A Potential Molecular Target for Caffeine in its Disruption of Ultradian Rhythms in Paramecium tetraurelia

Luke Piper
Indiana University of Pennsylvania

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A POTENTIAL MOLECULAR TARGET FOR CAFFEINE IN ITS DISRUPTION OF ULTRADIAN RHYTHMS IN PARAMECIUM TETRAURELIA

LUKE PIPER
A Potential Molecular Target for Caffeine in its Disruption of Ultradian Rhythms in

Paramecium tetraurelia

A Thesis

Submitted to the Robert E. Cook Honors College

In accordance with the requirements of the Biology Honors Program

Luke Piper

Advisor: Dr. Robert D. Hinrichsen

Department of Biology

College of Natural Sciences and Mathematics

May 2013
Acknowledgments

I would be remiss were I to not call attention to my advisor, Dr. Robert Hinrichsen. His guidance and mentoring throughout this process are chiefly the reason that this thesis exists in the first place. He has been supportive of my successes, and patient with my faults, and I do not think I could have asked for a better mentor in my research and in my undergraduate studies. In addition to being a superb educator, he is also a valued friend, and I thank him for all of his assistance and guidance over the course of my time at IUP.

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Finally, I must thank my family for its constant love and support. I firmly believe that my success at IUP had their foundations in my upbringing, with parents who infused me with a profound moral compass and a love of learning. Through their motivation, I never stopped challenging myself or pursuing excellence, and I believe it is this mindset that led me to all of my successes at IUP and to the completion of this project.
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Abstract

*Paramecium tetraurelia* has proven to be an effective model system for the study of biological rhythms, with an easily identifiable swimming behavior that fluctuates in frequency with an ultradian rhythm. Various substances have been implicated in disrupting the periodicity of this rhythm. In this study, we focused on the disruption caused by caffeine. Previous studies have demonstrated caffeine's effect on ultradian rhythm periodicity in *P. tetraurelia*, but did not investigate concentration efforts, nor potential molecular targets by which caffeine may induce this effect. As caffeine is a documented inhibitor of cellular phosphodiesterase, we investigated whether inhibition of phosphodiesterase expression via RNA interference would produce an ultradian rhythm disruption similar to what is seen with caffeine. Our results suggest that phosphodiesterase activity — and the resultant accumulation of cellular cAMP — plays a key modulatory role in the ultradian rhythms exhibited by *P. tetraurelia*. It is further suggested, therefore, that inhibition of this enzyme is at least one of the mechanisms by which caffeine exerts its disruptive effect.
Chapter I: Introduction and Literature Review

Ultradian rhythms describe oscillations in cellular processes and occur in almost all forms of eukaryotic life. These oscillations occur with a periodicity of less than 24 hours and maintain their periodicity in spite of fluctuating temperatures (1). Despite the large variety of examples of ultradian rhythms that have been discovered, including the rhythm of the courtship song used by the fruit fly species *Drosophila melanogaster* (2) as well as the defecation behavior of the nematode species *Caenorhabditis elegans* (3), the molecular mechanism responsible for ultradian rhythms is not well understood, nor is their biological significance (1). Previous research has identified genes involved in ultradian rhythms in *C. elegans* (4) and yeast (5), but these studies have been unable to identify the mechanisms by which these genes produce the oscillations characteristic of ultradian rhythms.

*Paramecium tetraurelia* is a single-celled, ciliated protist, and is an excellent model system for the study of biological rhythms, owing to its ease of maintenance, sequenced genome, and the ease by which it can be genetically manipulated and have its mutants isolated (6). The swimming behavior of *P. tetraurelia* is governed by a distinct ultradian clock that not only affects swimming speed, but also the rate of avoidance reactions (1, 7). These avoidance reactions, which will be the primary observation target for this study, are unique behavioral responses exhibited by *Paramecium* in response to obstacles and other stimuli encountered during locomotion. Avoidance reactions are defined as a brief (less than 1 second) episode of backwards swimming, after which the cell reorients itself and begins swimming forward again in an attempt to overcome the obstacle that triggered the response (8). These avoidance reactions are caused by the firing of a Ca$^{2+}$-based action potential that induces the cilia to rotate 180° and briefly propel the cell backwards (9). The length of time in which the backward motion is sustained is directly related to the strength of the action potential that induced the avoidance
response (10). The critical factor that allows these avoidance responses to be used as an observational target in this experiment is that the frequency of action potential firing in Paramecium varies with an ultradian rhythm (1). Furthermore, preliminary research has demonstrated that this ultradian rhythm can be disrupted by the addition of foreign substances, such as lithium administered in the form of lithium chloride (1, 11).

Caffeine is the most heavily consumed psychoactive substance in the world. Typically, caffeine is consumed for its stimulatory effect on the nervous system which, when taken in moderation, typically wards off fatigue and drowsiness while carrying few, if any, major health concerns. However, excessive caffeine consumption can lead to restlessness, anxiousness, irritability, headaches, and abnormal heart rhythms (12). Caffeine is also known to have a wide range of effects on other animals, and P. tetraurelia is no exception. Recent research in the Hinrichsen laboratory has demonstrated caffeine's ability to disrupt the ultradian rhythms of P. tetraurelia. Whereas the frequency of avoidance responses in P. tetraurelia normally oscillates with a periodicity of approximately 52.5 minutes, exposure to 1 μM caffeine reduces the average periodicity of these cells' ultradian rhythms to approximately 29 minutes (13).

In research investigating caffeine's effect on the ultradian rhythms of P. tetraurelia, the mechanism by which caffeine induced its effect was not investigated. However, caffeine produces other effects within cells that could implicate a plausible molecular mechanism in the regulation of ultradian rhythms. Caffeine is a well-documented inhibitor of cellular phosphodiesterases. These phosphodiesterases are enzymes that are primarily responsible for the breakdown of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). As a result of caffeine's inhibition of phosphodiesterases, cells which are exposed to
caffeine tend to experience a proliferation of cAMP and cGMP (13, 14). This leads us to suspect a potential connection between phosphodiesterase activity and ultradian rhythm regulation.

The primary focus of this study is to determine whether or not caffeine's ability to inhibit cellular phosphodiesterases is a significant factor in its ability to disrupt the ultradian rhythms of *P. tetraurelia*. We will be able to compare the effects on the ultradian rhythms of *P. tetraurelia* produced by caffeine to those produced solely by phosphodiesterase inhibition by using a technique called RNA interference (RNAi). In the context of our experiment, RNAi uses experimentally introduced double-stranded ribonucleic acid (dsRNA). Within the cell, dsRNA functions to inhibit the expression of homologous genes by either inducing degradation or preventing translation of homologous mRNA molecules (16). By using this technique to inhibit the expression of phosphodiesterase, we can observe the effects that phosphodiesterase inhibition has on the ultradian rhythms of *P. tetraurelia* and compare them to the effects of caffeine. This will allow us to ascertain whether phosphodiesterase inhibition plays a significant role in caffeine's ability to disrupt these ultradian rhythms, which will further allow us to determine whether or not cellular levels of cAMP and cGMP are significant in the cellular mechanism for ultradian rhythm regulation.

The significance of this study comes not only from the relatively unknown nature of the molecular mechanism governing the regulation of ultradian rhythms, but also from caffeine's status as the most heavily consumed psychoactive stimulant currently on the market. As mentioned previously, inferences made regarding the mechanisms controlling biological rhythms in *P. tetraurelia* can be applied to the mechanisms controlling biological rhythms in humans, since biological rhythms in general are controlled at the cellular level. Therefore, the goal of this
experiment is to provide insight that will not only prove useful to understanding how caffeine affects <em>Paramecium</em>, but also how it affects humans.
Chapter II: Materials and Methods

2.1 — Stock Solutions Used

<table>
<thead>
<tr>
<th>Material</th>
<th>Solvent</th>
<th>[Stock]</th>
<th>[Working]</th>
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</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>H₂O</td>
<td>50 mg/mL</td>
<td>2 µM</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>H₂O</td>
<td>50 mM</td>
<td>50 mM</td>
</tr>
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<td>H₂O</td>
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<td>0.5 µg/mL</td>
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<td>Caffeine</td>
<td>H₂O</td>
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</tbody>
</table>

*Table 1 – Chemical used, along with stock and working concentrations*

2.2 — Enzymes and Kits Used

<table>
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<th>Supplier</th>
</tr>
</thead>
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<td>QIAGEN Prep Spin Miniprep Kit</td>
<td>Qiagen</td>
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<tr>
<td>T4 Ligase</td>
<td>Promega</td>
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<tr>
<td>Xpn1</td>
<td>Promega</td>
</tr>
<tr>
<td>Kpn1</td>
<td>Promega</td>
</tr>
<tr>
<td>DNAeasy Kit</td>
<td>Qiagen</td>
</tr>
</tbody>
</table>

*Table 2 – Prepared enzymes and kits, along with supplying company*
2.3 – Gene Vectors and Primer Sequences

**Figure 1:** Schematic of pDrive and PL4440 cloning vectors
The primers used in this experiment were:

F1: 5' - AGATAATAGAACTGGTCATACTAA - 3'
R2: 5' - AGGTGACATCGAAAGCATGAATTAC - 3'

2.4 — Preparation of Growth Media

*Paramecium* cells were maintained in stock wheat grass media consisting of 5g Pines International Wheat Grass powder per 1L distilled water. Upon preparation, each solution was autoclaved for 10 minutes, then filtered. 5mL wheat grass buffer and 1mL stigmasterol were then added to the mixture. This solution was then separated into 200mL aliquots, which were each autoclaved on a liquid cycle for 20 minutes. Prior to the cells being fed, the media were inoculated with *Klebsiella aerogenes* and incubated at 26°C.

2.5 — Culturing and Stock Maintenance of Cells

The strain of *Paramecium tetraurelia* used in this study was designated as “51s.” The entire population used in this experiment is descendent from an original stock purchased several years ago and maintained in the laboratory of Dr. Robert Hinrichsen. All cells were grown in test tubes containing that aforementioned wheat grass media; new cells were generated by placing individual cells in 2-3mL of growth media while adding 1mL of growth media every other day until cells were reproducing at maximum capacity.

2.6 — Analyzing and Recording Avoidance Responses

Individual cells were isolated from large groups through careful extraction from a depression well with approximately 100μL of growth solution. A square central piece was cut from a square piece of filter paper approximately 2.54cm x 2.54cm, before being placed on a
standard microscope slide and moistened. A drop of solution containing one cell was placed in the middle of this cutout, with a cover slip being placed on top and petroleum jelly being applied to the perimeter of the cover slip to create a seal. This sealing was essential to ensure that the drop of solution did not dry out before the recording was complete. The completed slide was then placed under a compound-light microscope.

Recording of cells was accomplished via VHS recording. A video camera designed to fit into a microscope eyepiece was situated in the microscope such that it could record the microscope’s visual field. Recordings of the cells throughout the experiments were stored on VHS tapes for investigation.

The quantifier demonstrative of ultradian influence of swimming behavior are the cell’s avoidance responses. Avoidance responses were tallied according to the standard definition: any 180° reorientation of the cilia was counted as an avoidance response. To the observer, this was evident by a sudden, brief backward motion made by the cell. Avoidance responses were counted for one minute out of every five minutes for the duration of the experiment.

In this experiment, we first recorded data for three control cell (no chemical alterations or genetic inhibition) in order to confirm the expected ultradian periodicity for unaltered cells. The Hinrichsen laboratory has conducted numerous experiments that have established a typical ultradian periodicity for this strain as being between approximately 50 and 60 minutes. As mentioned, all cells used in the Hinrichsen lab are descendant from the same stock, so we had little reason to expect differences in the cells used for this experiment compared to cells used in previous experiments. One cell was recorded for 24 hours, and two others were each recorded for 3 hours.

In the experimental cells, we first performed a recording analysis of caffeine’s effects at varying concentrations. After executing the RNA interference procedure, we then recorded three
cells which were not expressing cellular phosphodiesterase. Besides the experimental alterations, the recording procedures were identical with the experimental cells as with the controls.

2.7 – Caffeine Analysis

We analyzed the effect elicited by caffeine on ultradian rhythms at varying concentrations of caffeine in order to compare how influence from this chemical compares with influence from selective inhibition of phosphodiesterase. Concentrations investigated were 125 μM, 250 μM, 350 μM, and 500 μM. At concentrations higher than 500 μM, cell mortality became too significant to merit usable data. Caffeine solutions were prepared using anhydrous caffeine from Sigma (lot no. 36F-0495) and distilled water. A stock solution of 1 mM was prepared, from which the experimental solutions were derived via dilution in distilled water. Cells were transplanted from their growth medium into a depression well containing a caffeine solution of the designated concentration, and were given 15 minutes to acclimate before recording began. Each cell was recorded for 180 minutes.

2.8 – Cloning Phosphodiesterase Gene

The genome of *P. tetraurelia* has been sequenced and well-characterized, and is readily accessible via the Paramecium Database at http://paramecium.cgm.cnrs-gif.fr/db/index. Once we located the sequence corresponding to the phosphodiesterase gene, we ordered forward and reverse primers from Invitrogen corresponding to the gene’s location in the genome. Two forward and two reverse primers were used, though only the F1 and R2 primers were used, owing to their superior yield in PCR. DNA was extracted from *P. tetraurelia* as per the
instructions provided in the Qiagen DNeasy kit, with a follow-up agarose gel screening verifying
the presence of purified DNA.

This DNA, along with the F1 and R2 primers, were used in standard PCR for
approximately 40 cycles, at temperatures of 94°C, 60°C, and 72°C for one minute each.
Subsequent gel analysis was used to confirm amplification of the PDE gene.

2.9 – Ligation of PDE Gene into pDrive Vector

The adenine overhangs characteristic of PCR products using Taq polymerases are
unsuitable for the RNA interference technique. The first step of the work-around to this problem
is the insertion of the gene into a DH5α strain of E. coli, via the pDrive cloning vector. A
diagram of this vector is provided in section III of the materials and methods. The uracil bases
present at each end of this vector allow the PCR product to be integrated into a circular genome
suitable for bacterial transformation. Ligation of the PDE gene fragment to the pDrive cloning
vector was performed as per the instructions of the Qiagen Cloning Kit, with subsequent gel
analysis verifying a successful transformation.

2.10 – Transformation of DH5α E. coli

Transforming the pDrive vector into a population of E. coli allows for a reliable
replication of the vector. This transformation was performed using transformation-ready
bacteria, which were kept frozen at -80°C until ready for use. Transformation using these cells is
considerably simpler than a typical transformation procedure. A small amount, usually around 2
µL, of the pDrive ligation mixture, is added to the bacterial solution after it has been slowly
thawed. The mixture is then incubated on ice for approximately 15 minutes before the solution
is spread on an LB agar plate containing ampicillin at a ratio of 100 µL ampicillin per mL LB
solution. Because the pDrive vector includes an ampicillin resistance gene, the colonies that
grow on this plate all contain copies of the vector containing the PDE gene. Cells were grown
on these plates for approximately 24 hours at 37°C.

2.11 — Extraction of PDE Gene via Restriction Digestion

In order to successfully perform RNA interference, a more appropriate vector, such as
pL4440, is necessary. The pL4440 vector allows us to produce the dsRNA needed for RNA
interference because it has T7 promoter regions on both sides of the gene insert location.
Transplanting the gene into this vector first requires its extraction from the pDrive vector,
following amplification in DH5α E. coli. We used the restriction enzymes XbaI and KpnI
because they also cut the pL4440 vector at the appropriate insertion site.

First, the plasmids had to be isolated from the E. coli containing them. This was done by
culturing a colony from the LB/amp plates in an LB broth solution containing ampicillin at a
ratio of 100 µL/mL, and allowing the culture to grow under gentle spinning for 12 to 16 hours.
The plasmid was purified from this solution as per the instructions in the Qiagen miniprep kit,
with gel screening again being used to verify successful purification.

The restriction digest was accomplished by combining 13 µL of nuclease-free water, 2
µL of 10x RE buffer, 2 µL of a 1:10 dilution of acetylated BSA, 1 µL of the isolated plasmid,
and 1 µL of each restriction enzyme solution in a microcentrifuge tube. Alongside this reaction,
a second reaction was initiated in identical fashion, except using the pL4440 vector instead of the
isolated plasmid. These reactions were allowed to proceed overnight, and were deactivated the
next day by submerging the tubes in a 65°C water bath for 5 minutes.
2.12 - Ligation of Gene Fragment into PL4440

With both the gene fragment having been extracted and the pL4440 vector having been prepared for reception of the fragment, the next step was ligation of the gene fragment in the pL4440 vector. While the pDrive vector was useful for amplifying the gene fragment, this vector is suitable for RNA interference because it positions promoters at both sides of the gene fragment, allowing for the production of the dsRNA.

The ligation was accomplished via the Promega T4 ligase kit. 1 μL from each of the two restriction digest solutions were combined (i.e., 1 μL containing the cut gene fragment solution and 1 μL containing the cut PL4440 vector). This combined solution was incubated at room temperature for 20 minutes with 5 μL of rapid ligation buffer and 3 μL of T4 DNA ligase.

2.13 - Transformation of pL4440 into HT115 E. coli

The final step before executing the RNA interference procedure was the transformation of the PL4440 vector (now containing the gene fragment) into an HT115 strain of E. coli. Unlike DH5α E. coli, this strain is engineered such that it does not produce RNases that would otherwise degrade the dsRNA produced by the PL4440 plasmid. Furthermore, this strain of E. coli produces the polymerase that recognizes both T7 promoters on the pL4440 vector and transcribes the dsRNA when activated by isopropyl β-D-1-thiogalactopyranoside (IPTG).

Transformation was executed by suspending single colonies of HT115 cells in 250 μL of calcium chloride (CaCl₂) and incubating on ice for 15 minutes. Then, we added 2 μL of the pL4440 solution to this tube, and allowed this mixture to incubate on ice for 15 minutes. The cells were then heat shocked in a 42°C water bath for 90 seconds, before being returned to ice for 2 minutes. This mixture was then added to a 500 μL LB broth, which was then shaken at 37°C for one hour. These transformed cells were spread an an LB agar plate containing ampicillin and
tetracycline. HT115 *E. coli* possess an engineered resistance to tetracycline, and the pL4440 vector grants resistance to ampicillin. Thus, colonies would only propagate on these LB/Amp/Tet plates if they both possessed the plasmid, and were the appropriate HT115 strain.

2.14 – *Inhibition of cAMP-PDE expression via Feeding Protocol*

Following transformation and incubation of HT115 *E. coli*, a pre-culture was prepared containing 3 mL LB broth, 6.0 μL ampicillin, 7.5 μL tetracycline, and a sample of transformed bacteria from an agar plate. This mixture was incubated overnight. The next day, a 250 μL aliquot was added to 25 mL of LB broth containing ampicillin (100 mg/mL). This mixture was briskly shaken for 4 hours at 37°C, in order for the cells to reach log phase growth. Next, transcription of the dsRNA was activated by introducing 62.5 μL of IPTG to the mixture, and shaking the cells for another 4 hours at 37°C.

Meanwhile, *P. tetraurelia* were prepared. At hour 3 of the *E. coli* incubation, they were washed in pure wheat grass media in order to remove the *K. aerogenes* typically present in the growth media.

Following the shaking the *E. coli* cells activated by IPTG, the culture was centrifuged at 3000 rpm for 10 minutes, with the resulting pellet being resuspended in an uninnoculated wheat grass medium. A 10 μL aliquot of this resuspension was added to a sterile depression slide, along with 90 μL of wheat grass medium, and 5 – 10 of the washed *Paramecium* cells. This final mixture was grown overnight at 26°C. As *Paramecium* ingested the transformed *E. coli*, the dsRNA would be released, recognizing and silencing expression of the complementary mRNA for *Paramecium* phosphodiesterase. Following RNA interference, recording analysis was performed to observe the effects of phosphodiesterase inhibition on the cells' ultradian rhythms.
Chapter III: Results

3.1 – Control Cells

The analysis of unaltered cells from our experimental sample demonstrates that these cells exhibit a similar standard ultradian periodicity to those used in past experiments in the Hinrichsen laboratory. The first cell was recorded for a period of 24 hours with the second and third cells being recorded for three hours, due to time constraints. Among the cells, the average ultradian periodicity was approximately 52.9 minutes, well within the expected parameters.

Figure 2a: Control Cell 1; 24 hours; period of approximately 58.75 minutes
Figure 2b: Data from control cells 2 and 3; 3 hours; periods of approximately 50 minutes
3.2 – Effects of Caffeine

Analysis of cells at varying caffeine concentrations reveals a clear trend: ultradian periodicity is inversely related to the concentration of caffeine in the cell’s solution. As caffeine concentration increases, the periodicity of the cell’s ultradian rhythm falls. It appears that this decrease is most potent around 350 μM caffeine, where the period was recorded at 12.85 minutes.

![Graph showing ultradian periodicity at varying concentrations of caffeine.](image)

**Figure 3** – *Ultradian Periodicity at Varying Concentrations of Caffeine*

3.3 – PDE Knockout Cells

Beginning approximately one day after the RNA interference procedure, three cells from the experimental group were analyzed using the same recording and analysis procedure. Each
cell was recorded for 24 hours. The average periodicity among these cells was demonstrably lower at approximately 35.7 minutes. We offer a potential explanation for the variance in this data, along with our interpretations of the results overall, in the discussion section.

**Figure 4a:** Experimental Cell 1, 24 hours. Period of approximately 40.1 minutes

**Figure 4b:** Experimental Cell 2, 24 hours. Period of approximately 37.2 minutes
Figure 4c: Experimental Cell 3, 24 hours. Period of approximately 29.8 minutes

3.4 – Statistical Analysis

An unpaired t-test reveals a statistically significant difference in the ultradian periodicities between the control cells and the phosphodiesterase knockouts, with a p-value of .0152.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Mean Period (min.)</th>
<th>Standard Dev.</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>52.9</td>
<td>5.05</td>
</tr>
<tr>
<td>(-)PDE</td>
<td>3</td>
<td>35.7</td>
<td>5.31</td>
</tr>
</tbody>
</table>

Table 3: Statistical analysis of data
Chapter IV: Discussion

4.1 — Overview

Our results appear to strongly confirm our initial suspicions going into this study. There is a clear reduction in the periodicity of *P. tetraurelia* ultradian rhythms resulting from the inhibition of cAMP-phosphodiesterase gene expression and the subsequent buildup of cAMP within the cell. Given cAMP’s role in protein kinase A cellular activation pathways (17) it is further suggested that these cellular pathways govern the oscillatory behavior patterns associated with biological rhythms.

4.2 — Caffeine’s Role

The inspiration for this project was to identify a molecular target to explain how caffeine exerts its disruptive effect on biological rhythms. At the very least, we have implicated caffeine-mediated inhibition of phosphodiesterase as a source of ultradian disruption. Unfortunately, there exist a number of ambiguities that render us unable to say with certainty if cAMP-PDE inhibition is the sole mechanism by which caffeine exerts this disruptive effect.

For one thing, our results show that caffeine’s effect on *P. tetraurelia*’s ultradian rhythms is not an all-or-nothing response. Rather, the magnitude of disruption increases as the concentration of caffeine increases. RNAi, by contrast, is by design an all-or-nothing approach. In addition, while our results from RNAi clearly implicate cAMP-PDE as a regulator of ultradian rhythms, this technique may not have completely inhibited expression of the gene, and indeed would not have any effect on PDE molecules already produced. Thus, the results seen in these cells may not be analogous to what we would see with caffeine, which targets active PDE molecules. Finally, whereas our RNAi procedure specifically targeted cAMP-PDE, caffeine is a nonspecific inhibitor of both cAMP-PDE and cGMP-PDE. It is possible that in our knockout
cells, cGMP-PDE may still have been executing its normal regulatory functions, whereas in the
cells treated with caffeine, this function would have been knocked out as well. Nevertheless, our
results show a clear linkage between caffeine's inhibition of cAMP-PDE and its effect on
cellular ultradian rhythms. The mystery that remains is whether this is the only mechanism by
which it executes this effect.

4.3 - Avenues for Future Study

This study is but one piece of an admittedly complex puzzle. Just as this study was based
off of a previous study, so too can future studies incorporate what was learned here. We have
strong evidence implicating cAMP-phosphodiesterase and cAMP levels in ultradian rhythm
control, which suggests that cellular signaling pathways somehow produce the oscillating
behavior patterns that we observe as ultradian rhythms. Future studies may account for
caffeine's nonselectivity as an inhibitor of phosphodiesterase by attempting to simultaneously
target genes for cAMP-PDE and cGMP-PDE. Future studies could also investigate other known
components of cellular signaling pathways to see if they influence these rhythms, or they may
investigate the basis for how the cell keeps track of time (i.e., the "molecular clock"), perhaps
using cellular signaling pathways as a guide point. This type of study could be improved
through methods that can quantify mRNA levels, such as real-time PCR, as well as through
techniques that can analyze phosphodiesterase activity within the cell. Both of these
improvements could help clarify the ambiguities presented in this study between caffeine and
phosphodiesterase inhibition. It is doubtful that any one study will achieve great clarity on the
mystery of biological rhythms, but by continuously piecing together the puzzle, we may one day
crack the code.
Chapter V: References


Thesis Approval

Thesis Title: A Potential Molecular Target for Caffeine in its Disruption of Ultradian Rhythms in Paramecium tetraurelia

Advisor: Name: Dr. Robert Hinrichsen
Signature: Date: May 17, 2013

Member 1: Name: Dr. Robert Major
Signature: Date: May 17, 2013

Member 2: Name: Dr. Megan Knoch
Signature: Date: May 17, 2013

Honors Program Completion Approval

Honors Program Coordinator:

Name: Dr. Sandra Newell
Signature: Date: May 17, 2013

Biology Department Chair:

Name: Dr. Carl Luciano
Signature: Date: May 17, 2013

Robert E. Cook Honors College Approval

Director:

Name: Dr. Janet Goebel
Signature: Date: May 17, 2013