of 23 probes targeting various transcripts of interest as per manufacturer-supplied protocol (Geiss et al., 2008), Nanostring Technologies, Seattle, WA, abbreviated target sequences summarized in Table 3). Each sequence-specific probe also contained a multi-unit fluorescent barcode for downstream identification. RNA/probe hybrid structures were placed on a charged substrate to allow presentation of fluorescent signal to a CCD-cooled camera. Images taken from the camera were analyzed *in situ* and raw counts of positive controls, negative controls, and sequence specific probes were determined. Total counts of RNA species were normalized using the R package NanoStringNorm (Waggott et al., 2012).

Statistical Analysis

Normalized transcript levels obtained from qPCR or NanoStringNorm were compared among temperature groups and among timepoints within each group using one-way ANOVA. *Bona fide* circadian rhythmicity was defined by significant difference between timepoints spaced ~12 hours apart and by adherence to putative transcriptional relationships; e.g. *gBmal1* and *gPer3* transcript should be anti-phase to one another. Statistical tests were carried out using Systat software.

3.3 Results

Gene Expression in Early Embryos

To determine the early progression of clock gene and melatonin receptor gene expression, RT-PCR was performed on cDNAs derived from whole embryos at embryonic days 1 and 4 (E1, and E4, respectively), and whole brain from E10. At E1, all clock genes and melatonin receptors showed expression, however melatonin biosynthesis genes *gAanat* and *gHiomt* were not detected (Figure 8A). E4 and E10 samples also showed expression of all clock genes (Figure 8B), however E4 embryos only expressed *gMel1a* (Figure 8C). Samples were also taken from newly hatched pineal and retinae for comparison and processed in parallel with E4 and E10 samples. *gmel1b* and *gMel1c* expression was also detected in E7 brain tissues, in addition to *gMel1a* (Figure 8D).

Temperature Entrainment

To determine if temperature affects clock gene expression in early

Table 2. Primers used for qPCR on chick embryos

Gene	Forward Primer Sequence 5'-3'	Reverse Primer Sequence 5'-3'
gAanat	*AGCCTTCATCTCCGTCTC*	*GTACCGCCACATCAGGAT*
gBmal	*GCCATCACGACTATGTTCT*	*AGCGACTCCTCAAGGTAA*
gClock	*GTTACTGTTCCTGAGATGTTC*	*TGTTGCTGCTGTGTTACT*
gCry1	*ATGTGGCTATCCTGTAGTTC*	*AAGTGTGATTGCTGATTGTC*
gCry2	*CATTCTTCCAGCAGTTCTTC*	*TTCAGCACTCTCATTCTTCA*
gHiomt	*TCTACCTTACGAAGTCAAGTC*	*CTCTCCAGCATCAGTCAG*
gMel1A	*ATGGGCCTAAGTGTCATTGG*	*AGGTTGGGCACAATAGCAAC*
gMel1B	*AGTGGCTTTGTGATGGGACT*	*GGAGCTTGCAGTCTGAACAA*
gMel1C	*CTCTTTGTTGGCTCCTTGC*	*GTCTCACCCGGTGTTTGACT*
gPer2	*TGTTCCAATCAAGGTGTAGT*	*ATCATAACGGTGTCATCTGT*
gPer3	*ATGGTAGCCTTGGTAGCA*	*ACTTGGAGACCTGTTGGA*

Table 3. Probes used for NanoString Analysis

Gene	Target Sequence
gAanat	CAGCGAGTTCCGCTGCCTCAGCCCCGAGGATGCCGTCAGCGTGTTC
gβ-Actin	CTCTGACTGACCGCGTTACTCCCACAGCCAGCCATGGATGATGATATT
gVaOpn	CTGGTGGCACCATCAGCTTTTTAGCCAACCTAAAAGGCTATTTCTATA
gBmal1	CAGTTAAAACTGATATAACACCCGGGCCATCACGACTATGTTCTGGA
gClock	CAGTCTCAACACCTTTGTATAACACTATGGTGATTTCTCAGCCAACAG
gCry1	TTTTGGCAGAAGAACTGACCCAAATGGAGATTACATCAGACGTTACT
gCry2	CCAGCAACTATCACGCTACAGGGGGCTCTGTTTATTGGCATCAGTCC
gCycA	GCGTCACCTTCGAGCTCTTCGCTGACAAGGTGCCCATAACAGCAGA
gFblx3	ACGAGGAGCTGATCCGTATCGCAGAGCGGTGCAAGTATTTGTCAGCT
gGlut1	TCTACTACTCCACCAGCATCTTTGAGAAGTCAGGTGTGGAGCAGCCC
gHsp90	GGAAGTGATCTACATGATTGAGCCTATTGATGAATATTGTGTGCAGCA
gHiomt	TACTGCATTTGACCTTTCCCCTTTCACACAGATTTATGACCTGGGAGG
gMel1c	TCTGGATTTTGGTGATTCAAGTCAAACACCGGGTGAGACAAGACTG
gRor-a	AGTTCACTGCCCAGACTTCTGACAGAACAATCAATTTGTTGATCAGA
gMel1b	TGGTGGCCTTGTATCCATACCCACTGGTGCTCTTAGCTATTTTCCACA
gMel1a	TGGTCTTATCTGGGTCCTAACAGTTGTTGCTATTGTGCCCAACCTGTT
gOpn4	ATTTTTGGTGAGAAAGGCTGTGAGCTGTATGCCTTCTGCGGAGCTCT
gOpn5	GCCTTATTACCATGACAGCCGTCAGCCTGGATAGATACTTAAAAATCT
gPer2	AGCGTGAAAATGAGATTTGCTATCACCCATTTCGGATGACTCCTTACC
gPer3	TCCAGGAAATCAAAAAGTGTTTGCCTGAAGAGAAAAGGAGTTCCAG
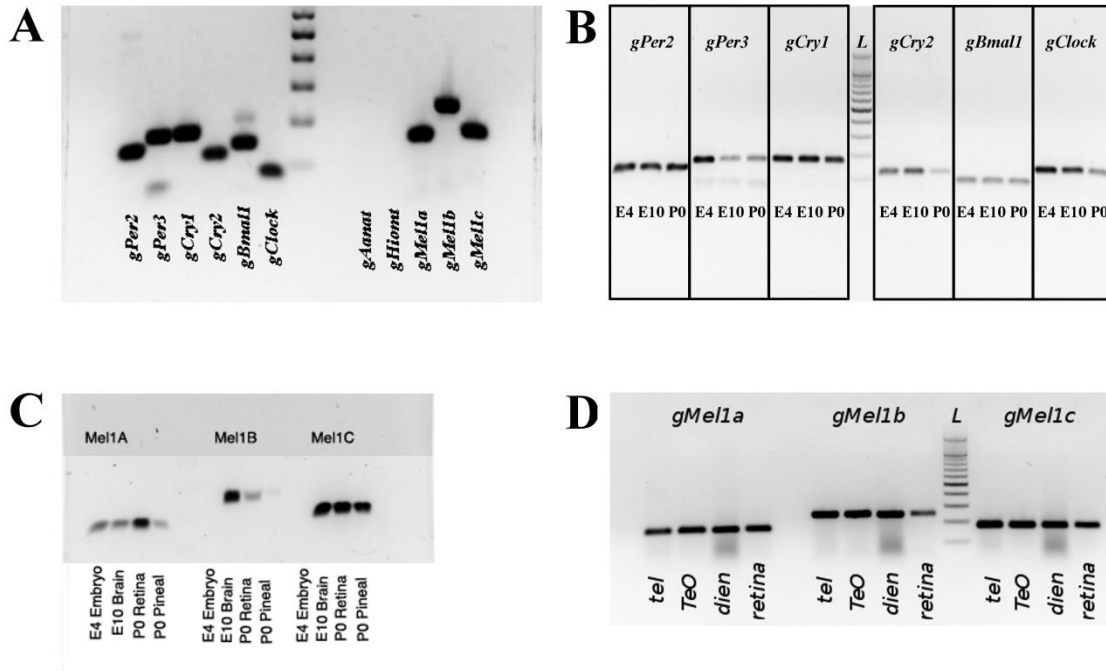
Figure 8. Clock genes and melatonin receptor transcripts are present throughout early chick development. Gel electrophoresis of RT-PCR against A) clock genes, melatonin receptors, and melatonin biosynthesis genes in E1 whole embryos, B) clock genes in E4 whole embryos, E10 brains, and P0 pineal glands, C) melatonin receptors at E4, E10, P0 retina and pineal, and D) melatonin receptors in E7 telencephalon (tel), optic tecta (TeO), diencephalon (dien), and retina show presence of all transcripts, with the exception of gMel1B and gMel1C in E4 embryos.
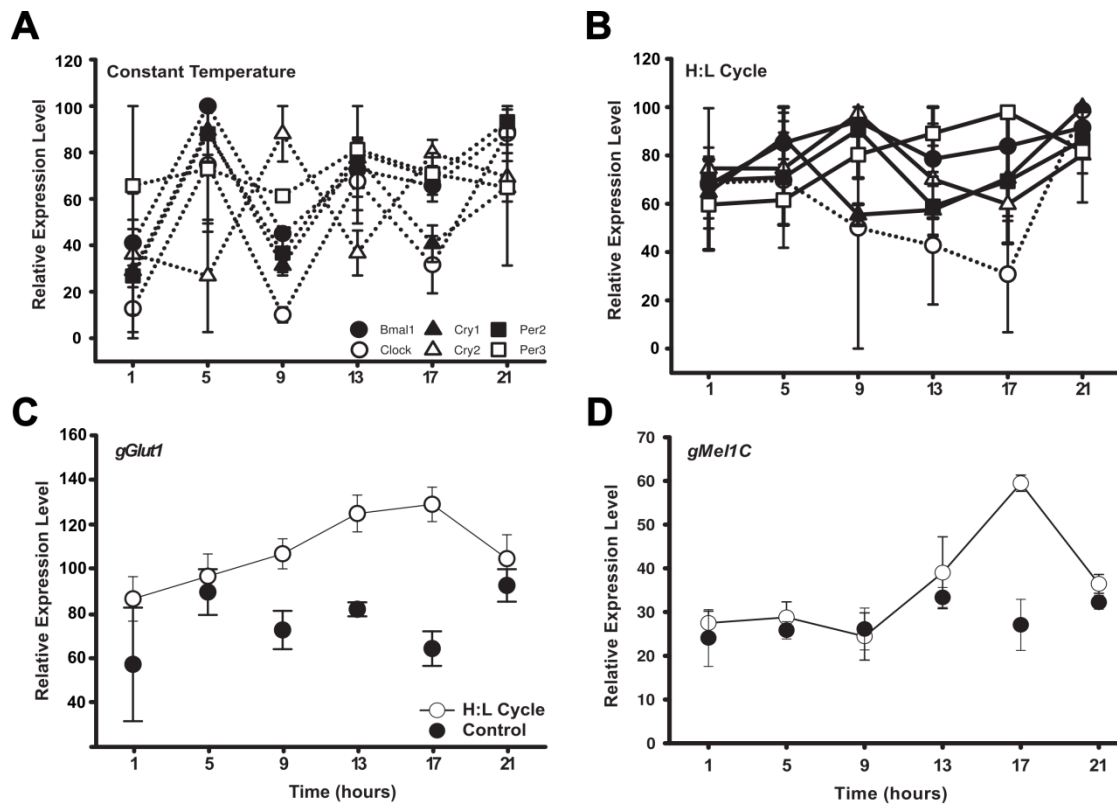
Figure 9. Temperature cycles drive rhythmicity in putative clock genes and glucose transporter expression. Clock gene expression under constant temperature conditions (A) varied over time, but not in a circadian fashion. Exposure to temperature cycles (B) drove rhythms in all clock genes except gClock. Temperature cycles (black circles) also drove gGlut1 (C) and gMel1C (D) rhythms while no rhythms were apparent in constant conditions (white circles). Solid lines indicate rhythmic expression as determined by Circwave software from n=5 embryos per timepoint per condition.

embryogenesis, fertilized embryos were placed under a temperature cycle for 3 days

before collection of whole embryos at four hour intervals. In constant incubation

temperature of 37.5º C, embryos showed no rhythms in any clock genes (Figure 9 A). In

contrast, exposure to 12 hours of 39º C followed by 12 hours of 36º C drove rhythms in

almost all clock genes, with the exception of *gClock* (Figure 9 B). *gGlut1* was also

rhythmic during temperature entrainment, exhibiting a nearly identical phase to that of

gPer3 (Figure 9 C).

Morphometrics and Transcriptomics Under Free-running Conditions

In order to determine whether early embryos were entraining to imposed

temperature cycles, as opposed to being driven by them, E0 embryos were incubated in a

temperature cycle as before and subsequently released into constant 39ºC, at which point

sample collection commenced. Control groups included embryos incubated at constant

high and constant low temperatures. At each timepoint, prior to collection in Trizol

reagent, each embryo was photographed to estimate developmental age by the

Hamburger and Hamilton scale (Figure 10 A, B, C). Averaged estimates from three

independent observations showed that constant low temperature incubation significantly

delayed development, however linear regression analysis showed no difference in the

rate of development among the three incubation conditions (Figure 10 D). RNA

expression analysis of canonical clock genes showed no discernible free-running rhythm

after three days of entrainment to temperature cycles (Figure 11A), however *gPer3* and

gBmal1 showed slight, anti-phase oscillations. Although the expression of *gCry1* was

not rhythmic, its abundance in all three treatments was significantly higher in all three

46
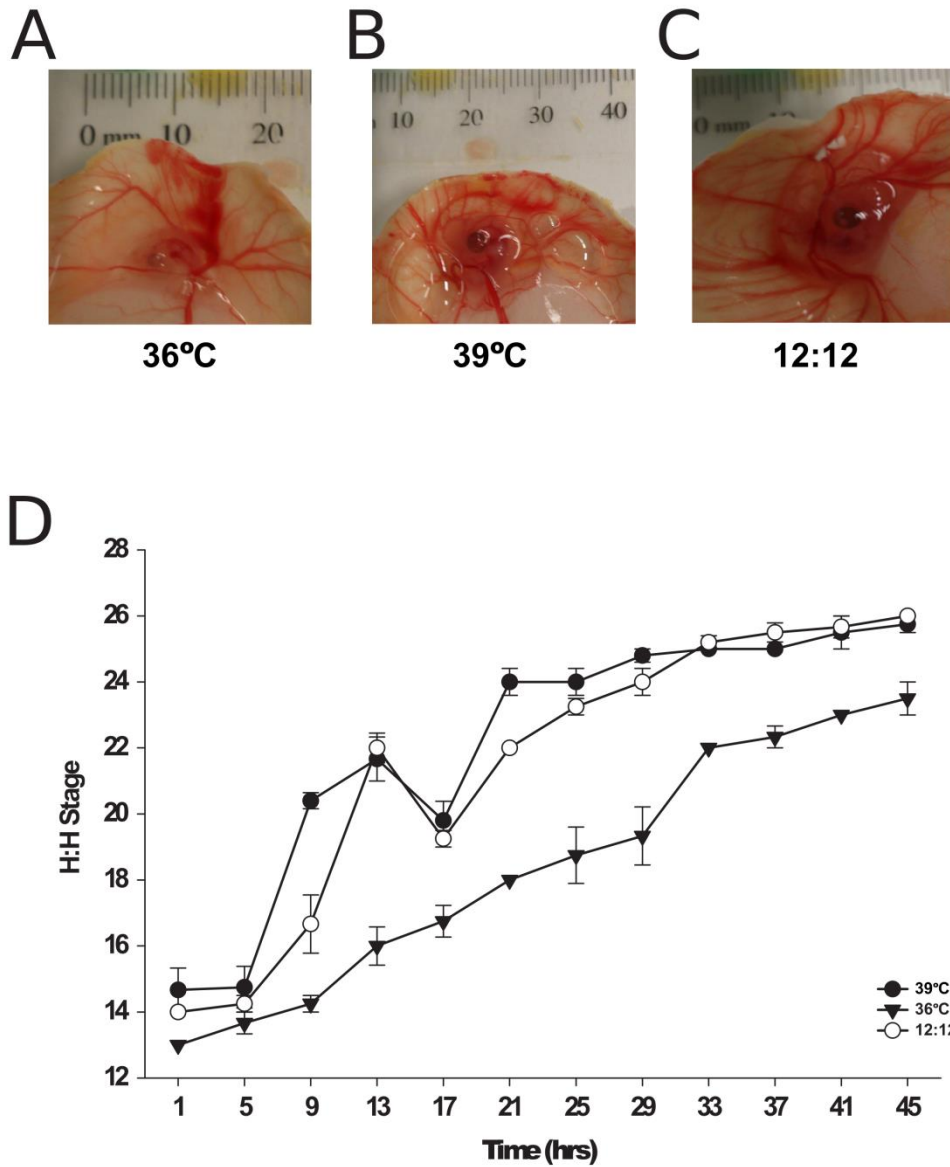
Figure 10. Lower temperatures delay early developmental progression; rhythmic temperature has no effect. Photographs of embryos sampled at 29 hours showed that embryos incubated at 36°C (A) were developmentally behind compared to embryos incubated at 39°C (B) or on temperature cycles (C). Time-series analysis across 48 hours showed that embryos incubated at a lower temperature were consistently delayed compared to embryos incubated at constant high temperature and cyclic conditions (D).
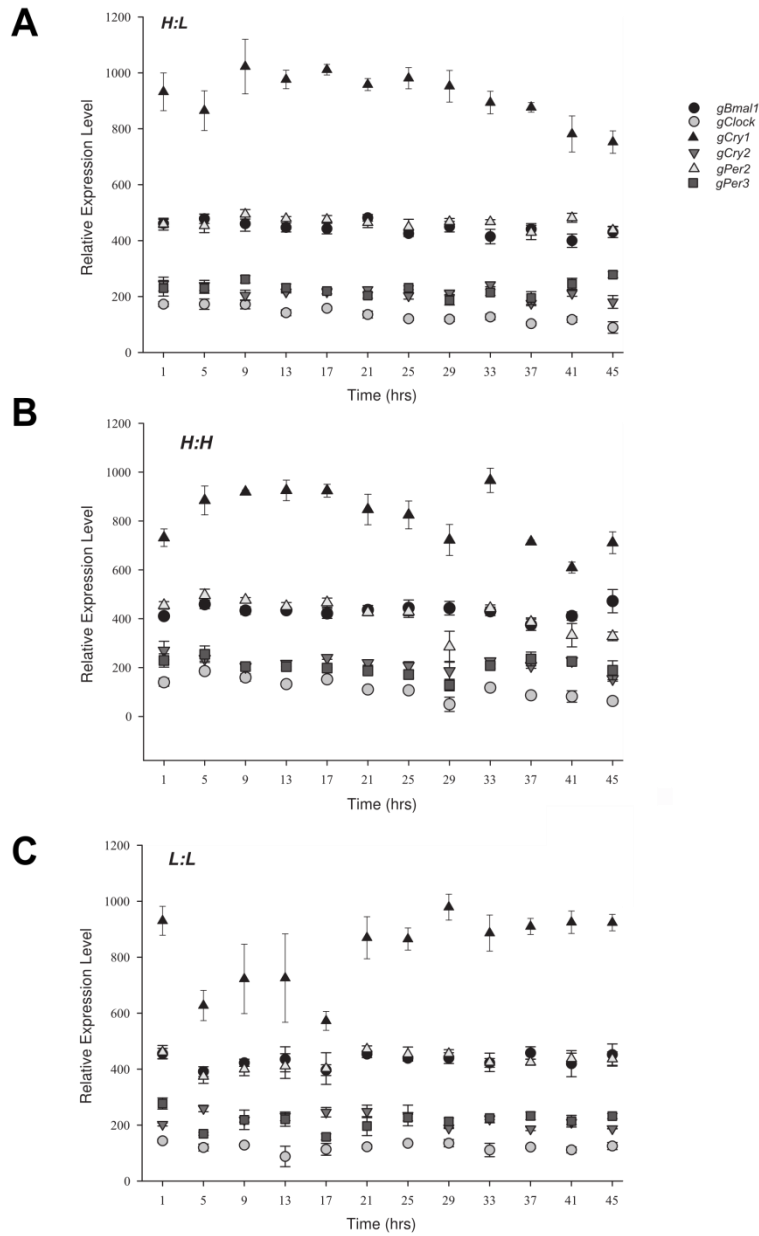
Figure 11. Clock genes are not rhythmic in free-running or constant conditions.
Clock gene expression in A) free-running conditions after 12:12 high:low
temperature cycles, B) constant high temperature, and C) constant low
temperature conditions revealed no circadian patterns of expression.

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groups compared to other clock genes.

As expected, neither *gAanat* nor *gHiomt* transcripts were detectable (not shown), however mRNA of all three melatonin receptors was present in all temperature conditions. In both H:H and H:L treated embryos, *gMel1a* and *gMel1b* were expressed in similar patterns over time, exhibiting early peaks of expression and low to nearly undetectable levels by day two of sampling (Figure 12 A,B). Conversely, those transcripts in L:L embryos exhibited low amplitude oscillations that increased on day two of sampling (Figure 12 C). Similar to *gCry1, gMel1c* mRNA was consistently higher than the other two receptor transcripts.

Although none of the clock genes were statistically rhythmic, Circwave analysis showed *gGlut1* to be rhythmic in constant low conditions for at least one day, and a clear peak is seen between 5 and 9 hours of sampling. The oscillations are not apparent on the second day of sampling, at which point expression levels match that of free-running embryos. In contrast, embryos kept at 39 C showed non-oscillating expression over 41 hours, with a single peak 4 hours before the final time point (Figure 13).

3.4 Discussion

Similar to the case in mammals, clock gene transcripts, as well as melatonin receptor transcripts, are all expressed from the very beginning of development in chicks. The molecular clockworks in birds is nearly identical to that in mammals, with the exception of *period* gene variants (*per2* and *per3* in birds vs. *per1*, *per2*, and *per3* in mammals, (Bell-Pedersen et al., 2005), which makes the chick embryo a suitable model
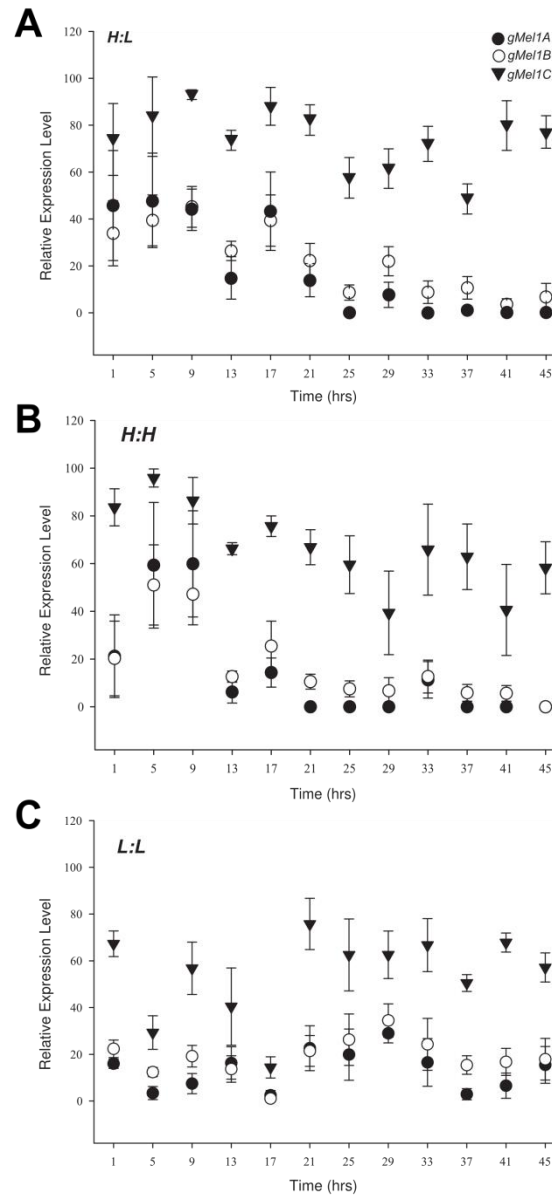
Figure 12. Melatonin receptors are differentially expressed, but not rhythmic in early chick embryos. *gMel1A* **(filled circles),** *gMel1B* **(white circles), and** *gMel1C* **(filled triangles) were expressed in A) free-running after 12:12 high:low temperature cycles, B) constant high, and C) constant low temperature conditions.**
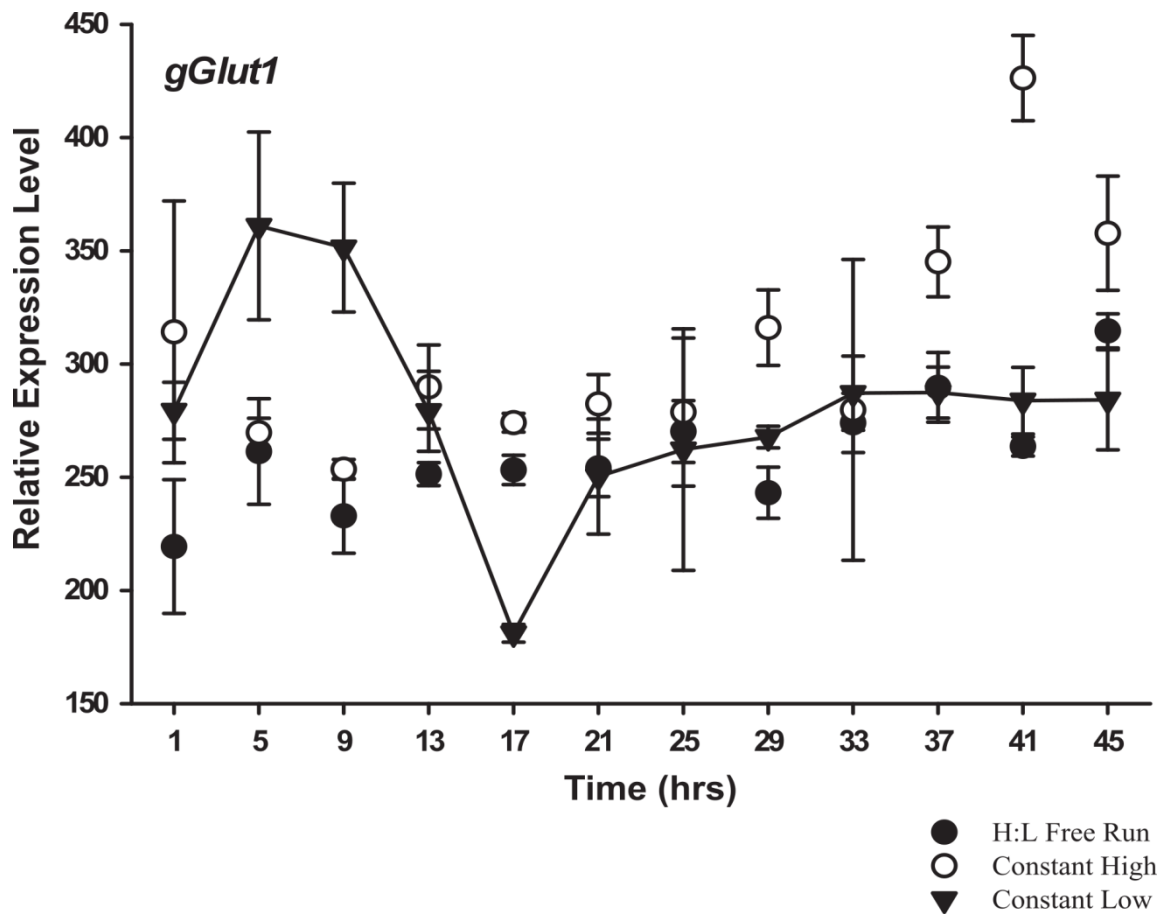
Figure 13. Glucose transporter expression exhibits circadian rhythmicity in embryos incubated at 36° C. Neither free-running embryos (filled circles) nor embryos incubated at 39° C (white circles) exhibited rhythmic expression of *gGlut1*. Circwave analysis (significance indicated by connected plots) did show significant rhythms in embryos incubated at constant low temperature (36° C, filled triangles).

to compare the developmental origin of the circadian mechanism. The previous study examined mammalian circadian development, however in the artificial environment of a culture dish. While this approach eliminated all influences of the maternal clock, it was also, by nature, devoid of any physiological context. The fertilized chick egg was chosen because it is its own incubator and contextually more appropriate to study the development of circadian rhythms. Notwithstanding, the chick embryo also presented disadvantages compared to mouse embryonic stem cells, namely that 2-DG could not be reliably administered and subsequently measured due to variability in embryo position and yolk volume. However, *glut1* expression was used as a proxy for glucose utilization, as the previous study established a correlation between the two. Similarly, the "egg as a womb" is not an equivalent situation to that in mammals: the egg is not a euthermic organ, and avian embryos are ectothermic until late gestation (Seebacher et al., 2006). This paradigm does however allow for the administration of natural zeitgebers, such as light and temperature.

The presence of clock gene and melatonin receptor mRNAs in the early embryo is not surprising. As previously stated, *gClock* and *gBmal1* transcripts appear to be provided maternally and are expressed by the embryo from the initial stages of development (Gonçalves et al., 2012). Here we expand upon those data and show that E1 embryos express all putative clock gene transcripts as early as E1 (figure 8A), and are expressed throughout early gestation. It is surprising that all of the melatonin receptors are expressed at E1 even though neither *gAanat* nor *gHiomt* transcripts were detectable (Figure 8A). Given the previous data – that maternally-contributed melatonin and its

formative enzymes are present in the yolk and decrease over time (Olszańska et al., 2007) – it could be that the early embryo utilizes the deposited melatonin as well as its own. The previous study showed a coincident presence of melatonin and enzyme activity, with the latter levels decreasing with time through HH stage 11. The absence of *gAanat* and *gHiomt* transcripts in this study would confirm a lack of melatonin *production* by the embryo at this stage, while the persistence of receptor transcripts suggests that melatonin is still present. This is confirmed by the variable expression of receptors in E4-E5 embryos (Figure 12). Surprisingly, the greatest variation over time is observed under constant conditions, where expression of *gMel1A* and *gMel1B* appear circadian, but for the lack of statistical significance (Figure 12). *gMel1C* expression is more highly expressed regardless of temperature, which is consistent with data from adult zebra finch (Jones et al., 2012), however differential expression patterns are observed depending on temperature treatment.

Similar to clock gene expression, *gGlut1* was driven to rhythmicity by temperature cycles given from day 0 to day 3 of incubation, while no significant variation was observed in constant conditions. Surprisingly, *gGlut1* expression in constant low temperature conditions was rhythmic for at least one day of sampling, and contrasted almost exactly with *gCry1* expression as well as *gMel1C*. Although neither of the latter two transcripts passed Circwave analysis for rhythmicity, and several data points within both data sets exhibited much higher variability, the coincidence of phasing between clock genes in both entrained embryos and low-temperature compensated embryos – which would have been at the same developmental age –

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suggests that glucose utilization and clock gene expression are, as seen in mouse embryonic stem cells, tightly coupled. Even more interesting, in both entrained and free-running conditions, glucose transport – and likely glucose utilization, is also tightly coupled to melatonin receptor expression.

When viewed in the light of natural conditions, so to speak, the presence of an entrainable clockworks in oviparous embryos is not so surprising. Eggs in a nest would be exposed to the naturally-occurring light:dark cycle automatically, and even dim light cycles are sufficient to entrain melatonin secretion in starlings (Gwinner et al., 1997). The studies here show that, with the exception of *gVaOpsin*, none of the photoreceptors are expressed early on in embryogenesis. Furthermore, imposition of a light:dark cycle had no effect on clock gene expression. It could be postulated that hens might impose a temperature cycle on embryos; the periodic attendance of eggs interspersed with foraging behavior by hens could raise the incubation temperature, with highest temperatures occurring during the night phase when hens sleep on their nests. This hypothesis is supported by a comparative study investigating nest temperatures in auklets, gulls, and albatrosses that found highly variable, sometimes circadian, patterns of daily temperatures (Shaffer et al., 2014). Previous to this, Cooper and Voss demonstrated that thermogenesis in the growing embryo is itself a modulator of egg attendance in chickadee hens (Cooper and Voss, 2013). Progression of development naturally changes the thermogenic properties of the embryo, the heat retention and radiation of embryos affects nest attendance, which therefore affects embryo temperature. Taken together, these data suggest that the temperature of the embryo

proper strives to remain constant, albeit surrounded by a nest environment with a (potentially) diurnally fluctuating temperature, which would impose temperature cycles early on in embryogenesis. It is likely not coincident that pineal melatonin production and the transition to homeothermic metabolism occur at the same time during incubation. The results here indicate that melatonin sensitivity and metabolism are coupled long before the embryo makes its own melatonin.

4. EFFECTS OF PINEAL MELATONIN ON ASTROCYTE CIRCADIAN RHYTHMS AND DEVELOPMENT[*]

4.1 Introduction

The avian circadian system comprises multiple circadian oscillators, pacemakers, and photosensitive structures (Cassone and Menaker, 1984, Cassone, 1990). These include the retina (Ebihara and Kawamura, 1981, Underwood et al., 1984), the avian homolog of 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 (Hamm and Menaker, 1980, Adachi et al., 1995, Klein et al., 1997). However, the molecular mechanisms by which melatonin influences physiology and circadian behavior are not completely known. Birds express three melatonin receptor subtypes: the Mel1A or MT1 receptor, the Mel1B or MT2 receptor, and the Mel1C receptor (Natesan and Cassone, Reppert et al., 1995, Adachi et al., 2002). Based on receptor binding studies and in situ hybridization of the three receptor subtypes in chick diencephalic astrocytes, our laboratory (Adachi et al., 2002) demonstrated that approximately 25% of these cells express Mel1A receptor mRNA, none express Mel1b receptor mRNA, and

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nearly all express Mel1C receptor mRNA. Additionally, when melatonin was rhythmically administered to these cultures, rhythms in glucose utilization and in the release of the glycolytic end products pyruvate and lactate were observed, with a circadian phase that corresponds to that of the melatonin cycle (Adachi et al., 2002). There is also evidence that rhythmic melatonin administration can entrain some clock genes in astrocytes (Peters, 2005).

One of the great surprises in the discovery of molecular components of circadian oscillators of animal species ranging from Drosophila to mammals (Bell-Pedersen et al., 2005) was the observation that clock gene rhythmicity is not restricted to pacemaker tissues such as the pineal gland, retina, and SCN. Rather, rhythmic clock gene expression is a global phenomenon raising the current consensus view that the role of pacemaker tissues is to entrain peripheral oscillators rather than to drive them. In Drosophila, for example, the molecular clockworks within olfactory tissues are sufficient to drive rhythms in olfactory sensation (Tanoue et al., 2004). Consistent with this view, certain peripheral tissues including cornea, liver, and lung explanted from transgenic mice containing an mPER2 promoter- driven luciferase gene exhibited rhythmicity in culture for several days before damping out. Furthermore, peripheral tissues from SCN-lesioned mice maintained PER2 rhythms that could be sustained by media exchange, although with some disruption in phase (Yoo et al., 2004). To differentiate pacemaker from downstream oscillators, our laboratory has previously employed a coculture preparation of immortalized SCN cells and target fibroblast cell lines (Allen et al., 2001, Allen et al., 2004). Here, we cocultured chick pinealocytes with target diencephalic

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astrocytes. These studies support the view that the pineal gland regulates target cells through the rhythmic secretion of melatonin, which may involve rhythmic clock gene expression. These data also indicate that pineal melatonin can regulate astrocyte development and mitosis.

4.2 Materials and Methods

Astrocyte Cultures

Astrocytes were harvested from embryonic day 17 chick brains (Hyline Hatcheries, Bryan, TX, USA). Embryos were extracted and the brains were removed. Diencephalic tissue was dissected and placed in Dulbecco's modified Eagle's medium (DMEM; Sigma Corp., St. Louis, MO, USA), then dissociated by triturating with 1% trypsin. Cells were filtered through nylon mesh (180 μm pore diameter) and centrifuged for 5 min at 1000 x g. The supernatant was removed and the pellet was resuspended in DMEM with 10% fetal bovine serum and a 1% PSN antibiotic cocktail (penicillin, streptomycin, and neomycin). 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. Confluent cultures were processed for immunocytochemical analysis of glial fibrillary acidic protein (GFAP) to determine the purity of the cultures. Cultures were plated on glass-bottom dishes for 5–7 days. Cells were fixed with 4% *p*-formaldehyde (PFA) for 10 min, washed, and processed for immunocytochemical identification of

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GFAP (1:1000; Incstar, Stillwater, MN, USA) as previously described (Peters, Cassone, et al. 2005). Differential interference contrast (DIC) microscopic images of cell cultures were captured using Simple PCI software (C-imaging, Hamamatsu Corp., Sewickley, PA, USA), and dual captures of DIC images and GFAP-immunoreactivity were superimposed to estimate the percentage of cultured cells that were actually GFAP immunoreactive astrocytes. In these parallel cultures, 87.1±4.8% of the cells was immunoreactive to GFAP. The remaining cells may have been fibroblasts or astrocytes that did not react to the antiserum.

Pinealocyte:Astrocyte Coculture

Confluent astrocyte cultures were passaged into six-well plates (BD Biosciences, San Jose, CA, USA) designed to accommodate insert dishes for coculture and then cultured for 2days to allow sufficient time to adhere. Pinealocytes were cultured as previously described (Zatz et al., 1988) with modifications. Briefly, newly hatched White Leghorn chicks were killed by decapitation and the pineal glands transferred to sterile Dulbecco's phosphate-buffered saline (PBS) with d-glucose. After 30 min of digestion in 0.05% trypsin with periodic trituration, the remaining debris was removed from the media and the cells were pelleted at 1000 x g for 10min. The pellet was resuspended in sufficient volume of McCoy's 5a media supplemented with 1% KCl and 1% PSN to allow for the plating of two glands per well. Pinealocytes were cultured directly onto 0.2 μm mesh inserts (BD Biosciences) which were subsequently returned to their companion well on the six-well plate containing the 2-day-old astrocyte cultures

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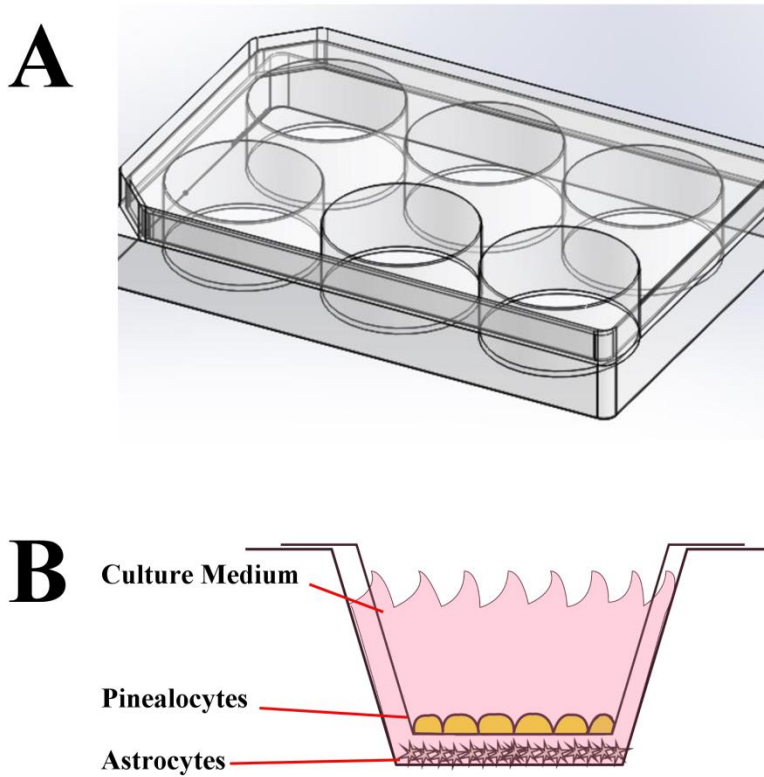
Figure 14. Coculture of chick pinealocytes and diencephalic astrocytes. A) Astrocytes were cultured in standard 6-well plates. B) Cross-sectional diagram of trans-well inserts containing dispersed pinealocytes suspended over astrocytes showing media exchange in the absence of physical contact.

(Figure 14). Control plates contained inserts without pinealocytes. Astrocytes were maintained in 3 mL of the DMEM-based medium while pinealocytes (and empty inserts) were administered 2 mL of the McCoy's 5A-based medium. All media and supplements were purchased from Gibco/Invitrogen Corp (Grand Island, NY, USA) unless otherwise stated. Cultures were placed in 12:12 light:dark cycles for 7 days, as we have previously determined this to be sufficient time for the pinealocytes to confer rhythmicity to the astrocytes. On the eighth day, cultures were placed in constant darkness and samples taken every 4 hours for 48 hours.

2-Deoxy [^{14}C]-Glucose Collection and Quantification

One hour prior to sampling, the cells were incubated with [14C]-2-deoxyglucose (2DG; 0.2 μCi/mL; American Radiolabeled Chemicals, St. Louis, MO, USA). Following incubation, media was removed and saved for melatonin quantification. Cells were rinsed twice with Dulbecco's PBS (Gibco/Invitrogen Corp.), and harvested in Trizol reagent (Invitrogen Corp.) to extract total cellular RNA and soluble protein. Duplicate aliquots of cell lysate (200 μL) were placed in scintillant and counted on a Beckman scintillation counter. Uptake of 2DG was normalized to protein or total RNA content.

Melatonin Quantification

Melatonin was measured using a radioimmunoassay method as in Fraser, et al. (1983) with modifications. Briefly, media samples from cocultured and control astrocyte cultures were incubated with melatonin antibody (Stockgrand Ltd., Surrey, UK) in tricine buffer (Sigma-Aldrich, Inc., St. Louis, MO) followed by incubation with competing [H3]-melatonin (Perkin Elmer, Waltham, MA, USA). After incubation, free

melatonin was separated from bound melatonin using dextran-coated charcoal (Sigma-Aldrich, Inc.) and the bound melatonin counted using the above scintillation counter. Quantities are reported here as relative to the maximum amount of melatonin among all timepoints.

Astrocyte Growth Study

For cell growth studies, astrocytes and pinealocytes were cocultured as before. Images of the astrocytes were taken at zeitgeber time (ZT8) every day for 7 days using an Olympus (Center Valley, PA, USA) IX-70 inverted microscope fitted with a Hamamatsu camera controller and processed using Simple PCI software. Quantitative measurements of cell coverage were analyzed using the public domain ImageJ program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/) and were determined as the ratio of the number of pixels in a specific area of the well covered by cells to the total number of pixels in that area. Six areas per well were quantified. To evaluate the rate of cell growth, confluent astrocyte cultures were passaged into 24 well plates (BD-Falcon, San Jose, CA, USA) and cultured for 2 days in complete darkness (DD). On the third day, and every 24 hours thereafter, the media was replaced at ZT12 with astrocyte media alone, or with media supplemented with 10 μm, 1 μm, 100 nm, 10 nm, 1 nm, 100 pm, or 10 pm melatonin (Sigma Corp.) in dimethyl sulfoxide (DMSO). Six wells per concentration of melatonin were analyzed. Beginning on day 3, samples were taken at the time of media replacement (ZT12) every day for 6 days to observe mitotic growth by DAPI staining. Cells were fixed in 4% PFA overnight at 4°C, washed with PBS, and incubated in 4′,6-

Diamidino-2-phenylindole (DAPI) for 20 minutes. Staining was visualized on the same imaging system fitted with a DAPI filter. Images were analyzed using ImageJ and stained nuclei were counted by hand from six regions per well, each region being 2.025 mm2 in area.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA, isolated with Trizol as above and subsequently treated with DNase I (Invitrogen Corp.), was primed with random hexamers and cDNA was synthesized by reverse transcription using a Superscript II RT polymerase chain reaction (PCR) kit (Invitrogen Corp.). An additional control reaction was performed in each experimental run by replacing reverse transcriptase enzyme with water for a selected duplicate sample. Relative quantification of selected genes was achieved by performing SYBR green-based real-time PCR using an ABI Prism 7500 Fast Sequence Detection instrument following the standard curve method outlined in the user protocol from Applied Biosystems (Foster City, CA, USA). Primers optimized for SYBR green real-time PCR amplification were designed for selected genes using Primer Express software (Applied Biosystems), and synthesized at our local gene technologies laboratory. Primer sequences used are as follows: *gCry1*, forward primer: CCGGGAAACGCCCAAA and reverse primer: TGCTCTGCCGCTGGACTT; *gPer3*, forward primer: CAGAATGGAAACGATCAGCCTAT and *gPer3* reverse primer: TCGGGAGAAAACAGGAAGCA; and *gPer2* forward primer: CCCCAGTAGTTGGTGCTCACTT; *gPer2* reverse primer: GACTGGTGAGCGATACAACACTTT. Standard curves were generated from target

gene cDNAs diluted at 1:50, 1:100, 1:250, 1:500, and 1:1000. Identical standard curves

were generated for *gCyclophilinA* used here as an endogenous control. The cDNA for

each timepoint was loaded in triplicate at 1:100 dilution, amplified using a preloaded,

9600 emulation protocol, and quantified based on the standard curves generated for each

target gene. Target gene expression levels were first normalized to corresponding

endogenous control values, and the resulting value normalized to a calibrator (a mixture

of each timepoint cDNA). Each plate included a no template control set (replacing

sample with water) and a RT-control set (no reverse transcriptase added) to check

against contamination.

Statistical Analysis

Changes in 2DG uptake and of relative levels of clock gene mRNA were tested

with ANOVA. When significant changes ($P < 0.05$) were found a Newman–Keuls post

hoc test was performed on those data. Astrocyte growth studies were analyzed by one of

two methods: for the growth area study, cocultured astrocytes were compared with solo

cultures using a Student's *t*-test. For the melatonin dose–response study, the number of

DAPI-positive cells was plotted over time for each concentration. The resulting data

points were fitted to a nonlinear polynomial using SigmaPlot software (Systat Software

Inc., Richmond, CA, USA). These regressions yielded equations that contained a

coefficient of slope, and the respective slopes at each dose were compared with that of

the vehicle treated control by ANOVA. A Holm–Sidak post hoc test was performed on

significantly different data sets. All statistical tests were performed using SigmaStat

software (Systat Software Inc.).
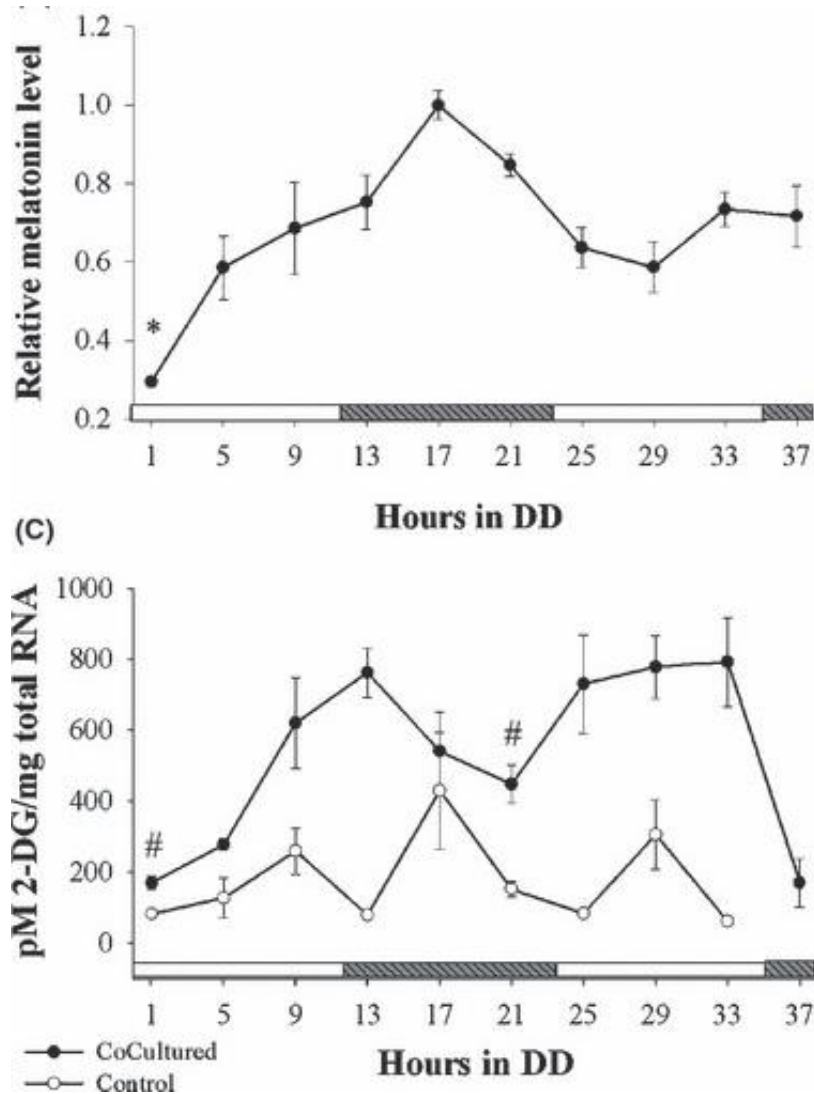
Figure 15. Rhythmic melatonin release from dispersed pinealocytes confers rhythmicity to co-cultured astrocytes. Melatonin release from cultured pinealocytes (top, n=6) remained rhythmic after 7 days of co-culture with astrocytes, with a decrease in amplitude by the second day of collection. 2-DG uptake in astrocytes (bottom n=6 per treatment) is rhythmic when cells are in co-culture (black circles), and not rhythmic in solo culture (white circles). Symbols indicate statistical significance (*=p<0.001, #=p<0.05) when compared to succeeding peak as tested by ANOVA.

4.3 Results

The rhythm of melatonin release from the pinealocytes in pineal–astrocyte cocultures persisted for 2 days of constant darkness (Figure 15, top). As expected, the first peak in melatonin levels occurred at circadian time 17, near the middle of subjective night. Two factors impeded a clear resolution of the second peak in melatonin production: the melatonin levels did not return to baseline levels after the initial peak, and the rhythm damped out by the end of the second day. This may be due to the static nature of the culture as the medium was not changed/removed during the course of the sampling. 2DG uptake from astrocytes also exhibited a circadian rhythm when cocultured with pinealocytes, but not in astrocytes cultured alone (Figure 15, bottom). On the first cycle, peak uptake coincides with increasing melatonin levels, but by the second day in DD, 2DG uptake and melatonin levels were near anti-phase to each other (29 hours in DD, Figure 15). Interestingly, the basal metabolic rate was higher in astrocytes cocultured with pinealocytes than in astrocytes cultured alone. The expression of two clock genes was profiled from astrocytes cultured alone and in coculture with pinealocytes (Figure 16). Unlike the clear metabolic rhythm, real-time PCR analysis of *gper3* and *gcry1* revealed differential effects of pineal-secreted melatonin on astrocyte gene expression. *Gper3* expression in cocultured astrocytes entrained to pineal-secreted melatonin for one cycle, with mRNA levels peaking between 13 and 17 hours and subsequently dropping before the onset of the second day (21 hours into DD, (Figure 16A). Surprisingly, *gcry1* expression in co-cultured astrocytes did not differ significantly from monocultures (Figure 16B). It was noted throughout the course of the entrainment
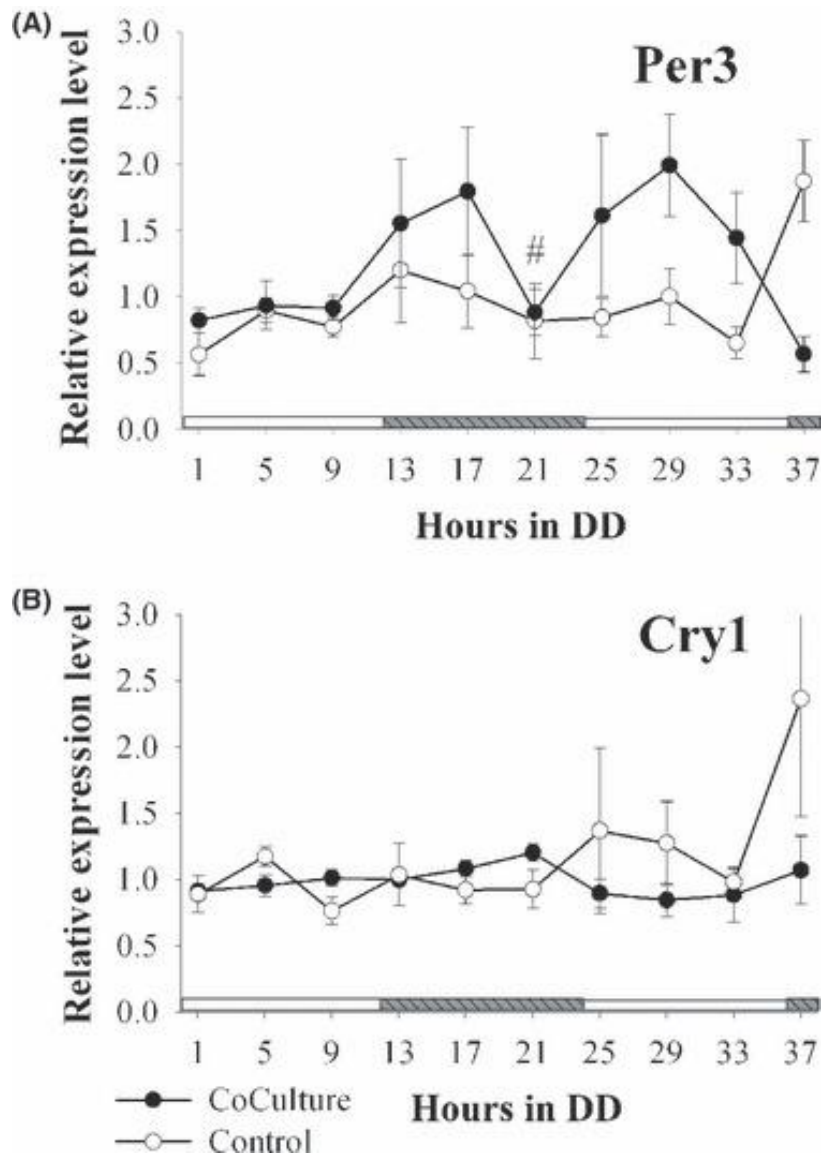
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Figure 16 Clock genes are differentially affected by melatonin cycles. *gPer3* **(A) expression in astrocytes co-cultured with pinealocytes (black circles, n = 6) showed statistically significant rhythmicity on the first day of sampling compared to solo cultures (white circles, n = 6).** *gCry1* **(bottom) expression showed no significant change in either culture condition.**

studies that the astrocytes co-cultured with pinealocytes grew to confluence much more quickly than did monocultured astrocytes. When confluence of the cultures was compared over time (Figure17A), an increase in astrocyte density was observed as early as 2 days after pinealocyte introduction. When compared with control cultures, the increase in density remained statistically greater from days 3 to 6 at which point the monolayer of astrocytes in co-culture reached 100% confluence, defined here as when cultures begin to lift off the plate for lack of space. We hypothesized that melatonin may be responsible for this increased growth rate. To test this hypothesis and determine whether the increased cell density was due to increased cell size or an increase in cell number, different doses of melatonin were administered to primary astrocytes daily at ZT12. Several cultures treated with different concentrations of melatonin were stained with DAPI and compared (Figure 17B). The number of astrocytes increased in a dose-dependent fashion (Figure 17C). While picomolar and 1 nM concentrations of melatonin had no effect on mitotic growth when compared with DMSO-treated controls (data not shown), the growth rate in cells treated with 10 nm, 1 μm, and 10 μm melatonin differed significantly from control cultures, as measured by regression analysis (Figure 17C). The rate of growth of the astrocytes, as measured by the slopes calculated from Figure 17C was also dependent on the concentration of melatonin administered throughout the 7 days of sampling (Figure 17D).
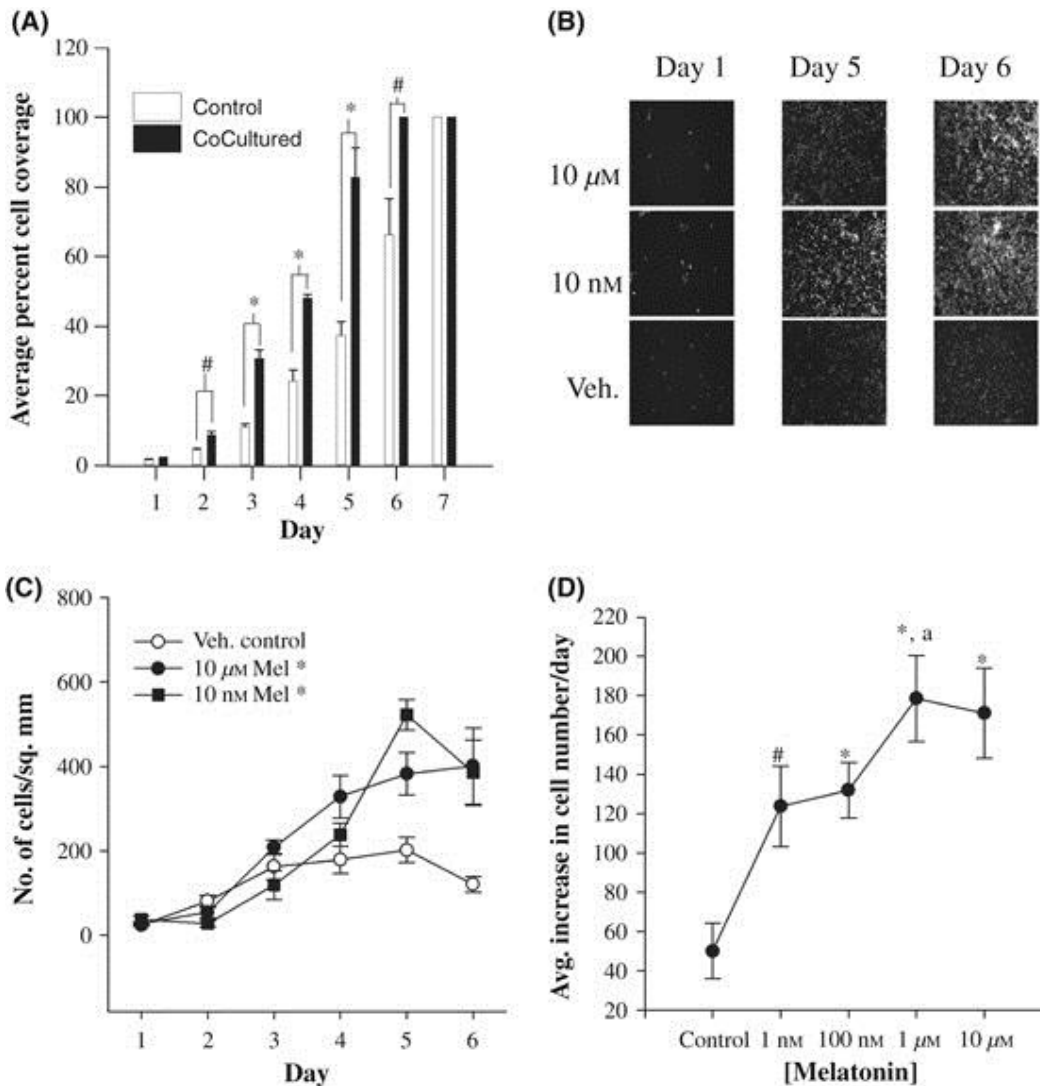
Figure 17. Exogenous melatonin stimulates mitotic growth in astrocytes. A) Cocultured astrocytes (n = 6 replicates) grew to confluence faster than monocultures, as measured by the average percent of cell coverage over six regions of interest per replicate; *P < 0.001, #P < 0.05 using t-test. B) Representative images of DAPI stained astrocytes administered 10 μm melatonin (top panel), 10 nm melatonin (middle panel), or vehicle control (bottom panel). C) Mitotic growth in astrocytes administered 10 μm melatonin (black circles), 10 nm melatonin (black squares), or DMSO control (white circles) as measured by number of DAPI stained cells on a given day; *P < 0.001 comparing slopes to vehicle control. D) Growth rates, calculated as the coefficients of slope of the respective line equations from (C), compared among cells given increasing doses of melatonin; *P < 0.001 compared with control (no melatonin), #P < 0.05 compared with control, and a P < 0.05 compared with 1 nm melatonin. Other doses are mentioned in the Materials and methods section.

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4.4 Discussion

In the present study, rhythmic exposure to melatonin synchronized rhythms in glucose uptake, corroborating and extending our previous observations that timed melatonin administration entrains and/or drives 2DG uptake rhythms (Adachi et al., 2002). Exogenous melatonin cycles also synchronized rhythms in clock gene expression and induced an increase in growth rate in cultured chick astrocytes. Consistent with the hypothesis that melatonin is an 'internal Zeitgeber' for downstream oscillators within the circadian system and in peripheral tissues (Adachi et al., 1995), precise melatonin cycles synchronized rhythms of *gper3* and *gper2* mRNA levels. This is the first demonstration that melatonin affects clock gene expression in birds, in spite of the fact that melatonin is known to profoundly affect circadian patterns of behavior and physiology in several vertebrate groups, such as birds, reptiles, and mammals, including humans (Cassone, 1998). These data stand in sharp contrast to two studies that report no role for melatonin in the regulation of clock gene expression. First, Yasuo et al. (2002) showed that while Japanese quail that received intra-peritoneal implants of crystalline melatonin in silastic capsules had elevated or constantly high levels of blood melatonin, there was no effect on *per2* or *per3* expression in the medial SCN (mSCN) of the mediobasal hypothalamus. These authors suggested that the circadian clock within the quail mSCN was 'tightly coupled,' making it immune to external signals. Secondly, Abraham et al. (2003) have shown in house sparrows that hypothalamic expression of *per2* is not affected by pinealectomy after 1 day in constant darkness. However, pinealectomized house sparrows exhibit rhythmic locomotor behavior and brain glucose utilization after 1 day

in DD (Abraham et al., 2003), and neither of these processes become arrhythmic until 10 days in DD.

Although the present study appears to contradict the previous findings, the previous studies' direct relevance to the present work is only marginal. While it is true that melatonin can acutely affect rhythmic processes in behavior (Lu and Cassone, 1993a) and physiology (Cassone et al., 1986), most of its effects on circadian and seasonal rhythms require multiple days or even weeks of rhythmic administration in vivo (Cassone et al., 1986, Cassone et al., 1988, Cassone, 1990, McArthur et al., 1991, Peters, 2005).

The fact that *gper3* expression rhythms are entrained by exogenous melatonin cycles in astrocytes is intriguing for several reasons. First, in spite of the fact that *per3* does not appear to contribute to circadian rhythm generation in mice (Adachi et al., 2002), recent studies have shown that polymorphisms in the human *per3* gene are associated with several sleep disorders, including delayed sleep phase syndrome (Bae et al., 2001). Secondly, the long transcript allele in this polymorphism is associated with preference for diurnal activity (Ebisawa et al., 2001). Along these lines, it is interesting that astrocytes are the sole source for brain glycogen biosynthesis, which has been shown to increase during sleep and anesthesia (Pereira et al., 2005). Since we have previously shown that exogenous melatonin administration increases glycogen biosynthesis in addition to synchronizing metabolic coupling in chick astrocytes (Brown, 2004), it is intriguing to speculate that regulation of this particular clock gene by melatonin is linked to birds' sleep wake cycle.

Whether or not astrocyte metabolism and clock genes are affected by melatonin in vivo is still under investigation. Several areas of the brain, such as the visual SCN, exhibit 2DG uptake rhythms such that uptake is higher during the day during the night (Adachi et al., 2002) when melatonin is present (Yasuo et al., 2002). Pinealectomy abolishes 2DG uptake rhythms in house sparrows (Klein et al., 1997), and rhythmic administration of melatonin to arrhythmic sparrows restores daily patterns of both locomotor behavior and brain 2DG uptake (Lu and Cassone, 1993a). Finally acute administration of melatonin to both house sparrows (Lu and Cassone, 1993a) and chicks (Cassone and Brooks, 1991) inhibits hypothalamic 2DG uptake. Since vertebrate astrocytes are known to mediate most glucose uptake and glycolysis in the central nervous system (Cantwell and Cassone, 2002), it is likely that these effects are at least partially mediated by these cells.

The mechanisms by which melatonin entrains *gper3* and *gper2* mRNA rhythms are less clear as we know little about the promoter regions of these genes in this species. In mammals, the *mper1* and *mper2* promoters contain bona fide $3',5'$-cyclic adenosine monophosphate (cAMP)-responsive elements (CREs) that link gene expression to dynamic changes in cellular cAMP (Magistretti, 2000). Since the known melatonin receptors in chicks are associated with G_i-guanosine triphosphate-binding proteins, (Travnickova-Bendova et al., 2002), it is possible that activation of the Mel1A and Mel1C receptors found on chick astrocytes (Reppert et al., 1995) may directly affect *gper3* expression by decreasing cellular cAMP levels. However, the mammalian *mper3* promoter does not contain a known CRE, and the times at which both *gper2* and *gper3*

mRNA peak in a melatonin cycle coincide with peak melatonin levels, making a direct regulation of these clock genes' expression through these inhibitory receptors unlikely. Alternatively, there is some controversial evidence that melatonin may also act via binding to a retinoid-related orphan receptor (RZR/ROR) intracellularly (Adachi et al., 2002). This is interesting, since ROR has recently been implicated as a regulatory element controlling mammalian *Bmal1* (Carlberg, 2000), which controls the expression of the *per* genes in all animal species studied (Sato et al., 2004).

Another question that arises from these studies is whether mammalian astrocytes are similarly sensitive to melatonin. Exogenous melatonin inhibits 2DG uptake within the rat SCN in vivo in a dose- and phase-dependent fashion (Cassone et al., 1986), and the rat SCN is replete with astrocytes (Cassone et al., 1986). Furthermore, our previous demonstration of melatonin receptors in mammalian astrocytes, (Van den Pol, 1980, Güldner, 1983) coupled with other studies demonstrating that the response of mammalian astrocytes to oxidative stress is ameliorated by melatonin (Peters et al., 2005b), indicate a possible function for melatonin in the regulation of various astrocyte functions, which could thereby affect metabolic activity in the SCN. However, Poirel et al. have shown that a single injection of melatonin to rats in the late subjective day, which is known to phase shift circadian activity patterns (Poirel et al., 2003), has no effect on SCN clock gene expression. These authors suggested that melatonin might only affect posttranscriptional events within the clock.

Remarkably, both pineal secreted and exogenously administered melatonin also affected the development of astrocytes by increasing mitotic growth in a dose-dependent

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manner (Figure 15). This observation contrasts with previous studies that report an inhibitory effect of melatonin on mitotic division of various tumor cells (Warren et al., 1993, Yasuo et al., 2002), and reviewed in (Jung and Ahmad, 2006). Perhaps, the effect of melatonin is different between neoplastic cells and diencephalic astrocytes. However, a role for melatonin as a potential developmental factor has been implicated in several systems, most notably in a mouse neural stem cell line, which expresses a functional MT1 receptor and GFAP (Niles et al., 2004). Exposure to physiological doses of melatonin in these cells induced glial-derived neurotrophic factor mRNA expression, implicating a direct link between melatonin and astrocytes in a developmental context. Another explanation for these contradictory data may lie in the concentrations of melatonin used. (Sainz et al., 2003) showed that melatonin reduces cell proliferation in Chinese hamster ovarian cells in a dose-dependent fashion, such that only millimolar concentrations were sufficient to inhibit mitotic growth. Indeed, in previous experiments low physiological concentrations of melatonin (10 fM and 10 pM) increased resting calcium levels in chick diencephalic astrocytes, whereas pharmacological doses of melatonin decreased resting calcium levels (Peters et al., 2005a) and Peters, unpublished data). More recently, nanomolar concentrations of melatonin were shown to increase the rate of in vitro development of porcine embryos (Sainz et al., 2003). Perhaps, the stimulatory effects we see here are linked to a global role for the pineal hormone in the maintenance of astrocyte function and the temporal organization of the avian brain during development.

5. CONCLUSIONS

The importance of the endogenous circadian clockworks in various physiological processes and behaviors is well-established and continues to be tested at various biological levels. However, the role of the clock in a developmental context is only now being investigated in earnest. The experiments presented above provide a comparative framework to further advance our knowledge of how this conserved mechanism may influence – or even direct – the developmental programming of early embryogenesis. Our hypothesis in chapter 2, regarding the ontogeny of mammalian circadian rhythms, focused on the earliest possible stage of development: the pluripotent embryonic stem cell. We posited that undifferentiated ESCs would not exhibit circadian rhythms, and that acute differentiation would initiate circadian expression of clock genes and glucose utilization. Coincident studies done by Yagita et al. (2010) and Kowalska et al. (2010) corroborate our findings that clock gene expression in ESCs is not rhythmic. However, glucose utilization was found to be rhythmic in the undifferentiated cells. Furthermore, the expression of glucose transporter *mglut8* was also rhythmic. The presence of a circadian rhythm exclusive of the putative molecular clockworks is not unheard of, however this study does show the earliest incidence of circadian rhythms in development. Most recently, two groups have shown that peroxiredoxin abundance is regulated in a circadian manner in the absence of any transcription (O'Neill and Reddy, 2011, O'Neill et al., 2011, Reddy and O'Neill, 2011, Edgar et al., 2012). In contrast here, there *is* a transcriptional rhythm that correlates highly with glucose utilization. Indeed, the phases of *mglut8* and 2DG content in both ESCs and dESCS would suggest a

common regulatory pathway. It is impossible to know if the transcriptional rhythm precedes the physiology in this case, as glucose levels are also known to modulate *glut* transcription in ESCs (Tonack et al., 2006). However, the lack of rhythmicity in all clock genes measured could suggest that the glucose utilization rhythm is driving the rhythm in transporter expression. Upon differentiation, all clock genes become rhythmic and 2DG rhythms increase in amplitude. Surprisingly, the phase of 2DG uptake is unchanged during this process. Further differentiation to neural progenitor cells results in maintained rhythmicity and, if the presence of harmonics is set aside, the phase of glucose utilization is still maintained. This would suggest that glucose metabolism as a process does not change during early embryogenesis, and perhaps even that this rhythm remains unmodified while individual cells are undergoing fate determination. If this is the case, one would predict that glucose utilization would be rhythmic during the retinoic-acid induced cultivation of nSCs, when *mBmal1* is not rhythmic (Yagita et al., 2010), and would maintain its phase from pluripotency throughout neural differentiation. Alternatively, the 12-hour rhythm observed in nSCs could be the result of two separate populations of cells, however the immunocytochemistry data indicate that all of the cells are committed to a neural fate.

   While the evidence above clearly shows that clock gene rhythms are not necessary for metabolic rhythmicity during early ontogenesis, the biological context of these cells is wholly removed: embryonic stem cells do not naturally grow in monolayer or as separated embryoid bodies, nor are they capable of completing development within a dish. In order to test the refined hypothesis that glucose utilization rhythms appear

early in embryogenesis and independent of putative clock genes, the domestic chick embryo was chosen as a model due to its well-characterized and mammal-like molecular clockworks (Bell-Pedersen et al., 2005). After confirming that clock genes, as well as melatonin receptors, are expressed from the earliest stages of development, it was shown that transcript abundance was not affected by light – dark cycles. Although attempts at measuring C14-labeled 2DG ultimately failed, the expression of *Glut1* transcript served as a proxy to glucose utilization rhythms in further studies. Time-series analysis from day 3 to day 5 of development under different temperature conditions revealed that, while temperature cycles can drive rhythms in clock gene and glucose transporter rhythms, those rhythms are lost upon release into constant conditions. Remarkably, the comparisons of free-running embryos vs. those in constant conditions revealed that glucose transporter expression and the expression of one melatonin receptor subtype correlate very highly, suggesting that the two processes are coupled. This hypothesis was confirmed in chapter 4 using chick embryonic astrocytes, in which rhythmic melatonin from free-running pinealocytes in coculture was able to entrain glucose utilization. These rhythms were, as in the mammalian study, not dependent upon an intact molecular clockworks, as *gCry1* was not entrained by melatonin cycles. Unfortunately, the fact that chick embryos are ectothermic until hatching is likely a major contributor to the inability of the embryos to entrain to temperature cycles. However, the data do show that the two arms of the transcriptional-translational feedback loop behave as they should when driven by temperature cycles, indicating that the clockworks are functional at such an early stage.

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The persistence of glucose utilization rhythms, as seen in mammalian ESCs and chick embryonic astrocytes, in the absence of an intact molecular clockworks would suggest that the two pathways are separable. Furthermore, the correlation between glucose transporter expression and melatonin receptor expression also points to a relationship involving circadian regulation of metabolism that is governed by a maternally – sourced signal. Lastly, melatonin itself is capable of entraining glucose utilization, but not clock genes. These three relationships share similarities despite occurring in different organisms. Melatonin is produced in uterine tissues as well as by the placenta, but these factors are removed in the case of ESC culture. Similarly, the maternal source of melatonin is constantly available – if not diminishing over time – to the chick embryo *in ovo*. It would also appear that the presence of melatonin in the egg may abrogate the need for multiple days of entrainment seen in astrocytes. If the pathways of glucose utilization and melatonin signaling are assumed to be interactive, one possible explanation of the data would be that the origin of these rhythms (or the point of entrainment) is the mitochondria.

The mitochondrion itself displays circadian rhythms of ATP accumulation and release that is mediated by rhythmic calcium signaling and is absent without a functional molecular clock (Womac et al., 2009, Burkeen et al., 2011, Marpegan et al., 2011). The loss of rhythmicity in clock gene mutants could be an immediate indicator that the molecular clockworks is solely responsible for regulating mitochondrial rhythms, but Marpegan and colleagues observed circadian rhythms in individual astrocytes taken from clock mutant mice. The luciferase reporter used in that study was linked to *Bmal1*

expression, which may provide a stronger link between mitochondrial rhythmicity and the nuclear transcriptional-translational feedback loop (Marpegan et al., 2011). The rhythmic expression of *Bmal1* is regulated at its promoter by ROR elements, which are bound by ROR and REVERB proteins to activate and inhibit transcription, respectively (Bell-Pedersen et al., 2005, Guillaumond et al., 2005). Remarkably, *Reverb-α* has been shown to modulate mitochondrial function in skeletal muscle, acting through a known pathway that links nuclear and mitochondrial gene expression (Woldt et al., 2013). That is not to say that *Reverb-a* – or any of the RORs for that matter – are the only link between the mitochondria and the molecular clockworks. Indeed, studies from multiple taxa including *Arabidopsis thaliana*, where mitochondrial genes interact with nuclear-encoded genes (Giraud et al., 2010), in *Neurospora crassa*, where resistance to a mitochondria – inhibiting antibiotic results in a shortened period (Diekmann and Brody, 1980), as well as in the mammalian SCN, where circadian expression of enolase is dependent upon functioning mitochondria (Isobe et al., 2011) show that there are multiple pathways through which such communication may occur. More specifically, however, is that several of these intersects involve redox-sensitive reactions and oxidative stress. With respect to the studies performed here, the proliferative capacity of embryonic stem cells has been shown to rely heavily on mitochondrial activity, as an increase in oxidative phosphorylation (OXPHOS) is critical for differentiation, while a switch to glycolytic activity, which decreases OXPHOS, appears to be important in cellular reprogramming (Xu et al., 2013). The latter pathway is of critical interest here, as both acute and directed differentiation, i.e. reprogramming, coincide with large

increases in glucose utilization.

The relationship between mitochondrial function and the clock within the context of redox-sensing is not limited to rhythmic mitochondrial activity. Several reports have indicated that the antioxidant properties of melatonin play a role in the daily sequestering of potentially damaging free radicals generated by mitochondrial respiration (Hardeland et al., 2003, Acuna-Castroviejo et al., 2007, Luchetti et al., 2010, Sarti et al., 2013). While some studies have shown that rhythmic mitochondrial function requires an intact molecular clockworks (Masri et al., 2013, Peek et al., 2013), the recent discovery of transcriptionally untethered rhythms in a class of peroxiredoxins (O'Neill and Reddy, 2011, O'Neill et al., 2011, Edgar et al., 2012) suggests that the mitochondria may be capable of self-sustaining rhythmicity, given the right conditions. It may be that the rhythmic availability of melatonin is capable of entraining such activity. In the data presented here using pineal melatonin, as well as in previous studies using exogenous melatonin (Adachi et al., 2002, Peters, 2005), entrainment of glucose utilization, as well as release of lactate and pyruvate, require 6 – 8 days of exposure to rhythmic melatonin to entrain. If the sites of action for melatonin are the mitochondria (incidentally also a site of lactate and pyruvate metabolism), it is plausible that several days are required to synchronize the multiple hundreds to thousands of mitochondria within each cell and then each cell to its neighboring cells, before a consolidated rhythm can be seen. Moreover, this process would be in response to the rhythmic change in redox state of the immediate environment of the mitochondria, for which the putative transcriptional oscillator is not necessary.

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While the above postulates address the results observed in ESCs and embryonic astrocytes, mitochondria also play a significant role in the transition from ectothermy to endothermy in developing birds. As demonstrated by Walter and Seebacher, the increase in mitochondrial uncoupling that is partly responsible for homeothermic transition is regulated by the action of thyroid hormone on PGC1-a, which is a known circadian controlled gene (Walter and Seebacher, 2009).

As previously stated, redox state-related circadian rhythms are broadly seen across taxa, including as yet not mentioned prokaryotes. Until recently, *Synechococcus elongatus* was the only prokaryote to exhibit demonstrable circadian rhythms, and the molecular/genetic basis of its oscillations have been well-characterized (Dunlap, 1999, Bell-Pedersen et al., 2005, Kondo, 2007, Johnson et al., 2008, Ito et al., 2009). However, the discovery of transcription-independent peroxiredoxin rhythms in eukaryotes was accompanied by the discovery of similar rhythms in cyanobacteria bacteria and one member of archaea (Edgar et al., 2012). This apparently universal rhythm is relevant to this discussion as it hearkens to the evolutionary development of circadian rhythms, as postulated by the authors. If further study into redox-state sensing mechanisms in other prokaryotes results in confirmation of this hypothesis, then it could be extrapolated that the bacterial ancestor of the mitochondria also possessed such a mechanism which would have been beneficial to the developing eukaryote. As an independent, but potentially related line of study, the data in Appendix A show a novel rhythm of motility in a human commensal bacterium, *Enterobacter aerogenes*. Although the mechanism of this rhythm is still being ascertained, it is intriguing that the

swarming response and synchronization of motility rhythms in *E. aerogenes* is directly caused by melatonin, an agent of potent antioxidant capability (Bubenik, 2002, Sainz et al., 2003, Juknat et al., 2005, Deng et al., 2015). Furthermore, the *E. aerogenes* genome contains several proteins similar to those found in the KAI complex as well as orthologues to the class of peroxiredoxins.

Although the independent rhythm of glucose utilization observed here is remarkable in its novelty, the results should be cautiously interpreted as the source of the data is wildly out of biological context. ESCs are never spread in a monolayer and, although LIF is secreted from the uterine walls to promote implantation, the developing embryo is not subjected to prolonged exposure to a differentiation-inhibitor. In order to test the hypothesis that the phase of ESC rhythms is inherited via the mitochondria of the oocyte one could use an *in vitro* preparation to establish pre- and post- fertilization phases. Relatively new and medium – throughput respiration monitoring devices such as the SeaHorse or Oroboros systems measure several outputs of mitochondrial and cellular physiology and could be used to assay sperm, and oocytes separately, and then together during the fertilization event and afterwards. This would provide a phase reference, presumably from the unfertilized oocyte, that could then be compared to the fertilized zygote. As an extension, multiple oocytes from the same donor could be synchronized at different phases and assayed simultaneously, as both of the above systems utilize multi-well platforms. Some caveats to this proposal are that the duration of time needed to measure any potential inheritance of rhythm might be longer than the commercially available systems allow. Alternatively, if the fertilization event itself is the zeitgeber,

multiple zygotes from synchronized oocytes would end up having multiple phases. This hypothesis seems less likely, as the data gathered here were cultured from different trophoblasts and the culture conditions were such that no feeding/passaging event occurred at the same time.

As stated previously, the experiments performed here are by no means definitive in defining "the beginning of time". However, they do provide concrete evidence that embryos, both avian and mammalian, are inherently endowed with the ability to perceive time of day and possess the molecular components and physiological pathways to initiate circadian timing. In the case of avian embryos, a consistent relationship was observed between glucose transporter expression and melatonin receptor expression, which was extended to show that melatonin affects circadian rhythms of glucose utilization as well as mitotic development in embryonic astrocytes. Furthermore, the evidence from these data suggest that, as seen in every other taxa, the circadian clockworks may be a requirement for anticipation of redox-state changes that are ever present in the differentiating environment of the embryo, be that *in ovo* or *in utero*. Indeed, it seems that every taxa in which circadian mechanisms are studied, there is at least one pathway that involves redox sensing, including the Cyanobacteria that do not possess mitochondria. It may be that the mitochondria, some different cytosolic machinery, or both are the true origins of circadian rhythmicity; surely, time will tell.

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Introduction

In contrast to the situation in the animal clock, which involves a transcriptional, translational feedback of "clock genes" such as *Per*, *Cry*, *Bmal1* and *Clock*, prokaryotic circadian clocks, demonstrated only in the cyanobacterium *Synechococcus elongatus*, are post-transcriptional in nature. These bacteria express circadian patterns of gene expression, photosynthesis and nitrogen fixation (Bell-Pedersen et al., 2005, Johnson et al., 2008, Mackey et al., 2011), but the molecular mechanism for this cyanobacterial clock is the result of rhythmic autokinase activity of the hexamer-forming ATPase KaiC that is enhanced by KaiA binding and subsequent autophosphatase activity of KaiC that is modulated by KaiB binding to the KaiA-KaiC complex (Kitayama et al., 2003). Remarkably, the three purified proteins, when provided free ATP, exhibit rhythmic phosphorylation of KaiC *in vitro* for many cycles (Nakajima et al., 2005). Although *S. elongatus* is the only cyanobacterium studied in much detail, the Kai proteins are found extensively within the Phylum Cyanobacteria (Axmann et al., 2014).

As stated above, vertebrate circadian organization results from the rhythmic expression of "clock genes" whose products interact in a dynamic transcription/translation feedback loop (Bell-Pedersen et al., 2005, Partch et al., 2013). "Positive elements" *Clock* and *Bmal1* are transcribed and translated in the cytoplasm,

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where they dimerize, reenter the nucleus and activate expression of genes containing an E-box element in their promoter regions. Among these, "negative elements" *Period* (*per1*, *2* and *3*) and *Cryptochrome* (*cry1* and *2*) are transcribed, translated and then feedback within the nucleus by interfering with *Clock/Bmal1* transcriptional activation (Panda et al., 2002, Bell-Pedersen et al., 2005, Partch et al., 2013). The molecular feedback loop is expressed in multiple tissues in the body, where they regulate rhythmic processes locally, but they are coordinated by pacemakers such as the hypothalamic suprachiasmatic nucleus (SCN) in mammals (Reppert and Weaver, 2002).

Among the vertebrate peripheral tissues that express circadian rhythms is the gastrointestinal system, which exhibit circadian rhythms in gene expression (including clock genes), motility and secretion *in vivo* and *in vitro* (Hoogerwerf et al., 2007, Hoogerwerf et al., 2008, Hoogerwerf et al., 2010). These rhythms depend upon a patent molecular clock, since they are abolished in *per1/per2* double knockout mice (Hoogerwerf et al., 2010). They are also coordinated by SCN input via the sympathetic nervous system (Malloy et al., 2012).

The emerging role of the gut microbiome as an important modulator of gastrointestinal function has recently included the role of circadian rhythms. Recent studies have suggested that microbial signaling plays a critical role in homeostatic maintenance of intestinal function along with the host circadian mechanism (Mukherji et al., 2013, de Kivit et al., 2014). Further studies have expanded this view and have shown that disruption of the circadian clock, either via dietary restriction or phase shifting (e.g. jet-lag) affects temporal distribution of the gut microbiome constituents

(Liang et al., 2014, Thaiss et al., 2014, Voigt et al., 2014, Zarrinpar et al., 2014). While it is clear from these studies that commensal bacteria and gut tissues do communicate, it is not clear which signaling modality or modalities the microbiome exploits to sustain its own homeostasis.

Here we present evidence for one possible modality, the indole hormone melatonin, which is present at high levels in the gut and which induces swarming activity in a clinical isolate of *Enterobacter aerogenes*. Further investigation of the motility patterns in this bacterium evinced an endogenous circadian rhythm within cultures, which is enhanced and synchronized by melatonin.

Materials and Methods

Strains and culture conditions

E. aerogenes and *E. coli* clinical isolates (gift from Dr. John Seabolt, U. of Kentucky), *K. pneumoniae* Isolate-1 (NR-15410, BEI resources, NIAID, NIH), and DH5α with *luxcdabe* driven by the promoter region of *MotA* ((Goodier and Ahmer, 2001), gift from Brian Ahmer, Ohio State University), were initially cultured in LB Broth at 37°C in a shaking incubator. Motility assays were conducted on Eosin-Methylene Blue Agar (EMB) plates (Difco, 1984) with a 50% reduction in agar to facilitate motility. All chemicals used in motility assays were purchased from Sigma (St. Louis, MO) and diluted in water.

Motility Assays

100mm petri dishes were visually divided into quadrants, filled with 30mls of EMB agar with or without specified concentration of chemicals, and allowed to dry for ~4 hours in a sterile hood. 2μl of overnight culture were stabbed and released into the center of each quadrant and allowed to grow for 48 hours at 37°C. Each plate was imaged on a light box by digital camera using qCapture Pro software (Media Cybernetics, MD) and areas measured by ImageJ (Rasband).

Transformation of MotA::luxcdabe into E. aerogenes

E. aerogenes were made competent by $CaCl_2$ method and *MotA:luxcdabe* plasmid extracted from the host strain was transformed into *E. aerogenes* by heat shock. Transformants were selected for on tetracycline-supplemented medium and stored as glycerol stocks for future studies.

Bioluminescence monitoring

2μl of overnight cultures were stabbed and released into the center of 35mm culture dishes containing 5mls of EMB agar with or without 1nM melatonin. Plates were sealed with 40mm cover glass by sterile vacuum grease and placed into an automated photomultiplier-based bioluminescence recorder (Lumicycle, Actimetrics, IL). Each sample was counted for 70 seconds on a rotating platform. Raw bioluminescence baselines were subtracted using a 24-hour running average via Lumicycle Analysis software (Actimetrics, IL). Cultures were photographed as above and used for illustrative purposes here.

Bioinformatics

Initial protein searches were performed by BLASTp (NCBI) using human

MEL1A and MEL1B protein sequences against a protein database from the curated

human microbiome project (HMP) repository (NCBI). Clock protein comparisons were

performed by PsiBLAST program (NCBI) using Uniref_50 clusters against the available

proteomes of *E. aerogenes* strains KCTC2190 and EA1509E (Uniprot.org taxonomy IDs

1028307 and 935296, respectively). Unique microbial proteins were aligned to the

original clock gene clusters using MUSCLE and trees generated by PhyML software

with 100 bootstraps.

Motif analysis

KAI complex protein sequences, including positive PsiBLAST hits above, were

entered into the online MEME suite (Bailey and Gribskov,

1998),http://meme.nbcr.net/meme) under Multiple Em for Motif Elicitation (MEME)

and subsequent Motif Alignment and Search Tool (MAST,(Bailey and Elkan, 1994)).

The output of MAST for each protein is included here in supplemental data.

Statistics

Circadian rhythmicity was determined by Circwave, Circwave Batch software

v3.3 (Oster et al., 2006) and periodogram analysis (Wichert et al., 2004). Each day of

bioluminescence recording was separated and analyzed for periods of 24 hours

(Circwave and periods between 19 and 28 hours (Circwave Batch, $p<0.02$).

Periodogram analysis was performed using R statistical program and the GeneCycle

package (Wichert et al., 2004) followed by Fisher's exact g Test to obtain p-values of

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each culture. Spread/motility measures, periods, amplitudes, and damping coefficients were compared by 1- or 2-way ANOVA, where appropriate. All analyses were performed using SigmaStat software (Systat, CA).

Results and Discussion

We hypothesized that one potential human signal that may affect gastrointestinal microbiota is the secretion of melatonin into the lumen of the gut. Although melatonin is widely regarded as a pineal and retinal neuromodulator of circadian and photoperiodic function (Acuña-Castroviejo et al., 2014, Brzozowski and Jaworek, 2014), it is present throughout the gastrointestinal system (Bubenik, 2002, Acuña-Castroviejo et al., 2014, Brzozowski and Jaworek, 2014), in part from pineal melatonin secretion (Bubenik and Brown, 1997, Reiter et al., 2011), but there is evidence for melatonin biosynthetic enzymes in biliary cholangiocytes, enterochromaffin cells and intestinal mucosa (Chen et al., 2011, Reiter et al., 2011). In addition, many foods contain melatonin (Reiter et al., 2011, Acuña-Castroviejo et al., 2014). In all, melatonin content has been reported to be 10-400x levels found in the serum (Chen et al., 2011, Reiter et al., 2011). We identified from metagenomics data in GenBank several enteric bacteria that expressed sequences with 24-42% identity to known melatonin binding sites in the human genome (Figure A1). These included receptors in *Enterobacter aerogenes*, but not in *Escherichia coli* or *Klebsiella pneumoniae*.

Colonies formed by clinical isolates of *Enterobacter aerogenes*, a Gram negative, indole-negative motile bacterium, proliferated on semi-solid Agar significantly

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more rapidly in the presence of melatonin in a specific, dose-dependent fashion, with maximal response coinciding within the physiological range of gut melatonin levels (Figures A2 and A3 A and B). This effect was specific for melatonin, as *E. aerogenes* spread further in the presence of melatonin than in the presence of equimolar concentrations of tryptophan, serotonin or N-acetylserotonin (Figure A3 D). In contrast, *Klebsiella pneumoniae,* a closely related but non-motile, indole-negative member, and *Escherichia coli,* an indole-positive but motile member of the Enterobacteriacea Family, do not respond to melatonin or the other indoles tested (Figure A3 E and Figure A4).

The larger cultures of *E. aerogenes* in the presence of melatonin exhibited patterns of swarming within the cultures, evidenced by stereotypical, concentric rings of colonies (Figure A3 A), similar to recently reported diurnal swarming in *Listeria monocytogenes* (Kaval et al., 2015) and identical to the bulls-eye pattern of swarming commonly seen in *Proteus mirabilis*, another intestinal commensal that is also in the Enterobacter family (Pearson et al., 2010). These patterns were less apparent in the smaller, control cultures of *E. aerogenes* in melatonin's absence (Figure A2). Remarkably, the number of rings consistently coincided with the number of incubation days. Calculation of banding periodicity – the number of bands visually observed divided by the number of hours of incubation – revealed a period of much greater than 24 hours in control-treated cultures. In contrast, in 1nM melatonin's presence, the period of swarming behavior was 25.1 ± 1.4 (S.D. hours (Figure A3 C).
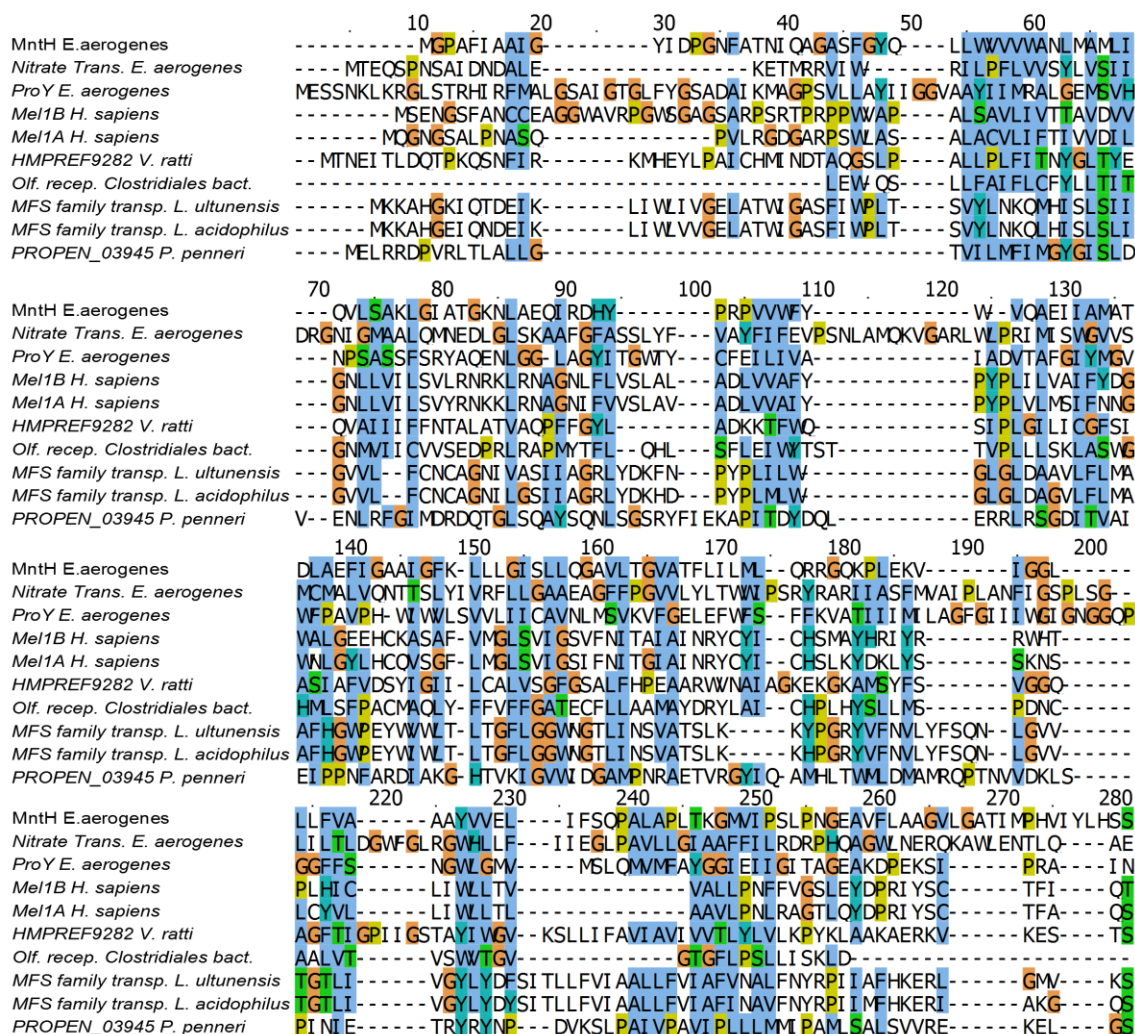
Figure A1: Multiple sequence alignment of protein BLAST hits to human MEL1A and MEL1B receptors show several areas of identity with protein sequences taken from the Human Microbiome Project (HMP). Alignments shown are a selection of positive BLAST hits (e-value < 0.001) aligned using MUSCLE that show several conserved residues and regions of high identity.
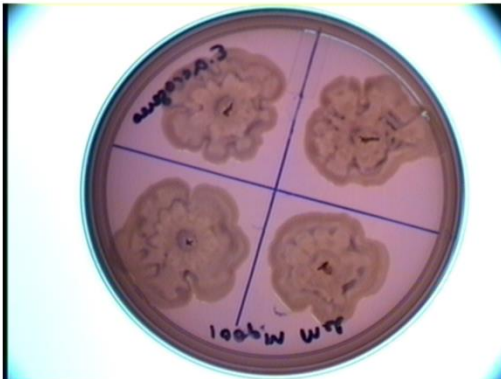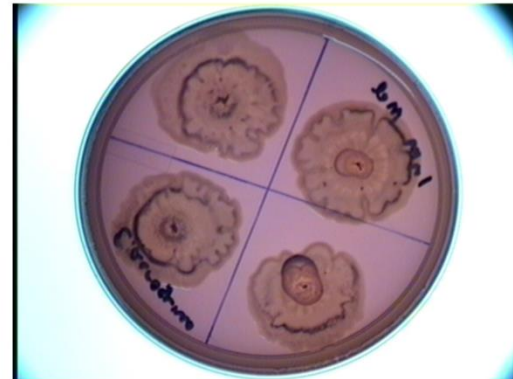
EMB Agar
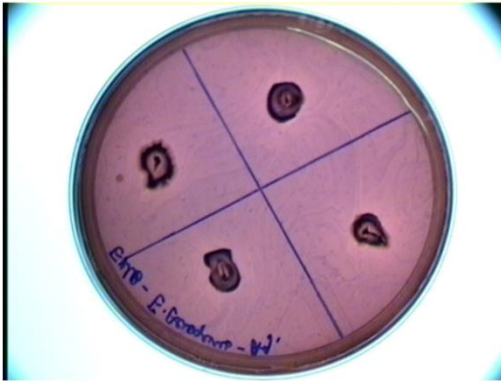
1pM Melatonin
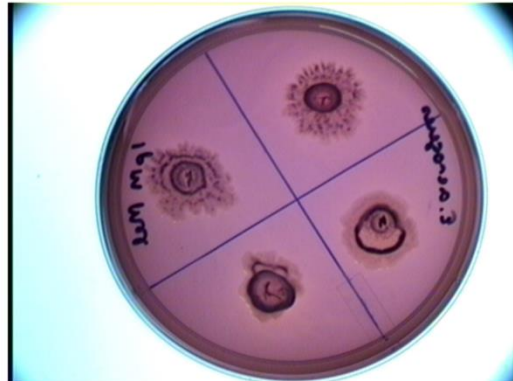
100pM Melatonin

1nM Melatonin

Figure A2: Exposure to physiological levels of melatonin induce swarming in *E. aerogenes*. 100mm EMB agar plates were inoculated with 2ul of overnight cultures (n=4/plate, replicated with 4 different starter cultures) and incubated for 48 hours. Rosette patterns of swarming increased with increasing concentrations of melatonin on the plates.
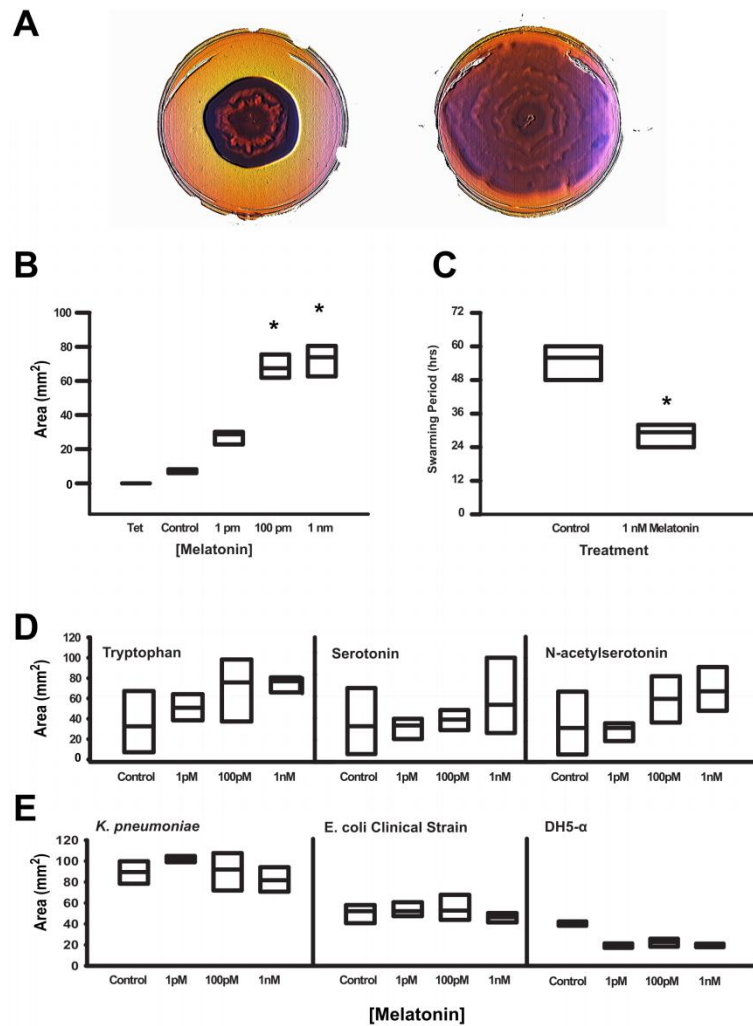
Figure A3: Swarming behavior in *E. aerogenes* is induced by melatonin and occurs with a circadian frequency. A Swarming behavior in control treated (left cultures vs. treatment with 1nM melatonin (right. Images were equally enhanced using "Bump Map" in GIMP software to highlight banding patterns. **B** The increase in swarming was only seen at 100pM and 1nM concentrations of melatonin, * = p value < 0.001 compared to vehicle treated cultures, n=16 cultures per treatment. **C** Period of swarming as calculated by the number of rings observed per culture period of 4 days in n=16 cultures per treatment, * = p value < 0.001. **D** Area of bacterial spread was unaffected by tryptophan (left, serotonin (middle and N-acetylserotonin (right, n=16 cultures per treatment. **E** Melatonin did not affect growth in *K. pneumoniae* (left or clinical or lab strains of *E. coli* (middle and right, respectively, n=16 cultures per strain per treatment.

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From the above banding period data, we hypothesized that the swarming rhythms might represent the output of a circadian clock. To test this, cultures of *E. aerogenes* were transformed to express luciferase using a *luxcdabe* construct driven by the MotA promoter ((Goodier and Ahmer, 2001), Figure A5 A). Bioluminescence from these cultures measured in a Lumicycle photomultiplier system indicated robust circadian patterns in 31-44% of cultures when maintained in temperatures ranging from ambient 27^0C to those corresponding to human body temperatures (T_B) of 34^0C, 37^0C and 40^0C (Figures A6 A-C). The circadian periods of these bioluminescence rhythms were temperature compensated with a $Q_{10} = 0.96$ from 27^0C to 40^0C. While there was no effect of melatonin on circadian period (Figure A5 C), there was a significant effect of melatonin on the phase of peak bioluminescence (Figure A5 B). In the absence of melatonin, the circadian phases of multiple replicates were highly variable. However, in the presence of 1 nM melatonin the phases of these rhythms were synchronized, especially at temperatures closely corresponding to body temperature (34-37^OC) (Figure A6 A, B). In contrast, the plasmid donor strain of DH5-α *E. coli* failed to exhibit daily patterns of bioluminescence in the presence or absence of melatonin, despite having a 5-fold higher raw bioluminescence level (Figure A5 B, C), which may be attributed to a higher plasmid copy number.
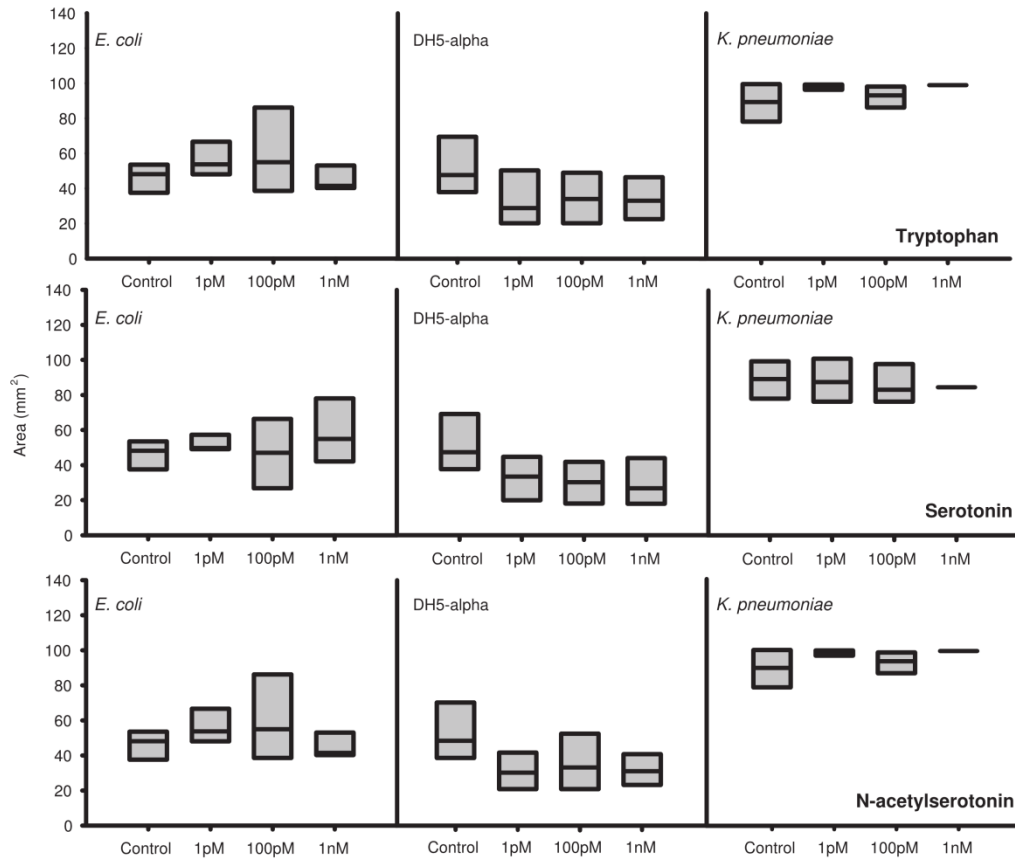
Figure A4: Neither lab nor clinical strains of *E. coli* nor *K. pneumoniae* show swarming response to other indoles. Cultures of clinical isolates of *E. coli* (left panels, DH5-α (middle panels, and *K. pneumoniae* (right panels were tested for swarming/growth in the presence of tryptophan (top row, serotonin (middle row, and N-acetylserotonin (bottom row, n=16 cultures per strain per treatment.
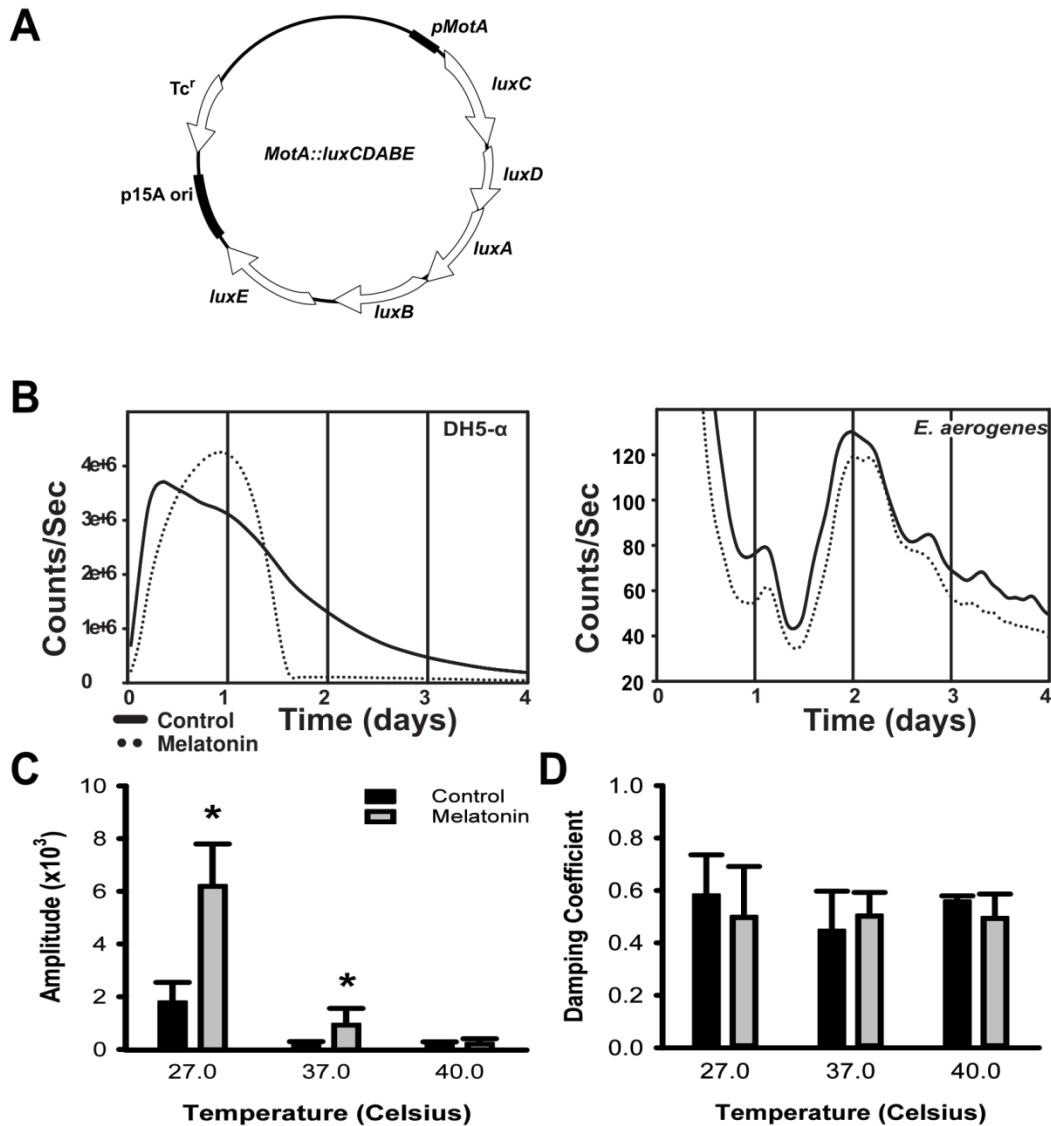
Figure A5: *MotA::luxcdabe* is expressed rhythmically in *E. aerogenes,* not in DH5-α. **A** Representative map of plasmid pRG19 showing MotA upstream of *luxcdabe* complex and tetracycline resistance. **B) DH5-α cultures (left) are not rhythmic regardless of presence of melatonin, however, raw trace of *E. aerogenes* cultures (right) transformed with *MotA::luxcdabe* plasmid show rhythmic expression with damping over time both in the presence and absence of melatonin. C) Melatonin increased the average amplitude of cultures exhibiting circadian rhythms at 27°C and 37°C, but not 40°C, * = p value < 0.05 as tested by one-way ANOVA. D) Neither temperature nor melatonin affected the damping rate of the cultures exhibiting circadian rhythms.**

This is the first demonstration of a circadian clock in a prokaryote outside Phylum Cyanobacteria. The fact that this species exists primarily as a commensal bacterium raises the possibility that the circadian clockworks driving these rhythms in *E. aerogenes* may have arisen from horizontal gene transfer of human and/or ancestral vertebrate clock genes into these bacteria (Jones, 2010, Aminov, 2011). However, comparison of the *E. aerogenes* proteome to known members of the vertebrate biological clock mechanism revealed no relationship between BMAL1, CLOCK, OR PER1 and any sequence within the *E. aerogenes* proteome (Figure A7 A, B, and C, respectively).

On the other hand, comparison of the *E. aerogenes* proteome data set to the cyanobacteria *KAIABC* complex revealed several sequences nested within trees for each of the *Kai* proteins (Figure A7 D, E, and F). Although position-specific iterated BLAST (PsiBLAST) provided significant alignments, motif-specific analysis using MEME and MAST software showed little similarity to conserved motifs within the KAI proteins (Figure A8). Despite a lack of similarity at the sequence level, there may be an underlying similarity in cellular functions of related proteins. One KaiC ortholog found here, Dephospho-Coa Kinase, is also known to act with a phosphatase in bacteria and mammals, with the latter relationship in the form of a bi-functional single enzyme (Worrall and Tubbs, 1983, Martin and Drueckhammer, 1993, Mishra et al., 2001). In *S. elongatus*, the Kai complex drives circadian rhythms of multiple processes through a post-translational molecular mechanism that persists even outside the bacterial cell;
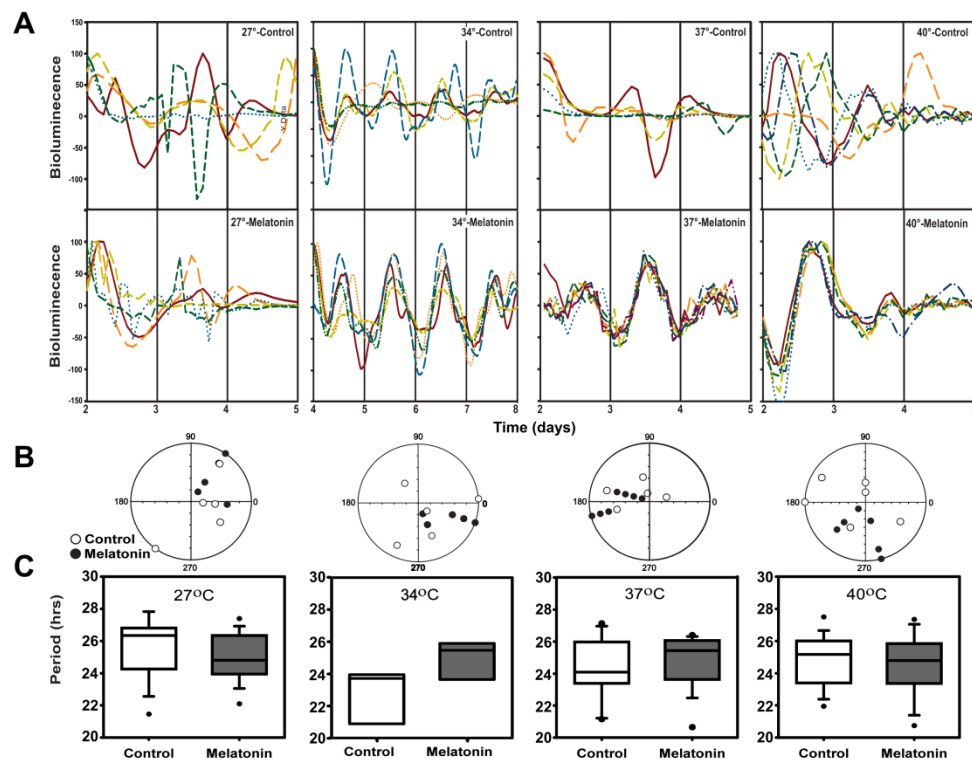
Figure A6: Bioluminescence recording of MotA::luxcdabe transformed E. aerogenes confirms a temperature compensated circadian rhythm.
A) Normalized bioluminescence rhythms from control-treated (top panels) and melatonin-treated (bottom panels) cultures show circadian rhythms at (from left to right) 27° (n=5/treatment), 34° (n=5/treatment), 37° (n=5 control and 7 melatonin-treated) and 40° (n=6 control and 6 melatonin-treated). Time scales represent days after plates were inoculated with bacteria, which varied in the amount of time needed to stabilize and begin outgrowth. B) Periodogram analysis-derived peak phases of rhythmic cultures from (A) reveal that control-treated cultures (white circles) show greater variation in peak phase than melatonin-treated cultures (black circles), which are more synchronized at all three temperatures. C) Periods of rhythms varied between 22 and 28 hours among temperature and melatonin treatments, but were not significantly affected by temperature or melatonin.
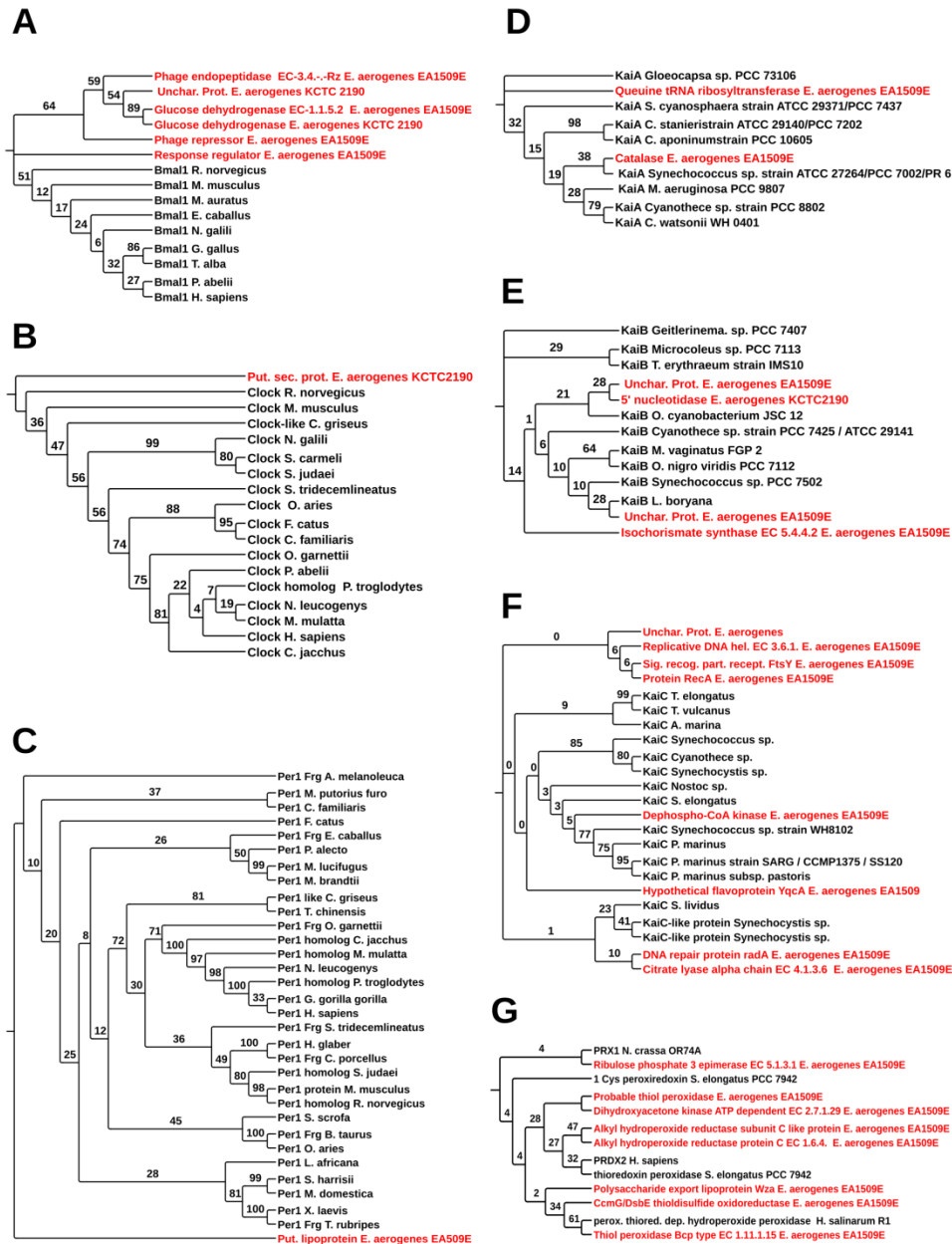
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Figure A7: Phylogenetic relationships exist between Cyanobacteria clock proteins and E. aerogenes, not vertebrate clock proteins. Bootstrapped trees (iterations shown between branches) show no homology among E. aerogenes proteins and vertebrate clock proteins BMAL1 (A), CLOCK (B), or PER1 (C). Similar analyses using Uniprot clusters of KAI A (D), KAI B (E), and KAI C (F) show potential homology with specific E. aerogenes proteins. G) E. aerogenes proteins share conservation with redox-related proteins across several taxa.

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the combination of the three Kai proteins and ATP reconstitutes a circadian pattern of

phosphorylation and dephosphorylation for many cycles *in vitro* (Nakajima et al., 2005).

The major component of the complex, KaiC, expresses both kinase and phosphatase

activities, the latter of which occurs in a manner similar to ATP synthase (Egli et al.,

2012). *In vivo*, this oscillator responds to light, temperature, and metabolic state through

the *CikA*, *LdpA*, and *Pex* pathways, each of which can entrain the Kai oscillator to

environmental cues (Kondo, 2007, Johnson et al., 2008, Mackey et al., 2011). This

relatively simple oscillator in turn regulates a wide array of processes through

transcriptional regulation (Ito et al., 2009, Hosokawa et al., 2011). Other factors must

influence this oscillator, however, since *in vivo*, the periods of multiple circadian

rhythms differ, depending on the promoter, the presence or absence of promoter

recognition subunits of RNA polymerase, and environmental conditions, including light

intensity and growth phase of the culture (Clerico et al., 2009). Our data cannot exclude

this possibility in *E. aerogenes*, as we have only examined rhythmicity as it manifests in

MotA motor protein expression, which – although an established proxy for motility – is

likely to be an output of the mechanism. Alternatively, but not exclusively, circadian

rhythms in *E. aerogenes* may derive from rhythmic peroxiredoxin activity, since this

mechanism has been identified only recently in eukaryotes as well as prokaryotes (Edgar

et al., 2012). Bioinformatics analysis reveals several sequences that share similarity to

peroxiredoxin and thiol redoxins from various taxa (Figure A7 G). Contrary to earlier

studies investigating the structure and function of KaiB and SasA proteins (Klewer et al.,

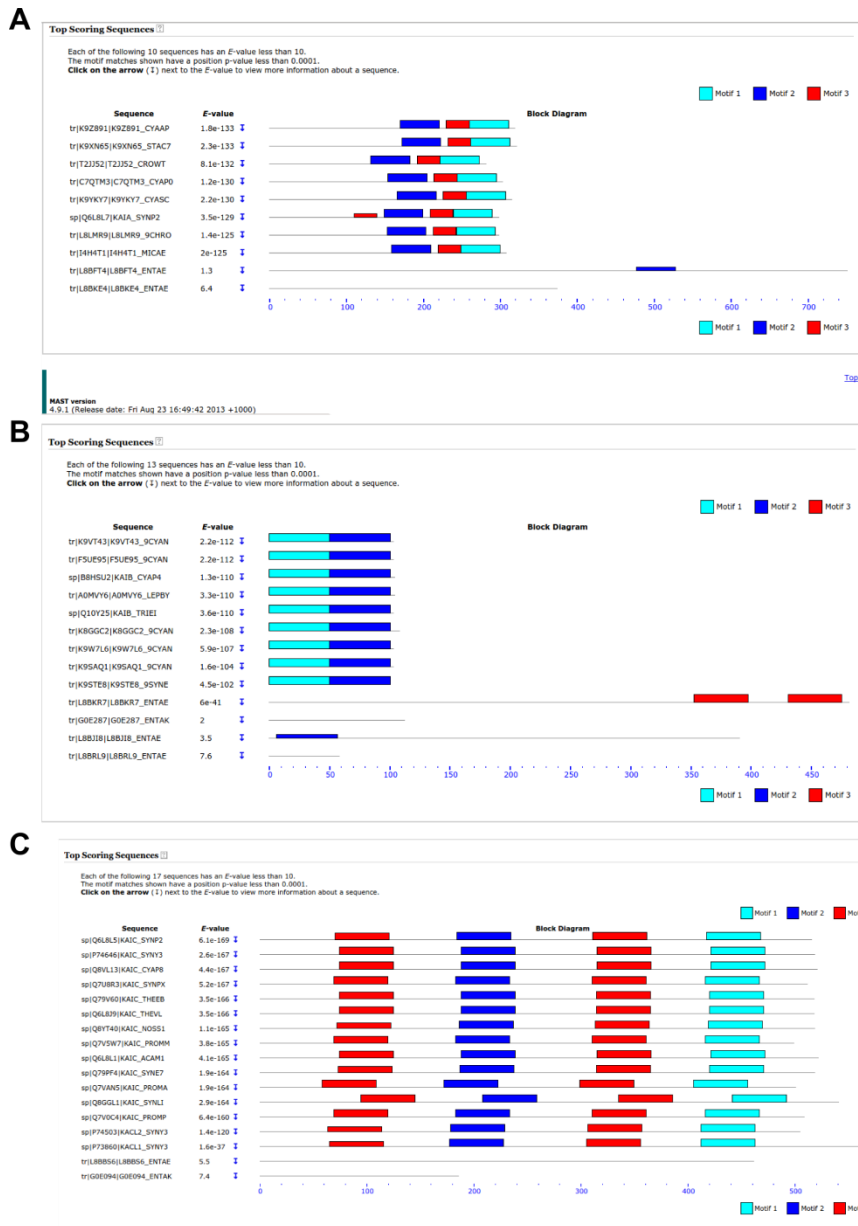Hitomi et al., 2005) our analysis showed no similarity between KaiB and thioredoxins or

Figure A8: Kai protein orthologs in E. aerogenes do not share motif-level similarity with other Kai proteins. Proteins with sequence homology via PSI-BLAST share some motif-level sequences with A) KaiA and B) KaiB, but not C) KaiC.

between KaiB orthologs and peroxiredoxin orthologs. However, the candidate proteins from our analysis are all linked to redox-sensitive pathways, including the manganese transporter MntH that initiated this investigation (Imlay, 2008). Recent reports of the anti-oxidant properties of melatonin in a neurodegenerative mouse model would support a mechanism involving melatonin and redox-state sensing (Deng et al., 2015). We are currently exploring these candidate proteins to determine the mechanism or mechanisms behind the observed rhythms.

Importantly, the present observations indicate that at least one member of the human microbiome may synchronize to its host through synchronization of an endogenous, temperature-compensated circadian clock. The detailed mechanism for this synchronization is at this stage not completely known. However, it is remarkable that the presence of melatonin in the culture medium synchronizes the periodicity and phases of multiple clonal populations across different culture plates (Figure 4). The latter phenomenon suggests melatonin as a novel source of host-commensal communication within the gut, if not the internal Zeitgeber itself. The existence of a circadian rhythm within a commensal bacterium that responds to an endocrine signal that is regulated by the circadian mechanism of the host gives further credence to the concept of the microbiome as a "meta-organism"; one with an endogenous clock that is entrained by its host's clock-driven signals. If we regard our own circadian mechanism as an evolved adaptation to environmental phenomena governed by 24-hour periods, organs and organ systems could be perceived as the entraining environment for resident microflora. As such, perturbations to the environment (i.e. circadian disruptions) will affect rhythms

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within the microbiome as previously demonstrated (Zarrinpar et al., 2014, Thaiss et al., 2015). However, it is not known whether or not, nor to what extent, the microbiome can recover from these challenges. Furthermore, the effect of host: commensal signaling is likely not relegated to one species, as we are limited to here, but to the community at large. If this phenomenon modulates quorum sensing, as is suggested by observations, there would be systemic alterations to the microbial community as a whole, as well as to the physiology of the host.