BIOLUMINESCENCE IMAGING OF TRANSGENE EXPRESSION AT THE WHOLE-MOUSE LEVEL AND IN THE MESENCEPHALIC TRIGEMINAL NUCLEUS

Daniel J. Hiler

A Dissertation

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

DOCTOR OF PHILOSOPHY

August 2009

Committee:

Michael E. Geusz, Advisor

William M. Scovell
Graduate Faculty Representative

Lee A. Meserve

Howard C. Cromwell

Robert Huber
ABSTRACT

Michael Geusz, Advisor

Bioluminescence imaging (BLI) of transgenic mice expressing the firefly luciferase gene \textit{luc} has been used to monitor continuous changes in gene expression in cultures and in the whole animal. This dissertation describes new techniques for bioluminescence imaging of gene activity at whole-animal and cellular levels. To record gene expression at the whole-animal level, the luciferase substrate luciferin is typically injected into mice prior to imaging. To avoid the effects of handling and stress from injection on expression of the transgene, a new method for delivering luciferin orally was developed and tested. Orally administered luciferin was found to be readily absorbed from the digestive tract and produced levels of bioluminescence in the whole animal that were similar to results following injection. Imaging at the cellular level focused on identifying a new model system for analyzing circadian rhythms of vertebrate neurons.

In the mammalian circadian system, multiple circadian pacemakers located throughout the body are synchronized to the external 24-hour day by a molecular circadian clock. BLI has been used to detect rhythmic expression of \textit{mPer1} and \textit{mPer2} genes in the “master” clock of the suprachiasmatic nucleus (SCN) which regulates daily physiology and behavior and also sends timing cues to peripheral oscillators outside the SCN. Because SCN neurons are small and the SCN contains many neuronal phenotypes, subcellular imaging in identifiable neurons is challenging. As a result of the limitations of the SCN, larger neurons with similar circadian and molecular properties were sought, resulting in a focus on the mesencephalic trigeminal nuclei (Me5). The Me5 are a heterogeneous group of large (30-40 \( \mu \text{m} \)) pseudo-unipolar primary sensory neurons and
multipolar interneurons that receive proprioceptive signals from spindle organs of the masseter muscles and periodontal ligaments of the teeth. Methods for Me5 cell culture and bioluminescence imaging of Me5 organotypic explants were developed using mPer1::luc and mPer2\textsuperscript{luc} transgenic mice. The period and phase of circadian rhythms in mPer1 and mPer2 gene expression in Me5 neurons were characterized. Most importantly, bioluminescence imaging and immunohistochemistry were used to provide evidence of a cell-autonomous circadian oscillator in Me5 neurons. The unique characteristics of the Me5 and its use as an alternative molecular model may provide opportunities to expand cellular studies of neural circadian pacemakers beyond the limitations of the SCN. Similarly, the ability to image bioluminescence after administering luciferin through drinking water could enable circadian rhythms in gene expressions to be monitored in various locations in animal models and more efficiently associate cellular circadian clock mechanisms with resulting animal behavior.
I dedicate this dissertation to my wife, Kate,
and to my family for their support
ACKNOWLEDGMENTS

I would like to thank several collaborators and individuals for their assistance and support throughout the past five years. First and foremost, I would like to thank Dr. Michael Geusz. His guidance and mentorship has allowed me to grow and develop into the thoughtful and innovative scientist I have strived to become. I would like to thank my committee members, Dr. Lee Meserve, Dr. Casey Cromwell, Dr. Robert Huber, and Dr. William Scovell for their assistance in developing and troubleshooting the present projects. Many thanks to the biology office staff, Lorraine DeVenney, Deb McLean, and Marsha Bostelman, for their assistance and last minute help over the years, as well as to the supply room staff, Linda Treeger and Chris Hess, for their help with travel forms and supplies for my various projects (and for the constant supply of candy). I would also like to acknowledge the animal care facility staff for their maintenance and care of the animal colony. I would like to thank the many undergraduates involved with the Geusz lab from 2004-2009 for their assistance in data collection and analysis. Finally, I would like to thank my family and friends for their encouragement, advisement, and understanding as I worked to complete my dissertation.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER 1. INTRODUCTION</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Evolutionary history of circadian clocks</td>
<td>1</td>
</tr>
<tr>
<td>Defining circadian clocks</td>
<td>3</td>
</tr>
<tr>
<td>Distinguishing circadian clocks from other oscillators</td>
<td>6</td>
</tr>
<tr>
<td>The molecular basis of the circadian clock in mammals</td>
<td>8</td>
</tr>
<tr>
<td>Bioluminescence imaging of transgenic mice expressing the gene <em>luc</em></td>
<td>10</td>
</tr>
<tr>
<td>Background information on the SCN</td>
<td>12</td>
</tr>
<tr>
<td>Background information on the mesencephalic trigeminal nuclei (Me5)</td>
<td>13</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CHAPTER 2. CIRCADIAN MPER1 GENE EXPRESSION IN MESENCEPHALIC TRIGEMINAL NUCLEUS CULTURES</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preface</td>
<td>16</td>
</tr>
<tr>
<td>Introduction</td>
<td>16</td>
</tr>
<tr>
<td>Material and Methods</td>
<td>18</td>
</tr>
<tr>
<td>Animals</td>
<td>18</td>
</tr>
<tr>
<td>Imaging brain slice cultures</td>
<td>19</td>
</tr>
<tr>
<td>Image Analysis</td>
<td>20</td>
</tr>
<tr>
<td>Results</td>
<td>22</td>
</tr>
<tr>
<td>Distribution and pattern of <em>mPer1::luc</em> expression</td>
<td>22</td>
</tr>
<tr>
<td>Period estimates of circadian rhythms</td>
<td>24</td>
</tr>
<tr>
<td>Phase estimates</td>
<td>25</td>
</tr>
<tr>
<td>Discussion</td>
<td>25</td>
</tr>
<tr>
<td>Imaging <em>mPer1::luc</em> expression in the brainstem</td>
<td>25</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Circadian period analysis</td>
<td>28</td>
</tr>
<tr>
<td>Possible circadian functions of the Me5 neurons</td>
<td>30</td>
</tr>
<tr>
<td>Me5 as a model circadian system</td>
<td>31</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>33</td>
</tr>
<tr>
<td>Figures and Tables</td>
<td>34</td>
</tr>
<tr>
<td>CHAPTER 3. CIRCADIAN GENE EXPRESSION IN INDIVIDUAL NEURONS OF</td>
<td>43</td>
</tr>
<tr>
<td>MESENCEPHALIC TRIGEMINAL NUCLEI EXPLANTS AND CELL CULTURES</td>
<td></td>
</tr>
<tr>
<td>Introduction</td>
<td>43</td>
</tr>
<tr>
<td>Material and Methods</td>
<td>46</td>
</tr>
<tr>
<td>Animals</td>
<td>46</td>
</tr>
<tr>
<td>Bioluminescence imaging</td>
<td>47</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>48</td>
</tr>
<tr>
<td>Cell Culture</td>
<td>49</td>
</tr>
<tr>
<td>Results</td>
<td>50</td>
</tr>
<tr>
<td>Growth of Me5 neurons in cell culture</td>
<td>50</td>
</tr>
<tr>
<td>Period and phase of circadian $mPer1::luc$ expression</td>
<td>51</td>
</tr>
<tr>
<td>in dispersed neurons</td>
<td></td>
</tr>
<tr>
<td>Immunolocalization of mPER1 in Me5 neurons</td>
<td>53</td>
</tr>
<tr>
<td>Period and phase of circadian $mPer2^{luc}$ expression</td>
<td>53</td>
</tr>
<tr>
<td>in organotypic cultures</td>
<td></td>
</tr>
<tr>
<td>Discussion</td>
<td>54</td>
</tr>
<tr>
<td>Me5 neurons in cell culture</td>
<td>54</td>
</tr>
<tr>
<td>Circadian $mPer1::luc$ expression in dispersed Me5 neurons</td>
<td>57</td>
</tr>
</tbody>
</table>
The distribution of mPER1-like immunoreactivity
in Me5 neurons ................................................................. 60

Circadian mPer2luc expression in Me5 organotypic cultures ........... 61

The Me5 as an alternative and complementary model to the SCN .... 62

Acknowledgements ........................................................................... 64

Figures and Tables .............................................................................. 65

CHAPTER 4. IMAGING GENE EXPRESSION IN LIVE TRANSGENIC MICE

AFTER PROVIDING LUCIFERIN IN DRINKING WATER .......... 79

Preface ............................................................................................... 79

Introduction ........................................................................................... 79

Material and Methods .............................................................................. 81

Animals .................................................................................................. 81

Bioluminescence imaging ..................................................................... 83

Results ..................................................................................................... 83

Oral administration of luciferin in anesthetized mice ....................... 83

Oral administration of luciferin in freely-moving mice ..................... 85

Discussion ............................................................................................... 86

Acknowledgements ............................................................................... 88

Figures ................................................................................................... 89

CHAPTER 5. CONCLUDING REMARKS ......................................................... 94

Additional questions regarding Me5 neurons in organotypic explants .... 94

Additional questions regarding Me5 neurons in cell dispersal ............. 95

Future work with Me5 neurons ............................................................. 96
Future work with orally administered luciferin and whole animal imaging of freely moving mice .................................................... 97

Future directions .......................................................................................... 98

REFERENCES ................................................................................................ 99
### LIST OF FIGURES/TABLES

<table>
<thead>
<tr>
<th>Figures</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chapter 1</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Double-plotted running wheel actogram of nocturnal and diurnal animals</td>
</tr>
<tr>
<td>2</td>
<td>Diagram describing the mammalian core circadian clock gene interactions</td>
</tr>
<tr>
<td>3</td>
<td>Luciferase-luciferin reaction</td>
</tr>
<tr>
<td><strong>Chapter 2</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Short-term cultures of coronal brainstem sections from ( \text{mPer1::luc} ) mice showing transgene expression in the Me5</td>
</tr>
<tr>
<td>2</td>
<td>Bright luminescence in PAG and pons corresponding with Me5</td>
</tr>
<tr>
<td>3</td>
<td>Circadian rhythms in ( \text{mPer1::luc} ) expression in Me5 of long-term explant cultures made from midbrain and pons of three different mice</td>
</tr>
<tr>
<td>4</td>
<td>Average phase of circadian rhythms in the Me5 according to four phase reference points</td>
</tr>
<tr>
<td><strong>Chapter 3</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Me5 neurons in dispersed cell culture</td>
</tr>
<tr>
<td>2</td>
<td>Bioluminescence and recovery of Me5 cells in culture</td>
</tr>
<tr>
<td>3</td>
<td>Circadian rhythms in ( \text{mPer1::luc} ) expression in dispersed Me5 cultures imaged for several days</td>
</tr>
<tr>
<td>4</td>
<td>Phase plots of circadian rhythms in dispersed Me5 neurons cultures based on four types of phase reference points</td>
</tr>
<tr>
<td>5</td>
<td>Immunohistochemistry showing mPER1-like immunoreactivity within Me5 neurons</td>
</tr>
</tbody>
</table>
6 Rhythms in \textit{mPer2}^{luc} in mouse Me5 of explant cultures persisted over several cycles ................................................................. 74

7 Lomb-Scargle analysis of rhythms in Me5 neurons of \textit{mPer2}^{luc} mice .......... 76

Chapter 4

1 Live hairless albino mice expressing firefly luciferase ........................................ 89

2 The 12 body areas used in analysis of luminescence distributions ............... 90

3 Relative luminescence in body areas of mice after oral or injected luciferin delivery. ................................................................. 91

4 \textit{Hr-CMV} imaged while mouse freely moving after oral luciferin administration ................................................................. 92

5 Control \textit{Hr-CMV} mouse imaged while freely moving after luciferin injection. 93
### Tables

#### Chapter 2
1. Rostral-to-caudal distribution of $mPer1::luc$ expression in the mesencephalic trigeminal nucleus .......................................................... 41
2. Period estimates of circadian rhythms in Me5 cell cultures .................. 42

#### Chapter 3
1. Number of Me5 neurons expressing $mPer1::luc$ during the first cycle .......... 77
2. Period estimates for $mPer1::luc$ gene expression in dispersed Me5 neurons . 78
CHAPTER 1: INTRODUCTION

Circadian biology is the study of the rhythmic circadian output of endogenous clocks present throughout nature and the mechanisms by which these rhythms influence complex physiology and behavior such as hormone levels (70), temperature (3), sleep (108), and feeding (89). In the mammalian system there exists a widely distributed circadian network consisting of the suprachiasmatic nucleus (SCN) and other peripheral circadian oscillators that provide the timing signals to regulate these complex behaviors (30). Several studies have examined the molecular properties of the mammalian circadian clock and have identified many core circadian clock genes, which are orthologous to the clock genes identified in Drosophila (7, 9, 67). Briefly, the mammalian molecular clock consists of a negative and positive autoregulatory feedback system involving the genes mPer1/2, Cry 1/2, Clock, and Bmal1 (46, 50, 125, 131, 133).

The SCN, widely considered to be the master clock of the brain, has an endogenous, temperature-compensated clock which produces a near 24-hour rhythm in core clock gene expression (63). The molecular clock in SCN neurons can be synchronized to environmental timing cues, most notably light from the retina, and can send this timing information to extra-SCN oscillators in the organism (1, 15, 43, 68, 91).

Evolutionary history of circadian clocks

In nature, daily and predictable fluctuations exist in the environment to which organisms have had a strong selective evolutionary pressure to anticipate these changes (30). Organisms anticipate these changes by coordinating their physiology or behavior and limiting when these different functions occur during the day. The most
overt rhythm in the environment is the 24-hour light-dark cycle and, indirectly, the consequential changes in temperature and humidity as a result of the earth’s rotation (110). The evolution of the circadian clock appears to have an ancient origin with geological records of cyanobacteria indicating the first known origin of a clock of approximately 3.5 billion years (28). Molecular circadian clocks are prevalent throughout Eukaryote and Bacteria domains of life, however to date, are not found in the Archaea domain. Clocks appear to have evolved several times in many different organisms through convergent evolution (30, 110).

Many theories have suggested several selective pressures on early organisms which caused the development of a highly conserved circadian clock. The “escape from ultraviolet (UV) radiation” theory suggested early organisms developed a circadian clock to anticipate the daily fluctuations of the sun’s UV radiation to protect against and limit DNA damage. These early single-cell organisms lacked any formal method of UV protection and therefore limited mitosis, specifically the S1 phase, to times at which UV radiation was less in order to protect their DNA replication from mutations (80). Another theory for cyanobacteria suggests that the selective pressure to separate incompatible chemical reactions resulted in the development of a circadian clock (28, 29, 60, 83). It was first predicted that because prokaryotes, such as cyanobacteria, lacked a nucleus and divided several times during the day they would have shorter oscillatory rhythms (29). However, because of the incompatibility of photosynthesis (oxygen producing) with nitrogen fixation (oxygen sensitive), cyanobacteria developed a circadian clock through protein phosphorylation to limit each of these reactions to separate times of day (28, 29, 60, 83). The circadian clock in cyanobacteria is considered the simplest form of
a circadian clock because it can occur in a cell-free system without the need for gene transcription (28, 29). In cyanobacteria, the 24-hour circadian clock is generated by the autophosphorylation and dephosphorylation of KaiC. Two accessory proteins, KaiA and KaiB regulate and synchronize the phosphorylation of KaiC to the solar light cycle (28, 29, 60, 80, 83). The timing cues produced by this simple circadian clock are passed on to the rest of the cell to limit photosynthesis to the day and nitrogen fixation to the night, thus separating these two incompatible biochemical pathways (80).

### Defining circadian clocks

Several terms are used to describe circadian clocks and the rhythmic output they produce. *Period* ($T$, tau) describes the length of the oscillation from one specific point on the wave to the same point occurring on the next wave; for circadian clocks the period is ~24 hours (Figure 1) (30, 84, 110). *Phase* is used to define a particular activity that occurs at the same point in a cycle from cycle-to-cycle (Figure 1) (30, 84, 110). An example of phase is the resting and sleeping activity in rodents kept on a 12-hour light and 12-hour dark cycle. Rodents, being nocturnal, are active during the dark phase and when given a running wheel, will run during their active phase and rest during the light phase; the opposite is true for diurnal organisms (99). In the above example, the regulation of the rodent’s self-sustained, endogenous circadian clock period ($T$) and phase relationship to the light-dark (LD) cycle is an example of *entrainment* (30, 84, 110). The endogenous circadian clock can entrain to widely different types of light schedules, such as a 10:14 or 8:16 LD cycle or even a skeleton photoperiod, where only a pulse of light is given at dawn and dusk (30). The environmental timing cue of
the light, which entrained the rodent’s endogenous clock to a particular phase, is called a zeitgeber, German for time giver (30, 80, 84, 110). The entrainment cue of the zeitgeber is often used as the starting point for the circadian oscillation leading to a timing scale called zeitgeber time (ZT) (30, 110). The period of the free-running endogenous clock, placed in constant darkness (DD), can also be recorded by monitoring running wheel activity of the rodent (30, 110).

Figure 1. Double-plotted running wheel actogram of nocturnal and diurnal animals. Dark lines indicate the period of activity during the LD cycle with running wheel activity occurring during the dark phase. June 11 indicates time in which animals were placed
in DD and allowed to free run. Deer mice (*P. maniculatus*), have a period shorter than 24-hours in constant darkness and begins running wheel activity earlier each day. The light zeitgeber must delay the rodent’s endogenous clock to match the LD cycle. Siberian hamster (*M. auratus*), right, has a period longer than 24-hour in constant darkness and begins running wheel activity later each day. The light zeitbeiber must advance the rodent’s endogenous clock to match the LD cycle. Figure adapted from Pittendrigh and Daan 1978, (99)

As with the rodent example, changes in the zeitgeiber produced a shift in the phase of the circadian period to entrain to the new timing cue. This shift in period of the rodent’s endogenous clock can produce an *advance*, moving the circadian period to an earlier ZT, or *delay*, moving the circadian period to a later ZT (30, 110). An example of the phase shifting effects of the circadian clock can be observed in the shifts of sleeping behaviors in humans after experiencing jet lag when traveling to a new time zone. Such is the case when traveling from the Eastern Time zone (GMT -05:00) of the US to the Pacific Time zone (GMT -08:00), a difference of three hours. Upon landing the eastern visitor will still experience behaviors, such as eating and sleeping, as if they were still on Eastern Time. However, during several days on the Pacific coast, the eastern traveler will delay their eating and sleeping behaviors to entrain to the Pacific Time zeitgeiber. Circadian clocks will respond differently to changes in the environmental zeitgeiber depending on when the clock receives a light pulse. The turning on of light, or the *non-parametric* effects, during the late day/early night produce a delay in the onset of activity while pulses of light during late night/early day will produce an advance in activity (30,
This responsiveness of the circadian clock to phase shifts can be plotted as a phase response curve (PCR) (30, 110).

Jurgen Aschoff, one of the founders of circadian biology, was particularly interested in how the duration of light affected the circadian output of the clock. Aschoff’s research into light duration and intensity showed that diurnal animals, such as humans, would display shorter periods of activity when exposed to constant light (LL) conditions, and longer periods of activity when exposed to constant darkness (DD); nocturnal animals showed the opposite effect (3). The discovery of the effects of light intensity on the circadian clock has since been described as Aschoff’s Rule (100).

Distinguishing circadian clocks from other oscillators

There are several unique properties which separate circadian clocks from mere oscillators. The cyclic nature of the output of the circadian clock is produced by an endogenous pacemaker that exists without any external timing cues from the environment (30, 110). This phenomenon was first noted in 1729 by De Mairan who first observed a daily rhythm of leaves on plants turning over during the day. When these plants were removed from light and placed in constant darkness, the leaves would continue to turn daily. Oscillations are rhythmic and can be a sustained over long periods of time, however, oscillations quickly damp and cease when the rhythmic or non-rhythmic input into the system is removed (30, 110). Pacemakers and clocks are self sustain oscillations which persist without rhythmic or non-rhythmic inputs into the system and are different from other oscillators because the rhythmic output provides timing information to other systems (30, 110). Another key component of circadian
clocks is the near 24-hour rhythm which can be entrained to external environmental timing cues. The most prevalent of these environmental timing cues are light cycles, which act as a synchronizing cue for the mammalian system (16, 91, 116, 117). However, temperature (80) and feeding (25) have been shown to influence and synchronize circadian clocks. Finally, circadian clocks are temperature compensated (30, 98, 110), in that the clock does not speed up or slow down as physiological conditions increase or decrease in temperature. Physiological reactions that are not temperature compensated roughly double their rate of the reaction with every 10°C increase in temperature (30, 110).

Colin Pittendrigh, another founder of circadian biology, demonstrated many of these circadian properties in his early work with the fruit fly, *Drosophila melanogaster* (98). Pittendrigh was particularly interested in the *Drosophila* behavior of eclosion (emergence of the adult from the pupal case) which primarily occurs during the early morning. By emerging in the early morning, adult *Drosophila* have time to allow their new, soft wings to harden before the heat of the day (7, 9, 98). After the wave of morning hatching, fly pupae from the same population wait until the following morning before undergoing another wave of eclosion. When the light-dark cycle of the pupae was changed during development, the time of eclosion entrained to the new light cycle and would occur immediately after dawn (98). The endogenous pacemaker in *Drosophila* altered the physiological state of the fly to predict changes in the environmental light cycle. Pittendrigh also demonstrated that the endogenous pacemaker controlling eclosion had a near 24-hour period when placed in constant
darkness and was temperature compensated when placed in a range of temperature conditions (7, 9).

The molecular basis of the circadian clock in mammals

The discovery of PER protein and sequential genetic components of the clock led to a greater understanding of the endogenous circadian clock by demonstrating that certain genes are necessary for specific behaviors in the organism. Ronald Konopka and Seymour Benzer discovered the *per* (period) gene in *Drosophila* while genetically screening flies exposed to DNA mutagens (67). *Drosophila* that had mutations in *per* had significant alterations to the timing of the endogenous clock and consequentially had shorter (19-hours) or longer (29-hours) periods of activity (67). Additional mutation of the *per* gene resulted in a strain of *Drosophila* which were arrhythmic. They had no defined phase of activity and behavior. Further analysis of *per* mutants in *Drosophila* and additional mutagenic genetic screens eventually led to the discovery of additional clock genes (*Timeless* (*Tim*), *Clock*, *Cycle* (*Cyc*), and the *Double-time* (*Dbt*)) gene, (7, 9, 80) and the identification of the underlying genetic mechanisms driving the circadian rhythms of the endogenous clock.

In the mammalian system, a similar set of orthologous core clock genes *mPer1/2/3, cryptochrome* (*Cry*) 1/2, *Clock, Bmal1*, and *Rev-erbα* etc.) (46, 50, 125, 131, 133) make up the genetic component of the clock. In the simplest form, mammals contain a circadian system that receives environmental timing cues from light input via the retina (91), and an entrainable circadian pacemaker in the SCN (63), that, through output pathways, sends timing cues to the rest of the body to direct physiology and
behavior (1, 43). The core clock genes and their gene products maintain the mammalian circadian pacemaker through positive and negative auto-regulatory transcription and translation feedback loops. The positive limb of the molecular clock consists of two core clock gene proteins, CLOCK and BMAL1 that are basic-helix-loop-helix (bHLH) transcription factors (80). In mammals, CLOCK protein is constitutively expressed in the cell cytoplasm (124), while BMAL1 has a peak expression level at night (54). CLOCK and BMAL1 proteins form heterodimers in the cytoplasm and undergo translocation to the nucleus where they target the E-box in the promoter region of target genes, in this case \textit{mPer1/2/3} and \textit{Cry1/2} (80). The binding of CLOCK and BMAL1 proteins to the E-box of the \textit{Per} and \textit{Cry} genes increases their gene transcription and translation (80). PER and CRY proteins, through delays imposed by transcription, translation, posttranslational modification, and phosphorylation degradation by casein kinase (CK), enters the cytoplasm and form multidimers before undergoing translocation to the nucleus and binding to the E-box in the promoter regions of their own genes, down regulating gene transcription (80, 110). \textit{Per} and \textit{cry} represent the negative limb of the molecular clock as a result of the direct inhibition of their own gene transcription by their gene products. For a more complete review of the molecular components of the circadian clock, see (80).
Figure 2. Diagram describing the mammalian core circadian clock gene interactions.
Abbreviations (for clock genes mentioned in dissertation text): PER (P), CRY (Cr), BMAL1 (B), CLOCK (C), casein kinase (CK). Gray circles represent phosphorylation.
Figure adapted from Lowery and Takahasi 2004 (80).

Bioluminescence imaging of transgenic mice expressing the gene luc

In circadian research, gene reporter systems based on the firefly luciferase gene luc, have been widely used to characterize the molecular activities of the core circadian clock genes in the SCN and peripheral oscillators. Several transgenic mouse lines have been created using the promoter for the core clock gene to monitor and record circadian rhythms in gene activity in cell culture (31, 49, 53, 127), and in explants (1, 43), and to monitor and record gradual or rhythmic changes in gene expression in whole animals.
Luciferase bioluminescence is an indirect measure of core clock genes promoter activity and provides non-invasive assays without destruction of cells or the animal. Luciferin is typically injected intravenously (iv) into whole transgenic mice via tail vein, or intraperitoneally (ip) (19, 111), or by adding mM concentrations of luciferin to the culture media (53). When luciferin, the substrate, reacts with luciferase an enzymatic light reaction occurs, producing luminescence detectable with low-light charged-coupled device (CCD) cameras (19).

$$\text{Luc} + d\text{-LH}_2 + \text{ATP} \overset{\text{Mg}^{2+}}{\longrightarrow} \text{Luc} \cdot \text{LH}_2\text{AMP} \overset{\text{O}_2}{\longrightarrow} \text{Luc} + \text{OxyL} + \text{AMP} + \text{CO}_2$$

Figure 3. Luciferase-luciferin reaction. Adapted from Geusz, M. 2001 (38)

Luciferin is light sensitive and must be kept in constant darkness or red light conditions to prevent degradation and phosphorescence when exposed to room light, which can obscure the light produced from the reaction (38). Bioluminescence imaging with luciferase has been an integral part in characterizing the molecular circadian clock through transgenic mouse models (19, 38). With the improvement of camera and detection technology, along with the creation of core clock gene knockout/knockin mice, bioluminescence imaging is advancing the field and providing more reliable and more rapid methods of directly detecting changes in gene expression (105). Bioluminescence imaging with luciferase has evolved from a simple bioreporter system of genes and is currently being used to monitor the progression of disease in rodents (21, 22, 57).
Background information on the SCN

The SCN has been identified as the “master” circadian clock (63) that provides the timing cues to other peripheral oscillators to synchronize the organism’s physiology and behavior. The SCN also appears to provide the rhythmic input to several “slave” peripheral oscillators, which only have a circadian rhythm in output while receiving neuronal (103) and humoral (112) entraining cues from the SCN. The SCN is a diverse and complex cluster of approximately 10,000 10-µm GABAergic (14) neurons in rodents located bilaterally along the third ventricle in the hypothalamus, superior to the optic chiasm (36). The SCN in rodents is anatomically divided in two regions, a dorsomedial shell and a ventrolateral core, based on the cell types present (2, 90). Vasoactive intestinal polypeptide (VIP) neurons are primarily located in the shell region of the SCN and arginine-vasopressin (VP) neurons are located in the core region (92). However, because of the complexity of the neuropeptides expressed in SCN neurons and the poor delimitation of these two anatomical regions, the dividing of the SCN into two discrete regions has been questioned (90).

SCN neurons are capable of generating self-sustained circadian rhythms in the core clock genes as isolated neurons (47, 49, 53, 104). SCN neurons have also been shown to have circadian modulation of spontaneous cell firing (47, 65, 79, 102), calcium oscillations (20, 58), and humoral output (69, 112). SCN neurons can be further divided into smaller populations of input and output neurons of which a small population (26%) of output neurons is electrically coupled through gap junctions. These act to synchronize the population and provide a temporal summation of some of the inhibitory GABA output of the SCN (79). In very low density primary cultures, a small population
of VIP-SCN neurons maintains a robust circadian rhythm while the remaining population displays a poor circadian oscillation with large variations in period and phase (47, 49, 106, 123).

Photoreceptors in the retina receive light entrainment cues and send an glutamatergic signal through the retinohypothalamic tract (RHT) (91) which acts to synchronize the poorly oscillating SCN neurons that produce the observed 24-hour rhythm output of the nucleus (46, 123). Chemical and electrical lesion studies have demonstrated that animals become arrhythmic in some of the circadian output without the temporal timing cues provided by the SCN (80, 103). Replacing the SCN in lesioned hamsters restores rhythmicity and retains the period of the donor SCN (80, 85, 114), providing further evidence that the SCN is acting as a master clock. Interestingly, SCN lesioned animals or animals placed in constant darkness maintained a circadian rhythm in food anticipatory activity (FAA) which indicated the presence of a food entrainable oscillator not controlled by rhythmic input from the SCN (25, 89).

Background information on the mesencephalic trigeminal nuclei (Me5)

An example of a complex behavior influenced by timing cues from the SCN is the daily behavioral gating of feeding and drinking activities in rodents. The Me5 are part of the system of trigeminal ganglia that innervates the face, jaw, and retina (17, 74) contains the Me5 primary sensory neurons that receive proprioceptive signals from spindle organs of masseter muscles and periodontal ligaments in the teeth. Me5 neurons send the sensory information to the motor nucleus of the trigeminal (Mo5) which indirectly regulates jaw movement and, to some extent, human speech (17, 56,
In addition to its well characterized chewing circuit, the Me5 interacts with hypothalamic nuclei serving hunger and satiety via the GABAergic multipolar Me5 interneurons and the tuberomammillary nucleus (TMN) (107). This indirect connection, in conjunction with cervical neurons, mediates coordinated movement of the head and neck during biting and chewing behaviors (35, 107).

Me5 neurons are distributed within bilateral rostral-to-caudal columns or bands that originate in the lateral edge of the periaqueductal gray (PAG) in the midbrain and extend into the pons to a position between the locus coeruleus (LC) and parabrachial nucleus (PB) (17, 36, 56). Me5 neurons are functionally homologous across the rostral-caudal distribution (17) although there are developmental differences in receptor distribution and masseter muscle innervation between the rostral and caudal Me5 (37, 56, 66, 107). The Me5 are easily distinguished from neighboring tissue and other trigeminal sensory ganglia because they are large neurons (30uM – 40uM) and have a distinctive ovoid shape (17, 56, 74, 96).

Based on anatomical studies, the Me5 contains smaller multipolar GABAergic-interneurons and larger glutamatergic pseudo-unipolar sensory neuron which originate in the mesencephalic neural crest (17, 74), and are similar in embryonic origin to other sensory ganglia such as the dorsal root ganglia (52, 59). Me5 are only present in organisms with a movable jaw, and are similar to Rohon-Beard cells in amphibians (56, 73). The neurochemistry of Me5 neurons is well established (74) along with the neurotransmitter receptor types they express for serotonin (5-HT) (77), dopamine (DA) (78), glutamate (97, 118, 120), histamine (HA) (107), ATP (62), and γ-aminobutyric acid (GABA) (45). The ion channels involved in the dynamics of the spontaneous, up to 100
Hz impulses of Me5 has also been characterized through patch clamp recordings of brain slices (33) and isolated neurons (136). The Me5 neurons have recurrent axon collaterals and are electrically coupled via gap junctions, which have been suggested to synchronize the spontaneous firing of the cell membrane providing a summation of sensory output of the Me5 during chewing (78, 82, 96, 136)
CHAPTER 2: CIRCADIAN MPER1 GENE EXPRESSION IN MESENCEPHALIC TRIGEMINAL CULTURES

Preface
This chapter was originally published as a research report in Brain Research June 2008 (Hiler et al. 2008. Circadian mPer1 gene expression in mesencephalic trigeminal cultures Brain Res. 1214:84-93). The original publication has been altered to meet the formatting specifications of the dissertation. This publication encompasses the first few years of my dissertation research and the initial development of the Me5 as an alternative model to the SCN.

Introduction
The circadian pacemaker of the hypothalamic suprachiasmatic nucleus (SCN) controls much of the circadian timing system of mammals and entrains to external light cycles through retinal ganglion cells that project to the nucleus (91). The circadian timing mechanism of the SCN and other circadian clocks contains several core clock genes, including mPer1, that act in interlocking feedback loops of transcription and translation to produce the near 24-hour period of circadian rhythms (80). Acting through neural and humoral signals, the SCN appears to keep circadian pacemakers in the internal organs in a correct phase relationship with itself and with external daily cycles (30, 112). Some brain areas show evidence of endogenous circadian pacemakers similar to the SCN clock (1), and their oscillations are typically delayed in phase by several hours relative to the SCN (43). It is not known whether the cells of these
pacemakers make use of the same membrane channels and molecular timing processes found in SCN pacemaker cells. It is also not clear whether the brain relies on only a few or many different ways to couple the molecular timing loops to extracellular signals.

Although circadian changes in gene expression in the intact animal suggest that these areas play a part in daily timing of neural activity, the true test of whether the tissue contains an independent circadian timing ability is to examine it in isolation ex vivo over several days in culture. Circadian rhythms in several brain areas have been recorded using the luminescence from tissue explants made from transgenic mice or rats expressing firefly luciferase under the control of promoters of the genes acting within the timing loops, such as mPer1 (mPer1::luc) and mPer2 (mPer2::luc), etc. (1, 46, 50, 125, 130, 133). By imaging the circadian rhythm in luminescence from brain tissues of transgenic mice, small regions or individual cells displaying circadian rhythms can be identified that are not detected when collecting single-channel luminescence measurements from entire cultures (111). We chose to use luminescence imaging to search for large neurons expressing circadian rhythms that would be both abundant and readily accessible for subsequent cellular studies of circadian pacemakers. Larger neurons than those in the SCN, which are typically less than 10 µm in diameter, would facilitate many intracellular imaging experiments including studies of single-molecule interactions important in circadian timing.

Here we describe imaging of mPer1::luc midbrain and pontine sections maintained as explant cultures. High transgene expression and circadian rhythms attributed to mPer1 gene induction were observed at multiple positions along the
mesencephalic trigeminal nucleus (Me5) which extends from midbrain to pons. The Me5 receives sensory signals from gingival membranes, periodontal ligaments, and spindle organs of masseter muscles and relays this information to the motor nucleus of the trigeminal (Mo5), thereby regulating jaw movement (17, 33, 56, 129). Unlike the SCN, the Me5 contains primary sensory neurons and may provide an effective alternative preparation for comparative studies of neural circadian pacemakers.

Material and Methods

Animals

Transgenic mPer1::luc mice (81) were bred and maintained in cycles of 12hr light/12hr dark to entrain their circadian system and were fed mouse chow ad libitum. Animal procedures were approved by the BGSU Institutional Animal Care and Use Committee and met National Institutes of Health guidelines. All efforts were made to minimize animal discomfort. Adult male and female mice were anesthetized with isoflurane, overdosed with Nembutal (0.1-0.3 ml, i.p., 50 mg/ml sodium pentobarbital), and decapitated between 7 and 9 hours after dawn. Brains were removed and 200 µm-thick sections were made in ice-cold Hank’s Balanced Salt Solution (HBSS) using a vibroslicer (World Precision Instruments, NVSLM1). Additional midbrain slices were surgically reduced to a crescent-shaped explant (Fig. 2D) with ependyma and tissue lateral to the Me5 removed. Crescent-shaped sections included the periaqueductal gray (PAG), dorsal raphe, and Me5. Dotted lines in figure 2A indicate the type of cuts made on midbrain sections (n=7 mice). Caudal sections of the Me5 were also prepared from 3 of the 7 animals used in circadian studies. The caudal Me5 begins near -5.0 mm
from bregma, at which point it leaves the PAG and rotates to a ventrolateral position between the locus coeruleus (LC) and the parabrachial nucleus (PB) or superior cerebellar peduncle (36). Brain slices from neonatal mice (11 to 14 days-old) were used for long-term recordings from crescent and caudal Me5 sections.

**Imaging brain slice cultures**

Sectioned brain tissue was transferred under sterile culture conditions to Millicell well inserts containing porous (0.4 μm) Teflon membrane (Millipore) in 35-mm plastic petri dishes with 1.4 ml culture medium consisting of Hepes-buffered (10 mM) Dulbecco’s Modified Eagle Medium (DMEM, Cellgro, Mediatech Inc., Manassas, VA) without phenol red that was supplemented with B27 (Invitrogen), penicillin and streptomycin, and with bicarbonate adjusted for use in room air, as described previously (111). All medium contained 0.1 mM or 0.2 mM luciferin (Xenogen Biosciences, Cranbury, NJ). Cultures were maintained in darkness in a 37°C incubator overnight before imaging.

Cultures were moved to a temperature-controlled chamber at 37°C and imaged from above as described previously (111) using a liquid nitrogen-cooled, back-thinned CCD camera (CH360, Roper Scientific, Tucson, AZ) and a 50-mm Nikkor f/1.2 lens (Nikon) combined with two close-up lenses (+10 and +4 diopter) used together. Lights with red light-emitting diodes were used when focusing the camera and handling the cultures. Luminescence images were captured with 2 x 2 binning and 1-hr exposures and then analyzed with V++ (Roper Scientific, Tuscon, AZ), ImageJ (NIH), and Photoshop 6.0 (Adobe, San Jose, CA) software. Each 2 x 2-binned pixel captured an
area of tissue 60 by 60 µm (effectively, 0.8X magnification). Sequential 1-hr exposures were captured for up to five days. Following imaging, cultures were fixed with Histochoice fixative (Amresco, Solon, OH) and stained with neutral red or methylene blue to aid in characterizing tissue morphology.

**Image analysis**

The following steps were used to determine the spatial pattern of transgene expression in brainstem sections: To remove the camera sensor’s bias values, a series of 50 camera bias images were averaged and then subtracted from the luminescence images yielding intensity measurements in analog-to-digital units (ADUs). Dark current noise is negligible with this cooled camera and these exposures (1-2 ADUs). Read noise was approximately 8 electrons/pixel and pixel well depth was 16-bit. To analyze the spatial distribution of transgene expression (Fig. 1 and Table 1), noise from cosmic ray related events was removed by taking the minimum intensity of each pixel in two sequential 1-hr images. With ImageJ software (NIH), areas greater than a threshold set at two standard deviations above the mean pixel intensity were selected for further analysis. Bright areas in each brain slice were matched to a mouse brain atlas for further identification of the luminescent structures (36). Independently, each pixel of the image were log transformed to improve simultaneous visualization of both dim and bright areas. An unsharp-mask filter was used on the bright-field images to help identify structural features of the brain slices.

To identify circadian rhythms in transgene expression within the Me5, seven crescent-shaped sections and three surgically-reduced pontine sections were imaged
using sequential 1-hr exposures for up to 5 days with 2 x 2 binning. Circadian rhythms were measured using the ten pixel locations that were brightest at the midpoint of the recording session. The brightest pixels were chosen because these would be least influenced by camera read noise, which is a limiting factor at low light levels for the cooled CCD camera used here (95, 113), thereby providing the best opportunity to detect rhythms. Any pixel value that was greater than twice the previous value in the image sequence was considered to result from comic ray-type noise. The pixel values from these rare events occurred in only about 1% of the time points and were deleted.

The time-series for the intensity of each of these pixels was analyzed using methods similar to those of a previous study (1). Essentially, the data were adjusted to the time of the prior LD cycle of the mice and were detrended by subtracting a 24-point adjacent average. A 3-point adjacent averaging was then used to smooth the data and to estimate the phase of each circadian cycle. The crossing of the smoothed data with a second 24-point average of the smoothed data yielded the time of the rising and falling phases. The highest time point falling between a rising and falling phase was accepted as a peak for that cycle if the amplitude was greater than or equal to 30% of the amplitude of the peak occurring before and the one during the next peak following the cycle. The amplitude was calculated as the difference between the trough and peak, and the trough was defined as the previous minimum after the last falling phase. Only peaks with measurable rising and falling phases were included. These criteria removed effects from the transient signal at the start of the culture and the damping near the end of the time in culture. The circadian rhythm periods were estimated using
the time between consecutive maxima, minima, rising, and falling phases of cycles that met these criteria.

A Lomb-Scargle periodogram analysis was performed on the same set of 10 pixels, which provided a spectral analysis on any time series with missing data points (121, 122). In this case, 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).

Results

*Distribution and pattern of mPer1::luc expression*

To screen for regions of elevated *mPer1* gene expression, entire coronal brainstem slices from adult *mPer1::luc* transgenic mice (n=6) were imaged using two sequential 1-hr exposures between 18 and 30 hours after the cultures were prepared. Brain sections from the entire rostral-to-caudal range of the PAG showed high expression in several brain areas, defined as a signal greater than two standard deviations (SD) above the mean intensity for the tissue section (Fig. 1, Table 1). Luminescence was detected in several nuclei, most notably the Me5, medial raphe (MR), dorsal raphe (DR), substantia nigra (SN), inferior colliculus (IC), and undetermined cerebellar nuclei. A thin margin of luminescence at the edge of these midbrain sections, corresponding with the meninges, and the ependymal cells of the ventricles and aqueduct were also among the brightest areas present.
Of the several midbrain areas examined along the rostro-caudal axis, the PAG was particularly noteworthy because of the elevated signal from clusters of Me5 neurons near the PAG’s dorsolateral and lateral columns (Figs. 1, 2). Methylene blue staining, used to visualize the nuclei, confirmed that the Me5 neurons were the source of this luminescence at the lateral edge of the PAG (Fig. 2A-C). The entire range of Me5 neurons in the midbrain and pons (36) showed high transgene expression (Table 1).

Surgical reduction of midbrain sections to crescent-shaped Me5 cultures (Fig. 2D) confirmed that the signal did originate in this nucleus and was not the result of scattered light produced elsewhere in the tissue. Within the PAG, luminescence was primarily from the Me5 (Fig. 2A-C). The DR, which is contiguous with the ventral PAG, was present at one end of the crescent-shaped Me5 cultures. Nevertheless, the brightest areas corresponded with the Me5 and not the DR, as shown by post-imaging staining and microscopic analysis at higher magnification (Fig. 2D). The 30 to 40-µm, pseudo-monopolar cells identified after imaging had the distinctive ovoid shape of Me5 neurons (Fig. 2E) as described previously (82).

In cultures made from the caudal region, the brightest areas of these pontine sections corresponded with Me5 nuclei. No bright signal was present in the neighboring LC and PB, although any low-level expression in these nuclei might not have been obvious because of light scatter from the nearby Me5 (Fig. 2F). As in the midbrain, the brightest luminescence was located in the Me5, and these cells were visible in explants stained with methylene blue after luminescence imaging.
Period estimates of circadian rhythms

As with coronal sections imaged during only the first day in culture, the Me5 in crescent sections produced a bright luminescence signal, and this persisted for several circadian cycles (Figs. 3, 4). Using four phase estimates—the rising phase, maximum, falling phase, and minimum—the percentage of the 10 brightest pixels, averaged across the first four cycles, that provided a period estimate within the circadian range (19 to 29 hrs) was 43.03% (rise), 47.35% (max), 43.60% (fall), and 48.25% (min) for the rostral cultures (n=7), and 44.87% (rise), 45.71% (max), 43.10% (fall), and 48.07% (min) for the caudal cultures (n=3). There was no significant difference between the rostral (45.56% ±2.63 SD) and caudal (45.44% ±2.06 SD) cultures (p>0.05, Student’s T-test).

Period estimates based on these four phase reference points are shown in Table 2 for the first four circadian cycles of the imaging sessions (cycles 2 through 5 of culture). Average period estimates for the midbrain and pons Me5 were not significantly different when compared using the four phase reference points (23.32 vs. 22.88, p>0.05, Student’s T-test). Of the four phase reference points the maximum showed the lowest variance in both the rostral and caudal cultures (Table 2).

For an additional estimate of rhythmicity, the time-series for each of the 10 pixels used in this cycle-to-cycle interval analysis was then examined using the Lomb-Scargle method. Significant rhythms (p<0.05) were found in the Me5 of all cultures. The average periods of pixels with rhythms meeting significance ranged from 19.62 hr to 30.67 hr for the rostral cultures and from 19.70 hr to 33.56 hr for caudal cultures. Just as when the four phase reference points were examined, the Lomb-Scargle method did
not detect a significant difference in period between the rostral and caudal explants (25.72 \pm 4.18 hr vs 25.07 \pm 7.44 hr, p>0.05, Student’s T-test).

**Phase Estimates**

Using data from the rhythmic pixels, the mean phase for each of the four phase reference points was plotted for each mouse (Fig. 4). For both rostral and caudal cultures, the mean phase of all maxima occurred during the night. Two of the mice, however, showed maxima near midday (Fig 4). Phase estimates based on the four phase reference points for all 7 cultures were analyzed by Rao’s Spacing Test (Oriana software, Kovach Computing Services, Anglesey, Wales, UK). According to this test, there was a significant difference from even spacing throughout the day for the rising phases (p<0.05) and minima phases (p<0.01), but not for the two other phase reference points (Fig. 4).

**Discussion**

*Imaging mPer1::luc expression in the brainstem*

The observed circadian rhythms in surgically reduced cultures, containing little more than the Me5, suggest that these sensory cells contain an endogenous circadian pacemaker capable of functioning independently from other brain areas. Me5 neurons are distributed within bilateral rostral-to-caudal columns or bands that originate in the periaqueductal gray of the midbrain and extend into the pons (17, 36, 56). The sampling of brain sections throughout this range revealed bioluminescence at both ends of the Me5 and at each level that was imaged.
Along with the Me5 signal, imaging showed high luminescence along a thin band at the edge of intact midbrain slice cultures and from ependymal cells of the ventricle walls. The signal at the tissue edge appeared to be from blood vessels and non-neural tissue. Images of the PAG in the crescent cultures, surgically reduced with the edges and ependyma removed, showed that the Me5 signal was not caused by light scatter from these other areas and that the rhythms persist without connections to neighboring brain nuclei. Interactions occurring entirely within the clusters of Me5 neurons that remained could be important for rhythm generation. Complete isolation of the Me5 neurons would be needed to establish them as fully independent circadian pacemakers.

Although fewer sections were imaged, the Me5 of the pons also appears to function as a circadian pacemaker independent from any timing signals originating from the neighboring LC and parabrachial nuclei. Although we were unable to trim the Me5 in this region away from these nearby nuclei, there was no circadian rhythmicity in the considerably weaker neighboring signal. Nevertheless, imaging following cell dissociation is needed to verify that rhythmic or non-rhythmic inputs from other nuclei are not needed to maintain the rhythm in the pontine Me5. The same two cell types are present in the rostral and caudal Me5, and in both regions they serve as sensory neurons that control motor neurons (74), suggesting that the same rhythmic phenotype will be found throughout the length of the Me5.

According to anatomical studies of rat and cat brainstem, the Me5 contains both multipolar and pseudounipolar sensory cells sharing embryonic origins with neurons of sensory ganglia (52, 59). The pseudounipolar Me5 neurons are distinguished from neighboring neurons by their unique ovoid shape, large (approximately 30-40 µm)
diameter, and single bifurcating process (82, 96). Cells with these properties were visible in the Me5 of the rostral and caudal explant cultures at high magnification. They also showed robust \textit{mPer1::luc} expression (Fig. 2) along with cells that could not be definitively categorized as of either cell type.

The imaging of midbrain and brain stem slice cultures in the present study provided unprecedented views of luminescent brain areas that would not have been detected easily in previous studies (1, 40) in which the total luminescence from individual explant cultures was recorded for several days. In one project using transgenic rats in which a \textit{Per1} promoter controlled luciferase expression (1), only a single 24-hour cycle of luminescence and no sustained circadian rhythms were detected in the raphe, SN, or other brainstem structures, although the Me5 was not examined specifically. In this earlier study, any subtle rhythms in luminescence recordings could have been masked by bright non-rhythmic areas, particularly if only a small cluster of neurons were rhythmic.

Another possible reason why earlier studies did not detect sustained circadian rhythms in the midbrain is because they used the 1-8L \textit{mPer1::luc} mouse founder line (131) that is not as bright as the 10-8L founder line used in the present experiments (81). Greater signal strength can improve detection of rhythmic expression that rises above background noise levels in the detection system. In agreement with the study mentioned above, in which rat brain areas were examined, the present results did not reveal rhythms in the DR, SN, and IC, and these nuclei were much dimmer than the Me5. To maximize our chances of capturing circadian luminescence signals, we limited our Me5 analysis to the ten brightest pixels of each culture. The lack of significant
circadian rhythmicity in the fainter areas does not preclude detection of rhythms by a more sensitive method. The rhythms in the Me5 observed here and the lack of obvious rhythms in other midbrain areas may be attributed to the unique developmental origin of Me5 neurons relative to the surrounding tissue and their role as primary sensory neurons (4, 56). Circadian pacemakers in several organisms are located within primary sensory cells or in nearby cells (8, 12, 16, 27, 32, 116)

*Circadian period analysis*

As in the SCN, only part of the Me5 cell population may contain a circadian pacemaker. One possibility is that only the pseudounipolar neurons or the smaller neurons of the Me5 express circadian rhythms (74). The imaging approach shown here provides resolution near the single-cell level, an ability that has been demonstrated with isolated fibroblasts expressing circadian rhythms (128). Considering the area of tissue captured by each 2 x 2-binned pixel of the imaging sensor, the depth of focus, the tissue thickness, and the density of the cell clusters, we estimate that there are between 1 and 8 Me5 neurons within each pixel.

About half of the brightest pixels in luminescence images of the Me5 were rhythmic according to the time intervals between the four phase reference points and the Lomb-Scargle analysis. A pixel could be arrhythmic because it includes only arrhythmic cells. Alternatively, two or more rhythmic cells located within the pixel could be at different phases of their cycles causing their summed rhythms to cancel each other out or appear distorted. Finally, sustained elevated expression in one or more bright cells in the pixel might mask rhythms in other cells in the same pixel. With higher
magnification, single-cell imaging could be used to determine how many of the Me5 neurons are rhythmic. In addition, other isolated brain areas should be examined through long-term luciferase imaging to help describe the full distribution of circadian pacemakers in the nervous system.

The Lomb-Scargle method is a spectral analysis that was employed here because it was developed for use with a discontinuous time series. The use of Lomb-Scargle for circadian data analysis has been described previously in detail (121, 122). Although the Lomb-Scargle results agree with our peak-to-peak estimates of period, these period values are a function of the length of each time series, as with the Fourier transform, and consequently would not be expected to provide the same period estimates as the estimates based on peak intervals. Nevertheless, they confirm the presence of circadian rhythms in the Me5.

The phase of circadian pacemaker cells in culture can be influenced by the time of dissection, depending on the tissue type (137), and the presence of serum or other factors in the medium that can induce expression of core clock genes (6). The Me5 cultures were always maintained in a serum-free defined medium, although the B27 supplement or other serum components could have affected circadian properties. It will be important to compare our results with measurements of Me5 mPer1 expression in the mice. Similarly, dissection could be performed earlier to test whether this shifts the phase of Me5 cells relative to the ones that we imaged.

Finally, these initial studies did not show differences in circadian phase, period or the percentage of rhythmic pixels along the rostral-caudal distribution of the Me5. Reports have described functional differences in the Me5 along this axis (41, 75, 94,
Rostral Me5 neurons vary in their afferent connectivity and receptor composition compared to the middle and caudal Me5 neurons. Caudal Me5 somata have connections with the periodontal ligaments, whereas masseter muscle connections are found throughout the rostral-caudal distribution (41, 75, 94). During embryonic development, the glutamate receptor composition differs between the rostral and caudal Me5 neurons (119, 120).

**Possible circadian functions of Me5 neurons**

The Me5 interacts with hypothalamic nuclei serving feeding behaviors and motor neurons mediating coordinated movement of the head and neck during biting behaviors (24, 34, 35, 37, 59, 64, 77, 78, 107). The Me5 receives discharges from spindle organs of jaw muscles, regulates motor control of mastication, and sends sensory signals to the hypothalamus that appear to modulate satiety (59). Lesioning of the Me5 in mice decreases time to reach satiety and the amount of exploratory time when searching for food (59). Consequently, the Me5 are likely to be most stimulated by jaw movement during the most active phase of circadian feeding rhythms, corresponding with the animal’s nocturnal activity (56, 96). The Me5’s known interactions with the tuberomammillary nuclei (TMN) (75, 107) suggest that it could play a role in the daily timing of feeding behavior.

Pharmacological treatments focusing on histamine could be used to test whether TMN projections to the Me5 might alter the phase or period of a circadian clock in the Me5. The neurochemistry of Me5 neurons is well established (74) along with the neurotransmitter receptor types that they express for serotonin (5-HT) (77), dopamine
(78), glutamate (97), histamine (HA) (107), and γ-aminobutyric acid (GABA) (45).
Neurotransmitters known to depolarize Me5 neurons such as GABA (45), glutamate
(97, 118, 119), and ATP (62) should be tested for any ability to induce circadian clock
genes and evoke phase shifts. Although rapid neural firing (>90 Hz) is common in Me5
neurons (33, 96), it is not yet known whether the impulse frequency is modulated
throughout the day. For example, the activity of TMN neurons in live mice increases
during wakefulness (115) and might influence Me5 firing patterns.

Me5 as a circadian model system

The large size of Me5 neurons and their distinct morphology allow for easy
identification of the nuclei at the lateral edge of the PAG or in the pons (82). The vast
distribution of Me5, over the brainstem of rats and mice, provides a great number of
putative circadian pacemaker cells that could be collected and isolated for single-cell
analyses of the pacemaker. Ion channels involved in the dynamics of rat Me5 neurons
have been characterized through patch clamp recordings of brain slices (33) and
isolated neurons (136). Three sodium currents, high and low threshold calcium
currents, and the I_h current interact to produce slow oscillations in membrane potential
(33). The high threshold calcium current has properties of L-type calcium channels
(136) and could be responsible for transgene activation through the cyclic-AMP
response element (CRE) of the mPer1 promoter (50). A simple hypothesis would
predict that frequent jaw movement or biting behaviors induce a depolarization, followed
by a calcium influx in a subset of Me5 neurons. Therefore, similar to other circadian
systems (8, 71), depolarization might shift the phase of the Me5 circadian pacemaker, in
this case, allowing it to entrain to daily cycles of feeding. Other potentially entraining stimuli, such as HA or 5-HT, might also act on Me5 circadian pacemakers through this mechanism. Known electrical coupling between Me5 neurons (4) might mediate spreading of this timing information throughout the Me5.

Because Me5 cells are easily identified and accessible for patch clamp recordings, their membrane properties could provide additional means for characterizing the role of neuronal activity in the circadian timing mechanism, similar to explorations of neurons in the SCN and molluscan circadian pacemakers (71, 88). Comparative studies of Me5 and SCN neuronal properties are needed. The Me5 manages a different sensory mode from the SCN, which receives photic signals from the retina, and contains neurons with bursting firing patterns and a much higher spontaneous firing rate than SCN neurons (33, 48, 93). Thus, the Me5 may reveal a uniquely different evolutionary solution to the problem of coupling a circadian rhythm in gene expression to a neural output effecting timing control in target brain nuclei and organs.

Additional studies will be needed to determine whether the circadian pacemaker modulates Me5 sensory responses, perhaps to optimize sensitivity for a particular phase or behavior, or modulates motor neurons in the Mo5 to maximize muscle control during the night to coincide with nocturnal behaviors. Functional studies of the Me5 should take into consideration the presence of a circadian pacemaker that could alter spontaneous firing patterns, responses to neurotransmitters, or other physiological measures relevant to the role of these cells.
Acknowledgements

This work was supported in part by the BGSU J. P. Scott Center for Neuroscience, Mind, and Behavior. We would like to thank Denise Hook and Paula Carver of BGSU for their help with mouse breeding and proofreading, Dr. Jaak Panksepp of Washington State University for thoughtful early guidance, Dr. Erik Olofsen and Leiden University Medical Center for the Lomb-Scargle MATLAB program, and the Xenogen Corp., Hopkinton, MA for luciferin.
Figure 1. Short-term cultures of coronal brainstem sections from \textit{mPer1::luc} mice showing transgene expression in the Me5. \textbf{A}: Log scaled luminescence intensity as pseudocolor overlay on brightfield reference image. \textbf{B}: Log scaled intensity alone. Positions are shown relative to bregma. The Me5 nuclei (arrows) first appear at -4.1 mm bregma and continue from the midbrain into the pons. Images captured using 1-hr
exposures, 2 x 2 binning. Distortions in reference images are from glare on the imaging chamber window at -3.6 and -4.2 mm.
Figure 2. Bright luminescence in PAG and pons corresponding with Me5.  

A: Methylene blue-labeled reference image of midbrain culture at position -4.1 mm in Figure 1. Arrows indicate stained Me5 neurons at the lateral edge of the PAG. Shown are where cuts were made to isolate Me5 crescent cultures from the ependyma of the central canal and tissue lateral to the Me5 (dotted lines). Scale bar: 500 μm.  

B: Close up of Me5 neurons in A. Bar: 250 μm.  

C: Luminescence of the same rostral culture shown as a pseudo-color overlay on A. Bar: 500 μm.  

D: Luminescence from Me5 in a crescent-shaped culture used for long-term imaging (Fig. 3). Bar: 500 μm.  


F: Pseudo-color overlay of luminescence from a caudal culture on its corresponding reference image showing the parabrachial nuclei (PB), superior cerebellar peduncle (spc), Me5, locus coeruleus (LC).
Figure 3. Circadian rhythms in mPer1::luc expression in Me5 of long-term explant cultures made from midbrain and pons of three different mice. Rhythms recorded from individual pixels persisted for over four cycles during recordings lasting up to 6 days since last light onset. A, B: rostral Me5. C: caudal Me5. Hashed bars indicate the animal’s previous night phase in the LD cycle. Black line is after 3-point adjacent averaging. Gray line is after 24-point adjacent averaging used to find the rising and falling phase of each circadian cycle. ADU: analog-to-digital units of camera sensor.
Figure 4. Average phase of circadian rhythms in the Me5 according to four phase reference points. Rostral Me5, average phase per mouse, ±SD is shown by filled circles, solid curve. Caudal Me5, average phase per mouse, ±SD is shown by open circles, dotted curve. Arrows indicate the average phase and mean vector length for all rostral (filled) and caudal (open) Me5 cultures. Mean vector lengths were 0.525 (rise), 0.426 (max), 0.574 (fall), 0.466 (min) and 0.377 (rise), 0.433 (max.), 0.383 (fall), 0.431 (min) for rostral and caudal, respectively. Time 0 is dawn (light onset) and time 12 is dusk (dark onset) of the light/dark cycle.
Table 1. *Rostral-to-caudal distribution of mPer1::luc expression in the mesencephalic trigeminal nucleus*

<table>
<thead>
<tr>
<th>Bregma</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
<th>M5</th>
<th>M6</th>
</tr>
</thead>
<tbody>
<tr>
<td>-3.3</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-3.4</td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-3.6</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-3.8</td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-3.9</td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-4.1</td>
<td>++</td>
<td>++</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-4.2</td>
<td>++</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-4.4</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>-4.5</td>
<td>+</td>
<td></td>
<td>++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-4.6</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-4.7</td>
<td>++</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-4.9</td>
<td>++</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-5.0</td>
<td></td>
<td>++</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-5.2</td>
<td>++</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-5.3</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-5.4</td>
<td></td>
<td>++</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Luminescence signal in coronal sections according to position from bregma (mm). Shown for each of six mice (M1-M6) are Me5 areas with signal greater than 2 SD (++) from average section intensity, less than 2 STD but visible (+), and without detectable signal (-). Sections are approximated to atlas sections ± 120 μM. Note the lack of signal anterior to position -4.1 mm.
Table 2. *Period estimates of circadian rhythms in Me5 cultures*

<table>
<thead>
<tr>
<th></th>
<th>Rostral</th>
<th></th>
<th>Caudal</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rise</td>
<td>Max</td>
<td>Rise</td>
<td>Max</td>
</tr>
<tr>
<td></td>
<td>Cycle 1</td>
<td>Cycle 2</td>
<td>Cycle 1</td>
<td>Cycle 2</td>
</tr>
<tr>
<td></td>
<td>22.87</td>
<td>24.72</td>
<td>23.25</td>
<td>24.86</td>
</tr>
<tr>
<td></td>
<td>± 1.20</td>
<td>± 0.87</td>
<td>± 2.97</td>
<td>± 1.54</td>
</tr>
<tr>
<td></td>
<td>23.26</td>
<td>23.83</td>
<td>22.22</td>
<td>23.47</td>
</tr>
<tr>
<td></td>
<td>± 1.22</td>
<td>± 1.62</td>
<td>± 2.06</td>
<td>± 1.72</td>
</tr>
<tr>
<td></td>
<td>22.43</td>
<td>23.91</td>
<td>21.21</td>
<td>24.27</td>
</tr>
<tr>
<td></td>
<td>± 2.91</td>
<td>± 1.24</td>
<td>± 2.01</td>
<td>± 1.74</td>
</tr>
<tr>
<td></td>
<td>22.96</td>
<td>23.62</td>
<td>22.52</td>
<td>23.67</td>
</tr>
<tr>
<td></td>
<td>± 0.40</td>
<td>± 0.58</td>
<td>± 1.02</td>
<td>± 1.20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Min</th>
<th>Fall</th>
<th>Min</th>
<th>Fall</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cycle 1</td>
<td>Cycle 2</td>
<td>Cycle 1</td>
<td>Cycle 2</td>
</tr>
<tr>
<td></td>
<td>22.78</td>
<td>26.78</td>
<td>22.84</td>
<td>22.08</td>
</tr>
<tr>
<td></td>
<td>± 1.45</td>
<td>± 1.88</td>
<td>± 1.66</td>
<td>± 1.74</td>
</tr>
<tr>
<td></td>
<td>24.10</td>
<td>24.10</td>
<td>21.08</td>
<td>21.08</td>
</tr>
<tr>
<td></td>
<td>± 3.97</td>
<td>± 3.09</td>
<td>± 2.42</td>
<td>± 1.66</td>
</tr>
<tr>
<td></td>
<td>20.74</td>
<td>20.74</td>
<td>22.84</td>
<td>22.62</td>
</tr>
<tr>
<td></td>
<td>± 0.97</td>
<td>± 1.24</td>
<td>± 1.24</td>
<td>± 1.66</td>
</tr>
<tr>
<td></td>
<td>22.59</td>
<td>22.59</td>
<td>22.71</td>
<td>22.71</td>
</tr>
<tr>
<td></td>
<td>± 0.39</td>
<td>± 0.39</td>
<td>± 0.56</td>
<td>± 0.56</td>
</tr>
</tbody>
</table>

Average period for each analysis based on each of the four phase reference points ± standard deviation of the mean.
CHAPTER 3: CIRCADIAN GENE EXPRESSION IN INDIVIDUAL NEURONS OF MESENCEPHALIC TRIGEMINAL NUCLEI EXPLANTS AND CELL CULTURES

Introduction

In mammals, a distributed network consisting of the suprachiasmatic nucleus (SCN) and peripheral circadian oscillators directs circadian rhythms in spontaneous action potential frequency (65, 79), serum hormone levels (13), feeding activity (108), sleep (108) and many other behaviors. (See (30) or (80) for reviews.) Several studies have examined molecular circadian clocks in the SCN and peripheral oscillators and have identified several core clock genes (mPer1/2, Cry 1/2, Clock, Bmal1, etc.) that serve in generating the circadian rhythm and expressing this timing information to other parts of the cell and the rest of the body (1, 46, 50, 125, 131, 133). The circadian oscillator of the SCN entrains to daily external signals, particularly light cycles, and provides the timing cues that synchronize peripheral oscillators and behaviors to daily events (80, 91). Electrical recordings from the SCN have also identified a circadian modulation of the cell membrane potential in vivo and in vitro (47, 65, 79, 93). Nevertheless, the mechanism linking the molecular clock to circadian modulation of spontaneous cell firing or rhythmic behaviors remains unclear.

Cell cultures of SCN neurons have been a valuable tool for exploring modulation of membrane mechanisms by the circadian clock. Previous studies have suggested that between 50 to 70% of SCN neurons can exhibit a circadian rhythm in electrical activity (33, 47, 93). Unfortunately, the SCN is a complex collection of approximately ten thousand, 10 µM neurons with a diversity of cell phenotypes and neurotransmitters.
Although cells expressing arginine-vasopressin or vasoactive intestinal polypeptide make up the majority of neurons in SCN cell cultures, the cell diversity and fairly uniform cell morphology present challenges to understanding circadian timing mechanisms at the single-cell level (87, 90). This disadvantage is compounded by the lack of organization of the SCN into cell layers or substructures other than somewhat poorly delineated “core” and “shell” regions (2, 90). Because of these limitations caused by the complexity and anatomy of the SCN, we sought a relatively simpler, alternative cell model with circadian, molecular, and electrical properties that could be used to address the connection between circadian modulation of the cell membrane and the molecular circadian clock in mammalian neurons. It should also be useful for imaging studies of sub-cellular circadian processes that are less easily examined using SCN cells.

We previously identified a circadian rhythm in $mPer1$ gene expression in organotypic cultures of mesencephalic trigeminal nuclei (Me5) explants (51). The Me5 generated circadian bioluminescence rhythms in these cultures made from midbrain and pons brain slices of transgenic mice expressing the firefly luciferase gene $luc$ controlled by the $mPer1$ promoter. Typical of peripheral circadian oscillators, the Me5 neurons displayed a near 24-hour rhythm in $mPer1::luc$ expression with a peak in bioluminescence occurring between one to three hours after the time of dusk in the previous light/dark cycle experienced by the animal (51). Whether this circadian oscillator is synchronized to environmental cycles through timing signals from the SCN or is instead indirectly coupled to the rest of the circadian system through, perhaps, sensory stimulation during daily feeding is not yet known. Interestingly, the Me5
interacts monosynaptically with the tuberomammillary nuclei that play a major role in feeding behavior and satiety (75, 107).

Two types of Me5 neurons have been characterized in rats—glutamatergic pseudo-unipolar and GABAergic multipolar neurons that are 30 to 40 µM in diameter and have a distinctive ovoid shape (82, 96). Thus, Me5 neurons provide about a ten-fold advantage in cellular volume for intracellular imaging and cell exploration relative to SCN neurons. In both rats and mice, the Me5 are distributed within bilateral rostral-to-caudal columns or bands that originate in the lateral edge of the periaqueductal gray (PAG), and extend into the pons to a position between the locus coeruleus (LC) and parabrachial nucleus (PB) (17, 36). Me5 neurons, along with the mesencephalic trigeminal motor nucleus (Mo5), serve in a well-characterized neuronal circuit used in jaw movement during feeding. The neurotransmitters and membrane receptors of Me5 neurons have been examined in the rat (17, 36, 56, 129), but far less is known about mouse Me5 neurophysiology. Acute electrical recordings have shown a spontaneous, high frequency (100 Hz) firing rate in rat Me5 neurons, although no long-term electrical recordings have been performed to identify circadian properties (33, 96).

In the present study, we further characterized the circadian oscillator in the mouse Me5 and identified circadian rhythms in individual Me5 neurons in explant cultures and dispersed cell cultures. These results support use of the Me5 as a valuable model to complement SCN research on genetic and membrane properties of mammalian neurons,
Materials & Methods

Animals

Transgenic \textit{mPer2}^{\text{Luc}}\textsuperscript{L} knock-in mice (135) and \textit{mPer1}::\textit{Luc} transgenic mice (81) were bred and maintained in cycles of 12 hr light and 12 hr dark to entrain their circadian system. Animal procedures were approved by the BGSU or Morehouse School of Medicine Institutional Animal Care and Use Committee and met National Institutes of Health guidelines. All efforts were made to minimize animal discomfort. Male and female mice (ages 8 days to 4 months) were anesthetized and decapitated during the light phase. Brains were removed and 200 µm-thick sections were made in 4°C Hanks Balanced Salt Solution (HBSS, Invitrogen) using a vibroslicer. Slices were surgically reduced to an area just outside the PAG or further reduced to a crescent-shaped PAG with ependymal cells removed. Slices from the pons were reduced to a triangle-shaped section enclosing the locus coeruleus (LC), parabrachial nucleus, and Me5. (See (51) for more detail.) The tissue was transferred under sterile culture conditions to Millicell well inserts containing porous (0.4 µm) Teflon membrane (Millipore) in 35 mm plastic petri dishes with 1.4 ml culture medium consisting of Hepes-buffered (10 mM) Dulbecco’s Modified Eagle Medium (DMEM) (Cellgro, Mediatech Inc., Manassas, VA), pH 7.2, without phenol red that was supplemented with B27 (Invitrogen), penicillin and streptomycin, and 0.1 mM or 0.2 mM K\textsuperscript{+} salt of luciferin (Xenogen Biosciences, Cranbury, NJ). Bicarbonate was adjusted for use in room air and cultures were maintained in a 37°C incubator prior to imaging.
Bioluminescence imaging

$mPer2^{luc}$ organotypic cultures were moved to a temperature-controlled chamber at 37°C and imaged with an intensified-CCD camera (xr/mega-10z, Stanford Photonics camera) with a 10x objective lens and analyzed with custom Zeiss software (Carl Zeiss MicroImaging, Inc. Thornwood, NY). Luminescence images were captured by using a 5-min summation of 900 frames per minute. Frames with cosmic ray-related events were automatically eliminated from the series, and accounted for approximately 10% of the total captured frames. Five minute summations were then summed to 30-min or 60-min images and analyzed using V++ (Roper), ImageJ (NIH), Matlab (Mathworks) and Photoshop CS3 (Adobe) software. Dissociated cell cultures in Sykes-Moore dishes (Belco Glass Company, Vineland NJ) were maintained at 37°C and imaged using a liquid nitrogen-cooled, back-thinned, back-illuminated CCD camera (CH360, Roper Scientific, Tucson, AZ) and a 50-mm Nikkor f/1.2 lens (Nikon) combined with two close-up lenses (+10 and +4 diopter) were used together. Cell dispersals were illuminated by red light-emitting diodes when focusing the camera and handling the cultures. Luminescence images were captured with 2 x 2 binning and sequential 1-hr exposures over several days for a maximum of five days. Each 2 x 2-binned pixel captured an area of tissue 60 by 60 μm (effectively, 0.8X magnification). After imaging, cell cultures were fixed with methanol for 15 minutes, stained with hematoxylin, to aid in characterizing cell types, and mounted on glass slides for cell identification and recovery. Cell cultures which were imaged for more than a day were also imaged with a thermoelectrically cooled CCD camera (Micromax, Princeton Instruments, Trenton, NJ) using red light-emitting diodes and a red R2 filter (Promaster) for the camera light
source to create a 5x montage of the entire live culture while maintained at 37°C. After bioluminescence imaging, cell cultures were fixed and stained as described above. Image montages were created using Photoshop CS3 (Adobe) software and ImageJ (NIH) to identify individual Me5 neurons and glial cells. Period estimates were analyzed using Origin 7.5 (Microcal) and Excel (Microsoft), and phase estimates were analyzed by Rao’s Spacing Test (Oriana software, Kovach Computing Services, Anglesey, Wales, UK).

**Immunohistochemistry**

Adult *mPer1::luc* mice (n=5) were anesthetized with Nembutal (0.13-0.16 ml, i.p.) and transcardially perfused with phosphate buffered saline (PBS) followed by 4% formaldehyde in PBS, pH 7.4. Fixed brains were removed, blocked, and immersed in a 4% formaldehyde solution in PBS overnight, followed by 30% sucrose in PBS overnight before being cryosectioned at 25-50 µM the following day. Tissue sections were mounted on glass slides (Plus Gold, Fisher Scientific) and air-dried for 3 hours prior to rehydrating and endogenous peroxidase quenching (5% hydrogen peroxide in methanol). Sections were immunolabeled with a polyclonal antibody against PER1 (ab3443, Abcam), and processed using an HRP-conjugated secondary antibody from an Immunohistochemistry Accessory Kit (Bethyl Laboratories, Montgomery TX), or the primary antibody labeling was instead followed by amplification with the Vectastain Elite ABC kit (Vector Labs, Burlingame, CA). All sections were processed with diaminobenzidine (DAB). The number of positively stained Me5 cell nuclei and cytoplasm were counted from each section in addition to the total number of Me5
neurons present. Me5 neurons were identified based on cell morphology and location using light microscopy.

**Cell Culture**

Brain sections (200 µM thick) were made from the pons of 8-day to 30-day-old mPer1::luc mice (n=3), as described above, and were trimmed to just outside the Me5. Tissue was digested in 37°C preheated 5 mg/ml Dispase (Gibco) in HBSS for 30 min. Tissue fragments were then triturated in 2 ml preheated DMEM and plated on poly-d-lysine-coated, 25-mm round cover glasses using 400 µl of the cell suspension per cover glass. The cover glasses were coated with 500 µl 0.1 mM poly-D-lysine in sterile water overnight at 37°C to achieve the desired thickness to allow for Me5 attachment. Cell cultures were incubated for 10 minutes at 37°C after plating and then given a final volume of 2 ml Hepes-buffered DMEM supplemented with B27 per culture. Cell dispersals were incubated serum-free for 2 days at 37°C and then 1 ml of medium was replaced with preheated medium containing fetal bovine serum (FBS) and luciferin to produce a final concentration of 5% FBS and 0.1 mM luciferin. Cell dispersals were incubated overnight and then moved to a Sykes-Moore chamber for bioluminescence imaging lasting for 2-hrs or up to 5 days at 37°C. Cell cultures not being imaged were maintained for several weeks by exchanging 50% of the medium with 5% FBS in DMEM every other day.
Results

*Growth of Me5 neurons in cell culture*

To evaluate whether Me5 neurons are able to generate circadian rhythms when isolated from the neural circuitry of the brainstem, it was necessary to first test whether they could be maintained in long-term primary culture like those used to examine circadian rhythms in SCN neurons. Several standard methods for maintaining neurons in primary cultures were evaluated. Poly-D-lysine was selected as an attachment factor and a DMEM-based medium containing 5% FBS was found to be effective for cell survival. Me5 neurons survived for several weeks in these dissociated cell cultures where most developed long processes, in some cases reaching over 20 cell diameters in length (Figure 1). Nevertheless, these isolated cells did not interact synaptically because they were maintained at very low cell densities (approximately 10 per cm²). In a few cases, however, clumps of Me5 neurons were present and these also generated long processes (Figure 1, Figure 2B). Me5 neurons in cell cultures maintained a cell diameter (25-35 µm) that was similar to acute dissociated rat Me5 cultures (82) and mouse Me5 organotypic explant cultures (51). They also retained their distinctive ovoid cell shape (82, 96) and had a light-refractive property when observed with phase-contrast light microscopy. Me5 neurons in culture were differentiated into two categories (multipolar and pseudo-unipolar) based on axonal regrowth (Figure 1), as described previously for histological sections of the rat Me5 (82).

Regrowth of Me5 axonal processes in culture was used as an indicator of healthy cell growth, but was not necessary for individual neurons to be bioluminescent and did not indicate that a neuron would be bioluminescent (Figure 1, Figure 2). DMEM
supplemented with B27 was sufficient for Me5 neurons to begin to regrow axonal processes while preventing rapid glial cell proliferation. The axonal growth was, however, limited and no new growth was observed after 2 days in culture. The addition of 5% FBS after two days of incubation stimulated additional extensive regrowth of axonal processes when compared to Me5 neurons not given FBS, but it did not appear to stimulate additional growth from previously non-sprouting Me5 neurons (not shown). Me5 neurons displayed greater axonal growth when cultured with 10% FBS from the beginning of the cell dispersal, however rapid glial growth interfered with bioluminescence imaging of Me5 neurons. Four days after dispersal, the glial cell population had increased in response to 5% FBS, but remained low when compared with 10% FBS cultures. The addition of 5% FBS at day two allowed for continuous imaging of individual Me5 neurons for up to five days without a media exchange. Me5 dispersals maintained with 10% FBS became less suitable for analysis after five days in culture because of excessive glial growth obscuring individual neurons during imaging; thus, only 5% FBS cultures will be used for analysis.

*Period and phase of circadian mPer1::luc expression in dispersed neurons*

Primary Me5 cell cultures were imaged for several days to identify any circadian rhythms in *mPer1* gene expression. Me5 neurons in the cell cultures produced enough bioluminescence to be detected when imaging the entire cover glass at low magnification (Figure 2). Astrocytes were also conspicuously luminescent and often migrated away from a central point as they proliferated. These moving cells were distinguishable from the Me5 neurons that were stationary throughout the imaging.
procedure. The identity of the Me5 neurons was confirmed by examination of the cultures at higher magnification either before or after imaging.

Of the observed Me5 neurons in the culture, 57.87% ± 2.91% of the cells produced a bioluminescence signal sufficiently higher than background to be detected and analyzed for period and phase estimates. The percentage of the luminescing Me5 neurons that had a circadian period (between 19 and 29 hrs) during the first 3 cycles after imaging began (days 5-8 in culture) was determined (Table 1). Four period estimates were determined for each cell based on four phase reference points—the time of the peak luminescence signal, the time of the trough in the cycle, and the rising and falling phases. Rhythms persisted during imaging for at least the first 3 cycles (Table 2, Figure 3). Me5 neurons showed an average period of 22.90 ± 0.70 hrs for the first cycle during imaging and 22.32 ± 0.74 hrs and 22.16 ± 1.00 hours for the second and third cycles, respectively (Table 2).

Using the four phase reference points from the rhythmic cells, the mean phase for each reference point for each mouse was plotted (Figure 4). Dispersed Me5 neurons had an average period of 22.40 ± 0.81 hours over the first 3 cycles of imaging. When adjusted to for time of fetal bovine serum (FBS) stimulation, each individual culture had a wide variance around the mean ZT when the maximum was used as the phase reference point (Figure 4). When analyzed with a Rao’s Spacing test, which test the distribution of the phases to a uniform, evenly spaced (360\(^\circ\)/N) distribution, the minimum phase reference point was significant (p< 0.05) for cultures A, B, and C and the rising phase reference point was significant for culture A (Figure 4).
**Immunolocalization of mPER1 in Me5 neurons**

To determine whether the percentage of Me5 neurons in culture showing mPer1::luc bioluminescence is comparable to mPER1 expression in the Me5 before cell dissociation we performed immunohistochemistry using cryosections from mPer1::luc mice (n=5, 2-4 months old). The mPER1-like immunolabeling was present in both the cytoplasm and nucleus of Me5 neurons and in neurons of sections containing the SCN that were used as a positive control (Figure 5). Faint staining was present in neighboring brain areas. Of the total observed Me5 neurons, mPER1-like immunoreactivity was observed in the cytoplasm of 45.89% ± 7.89% of the cells and in 13.30% ± 8.78% of the cell nuclei. No immunoreactivity was observed in mouse Me5 neurons in which the primary anti-mPer1 antibody or the secondary anti-rabbit antibody was withheld. There was no detectable difference in the background staining when using either the Vectastain Elite ABC kit (Vector Labs, Burlingame CA) or the Immunohistochemistry Accessory Kit (Bethyl Laboratories, Montgomery TX) (Figure 5). The number of immunopositive neurons in each section was similar after either method. Additionally, there also was no noticeable difference according to the thickness of the sections (25 or 50 µm) or the dilution of the anti-mPer1 antibody (100-fold or 500-fold) when comparing the immunoreactivity staining within the cytoplasm or nucleus in Me5 and SCN neurons.

**Period and phase of circadian mPer2luc expression in organotypic cultures**

To determine whether other circadian clock genes are also expressed in Me5 neurons, explants cultures from mPer2luc knockin mice were imaged for several days
using the methods described previously for imaging the Me5 of mPer1::luc mice (51). In this case, however, a higher magnification imaging system and an intensified-CCD camera were used that can perform single-photon counting (26). As with the mPer1::luc mice, these stationary organotypic cultures made from the pons of mPer2luc mice produced a bright bioluminescence signal in the Me5 neurons which persisted for several circadian cycles (Figure 6).

Me5 neurons having a circadian rhythm in mPer2Luc expression were identified using the Lomb-Scargle method (Figure 7). The Lomb-Scargle method (121, 122) detected a significant (p< 0.05), near 24-hr rhythm in at least a dozen Me5 neurons in explants from each of four mice.

Discussion

Me5 neurons in cell culture

The morphology of mouse Me5 neurons in primary cell culture and in situ (82, 96) are quite similar. Me5 cells in culture grow a long pseudounipolar axon like that which innervates the masseter muscle of the animal. Some Me5 neurons also displayed a multipolar morphology in culture like that described for a subset of rat Me5 neurons (17, 82). We believe this is the first report of long-term (several week) cell cultures of any Me5 neurons and also the first description of mouse Me5 neurons in cell culture. Furthermore, these cultures were not restricted to tissue from neonatal mice; Me5 neuronal cell cultures were produced from 8 to 28-day old mice. This result indicates that mature neurons from functional circuits could be examined in cell culture for comparison with studies of mastication and other behaviors involving the Me5.
We were surprised to discover that Me5 neurons could be cultured and maintained at very low densities with a relatively simple medium of 5% FBS and B27-supplemented DMEM (Figure 1). We were able to culture a few Me5 neurons on uncoated cover glasses or plastic cell culture Petri dishes, however the greater success at culturing Me5 neurons was with poly-D-lysine-coated cover glasses. More sophisticated cell adhesion factors, such as laminin, might increase the yield of Me5 neurons and further stimulate axonal growth, however, an average of 32 neurons per animal was recovered from these dispersals.

Some of the experiments used two animals to prepare five cultures in an attempt to increase the Me5 yield, although the total number of neurons surviving remained about the same. One possible reason for this lack of improvement is that the effective doubling of Me5 neuron and glial cell density may have placed greater demands on the culture medium, which was unable to support more neurons.

Previous studies (33, 96, 136) have demonstrated cell dispersal and electrical recording of rat Me5 neurons, however these studies consisted of acute recordings and did not maintain Me5 cultures longer than the recording day. We were able to maintain cultures of mouse Me5 neurons for several weeks with a medium exchange every other day. Additionally, Me5 neurons remained viable and produced a detectable bioluminescence signal for up to five days without media exchange, as was the case during imaging (Figure 2, Figure 3). The apparent hardiness of the Me5 neurons could be due to their unique embryonic origins in that they are more similar to neurons of dorsal root ganglia and other peripheral sensory neurons than most neurons in the central nervous system (52, 59). The ability to culture Me5 neurons with a single cell
adhesion factor and simple culture media adds to their potential value as an alternative to the SCN mammalian clock model.

The regeneration and growth of Me5 axonal process indicates that these neurons are healthy and are able to maintain cell function in a low-density primary culture. The unique refractile light properties of the Me5 aided in their identification along with their distinct ovoid shaped cell bodies (Figure 1). The size of the Me5 in cell culture ranged from 25 - 35 μM which is similar to the observed size of Me5 neurons in other species (33, 74, 82, 96, 136). Me5 neurons maintained their distinct ovoid shaped soma (17, 82) in culture and could be identified via light microscopy as either pseudo-unipolar or multipolar based on their axon regrowth. A few hours after the cell dispersal, Me5 neurons attach to the cover glass and develop processes of about 3 cell body lengths eventually reaching 10 to 20 cell body lengths after a few days. This was expected because of the long afferent processes the Me5 posses in the intact animal (17, 56). The addition of FBS to the cell culture appears to be required for individual Me5 neurons to regrow extensive processes. The greatest axonal growth was observed when Me5 neurons were initially cultured with 10% FBS at the onset, however Me5 neurons were able to initiate regeneration of their processes in a serum-free, B27 supplemented growth media. The addition of 5% FBS on the second day of culture was required to further stimulate the sprouting Me5 neurons to aid in later identification for imaging.

Previous studies have shown that serum shocking the cells (with up to 50% serum) can synchronize the circadian clocks in astrocyte cultures (101). The time of the addition of FBS was staggered several hours for each mouse in order to determine
according to the phase analysis whether a serum effect was occurring. At this time, FBS stimulation does not drive the circadian clock to a specific ZT, but might have a PRC dependant on the time of stimulation. Whereas more axonal growth was observed from Me5 neurons taken from younger (8-16 day-old) animals, Me5 neurons were still able to regrow and maintain axonal process from older animals (20-28 days) (Figure 1).

In the intact mouse, Me5 neurons have recurrent axonal processes and are electrically coupled by gap junctions (78, 82). After the cell dispersal, a few Me5 neurons were able to remain connected together for the duration of the culture and therefore may have maintained their electrical synapses (Figure 1C). These cells could have relayed circadian timing cues within the connected population. Additionally, the extensive axonal growth of the Me5 could allow individual neurons to synapse on other Me5 within a more purified and condensed culture condition. By deliberately maintaining the gap junctions between Me5 neurons, or by encouraging chemical synapse formation following axonal regrowth, the coupling mechanism used in communication between circadian oscillators could be examined.

**Circadian mPer1::luc expression in dispersed Me5 neurons**

Me5 neurons dispersed and cultured in low-density primary cell cultures maintained a circadian rhythm in *mPer1::luc* expression (Figure 3). Like SCN neurons (46, 80), neurons of the Me5 appear to be autonomous circadian oscillators that can generate rhythms persisting for several cycles without damping. The average period of the rhythms for the first 3 cycles of imaging was shorter than those recorded from Me5 explant cultures of *mPer1::luc* mice (22.40 ± 0.81 hrs versus 23.35 ± 1.35 hrs), however
not significantly different (p > 0.05 Student’s T-Test) (Figure 3) (51). One possibility that should be further tested is whether uncoupling between the Me5 oscillators leads to a shortening of period. The wide distribution of periods in the isolated neurons within the circadian range that we observed also suggests that a more narrow range of periods present within Me5 neurons of the intact mouse could result from inter-oscillator coupling. Additional measurements of rhythms in Me5 explant cultures would be required to test this possibility.

Whereas the percentage of the Me5 neurons displaying a circadian rhythm was 27.95% ± 5.82% of the expressing cells for the first cycle of imaging, the number of Me5 neurons expressing detectable levels of bioluminescence in culture was 66.99% ± 11.57% (Table 1). The circadian oscillation was not specific to a particular sub-population (e.g., pseudo-unipolar or multipolar) based on the axonal regrowth (Figure 1, Figure 2).

The Me5 neurons have recurrent axon collaterals and are electrically coupled via gap junctions, which act to synchronize the output of the Me5 during chewing (78, 82, 96, 136). Within the SCN, a smaller sub-population of output neurons are electrically coupled via gap junctions, which serve to synchronize the GABAergic output of the SCN during the circadian day (65, 79). A similar hypothesis can be proposed for the Me5; a smaller population of circadian oscillating neurons might synchronize the GABAergic or glutamatergic output of the Me5 through electrically coupled gap junctions and recurrent axon collaterals. The circadian modulation of the cell membrane via the core clock genes would facilitate a temporal clustering of action potentials and synchronize the neuronal output, similar to SCN output neurons (65, 79). Synchronization of Me5 cell
membrane firing to coordinate neck and jaw movement during feeding has been reported (132), and the circadian oscillator could optimize these behaviors.

Previous studies have indicated that circadian pacemaker cells in culture can be influenced by culture conditions (137), such as the time of dissection or the presence of serum or other factors which can induce core clock gene expression (5). Phase estimates for dispersed Me5 neurons were analyzed to determine if such effects were present in the circadian oscillation of \textit{mPer1::luc} expression (Figure 4). Me5 neurons were maintained in a serum-free culture of DMEM supplemented with B27 for two days before the addition of medium with 5\% FBS and 0.1 mM luciferin. The present, limited study could not show definitive effect of FBS on phase (Figure 4). When adjusted for time of FBS stimulation, the mean ZT for the maximum phase reference point is not driven to the FBS ZT (Figure 4). For each culture there was a varying difference in the phase angle between the time of FBS stimulation and the mean angle for the maximum phase reference point. At this time, FBS does not appear to have a resetting effect on the Me5 circadian clock, but might shift the clock differently depending on the phase of the cycle when this potential stimulus was administered (Figure 4).

Unlike our explant study, the minimum phase reference point was the most reliable point in determining whether there was a significant cluster of phase in Me5 cell cultures (Figure 4). When comparing the maximum phase reference points between Me5 cell culture and explants, there is a difference in the mean ZT. In explants, Me5 neurons had a maximum expression occurring just after dusk (51), while in cell culture Me5 neurons had a wide variety in the maximum expression of \textit{mPer1::luc}. This wide distribution in phase was expected because of the additional time between culturing and
imaging allowed the individual free running Me5 neurons to separate in phase. The first three cycles of imaging for dispersed Me5 neurons correlates to cycles 5 to 8 for the culture, which were not observed in our previous study (51).

The average period (22.40 ± 0.81 hours) for each cell culture during the first 3 cycles during imaging was not significantly different (p>0.05, Student’s T-Test) from the first 3 cycles of explant cultures (23.35 ± 1.35 hours) (Table 2). Our previous study did not indicate any significant difference in period between the rostral and caudal Me5 in explant cultures, however the caudal Me5 tended to have a slightly shorter period than rostral Me5 (51). Cell cultures were primarily made from caudal Me5 sections, which could indicate why the cell cultures had a slightly shorter period than previously reported (51).

The distribution of mPER1-like immunoreactivity in Me5 neurons

Me5 neurons in brain sections showed mPER1-like immunoreactivity within the cytoplasm and nucleus in agreement with the mPer1::luc rhythms in Me5 neurons described in our previous study (51). The percentage of Me5 neurons which had mPER1-like immunoreactivity within the cytoplasm (45.50% ± 2.19%) was similar to the percentage of rhythmic pixels (45.89% ± 7.89%) previously reported (51). However, we should note that our previous study (51) measured rhythms in pixels that could contain more than one Me5 neuron. The presence of mPER1-like staining in the cytoplasm and nucleus of Me5 neurons is an indication that the rhythms in mPer1::luc expression observed in organotypic culture (51) are not an artifact of culture conditions and show that mPER1 is expressed in Me5 neurons in the intact animal (Figure 5). Similar
immunoreactivity within the cytoplasm and nucleus in the SCN control sections confirms
the anti-PER1 antibody was binding specifically to PER. The role of mPER1 has been
studied extensively in the SCN (46, 50, 80, 125, 131, 133) and likely serves a similar
function in the Me5.

The purpose of our study was to determine the presence of mPER1 in Me5
neurons within the intact animal, however, future work may be done to determine any
day/night differences in mPER1 concentration within the Me5 cytoplasm or nucleus. As
with the SCN, the nuclei of some of the Me5 were stained well. However, because of
the larger size of the neurons, subcellular localization of the mPER1 protein may be
possible because of the 10-fold size increase over the SCN. The SCN nucleus is made
up of 10 µm cells (79) whereas, Me5 neurons are 30-40 um in diameter (82, 96, 136).
The increase in cell size could facilitate subcellular measurements within circadian clock
neurons, such as imaging the translocation of key clock proteins serving in circadian
oscillations (see (80) for a review of circadian clock components).

Circadian mPer2luc expression in Me5 organotypic cultures

The circadian rhythm in mPer2luc expression in surgically reduced organotypic
cultures containing little more than the Me5 further strengthens the evidence that these
primary sensory neurons contain a circadian oscillator independent of the timing cues
from the SCN. Like the SCN (46), not all of the luminescing Me5 neurons displayed a
significant rhythm. However, the circadian expression in the Me5 produced a
detectable bioluminescence signal which persisted through several days of imaging.
Lomb-Scargle analysis of the Me5 indicated that the oscillation was significant (p<0.05)
and near 24 hours (Figure 6, Figure 7) in a large percentage of the Me5 cells but not in neighboring nuclei. Both \( mPer1::luc \) (51) and \( mPer2^{luc} \) showed peaks in expression in the Me5 that are delayed several hours compared to peak expression in the SCN (50), but are similar to that in other peripheral circadian oscillators (1, 43) (Figure 3, Figure 6).

Activation of L-type Ca\(^{2+}\) channels in the Me5 (136) during the depolarization associated with feeding could act as an entrainment cue by inducing either \( mPer1 \) or \( mPer2 \) (50). A simple hypothesis for the Me5 is that the resulting intracellular Ca\(^{2+}\) signals would act as an entraining cue for the circadian clock, as argued for other circadian pacemakers (8, 71). Although the Me5 could be receiving timing cues from the SCN, perhaps indirectly through the tuberomammillary nucleus (TMN) (75, 107), the depolarization from chewing could also serve as an entraining mechanism. Circadian oscillations in other primary sensory neurons have been reported across several species and may serve as a starting point for additional Me5 research (8, 12, 16, 27, 32, 116).

**The Me5 as an alternative and complementary model to the SCN**

Me5 neurons may serve as an alternative circadian model to the SCN for studying the cellular properties of the mammalian circadian clock. The results of this study and a previous work (51), have demonstrated that Me5 neurons have circadian oscillations in \( mPer1::luc \) and \( mPer2^{luc} \) expression, similar to other peripheral oscillators, and act as autonomous clock neurons when maintained in cell culture. Because of their size, Me5 neurons provide a much larger preparation for imaging subcellular or even single-molecule trafficking within a circadian mammalian neuron. Although similar
imaging might be performed with SCN neurons, Me5 neurons may provide the same opportunities without more sophisticated imaging techniques.

The Me5 neuron population is comparatively simpler than the SCN when considering cell phenotype and neuropharmacology. The SCN has diverse cell types expressing multiple neuropeptides and proteins including arginine-vasopressin, vasoactive intestinal polypeptide, gastrin-releasing peptide, dynorphin, calbindin, and others. (63) The SCN neurons are also not readily distinguishable by cell morphology alone, presenting challenges to single-cell studies of specific SCN cell types (65, 80, 92, 106, 109). The properties of Me5 neurons and their role in feeding behavior have been extensively studied across many different species (24, 34, 35, 37, 59, 64), and have been characterized well by pharmacology (45, 62, 77, 78, 97, 107, 118, 120). As with the SCN (47, 79), Me5 neurons have a spontaneous depolarization of the cell membrane (33, 96, 136) and the ion channels involved in the dynamics of rat Me5 membrane potential have also been characterized through patch clamp recordings of brain slices (33) and isolated neurons (136). Unlike SCN neurons, Me5 neurons show high frequency bursts of action potentials (up to 100 Hz) in acutely recorded cell dispersals (33, 96, 132). How these bursts might influence the output or entrainment of the circadian oscillator should be examined and compared with the SCN.

Another advantage of the Me5 as an alternative model is its unique location within the brain, The Me5 cells are primary sensory neurons receiving possible timing cues directly from the environment through chewing and mastication. While circadian rhythms have been identified in the retina and other primary sensory neurons (8, 12, 16,
27, 32, 116), Me5 neurons might have interesting functions because of their location in the brainstem and their proximity to vital regulatory areas.

In summary, the presence of a circadian oscillation in the expression of two of the core clock genes in organotypic cultures and \textit{mPer1} in dispersed cells provides additional evidence that Me5 neurons have an autonomous circadian oscillator. These results coupled with the extensive electrophysiological and neuropharmacological data from the Me5 make these neurons a potentially valuable alternative model to the SCN for studying the cellular properties of the circadian clock in mammalian neurons.

Acknowledgements

This work was partially supported by the BGSU J.P. Scott Center for Neuroscience, Mind, and Behavior. We would like to thank the animal care support staff at BGSU for their help with mouse breeding and colony maintenance, undergraduates Nicole Dusseau, Lee Krueger, Britany Sheard, and Jessica Zavadil for their diligence and help with data analysis and collection. We would also like to thank Dr. Erik Olofsend at the Leiden University Medical Center for the Lomb-Scargle MATLAB program and Caliper Life Sciences, Hopkinton, MA for luciferin.
Figures and Tables
Figure 1. Me5 neurons in dispersed cell culture. A,B,C: Me5 neurons showing their characteristic 30-40 µm, ovoid, light-refractile soma, and granular cytoplasm. Me5 neuron showing regrowth of a typical pseudo-unipolar process seen *in vivo* after 2 days in culture. C: An example of the few Me5 neurons that remained attached for the duration of the culture. Scale bars = 50 mm.
Figure 2. Bioluminescence and recovery of Me5 cells in culture. A: Bioluminescence image of a Me5 cell culture on a cover glass imaged at low magnification. Bright bioluminescence points were either clusters of astrocytes, large oligodendrites or Me5 neurons. Numbers indicate pattern of identification for cell recovery. B: After imaging, the cultures were fixed with methanol, stained with hematoxylin, and imaged as a series of overlapping brightfield images at 5x magnification (1300 x 1030 pixels each). Me5 neurons and astrocyte clusters were identified in the mosaic and then overlayed with the bioluminescence image. Patterns of Me5 neurons and larger astrocyte clusters matched the bioluminescence image. The green circle indicates a bright astrocyte cluster and red circles indicate bright Me5 neurons. Black circles indicate Me5 neurons identified in the mosaic that appear dark in the bioluminescence image. Scale bar = 500 mm.
Figure 3. Circadian rhythms in $mPer1::luc$ expression in dispersed Me5 cultures imaged for several days. A,B: Rhythms recorded from individual Me5 neurons lasted up to 3 cycles during imaging (cycles 5-8 of the culture). Hash bars indicate the phase of the previous light-offset in the LD cycle. Blue line is a 3-hour adjacent averaging and the green line is a 24-hour adjacent averaging. The 24-hour adjacent averaging was used to find the rise and fall phase reference points as determined by the time when the blue and green lines intersect (see (51)). ADU: Analog-to-digital units of the camera sensor.
Figure 4. Phase plots of circadian rhythms in dispersed Me5 neurons cultures based on four types of phase reference points. Rao’s spacing test indicated significant (p<0.05) for the minimum phase of each culture A,B,C and for the rising phase of culture A. The black line indicates the mean angle for each phase reference point. The standard deviation of the mean is shown by the arc on the outside of each plot. Time 0 is the animal’s dawn (light onset) and time 12 is dusk (dark onset) in the previous LD cycle.
Figure 5. Immunohistochemistry showing mPER1-like immunoreactivity within Me5 neurons.  A: Me5 neurons are found along the Me5 tract as shown in this caudal section labeled with a polyclonal antibody direct against mPER1.  Darkly stained nuclei can be seen in some neurons while others appear to have little to no staining.  B: Positive control showing staining within the SCN cell nuclei.  C: Close-up view of Me5 neurons showing localization of mPER1 protein in the nucleus.  D: Control SCN section showing robust staining within SCN cells.  Scale bar = 50 μm
Figure 6. A and B: Rhythms in mPer2^Luc in mouse Me5 of explant cultures persisted over several cycles. Lomb-Scargle analysis of the organotypic cultures isolated 13 to 36 cells which had a significant (p<0.05) circadian rhythm. Circadian rhythm plots were determined by measuring the region-of-interest determined by Lomb-Scargle and were
analyzed and detrended as previously reported (51). Blue line is after 6-point adjacent averaging. Green line is after 48-point adjacent averaging that was used to find the rising and falling phase of each circadian cycle. ADU: analog-to-digital units of camera sensor.
Figure 7. Lomb-Scargle analysis of rhythms in Me5 neurons of *mPer2^luc* mice.

A: Bioluminescence from a caudal Me5 slice culture containing little more than the Me5, LC, and PB. All pixels above a set threshold intensity were analyzed with a Lomb-Scargle algorithm to identify cells with significant 24-hour circadian rhythm (p<0.05) as shown by green pixels, e.g., cell at arrow.
Table 1. *Number of Me5 neurons expressing mPer1::luc during the first cycle*

<table>
<thead>
<tr>
<th>Cell Counts</th>
<th>Total</th>
<th>Expressing</th>
<th>Percentage</th>
<th>Rhythmic Me5</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture 1</td>
<td>42</td>
<td>23</td>
<td>54.76%</td>
<td>7.5</td>
<td>32.61%</td>
</tr>
<tr>
<td>Culture 2</td>
<td>38</td>
<td>26</td>
<td>68.42%</td>
<td>7.75</td>
<td>29.81%</td>
</tr>
<tr>
<td>Culture 3 *</td>
<td>36</td>
<td>28</td>
<td>77.78%</td>
<td>6</td>
<td>21.43%</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td></td>
<td></td>
<td><strong>66.99%</strong></td>
<td><strong>Average</strong></td>
<td><strong>27.95%</strong></td>
</tr>
<tr>
<td><strong>SD</strong></td>
<td></td>
<td></td>
<td><strong>11.57%</strong></td>
<td><strong>SD</strong></td>
<td><strong>5.82%</strong></td>
</tr>
</tbody>
</table>

The average number of rhythmic Me5 neurons during the first cycle for all of the phase reference points is shown. The percentage of Me5 neurons which displayed a rhythmic (19-29 hours) expression in *mPer1::luc* for all of the phase reference points for each culture was determined by dividing the number of expressing cells by the number of rhythmic cells.

* indicates cell cultures in which 2 animals were used in the cell dispersal.
Table 2. *Period estimates for mPer1::luc gene expression in dispersed Me5 neurons*

<table>
<thead>
<tr>
<th>Culture 1</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Period</td>
<td>23.36</td>
<td>22.90</td>
<td>22.82</td>
</tr>
<tr>
<td>SD</td>
<td>1.23</td>
<td>0.94</td>
<td>0.60</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Culture 2</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Period</td>
<td>22.10</td>
<td>22.05</td>
<td>22.65</td>
</tr>
<tr>
<td>SD</td>
<td>0.54</td>
<td>0.42</td>
<td>3.10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Culture 3 *</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Period</td>
<td>23.26</td>
<td>21.43</td>
<td>21.01</td>
</tr>
<tr>
<td>SD</td>
<td>1.47</td>
<td>1.09</td>
<td>0.46</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Total</th>
<th>Period 1</th>
<th>Period 2</th>
<th>Period 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Period</td>
<td>22.90</td>
<td>22.13</td>
<td>22.16</td>
</tr>
<tr>
<td>SD</td>
<td>0.70</td>
<td>0.74</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Average period estimates for each culture for all phase reference points. * indicates cell cultures in which 2 animals were used in the cell dispersal.
Chapter 4: IMAGING GENE EXPRESSION IN LIVE TRANSGENIC MICE AFTER PROVIDING LUCIFERIN IN DRINKING WATER

Preface

This chapter was originally published as a technical note in *Photochemical and Photobiological Sciences* in August 2006 (Hiler et al. 2006. Imaging gene expression in live transgenic mice after providing luciferin in drinking water. *Photochem. Photobiol. Sci.* 5: 1082-1085). The project began as a side project research project to my dissertation work and was used to train new undergraduates in the lab. The results of this study generated a very favorable reaction from the research community and numerous reprints have been sent around the world. To date, two publications on luciferin imaging in mice have cited this work, and current research using orally administered luciferin. For this dissertation, the original published text has been modified to include data from freely moving mice, which we felt was not publishable when we submitted the original article. Apparently our work in freely moving animals was ahead of its time and now imaging systems capable of low-light imaging with short exposure times have been developed and are being used to monitor gene activity in freely moving rats.

Introduction

Gene reporter systems based on the firefly luciferase gene luc have become an effective alternative to more costly and time-consuming methods to quantify gene expression. (23, 39, 44, 61, 86, 126, 134) Unlike methods that rely on tissue harvesting to isolate mRNA, luciferase bioluminescence can be imaged in whole animals or live
cells to provide non-invasive assays over the life of the organism or culture. Each animal or preparation can be used as its own control, thereby reducing error from inter-individual variation and reducing the number of animals used. This non-invasive technique is also useful for monitoring a single animal over several months to study gradual or rhythmic changes in gene expression. (19) To image luciferase expressing reporter genes in live mice, the substrate luciferin is typically injected intravenously (iv) into a rat or mouse tail vein or intraperitoneally (ip) or chronically administered through a surgically-implanted osmotic micro pump (42).

Any injection of luciferin could affect gene expression through the stress from handling or tissue damage from the injection needle. We were interested in whether orally delivered luciferin would be an effective alternative to injection by producing a comparable luciferin distribution while eliminating any effects from injection or handling. Orally administered luciferin might also allow for chronic luciferin administration without the need for repeated injections.

One study has suggested that iv injection produces a distribution pattern of isotopically labeled luciferin different from that of ip injection. (76) This difference may indicate that some tissues and body areas receive and distribute luciferin at different rates. Oral luciferin could provide a third distribution pattern that may allow visualization of body areas not possible by ip or iv injection. Continuous oral delivery may provide sustained luciferin levels throughout tissues that are not achieved by injection.

Previous studies have reported that the concentrations of anesthesia similar to the dosage used in animals reduced the activity of luciferase in solution (76). If luciferase is affected in a similar way in vivo, then luciferase images collected from
anesthetized animals may be misleading and may not truly reflect the luciferase signal in an untreated, freely moving animals. Furthermore, altered oxygen levels and metabolic rates as a result of the general inhibitory effects of anesthesia could lead to altered gene expression patterns in the anesthetized animal.

To aid in visualizing deep tissues, we used transgenic mice expressing luc controlled by the promoter and enhancer of the major immediate-early gene of the human cytomegalovirus. (10, 40, 55, 111) These CMV::luc mice were crossed with a hairless albino mouse line (HRS/J) to improve imaging of deep tissues (Hr-CMV). (18, 19) We found that luciferin was effective in providing luminescence when delivered orally, in water or apple juice, and the signal was similar to the signal seen in ip-injected mice, except for a slightly higher signal in the tail after oral luciferin. Mice administered luciferin orally could also be imaged while freely-moving within a confined space and produced a bioluminescence signal similar to injected mice.

Material and Methods

Animals

Hr-CMV transgenic mice (Mus musculus) (18) were raised and maintained in cycles of 12 h light/12 h dark with food (Mouse Breeder Diet, Harlan Teklad) and tap water provided ad libitum. Mice of both sexes between 1 and 7 months of age were used for imaging. The sodium salt of luciferin (1–5 mM, Invitrogen) was provided to the mice in 1–2 ml volumes of distilled water or pure apple juice without additives (pH 3.8, Everfresh, Sundance Beverage Co., Warren, MI). The luciferin solution was supplied in either a 35 mm dish placed on the floor of a clean cage or in a modified 15 ml
polyethylene centrifuge tube cut to allow access through a 5 mm hole about 15 mm from the base. This drinking tube was inserted through a hole in the side of a cage constructed from a 165 mm long black PVC cylinder that was mounted vertically on a clear Plexiglas sheet with black felt underneath to minimize light scatter.

Two hours after mice were administered luciferin laced drinking water, mice where placed in the PVC cylinder to limit their movement without restraint and imaged to detect any bioluminescence signal in the freely moving animal. Control mice injected i.p. with 100 uL of 0.1 mM luciferin in Hank’s Balanced Salt Solution (Invitrogen) were placed into the PVC cylinder 15 minutes after injection to record the bioluminescence signal in freely-moving animals. Several of the mice which were administered luciferin orally were also imaged as control animals. Animals used as their own control were given several weeks between imaging sessions to recover from the previous treatment.

After imaging freely-moving animals, mice were anesthetized with isoflurane and were immediately placed back into the PVC cylinder or on a wire mesh form (Wireform 6.3 mm diamond mesh, American Art Clay, Inc., Indianapolis, IN) covered with black felt to maintain their orientation during imaging. An isothermal pad (Braintree Scientific, Braintree, MA) was placed underneath the animal to ensure maintenance of body temperature during anesthesia and imaging. After imaging, mice were returned to their cage and allowed to recover under observation before returning them to the colony. All procedures were approved by the University’s Institutional Animal Care and Use Committee.
Bioluminescence imaging

A liquid nitrogen-cooled CCD camera (Roper Scientific CH360, 2.5 L dewar) with a back-thinned, back-illuminated sensor (512 × 512 pixels, each 24 × 24 μm) and a Nikkor 50 mm f/1.2 lens was used to image bioluminescence from the anesthetized mouse. Anesthetized mice were imaged using 20 to 60 second exposures and 4x4 pixel binning. V++ imaging software (Roper Scientific, Tucson, AZ) enabled continuous collection and processing of the images with a 16-bit dynamic range. The images were processed further with ImageJ (NIH) and Photoshop 6.0 (Adobe). Of the body areas selected, only luminescence signals greater than 10 times the bias corrected background intensity were used in the analysis, and these are referred to here as detectable signals.

For freely-moving non-anesthetized mice, imaging began immediately after introducing the mice to the PVC cylinder by taking 50 consecutive 5-second images with 4x4 binning every 10 minutes. Animals were unrestricted and allowed to freely move around the cylinder during the imaging session. Additional imaging sequences were also taken with 2x2 binning and at different durations to determine the optimal exposure time for freely moving animals.

Results

Oral administration of luciferin in anesthetized mice

Six Hr-CMV mice were injected intraperitoneally with luciferin, anesthetized with isoflurane five minutes later, and then imaged. Similar to ip-injected Hr-CMV mice imaged previously, 8 areas with thicker skin, such as the paws and snout showed high expression, and areas with thinner skin and little underlying tissue, such as the ear
pinna, showed very faint expression in all six mice (Fig. 1). Eyes, some skeletal muscles, ear canals, claws and several structures below the skin were also visible, such as tail vertebrae and internal organs.

When five Hr-CMV mice were provided luciferin in the drinking tube with water, only one showed luminescence. Nevertheless, this mouse could be imaged three times over a several month period using the tube for oral administration. Because of the low success rate with this approach, a 35-mm plastic petri dish containing 1 ml of 5 mM luciferin in water was tested as an alternative to the drinking tube.

Two of two Hr-CMV mice provided with luciferin and water in the 35 mm dish showed expression. The same technique was also assessed using luciferin in apple juice to test whether this would increase drinking rates. Using apple juice instead of water, three of three Hr-CMV mice tested clearly showed luminescence (Fig. 1). The time elapsed before detecting luminescence was 50–60 min after providing luciferin to the animal in the dish with water or apple juice. Some mice were provided luciferin for up to three hours prior to imaging.

Hr-CMV luminescence patterns in orally treated mice were much like those of ip-injected ones except for a slightly higher signal in the tail after oral luciferin. Areas showing consistent expression across mice were delineated by anatomical boundaries and used for comparing oral versus injected luciferin (Fig. 2). The areas showing detectable signals after oral delivery were (in order of brightness) the abdomen, proximal tail, front shoulder, rear leg, neck, sacrum, thorax, distal tail, ear canal, gonads, snout, and rear paws. For the injected mice, these areas were abdomen, rear leg, thorax, neck, sacrum, front shoulder, proximal tail, ear, distal tail, rear paws, snout,
and gonads. The injected mice displayed significantly brighter luminescence (6794.4 ± 2097.66, SD) than mice given oral luciferin (1149.80 ± 458.76, SD) when the abdomen, the brightest area, was used for comparison (5.9-fold, p < 0.05, Student’s t-test). To compare the distribution pattern of luciferin expression, average signals in the oral and injected groups were normalized to the abdomen signal (Fig. 3). The intensity of body areas did not show significant differences by one-way ANOVA within the oral (F = 0.924, p = 0.491) or injected (F = 1.497, p = 0.231) groups. Only body areas in which at least three mice showed a detectable signal were used in this analysis.

*Oral administration of luciferin in freely-moving mice*

As with the anesthetized mice, of the five Hr-CMV mice provided luciferin in a drinking tube with water, only one showed luminescence. During the sequential imaging session over a several month period, this mouse continuously drank sufficient luciferin to produce a detectable bioluminescence signal as a freely moving animal (Figure 4). However, because the animal was unrestrained and sometimes moving rapidly, only small percentage of the images captured were not blurred as a result of movement.

Of the anesthetized mice given luciferin in apple juice or water in a dish, three Hr-CMV mice consumed sufficient luciferin to be detected as a freely moving animal. However, unlike the mouse given luciferin via drinking tube, the bioluminescence signal from these animals was weaker, and because of the constant movement, an even smaller percentage of the images captured showed the shape of a mouse.
Control Hr-CMV mice injected with luciferin i.p. produced a similar distribution pattern of bioluminescence compared to orally administered mice when imaged while freely moving (Figure 5). The intensity of the bioluminescence signal was more impacted by the position of the animal and lack of motion than difference between the methods of luciferin administration. Similar problems with the rapid movement of the mouse and motion blur in the images were also prevalent during image sequences of control freely moving mice.

Discussion

These results show for the first time that luciferin will generate luminescence in the tissues of live transgenic mice containing luciferase-based reporter genes when it is provided in the drinking water. Luciferin is not degraded substantially when given by this route and may in fact be protected from degradation by the stomach contents; luciferin is known to be stable in acidic solutions. (11) An effective oral dose of luciferin for detecting expression was 1–5 mM in 1–2 ml distilled water provided for at least one hour. Furthermore, luciferin administered to the mouse in pure apple juice was also effective at generating luminescence, and the acidity of the juice may increase luciferin stability relative to water alone during prolonged treatments. Juice may also increase the palatability of luciferin for some mice, possibly accelerating the ingestion rate and could be useful for any mouse strains showing an aversion to luciferin in their drinking water.

We found that only a small percentage of the Hr-CMV mice given luciferin in water through the drinking tube drank enough to generate a detectable signal,
suggesting that variability between mice in their preferences could play a role in the effectiveness of oral delivery. Alternatively, mice may have avoided the tube because it was novel, whereas the dish on the floor of the cage may have been more accessible. Mice exposed to the dish on several occasions may become more accustomed to it and ingest the luciferin more rapidly, decreasing the time needed for imaging. The time of day that the animals are given luciferin in drinking water may also have an effect on how much water or apple juice they will drink. (72) Oral luciferin administration should be most effective just before the time that environmental light ends or just after the light resumes. Taking advantage of this daily rhythm in drinking rate might reduce the time elapsed before luminescence is detected.

The result of imaging freely-moving Hr-CMV mice highlighted the limitations of this CCD camera imaging system. While both orally administered and injected animals both produced a luminescence signal detectable by our CCD-camera (Figure 4, Figure 5), the limiting factor was the exposure time. To detect a bioluminescence signal, a 5-sec exposure time was required. However, because the mice were not anesthetized and were unrestrained, the mice did not remain still long enough to be imaged without motion blur. The small percentage of mice that readily consumed luciferin orally confounded the problem by limiting the number of freely moving trials. Imaging freely moving control mice also faces similar problems as those seen in orally administered animals because of the constant movement of the mouse. As with the drinking tube, being placed in a novel environment may have contributed to the exploratory behavior of the mice, which may be reduced by pre-exposing the mice to the imaging chamber several times prior to imaging. The most likely solution to whole animal imaging is the
development of more sensitive imaging systems which can require shorter exposure
times to record from a fast moving rodent. Despite the difficulties, these results show
that transgenic mice using a luciferase bioreporter produce detectable levels of
luminescence to be imaged as a freely-moving animal. Future work with more
sophisticated imaging systems will be able to monitor systems wide changes in the
whole animals without invasively altering the animal’s physiology and gene expression.

As with injected luciferin, mice could be imaged several times after giving
luciferin orally during separate experiments. These results suggest that voluntary
drinking of luciferin can be equally effective as i.p. injection for longitudinal studies
directed at observing changes in gene expression in response to drug treatments, tumor
growth, circadian rhythms, and other processes acting over several hours or days.

Acknowledgements

This work was supported in part by the National Institutes of Health grant
5R21RR12654-2 and the BGSU J. P. Scott Center for Neuroscience, Mind, and
Behavior. We would like to thank Denise Hook and Melinda T. Lynn for their help with
mouse breeding and initial experiments, respectively.
**Figure 1.** Live hairless albino mice expressing firefly luciferase. A: reference image of Hr-CMV mouse anesthetized after ip luciferin injection. B: corresponding luminescence. C: reference image of anaesthetized Hr-CMV mouse after drinking luciferin in apple juice. D: corresponding luminescence. Luminescence images were captured with 40 s exposures and 4 × 4 pixel binning and were log transformed. Scales to the right show luminescence intensity as analog-to-digital units of the imaging sensor.
Figure 2. The 12 body areas used in analysis of luminescence distributions. A: lateral view of reference image. B: dorsal view of reference image. 1 snout, 2 ear canal, 3 neck, 4 shoulder, 5 thorax, 6 abdomen, 7 rear leg, 8 rear paws, 9 sacrum, 10 gonads, 11 proximal tail, 12 distal tail. Only areas with reliably detectable signal (20-times background) were used. Signals from most of the head were faint.
Figure 3. Relative luminescence in body areas of mice after oral or injected luciferin delivery. Body area numbers correspond with Fig. 2. Shown is average relative luminescence ±SE (normalized to abdominal signal). Labels include the number of mice showing detectable signal. Maximum pixel values within the body areas were analyzed.
Figure 4. Hr-CMV mouse freely moving and imaged after oral luciferin administration. 5-sec exposure with 4x4 binning. This particular mouse was imaged at three different times over several weeks. Three other mice were imaged with this method but produced a weaker luminescence signal. Arrowhead indicates the location of the animal’s foot during one 5-second exposure. Arrow indicates the location of the tail. Long arrow: Luminescence signal produced by the underlying muscle located in the rear leg.
Figure 5. Control Hr-CMV mouse freely moving after luciferin injection. 5-sec exposures with 4x4 binning. Long Arrow indicates luminescence signal produced by the underlying muscle located in the rear leg.
CHAPTER 5: CONCLUDING REMARKS

Additional questions regarding Me5 neurons in organotypic explants

Organotypic explants of the Me5 used to image \textit{mPer1::luc} expression were reduced by dissection to an area containing only the Me5, parts of the PAG, and the dorsal raphe or the LC and PB in caudal sections. One question not addressed by the present study was any possible rhythmic or non-rhythmic input into the Me5 from these neighboring structures. Luminometer gene expression surveys of mPer1 expression in the midbrain has detected luminescence from the dorsal raphe nucleus (DR) \cite{1, 43}, and the Me5 neurons have 5-HT receptors and known projections from the dorsal raphe \cite{74, 77}. Although the expression of \textit{mPer1::luc} expression in the dorsal raphe is arrhythmic \cite{1}, non-rhythmic signaling from 5-HT pathways to the Me5 are still present in the reduced explants \cite{74, 77}. We would like to know if the input from non-rhythmic structures, such as the DR or LC, provides a non-rhythmic signal which modulates the circadian clock and rhythmic \textit{mPer1::luc} expression in Me5 neurons.

Our initial survey of \textit{mPer1::luc} expression in explants of Me5 neurons was only able to detect individual pixels of Me5 clusters (Chapter 2). Are additional Me5 neurons with rhythmic \textit{mPer1::luc} expression present in explants but not previously detected by our initial study? Our study was unable to achieve a suitable resolution to record from individual Me5 neurons. For the present study, we analyzed the 10-brightest pixels, chosen from the midpoint of the imaging sequence, within a given organotypic culture. Because of our method of analysis, there was some bias toward more healthy explants which had brighter Me5 neurons than adjacent explants and Me5 clusters which were reaching a peak midway through imaging. Based on the later results from \textit{mPer2^{luc}}
explants and the number of dispersed Me5 neurons expressing \textit{mPer1::luc} (where individual cells were resolved), revisiting \textit{mPer1::luc} explants of the Me5 and imaging at a higher resolution may produce better results.

**Additional questions regarding Me5 neurons in cell dispersal**

Preliminary work on the imaging of \textit{mPer1::luc} expression in dispersed Me5 neurons has revealed several questions that should be addressed by future studies. Through cell dispersal, Me5 neurons are isolated from each other synaptically and, in most cases, electrically by the removal of recurrent axon processes and culturing at a low-density in primary cell cultures. Based on the results of the present study (Chapter 3), synaptically isolated Me5 neurons can act as autonomous circadian clock neurons, similar to isolated SCN neurons (46, 49). However, in both cases, SCN and Me5 dispersals paracrine signaling from other circadian clock neurons has not been removed from the culture and could be synchronizing the circadian output of some of the neurons. Although Me5 neurons were cultured at a low density, around 10 per cm\(^2\), these neurons should be cultured as completely isolated, single neurons, thus completely removing any possible humoral signals, to be entirely confident that the Me5 neuron can function as an autonomous clock.

Another question not addressed by the present study is whether specific subtype (pseudo-unipolar or multipolar) of Me5 neuron display a circadian rhythm in \textit{mPer1::luc}. Or do both cell types express a circadian rhythm in \textit{mPer1::luc} equally? Pseudo-unipolar Me5 neurons are uniquely different from multipolar Me5 neurons in receptor distribution (56, 107), neuronal output (17, 74), and sensory modality (74).
Based on our preliminary analysis of dispersed Me5 neurons, the larger, pseudo-unipolar Me5 are easier to identify in culture and produce a circadian rhythm in mPer1::luc expression. However, our analysis was slightly biased toward the larger, pseudo-unipolar Me5 because of the presence of oligodendrocytes in the culture. After several days of culturing, oligodendrocytes appeared similar in shape and morphology to the multipolar interneurons of the Me5. A more conservative selection process was used in identifying expressing multipolar Me5 neurons for period analysis. Future studies using GABAergic and glutamatergic labels, such as glutamic acid decarboxylase (GAD), in immunohistochemistry or in situ hybridization to individually identify Me5 neurons would identify any differences in the cell types producing the circadian output of the clock in Me5.

Future work in Me5 neurons

Future research studies into the circadian properties of Me5 neurons have some very broad questions to address. What is the role of the circadian oscillator in Me5 neurons during feeding, and are circadian oscillators present in other parts of the trigeminal ganglia? As a result of the sensory similarity between the sensory trigeminal ganglion and the similarly large neurons in the trigeminal motor nucleus (74), future work may begin by surveying these areas for any detectable levels of mPer1::luc expression. One possible explanation for the presence of a circadian clock in the chewing circuit is to synchronize the sensory trigeminal ganglion to provide a peak in chewing sensory responsiveness at dusk. Rodents are known to begin their activity phase at night with several bouts of eating and drinking at dusk (72).
The results of the present study showed that \textit{mPer1::luc} expression had a maximum peak in gene expression just after dusk (light onset) in explants and just before dusk (dark onset) in dispersed neurons. The influx of Ca$^{2+}$ from depolarization of the Me5 stimulated by chewing could be responsible for the transgene activation through the cyclic-AMP response element (CRE) of the \textit{mPer1} promoter (50). The potential "Chewing Entrainable Oscillator (CEO)" of the Me5 clock could act to modulate the spontaneous neural impulses of the Me5 and provide the Mo5 with a temporal summation of Me5 output, leading to a peak responsiveness in chewing.

Future work in orally administered luciferin and whole animal imaging of freely moving mice

One of the questions unanswered by our orally administered luciferin project was the proper dosage required to produce a detectable luminescence signal in anesthetized Hr-CMV mice. To compensate for a potentially weak signal, the amount ingested by the animal, and the absorption of the substance by the digestive track, a substantial dosage of 1-5 mM luciferin, 10x – 50x greater than the typical injection, was used. The cost of using this dosage of luciferin in a constant water supply or large volume of water is great, and therefore, a dose response curve is needed to determine the optimal dosage required to produce the desired bioluminescence signal.

Motion blur occurring while imaging freely moving animals was primarily the result of limitations in the exposure time of our imaging system. Recent improvements in the sensitivity of camera imaging systems have lead to a decrease in the exposure time required to capture an image. The use of bioluminescence imaging has been
recently used to detect and monitor the spread and progression of disease at the whole
animal level (21, 22). The recent development of new camera imaging systems which
allow for shorter exposure times eliminates the primary problem we had during whole
animal imaging of orally administered luciferin (105).

Future directions.

While we have established the presence of a circadian oscillator in Me5 neurons,
utilizing Me5 neurons as an alternative model to study the molecular properties of the
mammalian clock will require more extensive work. One of the most promising avenues
of research is in the spontaneous 100-Hz impulses of Me5 neurons (33, 96, 132) and
determining whether there is circadian modulation of the cell membrane potential.
Voltage and current clamp recordings of day/night differences in the firing rate of Me5
neurons would be a starting point and would provide an analysis similar to work already
done in the SCN (47, 65, 79, 93). One of the key questions in circadian biology is how
the circadian clock interacts with other parts of the cell at the molecular level. Whereas
the SCN has been shown to be a valid model for studying these properties (65, 79), the
small size of individual SCN neurons and complexities of the nucleus may be a limiting
factor. Through the use of alternative cellular models, such as the Me5, the field of
circadian biology can gain a better understanding of the complex molecular interactions
of the circadian clock and develop methods to influence its actions.
REFERENCES


