EVIDENCE OF POSSIBLE COUPLING BETWEEN CIRCADIAN OSCILLATORS OF THE MOUSE LUNG

Paula Carver

A Thesis

Submitted to the Graduate College of Bowling Green State University in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2008

Committee:

Michael Geusz, Advisor

Carmen Fioravanti

Daniel Pavuk
ABSTRACT

Michael Geusz, Advisor

Circadian rhythms in gene expression have been detected in almost every organ of mammals, including the lung. These rhythms appear to optimize daily physiological functions and are regulated by peripheral circadian oscillators in the organs. A major circadian pacemaker in the suprachiasmatic nucleus of the hypothalamus also controls the timing of the peripheral oscillators in part through the vagus nerve. This study provided evidence of possible coupling between circadian oscillators in lung tissue maintained in culture. It also tested whether acetylcholine treatments would mimic timing signals from the vagus nerve. Circadian rhythms in gene expression were monitored by imaging the bioluminescence from lung cultures made from mice that contain the mPer1::luc transgene. Acetylcholine at 50 µM had no affect on the period, phase, or luminescence intensity of the lung cultures. Maps of the period and phase of rhythms in the cultures were determined at each pixel of the bioluminescence images. The most frequent periods were 19.50 and 25.75 hours in cultures from two mice. The spatial distribution of phases found in the maps showed a clustering, and these phases were compared with the timing of the prior light cycle experienced by the mice. Phases ranged from one hour after dusk to one hour after dawn, in agreement with previous studies that identified an average peak in the lung circadian rhythm during the night. Changes in phase distributions between circadian cycles suggested that lung oscillators may interact to maintain a closely coupled state. Desynchrony between these oscillators could play a role in diseases of the human lung.
This thesis is dedicated to my parents…
ACKNOWLEDGMENTS

I would like to thank everyone who has guided and educated me as I have traveled through to get my graduate degree. I would like to thank my parents for always encouraging me and never doubting my abilities. I am grateful for Dr. Carmen Fiorvanti and Dr. Stan Smith for being not only patient with me as I worked to complete my research but also guided me as to what steps I should be taking as I continue with my work. I am thankful for Dr. Daniel Pavuk for always being encouraging and compassionate as well as being a fun teacher and willing to sit in my committee for me. I would like to thank Dr. Underwood for keeping me on tract whenever I was distracted. I would also like to thank Sima Rahman, who trained me in the practice of research back when I was an undergraduate. I would like to show my gratitude to Denise Hook, who made tail clips fun. I would also like to thank many of the graduate students in the department who were continually encouraging and enlightening (especially Liz Morgan, Rostern Tempo, Melanie Bergolc, Michelle Cook, and Matt Hoostal—thanks for the company during all of those late nights). And lastly, I would like to show my gratitude for Dr. Geusz, who continually showed patience and guidance with me and whatever project I was working on.
# TABLE OF CONTENTS

INTRODUCTION ................................................................................................................. 1

What are Circadian Rhythms? ................................................................................... 1
What Controls the Circadian Rhythm? ...................................................................... 1
What are the Peripheral Circadian Oscillators? ......................................................... 4
Circadian Coupling .................................................................................................... 5
  Coupling and entrainment................................................................................... 5
  What is desynchrony?......................................................................................... 5
Types of coupling systems.................................................................................... 6
Coupling in peripheral tissues................................................................................ 7
Alterations in phase coupling............................................................................... 8
Questions concerning coupling between pacemakers in the lung ................. 9
Role of the Vagus Nerve and Acetylcholine in the Lung Circadian System........ 9
Acetylcholine ................................................................. ........................................ 10
  Role of acetylcholine in the lung ........................................................................ 10
  Muscarinic and nicotinic cholinergic receptors ................................................. 10
  Agonists ......................................................................................................... 11
  Cholinesterase inhibitors................................................................................ 11
Circadian Rhythms Present in the Lung ............................................................ 12
Questions about the Role of Acetylcholine ....................................................... 13
Luminometry versus Imaging ................................................................................ 13
The Mice.................................................................................................................. 14
METHODS ............................................................................................................................ 16

Housing and Care of the Mice .......................................................................................... 16

Explant Preparation and Agarose Gel Technique ....................................................... 16

Method for Holding the Explants ................................................................................ 17

Slice Preparation ........................................................................................................... 17

Experiment One: Imagining Lung Explants .............................................................. 17

Experiment Two: Acetylcholine Effects on Luminometry of Lung Explants .......... 18

Experiment Three: Imaging Lung Slices ................................................................... 19

Analysis of Imaging Data ............................................................................................ 19

Analysis of Luminometry Data .................................................................................. 20

RESULTS......................................................................................................................... 21

Experiment 1: Imaging Explant Tissue...................................................................... 21

Acute imaging—light was observed throughout explant tissue in bioluminescent images.................................................................................................................................................. 21

Long-term imaging—period and phase could be measured over four to five days .................................................................................................................................................. 21

Pixel-level measurements—phase and period varied throughout the explants.................................................................................................................................................. 24

Experiment 2: Acetylcholine Treatments .................................................................. 29

Experiment 3: Imaging Tissue Slices ........................................................................ 29

Acute image—light was observed throughout explant tissue in bioluminescence images.................................................................................................................................................. 29
Long-term imaging—period and phase could be measured over four to five days.

DISCUSSION

Analysis of Explant Imaging Results

The Lomb-Scargle period estimates

Phase estimates

How maximum pixel-level measurements and total intensity results compare

Acetylcholine Results

Potential Benefits of Slice Imaging

Circadian Rhythms in the Lung

Mouse versus Human Circadian Rhythms

Timing Disorders in the Lung Affect Disease State Patterns in the Lung

Tissue Types with Unique Circadian Patterns

What this Study has Shown

REFERENCES
LIST OF FIGURES/TABLES

Figure/Table                                               Page
1  Figure 1. Whole-animal bioluminescence imaging of an anesthetized *mPer1::luc* mouse ................................................................. 18
2  Figure 2. Examples of bioluminescence from *mPer1::luc* lung explants. .............. 22
3  Table 1. Imaging Experiments .......................................................... 23
4  Table 2. Luminometry Experiments ...................................................... 23
5  Figure 3. Circadian rhythm in total luminescence from a lung tissue explant. ...... 25
6  Figure 4: Lomb-Scargle estimated period maps of the lung explants .................. 26
7  Figure 5. Phase maps of two long-term explant cultures ................................. 27
8  Figure 6. Edge of lung explant. ..................................................................... 28
9  Figure 7. Luminescence recorded from explants administered acetylcholine. ...... 30
10 Figure 8. Slice luminescence images depicting lung structure ........................... 31
INTRODUCTION

What are Circadian Rhythms?

This project examined the circadian properties of mouse lung tissue maintained in culture. Before introducing the questions addressed and the novel procedures that were employed, a brief introduction to the unique features of circadian rhythms will be provided. Principles that apply to circadian clocks in all organisms will be followed by a focus on the complexity of inter-pacemaker coupling within animals.

Circadian rhythms are the near 24-hour rhythms in physiology or behavior generated by endogenous pacemakers. These rhythms control the daily flow of hormones, immune factors, and neural signals that in turn control other rhythmic functions in the body (Hastings, 1997). A way to examine how the rhythms are patterned is to look at how much gene activity is expressed throughout the day, a method that was used extensively in this project.

Both phase and period are important properties of circadian rhythms. The period of the rhythm can be found by measuring the time between maxima when this falls within the circadian range (about 19 to 29 hours). The phase of a circadian rhythm will be considered as the time when the tissue reaches maximum transgene expression in a 24-hr interval. In addition to phase and period there are also the attributes of rhythm shape and amplitude. Amplitude is the difference between the maxima and minima. Shape refers to how the rhythm appears, whether it is a sine wave, saw-tooth wave, square wave, etc.

What Controls the Circadian Rhythm?

The circadian clock synchronizes itself to the local environmental cycles of temperature, light, or other external “Zeitgebers” (literally, “time-givers”) (Devlin, 2002). This process is
known as “entrainment”. Nearly all circadian rhythms are entrained to the light/dark cycle. The
time corresponding with this light cycle is known as Zeitgeber Time (ZT) with dawn equal to ZT 0. Dusk is at ZT 12 in a light cycle of 12 hr light and 12 hr dark (LD 12:12). Circadian
pacemakers in organisms entrain to the light cycle of the environment though adjustments of
their endogenous circadian rhythm (tau) by daily advances or delays in phase. In the entrained
state, the daily phase shift equals tau minus the period of the light cycle. When tau is less than
24 hr, a daily delay (negative phase shift) is needed to entrain to the natural daily cycle, and a tau
greater than 24 hr requires a daily phase advance.

The part of the circadian system of mammals that controls nearly all entrainment is the
suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN is the primary and, in many
ways, the master circadian pacemaker in the body. The paired SCN of rodents are located
immediately dorsal to the optic chiasm and on both sides of the ventral third ventricle. Studies
have shown that if the SCN is lesioned, most circadian rhythms will cease and arrhythmic
activity will follow (Mendelson, 2003). The lack of a functioning SCN is detrimental because
instead of having a steady sleep/wake cycle an animal with both SCN lesioned will sleep
sporadically (DeCoursey et al., 1997).

It is interesting to note that it is light pulses in the subjective night and not the subjective
day that shift the phase of rhythms in the organism (Daan et al., 1977). The subjective day and
night refer to the two halves of the organism’s circadian cycle, and these coincide with the light
and dark phases of the LD cycle during entrainment. These phases are evident even when the
organism is placed into constant experimental conditions with no timing cues. In this state, the
organism expresses tau, its free-running circadian rhythm. Tissues express their free-running
rhythms after being excised from the animal and placed into culture (ex vivo).
Circadian time (CT) refers to the phases of the circadian pacemaker in which CT 0 (or hour 24) is the start of the subjective day and CT 12 is the onset of the subjective night. It should be obvious that when an organism or tissue free-runs, without the entraining influence of a Zeitgeber, its CT deviates progressively from ZT. Similarly, jet lag occurs in human passengers on aircraft when the phase of their internal circadian clocks differs from the local time of their destination, although this phenomenon is often a large abrupt phase shift rather than the typically smaller cumulative phase shifts that occur in animals as they entrain to changing day length throughout the year. Disruptions of the human circadian system by sleep disruption, diseases, or metabolic disorders cause altered circadian phase in the organs of the body. Lung tissue is one area where alterations in circadian time can be examined in relation to impaired lung functions and associated health effects.

The way in which the circadian clocks of mammals are regulated is not completely known, however it can be noted that the transcription factor CREB (Ca\(^{2+}\)/cyclic-AMP response element binding protein) is phosphorylated in the SCN in response to light during the organism’s subjective night (Wollnik et al., 1995). Because ionotropic glutamate receptor activation is required for phase shifts in the SCN in response to light, and because these ion channels provide an influx of cell Ca\(^{2+}\), intracellular Ca\(^{2+}\) is considered an important part of the circadian system (Colwell and Menaker, 1992). Glutamate is often essential to the modulation of neuronal activities that depend on Ca\(^{2+}\) (Innocenti, 2000). Additional evidence supports a role for a circadian rhythm in Ca\(^{2+}\) in the SCN pacemaker (Allen, 2003). Intracellular Ca\(^{2+}\) is important in the lung because the neurotransmitter acetylcholine (ACh) can induce calcium signaling and this can lead to smooth muscle contraction and other effects in the lung (Bergner and Sanderson 2002). Consequently, Ca\(^{2+}\) signals from ACh release might also phase shift the circadian
pacemakers of lung tissue and serve in synchronization of the lung clock to the SCN and the rest of the body.

**What are the Peripheral Circadian Oscillators?**

Along with the SCN, there are other circadian oscillators distributed throughout the tissues of mice, humans, and other organisms (Liu et al., 2007). Circadian activity has been found in almost every organ of the body when recorded *ex vivo*, including cornea, pancreas, liver, heart, skeletal muscle, spleen, lungs and thyroid (Peschke and Peschke, 1998; Zylka et al., 1998; Bittman et al., 2003; Bando et al., 2007). It is believed that the SCN has control over the pacemakers of these tissues. Studies have shown that when fetal SCN tissue from one hamster is transplanted into another hamster that has an ablated SCN, the second hamster will start expressing the circadian period of the first hamster (Ralph and Menaker, 1988; Sujino, 2007). This result shows that the SCN is not only required for these rhythms but also provides necessary timing information by establishing the period of the transplant recipient.

There is a noted phase delay between the circadian signals seen in the SCN and the signals observed in the peripheral oscillators of the circadian system (Yamazaki et al., 2000). Because the SCN appears to regulate the phase of the other circadian pacemakers in the body, these pacemakers gradually establish a new phase relationship with environmental cycles following a phase shift, whereas the SCN entrains more rapidly because it receives entraining timing signals directly from the retina (Yamazaki et al., 1999). The coupling between the SCN and the lung, possibly through ACh release, would be responsible for the lung’s re-entrainment, thereby maintaining temporal order between the organs.
Circadian Coupling

Coupling and entrainment

Entrainment in the circadian system depends on the information that the SCN receives from external influences, such as cycles in light and darkness, ambient temperature, local acoustic signals, and the social atmosphere that is revolving around the organism. These daily signals are processed by the SCN and information regarding their occurrence is then sent out to the other peripheral circadian oscillators that regulate almost all other systems in the body. These systems take in this information and use it to regulate the way cells, tissues, and other systems in the body are maintained throughout the day.

Daily entrainment usually occurs as light signals enter through the eyes, but in some animals, such as zebrafish, light entrainment can occur by light directly entering into the tissues (Dickmeis et al., 2007). Light entrainment is especially important in regulating the activities of animals that are night active or day active because the timing of their daily sleep cycles need to shift along with the seasons (Lowrey and Takahashi, 2004).

What is desynchrony?

Desynchrony refers to an abnormal circadian rhythm that results in an altered phase or period in the system that not only deviates from what was is normally found in the system but can also result in a system showing phase as occurring in double peaks as opposed to single ones per cycle. It can be distinguished as a system where different sections have vastly differing phases. Current questions on circadian coupling address how each part of the system interacts with itself and how it also interacts with neighboring parts. One feature that must be noted is that there can be desynchrony within the same organ in the body. Molluscan eyes have been
used to study this property. For example, a 1992 report by Page and Nalovic has shown that *Bulla gouldiana* eyes cannot maintain a stable phase if one of the eyes differs in tau by about one hour from the contralateral eye. This result can be useful in understanding other circadian systems because the snail eyes are examples of a bilaterally distributed pacemaker system (Page and Navolic, 1992). Lung tissue likely contains a much more complex system with its many tissues and cell types possibly having their own unique periods and phases (Bando et al., 2007).

**Types of coupling systems**

When looking at coupling between circadian pacemakers it is important to note which kinds of coupling systems are being used. In unidirectional coupling a top-down approach is used where some cells determine the types of timing signals the cells below them will have to follow (Page and Navolic, 1992). Another system, possibly more common in lung tissue, would be the multiple coupling system that works by letting many cells within a system interact with one another.

Coupling plays an important role in the circadian system and simply refers to the interactions between circadian pacemakers that alter their phase. There are two different regulators in cell gene expression, the systemic regulatory pathways and cell autonomous ones within the cells (Dickmeis et al., 2007). The cell autonomous pathways refer to the ways that cells regulate gene expression within themselves, most notably with a negative feedback loop. The systemic regulatory pathway refers to the way that cells interact with other cells to regulate them by using neurotransmitters and hormones such as glucocorticoids (Dickmeis et al., 2007). These processes act to regulate the system in conjunction with the negative feedback loop of the
circadian clock’s molecular mechanism, e.g., though BMAL1 and CLOCK genes (Dickmeis et al., 2007).

The SCN in particular alters endocrine, locomotor, and sleep-wake rhythms to continually alter phase throughout different regions of the body (Guo et al., 2006). The SCN also regulates the daily patterns of cell proliferation by controlling the regulation of cell cycle regulatory genes (Mendoza, 2007). This mitotic regulation is most likely to limit the damage received from hazardous daytime UV light (cited in Lowrey and Takahashi, 2004). Circadian genes are expressed in nearly all tissues and despite the fact that different tissues have different circadian patterns they rely on many of the same genes (Ko and Takahashi, 2006). These circadian rhythms in gene expression are found in organisms ranging from cyanobacteria, to Drosophila, to zebrafish and the mammalian system (Bell Pederson et al., 2007).

**Coupling in peripheral tissues**

The ways that systems interact with themselves are quite varied and these mechanisms can range from what is known as cell clustering to top-down directives. In cell clustering, each cell sends out chemical signals to all of the cells around it telling those cells how it is functioning and what is going on within the cell. These signals tell the cells around the cell in question to alter their phase and they react accordingly. Each one of these cells in return sends signals out to all of the cells around them and all of the cells in turn adjust their phase. In this way all cells within a tissue or organ might work in synchrony with each other. In top-down signaling one cell sends out signals to all of the cells around it in a hierarchical manner so that they alter their circadian phase and signaling, but this cell does not adjusts its own phase by input from the target cells.
When one looks at the behavior of peripheral tissues, it is obvious that they have their own sustainable rhythms that are not necessarily tied to the sleep-wake cycle and are not constantly subjected to the demands of the SCN (Lowrey and Takahashi, 2004). That observation implies that each system in the body may have some degree of regulation of its own distinct circadian system. This organizational structure is evidenced by the different levels of robustness in some of the circadian genes they express such as the differing levels of \textit{per1} and \textit{per2} gene expression.

\textit{Alterations in phase coupling}

Sometimes, when an animal is in an unnatural condition, such as constant light, an alteration in phase occurs. This can result in a complete obliteration of any discernable phase. Another possibility is that the rhythm can show two peaks per cycle, which is seen in “phase splitting”. This phenomenon results when the left and right halves of the SCN are no longer working in conjunction with one another and each starts to work independently of the other (Indic et al., 2007). It appears to be the SCN’s way of coping with a system that is working against it, causing the SCN to continually reset itself in response to the constant influx of light.

There are also other ways to study how circadian genes interact than to record from normal animals. Animals that are missing some of the core circadian genes have been shown to have various permanent alterations in period and phase (Mezendorfer, 2007). These organisms can be observed to understand the effects of circadian genes acting on one another.
**Questions concerning coupling between pacemakers in the lung**

This project asked whether there is a single period throughout lung explant cultures or, instead, multiple rhythms with different periods. It also determined whether there are different phases in the explants and how these change over time in culture. These results will be useful in determining whether there is inter-pacemaker coupling in the lung and how coupling alters phase of the circadian pacemakers.

**Role of the Vagus Nerve and Acetylcholine in the Lung Circadian System**

The vagus nerve is the 10th cranial nerve and starts in the medulla oblongata and descends into the neck, chest, and abdomen. Studies have shown that if the vagus nerve is obliterated, circadian rhythms in lung and respiratory system tissues are eventually eliminated (Bando et al, 2007). The vagus nerve plays an important role in the parasympathetic pathways that innervate the lung (Nadel and Barnes, 1984). Bando et al. (2007) showed that vagotomy did block circadian expression. A possible explanation for this may be that the vagus nerve is the nerve that the SCN uses to send signals down to the trunk of the body. Since Acetylcholine is one of the major vagus nerves in the body, there is a possibility that this may be the transmitter that is used to send the signal.

What role does the vagus nerve serve in the mouse circadian clock system? If the vagus nerve does indeed affect the way that mper1 activity is expressed, what neurotransmitter is used? What impact do culture conditions have on the regulation and activation of circadian gene expression?
Acetylcholine

Role of acetylcholine in the lung

ACh is a major neurotransmitter of the vagus nerve (Snyder, 2006). It works to modulate various processes throughout the lung. There is evidence that many tissues of the lung—smooth muscle, monocytes, macrophages, and epithelial cells—have both muscarinic and nicotinic cholinergic receptors. (Gwilt, 2007). Acetylcholine acts on all of these tissues. It is well noted that acetylcholine has effects on the tissues because it is found to be anti-inflammatory in asthma but is pro-inflammatory in chronic obstructive pulmonary disease (COPD) (Gwilt, 2007). If acetylcholine does have an effect on the circadian functions in the lung, it likely will be an alteration in the phase and/or period in the system. The alteration could be as simple as a phase or period shift, but it could be as complex as a shutdown in circadian signals sent to the rest of the body.

Muscarinic and nicotinic cholinergic receptors

Muscarinic receptors are G-protein-coupled receptors whereas nicotinic receptors are ion channels gated by ACh binding. Both of these types of receptors have different functions. The nicotinic receptors are triggered by both ACh and nicotine, and the muscarinic receptors bind to ACh and muscarine. When a nicotinic receptor is activated by ACh there is a rapid depolarization that excites the neurons. When the muscarinic receptor is stimulated this reaction takes longer to occur and that is why these receptors are used as mediators between the sympathetic and parasympathetic nervous systems (Gwilt, 2007).

In the lung, the muscarinic receptors are the ones that stimulate the secretion of mucus. With more ACh released there is a greater number of cells activated to secrete mucus (Bando et
al., 2007). Nicotinic receptors are those that regulate inflammation by limiting the production of tumor necrosis factor (Wang, 2003). Therefore, the stimulation of the nicotinic receptors will slow down inflammation because it tells the cells not to produce any more material while the muscarinic receptors tell the cells to increase mucus production (Bando et al., 2007).

**Agonists**

An agonist is any type substance that reacts and binds with the same receptor as an endogenous (naturally occurring) ligand and triggers the same response in the cell as the ligand. In contrast, antagonists bind to the receptors in the cell and inhibit the pathway normally activated by the endogenous ligand. Typical ACh agonists are substances such as carbachol, lobeline, aceclidine, pilocarpine, and anabasine. One unique factor of these agonists is that their cellular structure is different from that of ACh so it usually takes much longer for them to be broken down. ACh antagonists are used primarily to treat the symptoms of asthma and COPD. A commonly used muscarinic antagonist is ipratropium bromide.

**Cholinesterase inhibitors**

Naturally occurring enzymes, known as cholinesterases, break down acetylcholine in the body. These cholinesterases work by catalyzing the hydrolysis of acetylcholine into choline and acetic acid. They are responsible for keeping levels of acetylcholine at a minimum ensuring that receptors do not desensitize and that signals can be sent repeatedly. Cholinesterases can be inhibited by many pesticides such as bendiocarb and diazinon. Other cholinesterase inhibitors include muscle relaxants such as neostigmine or organophosphates such as metrifonate (Ogane,
et al., 1991). In cases where the levels of acetylcholine decrease much more rapidly than they should, cholinesterase inhibitors useful.

**Circadian Rhythms Present in the Lung**

A study in 2007 from Bando et al. shows that the *mPer1* and *mPer2* mRNA levels in a Northern blot analysis of the entire respiratory system can be seen rising and falling in accordance with the regular daily activity of the organism. Their immunohistochemical staining also shows that the protein activity peaks quickly after the mRNA levels peak. Bando’s study indicates that there is a definite rhythm in mRNA levels reaching a peak at CT12 in the lung of intact mice. In addition to this result, they demonstrate that there are definite rhythms in *mPer1* and *mPer2* in mouse respiratory systems. There is also a rhythm in albumin site D-binding protein (DBP), which is a clock-controlled gene that regulates the binding rates of Vitamin D, with a peak between CT12 through CT16 (Bando et al., 2007). This shows the complex interactions between the *per* genes. Bando’s study shows that *Cry* knockouts are arrhythmic, as is expected due to the role that Cry plays in the circadian system. CRY protein functions with PER protein, and the cry gene is controlled by a negative feedback loop involving *Bmal1* and *clock* genes.

The Bando study showed that there is substantial PER2 protein activity within the alveolar and the epithelial tissue, which was confirmed by immunohistochemical staining (Bando et al., 2007). Nevertheless, Bando’s study left some unanswered questions: Why was there minimal PER2 immunohistochemical staining at hours C14 and C16 in the nuclei of the alveolar cells, tracheal submucosal cells, and bronchial epithelial cells of SCN-lesioned mice? Does this
mean that it is not the SCN alone that drives the clock of the lung? Are the separate lung and SCN oscillators decoupled?

Questions Concerning the Role of Acetylcholine

How vagal acetylcholine release may alter the phase of circadian pacemakers throughout the lung during entrainment by the SCN is not known. This study asked whether ACh induces or represses expression of the transgene in lung explant cultures. Either result would help explain how ACh might phase shift the lung pacemakers. Additional experiments might identify any phase delay or phase advance of the lung rhythms in response to acetylcholine through imaging experiments using luciferase luminescence. These experiments also provided insight into coupling between circadian pacemakers within lung tissue that could depend on ACh.

Luminometry versus Imaging

Both luminometry and imaging were used for this study. While luminometry is a good method for running multiple samples at once it does have its downsides. Oscillators that are out of phase look distorted in luminometry. This is due to the way that luminometry captures data, with an entire culture being looked at simultaneously. It means that if more than one oscillator is present in a culture, both rhythms will be distorted. Rhythms may also be masked in luminometry due to bright areas of tissue that dominate the total signal recorded. The main problem with luminometry though is that even with the tissues stationary and embedded in agarose, the source of luminescence can move around within a tissue without the observer’s knowledge. That is, there is no way of being sure what signal the imaging station is detecting. Imaging is a preferred method for looking at the tissues in that imaging can be used to look at
different areas of the same explant at the same time and this can be used to show which tissues have unique periods and phases. Imaging can test whether individual cell and tissue types have the same or varied rhythms.

The Mice

The mice used in this experiment are C57BL/6 inbred transgenic mice that contain the \textit{mPer1::luc} transgene (Figure 1). The \textit{mPer1} promoter of the transgene controls expression of the firefly luciferase gene \textit{luc} and is activated when the endogenous \textit{mPer1} gene of the animal is expressed. A glow occurs when the transgene is activated in the presence of luciferin, the substrate for luciferase. The transgenic mice were provided by Dr. Hajime Tei of the Mitsubishi Kagaku Institute of Life Sciences, Tokyo, Japan.
Figure 1. Whole-animal bioluminescence imaging of an anesthetized $mPer1$:luc mouse. Note the intensity of light from areas not blocked by fur. The mouse was injected intraperitoneally with luciferin prior to imaging.
METHODS

Housing and Care of the Mice

Animals were maintained and bred in the Bowling Green State University Animal Facility. All animal procedures were approved by the University’s Institutional Animal Care and Use Committee. The mice were maintained in a 12-hour light/12-hour dark cycle in the animal facility to entrain their circadian system. They had constant access to food and water. Light onset was at 7:00 AM and offset was at 7:00 PM. The animals used in this study were between the ages of one and eight months. Both males and females were used.

Explant Preparation and Agarose Gel Technique

All dissections took place between ZT 5:01 and 8:15. Mice were euthanized using isoflurane inhalation followed by intraperitoneal injection with 0.1 ml Nembutal. Dissections took place immediately thereafter. After the left posterior lobe of the lung was removed, one-mm square sections were trimmed away along the outer left edge and about 2/3 of the distance to the posterior end of the lung. Meanwhile, a 0.5% w/v mixture of agarose in ultrapure (MilliQ) water was prepared. This agarose was kept warm, near 37° C, using a hot plate. Next, the agarose was mixed with a 2x solution of culture medium based on Dulbecco’s Modified Eagle’s Medium (DMEM). The medium contained 10 mM Hepes buffer to maintain pH, and the bicarbonate content was adjusted for use in room air. Penicillin, streptomycin, and the serum supplement B27 were added as described previously (Yamazaki et al., 2000). Hepes buffer was used because it does not require 5% CO2 during incubation of the tissue. The lung section was placed in this 50:50 mixture of 2x medium and agarose to keep it from moving during imaging. Next 5 ml of 1X culture medium and 10 µl of a 100 mM luciferin solution in water were added to the mixture. The culture was then maintained in a 37° C incubator for 24 hours and then
examined in one of two ways—low-light imaging with a liquid nitrogen-cooled CCD camera or luminometry with a multi-well plate reader. The explants were either imaged normally or they were treated with ACh.

Method for Mounting the Explants in Culture

To prevent movement of explants during recordings, they were placed in agarose in either 96-well plates for luminometry or in a stainless steel chamber, Sykes-Moore (Bellco), with a 1.8 mm aperture for imaging. A synthetic sapphire window was used to transfer heat and prevent condensation that would have obscured images. This sealed chamber was autoclaved before each session and was put together using silicone gaskets.

Slice Preparation

In this set of experiments the whole lung was removed and a tissue chopper was used to cut slices of chilled lung tissue. The lung was placed on the chilled surface of the chopper and sectioned at a thickness of 200 or 500 µm. Because these slices were cut from the lung, they had variable widths and lengths. These slices were also embedded in agarose and were provided with the same medium that used for luminometry. The liquid nitrogen-cooled camera was used for imaging.

Experiment One:

Imaging Lung Explants

The tissue was maintained at a constant temperature of 36° C and imaged continuously for several days. Bioluminescence was captured with half-hour or one-hour exposures. There
were two ways of imaging the culture. It was either placed in a 35-mm dish while embedded in agarose or placed into the Sykes-Moore cell culture chamber. The 35-mm dish was sealed with sterile vacuum grease to a glass slide. Heaters above and below this chamber maintained the temperature.

Images were collected from above the cultures with a liquid nitrogen-cooled, back-thinned CCD camera (CH360, Roper Scientific, Tucson, AZ) and a 50-mm Nikkor f/1.2 lens (Nikon) with two close-up lenses (+10 and +4 diopter) in combination. Luminescence images were captured (either full-frame or with 2 x 2 binning) during half hour or 1-hr exposures and analyzed with V++ (Roper Scientific, Tuscon, AZ), ImageJ (NIH), and Photoshop 6.0 (Adobe, San Jose, CA) and custom Matlab software. Each 2 x 2-binned pixel captured an area of 60 by 60 µm (approximately 0.8X magnification).

For some images, a 10X microscope objective was used with the camera to provide a better view of circadian rhythms in several regions of the tissue section. The Lomb-Scargle method was used to estimate the period of the luminescence rhythms. Period and phase estimates were made at each pixel to provide information from all points of the explants.

**Experiment Two:**

**Acetylcholine Effects on Luminometry of Lung Explants**

Other lung explants were examined using a plate reader that detects the bioluminescence of many small explants kept in 24-well or 96-well plates with black walls and clear bottoms. Being able to look at multiple pieces of tissue in quick succession was beneficial because it allowed for a large number of explants to be examined at once. This approach also enabled control and experimental explants to be recorded together. After recording, the explants were
examined with an inverted microscope to see if any major changes in tissue occurred during imaging.

ACH was used as a brief stimulus and was predicted to increase *mPer1* expression and possibly shift the phase of the lung oscillators. It was expected to act for a shorter time than ACh agonists such as carbachol. The intention was to stimulate only a small phase of the circadian cycle much like how a brief pulse of light can act on the SCN to cause a phase shift at a discrete time. In these experiments the lung samples were mounted the same way as before, but 50 µM ACh was added after an initial day of luminometry readings to get a baseline so that pairs of equally luminescent wells could be identified within each dish. Luminometry was used with about 36 explants recorded per plate. Half of the viable explants, that expressed luciferase, were used as controls and given medium alone and the other half were given medium with 50 µM ACh.

**Experiment Three:**

**Imaging Lung Slices**

Thin slices of lung tissue about 200 to 500 µm-thick were imaged to determine which areas displayed transgene expression. Images were only collected the day after the slices were placed in culture. Areas corresponding to major anatomical areas of the lung were identified and compared with the luminescence images.

**Analysis of Imaging Data**

ImageJ software (NIH) was used to subtract the bias signal from images. A median filter was used to remove noise caused by cosmic ray events. The Lomb-Scargle periodogram
analysis was performed on all pixels, which provides a spectral analysis on any time series with
missing data points (van Dongen et al., 1999a; van Dongen et al., 1999b). In this case, data
points were missing primarily because of time needed to add liquid nitrogen to the camera. The
Lomb-Scargle periodogram period estimates and probabilities were calculated using a program
developed by Erik Olofsen, Hans P.A. Van Dongen, Jan H. VanHartevelt, and Erik W. Kruyt at
Leiden University Medical Center that was written for Matlab (The Mathworks, Natick, MA). A
custom Matlab program was used to estimate phase at every pixel using the time of peaks.

**Analysis of Luminometry Data**

All luminometry data were gathered on a Wallac Victor2 1420 Multilabel plate reader
(Perkin Elmer) and analyzed using Origin. All wells with signal two standard deviations above
the average background (empty) wells were used in the analysis. The percent change in signal
from the second reading after adding the treatment was calculated for all usable wells. Controls
and ACh-treated wells were averaged for each mouse. One plate was prepared per mouse. Due
to differences in the treatment times of the groups, the ZT as well as the treatment times were
compared statistically.
RESULTS

Experiment 1: Imaging Explant Tissue

Acute imaging—light was observed throughout explant tissue in bioluminescent images

Light was observed throughout all explants that were imaged (Table 1). Regions of the tissue samples varied in the level of transgene expression (Figure 2). This result suggests that there are cells that for one reason or another activate mPer1 differently. Signal was detected in the cell cultures as early as 24 hours in tissue cultures. However, with live animal imaging signal can be seen in as little as 15 minutes perhaps because of faster luciferin distribution.

Long-term imaging—period and phase could be measured over four to five days

Signal activity was routinely measured over a period of days with very little change in robustness. These signals were sustainable with signal lasting in some cases as long as a week. Tissue cultures were able to be maintained and kept alive for up to 24 days. This means that long term imaging was possible. In one case, the period was recorded from the total luminescence in all images of a time series (Figure 3). When the period at each pixel was examined, the period was mostly consistent across the tissue (Figure 4). A rhythm was detected after less than 24 hours in culture. The period of the explants was 19.5 and 25.75 hours according to Lomb-Scargle (n=2).
Figure 2. Examples of bioluminescence from mPer1::luc lung explants. Shown is the variation in size and shape of these sections trimmed from the lung. Also, note the distribution of signal across each explant.
## Table 1. Imaging Experiments

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Gender</th>
<th>Age at Dissection</th>
<th>Time of Dissection (Local Time)</th>
<th>Duration of Recordings (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Female</td>
<td>14 Days</td>
<td>1:25 PM</td>
<td>115</td>
</tr>
<tr>
<td>2</td>
<td>Female</td>
<td>18 Days</td>
<td>1:31 PM</td>
<td>49.5</td>
</tr>
<tr>
<td>3</td>
<td>Female</td>
<td>3.5 Months</td>
<td>1:57 PM</td>
<td>59</td>
</tr>
<tr>
<td>4</td>
<td>Female</td>
<td>3.5 Months</td>
<td>6:21 PM</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>Female</td>
<td>5 Months</td>
<td>1:39 PM</td>
<td>78</td>
</tr>
<tr>
<td>6</td>
<td>Male</td>
<td>7 Months</td>
<td>1:09 PM</td>
<td>66.5</td>
</tr>
<tr>
<td>7</td>
<td>Female</td>
<td>7.5 Months</td>
<td>1:30 PM</td>
<td>159</td>
</tr>
<tr>
<td>8*</td>
<td>Female</td>
<td>11.5 Months</td>
<td>3:15 PM</td>
<td>90.5</td>
</tr>
<tr>
<td>9*</td>
<td>Female</td>
<td>12 Months</td>
<td>3:11 PM</td>
<td>66.5</td>
</tr>
</tbody>
</table>

*used for phase and period maps (Figures 2 and 3)

All nine cultures showed bright luminescence during imaging

## Table 2. Luminometry Experiments

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Gender</th>
<th>Age at Dissection</th>
<th>Time of Dissection (Local Time)</th>
<th>Duration of Recordings (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Male</td>
<td>14 Days</td>
<td>1:15 PM</td>
<td>140</td>
</tr>
<tr>
<td>11</td>
<td>Male</td>
<td>23 Days</td>
<td>2:44 PM</td>
<td>169</td>
</tr>
<tr>
<td>12</td>
<td>Female</td>
<td>28 Days</td>
<td>2:22 PM</td>
<td>153.5</td>
</tr>
<tr>
<td>13</td>
<td>Female</td>
<td>1 Month</td>
<td>1:50 PM</td>
<td>2</td>
</tr>
<tr>
<td>14**</td>
<td>Female</td>
<td>1 Month</td>
<td>12:10 PM</td>
<td>48</td>
</tr>
<tr>
<td>15</td>
<td>Male</td>
<td>1 Month</td>
<td>1:48 PM</td>
<td>48</td>
</tr>
<tr>
<td>16**</td>
<td>Female</td>
<td>5 Weeks</td>
<td>1:15 PM</td>
<td>24</td>
</tr>
<tr>
<td>17**</td>
<td>Male</td>
<td>4 Months</td>
<td>12:47 PM</td>
<td>48</td>
</tr>
<tr>
<td>18</td>
<td>Male</td>
<td>8 Months</td>
<td>12:01 PM</td>
<td>48</td>
</tr>
<tr>
<td>19**</td>
<td>Female</td>
<td>8.5 Months</td>
<td>12:56 PM</td>
<td>48</td>
</tr>
<tr>
<td>20</td>
<td>Female</td>
<td>9 Months</td>
<td>2:12 PM</td>
<td>72</td>
</tr>
</tbody>
</table>

*used in acetylcholine experiment (Figure 4)

*Pixel-level measurements—phase and period varied throughout the explants*
The phase also varied between the tissues (Figure 5). The estimated phases showed that they clustered in different regions of the tissue. Phase-mapping showed clusters of phase rather than random distribution of phase. After four days of being in a culture, the tissue from Mouse 8 was able to achieve a uniform phase that had a peak at around ZT 21. In Mouse 9 the phases clustered in different regions of tissue and the phase ranged from about ZT 13 to ZT 1 throughout the recording period.

Possible growth was observed in the tissue imaged at higher magnification (Mouse 7). As time passed, the dimensions of the tissue increased because of cell division and growth or because of flattening of the tissue (Figure 6).
Figure 3. Circadian rhythm in total luminescence from a lung tissue explant. The tissue was imaged for nearly five days and a region-of-interest was selected to include the entire explant and record intensity from all pixels. The explant shows a peak between ZT 17 and ZT 19 similar to other peripheral circadian oscillators.
Figure 4: Lomb-Scargle estimated period maps of the lung explants. The period map from Mouse 8 shows a dominant period of 25.75 hours (orange region). Period map from Mouse 9 shows periods of 19.5 hours (yellow) and greater than 30 hours (red).
Figure 5. Phase maps of two long-term explant cultures. Luminescence imaging began on day 2 of culture. These cultures are from Mouse 8 and Mouse 9. Above: The phase appears to synchronize over time suggesting inter-oscillator coupling is involved. In the culture from Mouse 9 there is a uniform phase throughout the tissue that peaks at about ZT 21 by the fourth day in culture. The second culture, from Mouse 8, has phases clustered around ZT 1 (turquoise) and around ZT 4 (blue). By the third day in culture, most of the tissue showed a uniform phase near ZT 1. Black indicates areas where phase could not be determined.
**Figure 6. Edge of lung explant.** As seen in this 10X magnified brightfield image from Mouse 7 explant, the lung explant spreads out between days 2 and 7 in culture, suggesting that cell growth can modify the luminescence image. Day two growth is represented by green and day seven growth can be seen by the red. Overlapping is represented by the orange area. Note that the red extends out the most.
Experiment 2: Acetylcholine Treatments

The acetylcholine treatments had no significant effect on luminescence (Figure 7). Four of six trials had usable data (Table 2). One trial was a non-expresser, and the other culture became infected with mold shortly after imaging. The four trials used were from Mouse14, Mouse 16, Mouse 17, and Mouse 19. Three of these trials showed small changes in signal, and one trial showed a more robust difference between treated and untreated.

Experiment 3: Imaging Tissue Slices

Acute image—light was observed throughout explant tissue in bioluminescence images

The slice imaging provided sharp views of structures in the tissue that were not visible in the explants (Figure 8). For example, images from Mouse 4 show distinct structures in the lung. One can make out what appears to be the bronchus and what looks like the blood vessel system passing through the lung.

Long-term imaging—period and phase could be measured over four to five days

The next phase in this research would use slice imaging to determine the phases and periods present in each of these tissue types within the slice. It would examine any clustering of circadian period or phase present during long-term recordings.
Figure 7. Luminescence recorded from explants administered acetylcholine. The red lines are from the explants treated with ACh, and the black lines are from explants given medium alone. The difference in the percent change in signal calculated from the second reading after the treatment began is shown as an average for each mouse. There was no significant difference between the ACh and control groups (n=4 mice each).
**Figure 8.** Slice luminescence images depicting lung structure. The image on the left is from 500 µm-thick slices, and the image on the right is from 200 µm-thick slices. The thinner sections appeared to remain viable and finer structures were visible. On the left, the following objects can be seen: A. One pulmonary artery in cross section. B and C. Bronchioles.
DISCUSSION

Analysis of Explant Imaging Results

The Lomb-Scargle period estimates

As the period maps show, there are regions with distinct periods within them. After a couple of days in culture, the whole explant shows the same period throughout almost the entire tissue. This synchrony is evidence that the explant may be stabilizing itself by organizing a system wherein all tissues have the same circadian period. This ability might provide the animal an advantage in dealing with stress or diseases of the lung. Coupling of different oscillators indicates possible interaction between different types of tissue.

Phase estimates

The phase generated by these explant cultures also shows that there is a degree of clustering of phase in the tissue as well. Clustering of phase indicates that there may be inter-oscillator coupling in the lung. Clustering of phase indicates that there may be coupling within different structures in the tissue. This is significant because there are nearly whole explants that have nearly the same phase after only four days in culture. As with the period estimates, the coupling of different oscillators indicates possible interaction between different types of tissue. Lack of coupling might be used as an indicator of disease states in assays of lung tissue taken from genetically modified mice that serve as disease models.

How maximum pixel-level measurements and total intensity results compare

The results from this study show that there is something to be gained from taking the pixel measurements from all areas in the tissue. One can see that by determining when each
pixel reaches its peak a useful measure of phase throughout the whole explant can be found. Deriving the phase and period from every pixel in all images of a time-series is a much better way of determining whether or not clustering occurs. In the future, more trials will be needed to determine whether there is a relationship between age or gender of mice and their phase maps as presented here.

**Acetylcholine Results**

There could have been many factors that might explain why this experiment did not produce an effect on the luminescence signal. Other than possible experimental error, the effect on the cultures may have been too small to detect. Also, the acetylcholine may need to have another molecule present to produce an effect. The molecule may be present in the animal but may be lacking in the culture medium. The last option is that cholinesterases in the tissue degraded the ACh too quickly and limited its effects. Perhaps a longer acting acetylcholine agonist such as carbachol should be used in future experiments.

**Potential Benefits of Slice Imaging**

The main advantage of using slices in imaging is that the slices are thin enough to see distinctive structures in the images. The slices of lung tissue provide the observer with a much better way of having the structure and detail needed to accurately identify circadian rhythms in different lung tissue types. Although the thinnest slices used here were 200 µm thick, they provided an astounding amount of detail. All additional cultures should be performed in this manner to better classify phases and periods. Future acetylcholine treatments and other neurotransmitter studies should be performed on these slices. In addition, long-term imaging
studies of the lung would benefit from use of this slice technique. A tissue culture may provide more detail about the circadian rhythms in specific structures with regards to phase and period.

**Circadian Rhythms in the Lung**

Circadian patterns have been discovered in various tissues throughout the body (Peschke and Peshke, 1998; Zylka et al., 1998; Bittman et al., 2003; Bando et al., 2007). The lung is of critical importance to study because lung cancer is a major disease in society today and circadian pacemakers play a role in tumor growth.

Gwilt (2007) showed that many tissues of the lung—smooth muscle, monocytes, macrophages, and epithelial cells—had both muscarinic and nicotinic cholinergic receptors (Gwilt et al., 2007). Wang showed that there were nicotinic receptors that are regulating inflammation by limiting the production of tumor necrosis factor (Wang et al., 2003). In stark contrast to this, Bando showed that muscarinic receptors in the lung tell the epithelial cells to increase mucus production (Bando et al., 2007). Acetylcholine has effects on these tissues and gives rise to different reactions because it is found to be anti-inflammatory in asthma but is pro-inflammatory in chronic obstructive pulmonary disease (COPD) (Gwilt, 2007). Because the same neurotransmitter has very different affects and on the same tissue, it is interesting to question what role circadian genes play in the activation of these responses.

Because there are known daily rhythms in COPD and asthma symptoms, it can be assumed that the circadian rhythm that controls them can be altered to lesson the symptoms. Mice are the ideal candidates to use for this because they develop lung diseases and show symptoms of lung diseases after being exposed to the same agents that are a threat to human health (Taraseviciene-Stewart et al., 2008). For this reason, mice make such good models for
how the disease develops and how it can be treated, which is especially needed in diseases like COPD where the problem is centrally located in the severely inflamed lungs (Taraseviciene-Stewart et al., 2008).

**Mouse versus Human Circadian Rhythms**

The question of whether or not rodent tissues have circadian rhythms that are similar to humans also needs to be addressed. Despite the fact that both are mammals, there is a distinctive difference in animals that are night-active or day-active. For example, peaks and troughs in the circadian pattern are at quite different times between the two species.

**Timing Disorders in the Lung Affect Disease State Patterns in the Lung**

Timing disorders have been known to affect the way that disease patterns express themselves. Many peripheral oscillators have been shown to have altered periods and phases in older animals (Davidson, et al., 2008). This could be why so many diseases that result from a lifetime of neglect are finally present in older individuals. Lung diseases, like asthma and COPD have been shown to have times of the day when mucus build-up is at its worst and this then poses a threat to the health and well-being of the person affected (Bando, et al., 2007). Furthermore, the same neurotransmitters act on different receptors (Gwilt, 2007). If there is a way of determining when which receptors will be most active, there may be a way to silence the unneeded receptor at times when symptoms become severe.

Asthma has a circadian pattern in which the peak of the intensity is from midnight to 2:00 AM local time (Bando, et al., 2007). COPD sufferers have symptoms at about the same time
(McCarley et al., 2007; Tsai, et al., 2007). Therefore, nighttime is when problems are likely going to arise and when drugs that are more active during the night are needed.

**Tissue Types with Unique Circadian Patterns**

Because clustering was seen throughout the tissue, it can be assumed that the differences in phase that were observed may be due to properties of the different tissues present. With slice imaging, these variations according to tissue may be detected. It can be used to show the unique phases and periods visible throughout the different structures of the lung. With this method more data can be obtained on the expressed levels of circadian genes in various tissues. That would enable the researcher to look at more than the mRNA for circadian genes expressed in tissues like the epithelium, bronchi, and blood vessels in addition to the macrophages. Unique periods and phase may be found in each structure.

**What this Study has Shown**

Here, it has been made evident that there is a traceable pattern in phase and period within the lung circadian system. Better analysis of these rhythms can be performed if thin slices of lung tissue that show detailed structure within the lungs are imaged. This method may even show what tissues in the lung are the most receptive to acetylcholine. The method of looking at individual pixels to get their periods and phases can also be used with other tissues in the body. Notably the pancreas and cornea have been looked at by Sima Rahman and Aritra Battacherjee in this lab. They have seen the pancreas having nearly uniform phase and period; and the cornea having clustered phase and period (Battacherjee, 2006, Rahman, 2005). This method should quickly become the standard as it allows for quick differentiation between different tissues.
REFERENCES


