AN ABSTRACT OF THE THESIS OF

Zoë J. Gombart for the degree of Honors Baccalaureate of Arts in Music presented on June 3, 2008.
Title: The Role of Circadian Transcription Factors in Gonadotropin-Releasing Hormone Gene Expression

Abstract approved:

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Barb Taylor

Circadian regulation is essential for many physiological functions, from sleep/wake patterns to hormonal rhythms. While the brain's biological clock, the suprachiasmatic nucleus (SCN), likely plays a role in reproductive rhythms, it remains unknown if intracellular circadian clocks elsewhere contribute to the neuroendocrine control of reproduction. Gonadotropin-releasing hormone (GnRH) stimulates pituitary gonadotropin secretion, which plays crucial roles in reproduction. My research examined if the clock may regulate GnRH gene expression by determining whether the intrinsic proteins of the clock regulate GnRH transcription in an *in vitro* model of GnRH neurons, GT1-7 cells. These cells were transfected with full-length and truncated regions of the GnRH reporter combined with luciferase reporters, and co-transfected with clock proteins. Using a luminometer, *GnRH-Luc* activity was quantified, revealing that overexpression of core clock proteins inhibit transcription of all GnRH constructs used. Since this is contrary to the established function of these clock components, we have concluded that transcriptional repressors activated by the clock machinery bind to the GnRH promoter, likely via multiple repressor sites, as revealed by truncations. Further study of the GnRH promoter, using electromobility shift assays, will likely determine where clock-controlled transcription factors are binding, and the use of stably-cloned secretable luciferase GnRH promoter plasmids will allow measuring of oscillatory

promoter activity. This knowledge could aid in a better understanding of the neuroendocrine regulation of reproduction and etiology of various reproductive disorders.

Key Words: circadian biology, GnRH, reproduction

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The Role of Circadian Transcription Factors in Gonadotropin-Releasing Hormone Gene Expression

by

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A PROJECT

Submitted to

Oregon State University

University Honors College

in partial fulfillment of
the reguirements for the
degree of

Honors Baccalaureate of Art in Music (Honors Scholar)

Presented June 3, 2008
Commencement June 2008

Honors Baccalaureate of Arts in Music project of Zoë J. Gombart presented on June 3, 2008.

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I understand that my project will become a part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Zoë J. Gombart, Author

ACKNOWLEGEMENT

I would like to acknowledge Patrick Chappell, first of all, allowing to me work in his lab and be a part of this research, and for all of his guidance and mentorship during these last several months. I am also very appreciative of the other members of the Chappell lab, in particular Kristin Latham, Karen Tonsfeldt, and Cheri Goodall, who have all so many times gone out of their way to assist and patiently explain to me procedures and tasks. Also, I would like to give thanks to Barb Taylor for agreeing to be my mentor for the project and to Jaga Giebultowicz for acting as part of my thesis committee.

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The Role of Circadian Transcription Factors in Gonadotropin-Releasing Hormone Gene Expression

1. INTRODUCTION

Gonadotropin-releasing hormone (GnRH), a peptide hormone, is produced and released by specific neurons in the hypothalamus, required for stimulating the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary. These hormones are both important for the basis of reproduction, regulating and the production of gonadal steroids and indirectly regulating gametogenesis. GnRH is secreted in a pulsatile fashion in both males and females, and, in females only, exhibits high amplitude mid-cycle surges, required for ovulation (1). This pulsatility is regulated by the circadian clock (2).

Recent studies have shown a link between the secretion of GnRH and the intracellular circadian clock. The circadian clock transcriptional feedback loop inside many different cell types in the body that is synchronized to changes in daylight by the body's main biological clock, the hypothalamic suprachiasmatic nucleus (SCN) (2). It is unclear, however, if the clock's influence lies at the level of transcriptional regulation of the GnRH gene, post-translational modifications, prepropeptide

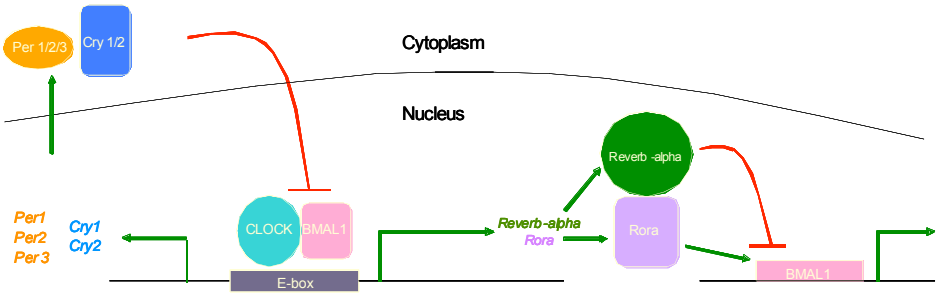

Figure 1: Circadian clock negative feedback loop

cleavage and processing, or secretion from vesicles at the level of the membrane. The circadian clock functions using a central negative feedback loop (**Figure 1**). CLOCK and BMAL1, basic helix-loop-helix (bHLH) transcription factors, heterodimerize and bind to E-boxes in the promoter region of *Period* genes, which exist as three homologs in mammals, *mPer1-3,* and the *Cryptochrome* genes, which exist in two homologs, *mCry1* and *mCry2,* effectively stimulating transcription of these genes (3). NPAS2, a transcription factor with high homology to CLOCK, has been shown to exhibit the same patterns of heterodimerization with BMAL1, and functions in circadian regulation similarly to CLOCK, but confined to neurons (4). PER and CRY proteins, during times of high concentration, translocate to the nucleus to inhibit their own transcription. As the levels of PER and CRY fall, transcription begins again, as CLOCK/BMAL1 heterodimers are free from inhibition by PER and CRY (5). Although transcript levels of *Clock* are constitutive, transcription of *Bmal1* is in antiphase to the transcriptional oscillations of of *mPer* and *mCry* mRNA and is regulated by *Rev-erb alpha*, a repressor, and *Rora*, a transcriptional activator. These two orphan nuclear receptors compete for response elements in the *Bmal1* promoter, keeping its rhythm in antiphase to those of *mCry* and *mPer* (6). These two "arms" of the molecular clock exist in the SCN, GnRH neurons, pituitary gonadotropes, and the gonads, suggesting multiple levels of control by circadian mechanisms (7).

GnRH neurons are not localized within one discrete area of the hypothalamus, but are instead dispersed along the mediolateral and rostrocaudal aspects, interspersed with multiple neuronal phenotypes. Because GnRH expressing-and -secreting neurons are very difficult to isolate *in vivo,* our laboratory uses GT1-7 cells, a representative immortalized

cell line, to study mechanisms regulating GnRH gene expression and secretion. The clock genes described above are also expressed in GnRH neurons and our model GT1-7 cells. In this cell line, clock gene expression and protein levels oscillate with a circadian (~24 hours) period, which has been measured by RNase protection assays, quantitative real-time PCR, and Western protein blotting (8). It has previously been shown, by transfection of luciferase reporter constructs, that activity of the *mPer*1 promoter also shows intracellular circadian oscillations in GT1-7 cells (9). Studies have shown that GnRH gene expression occurs in a circadian pattern, particularly in females in the proestrus phase of the estrous cycle, and that basal pulse secretion patterns are dependent on a functioning circadian clock (10).

While the activity of GnRH neurons can be regulated by exogenous adrenergic, GABAergic, and glutamatnergic signals, it has been demonstrated that the function of GnRH neurons is not dependent on a complex set of afferent neuronal signals (11, 12, 13). Explants of GnRH neurons isolated *in vitro* have been shown to secrete GnRH in the same pulsatile fashion as those *in vivo* (14), and perifused GT1-7 cells also exhibit the same pulse frequency as that observed *in vivo*. It is likely that molecular mechanisms underlie much of the regulation of GnRH, but the exact method of this regulation, whether due to transcriptional regulation, posttranslational or signaling mechanisms, or both, however, is unknown (1). Thus, the GnRH promoter needs to be closely examined to determine in what regions transcription factors bind and how they can affect basal and stimulated levels of transcription. My project examined the role of core clock regulatory factors NPAS2 and BMAL1 in regulation of GnRH transcription in GT1-7 cells.

2. METHODS

2.1. Cell culture and plasmids

GT1-7 cells, obtained from Dr. Pamela Mellon at the University of California, San Diego, were grown in Dulbecco's Modified Eagle's Media (DMEM) containing 10% Fetal Bovine Serum (FBS), penicillin, and streptomycin. Cells were incubated in 5% CO_2 in a water-jacketed incubator at 37°C. A combination of GnRH-luciferase reporter constructs and clock overexpression plasmids were used in transiently transfecting cells. Reporter plasmids consisted of full-length and truncated GnRH promoters ligated to a firefly luciferase reporter, resulting in expression of the protein luciferase under the control of the GnRH promoter. Reporter plasmids used include the characterized full-length 5-kilobase GnRH promoter and two truncated constructs of the GnRH promoter: a 2.1-kilobase and 520-base pair. pGL3, a promoterless luciferase vector, and an *mPer*1-luciferase vector were used as a positive control for circadian clock protein effectiveness. The circadian clock overexpression plasmids used were: NPAS2, BMAL1, CRY1, and their promoterless parent vector, pcDNA3.1. ß-galactosidase under the control of a minimal Rous sarcoma viral promoter (RSV) was also transfected into all cells as a control for transfection efficiency. After the concentrated stock plasmids were prepared by bacterial isolation via Maxiprep (Oiagen), all were diluted to proper concentrations for transfection. Dr. Pamela Mellon (UCSD) generously provided all GnRH-luc plasmids and Dr. Joseph Takahashi supplied *mPer1*-luc (Northwestern Univerity). pGl3 (Promega) and pcDNA3.1 (Invitrogen) were obtained through purchase from respective vendors.

Later experiments were designed to detect GnRH promoter activity in real-time within circadian tie windows by using the 5-kb GnRH driving the luciferase gene derived from *Metridia Longa,* a marine ostracod capable of secreting a stable luciferase into the media in which the GT1-7 cells are being grown. This differed from the standard GnRH-luc plasmids in that *M. Longa* luciferase expression can be measured by taking samples of media rather than lysing multiple plates of cells and measuring luciferase expression from the lysate.

2.2 Cloning

Creation of the GnRH-*Met*-luc promoter began with cloning the *Met*-luc sequence into the intermediate plasmid pBKS using HincII, followed by diagnostic digests and sequencing, screening for proper orientation. The firefly luciferase sequence was removed from GnRH-luc by XhoI and XbaI and it was replaced with the *Met*-luc sequence, effectively creating a GnRH promoter driving the expression of *Metridia Longa* luciferase. At the time this paper was written, the Chappell lab has only just cloned the *Met*-luc sequence into the full-length 5-kb promoter. Transient transfections of the plasmid have been performed that validate functionality.

3. TRANSIENT TRANSFECTION

3.1 Plating

For the first step of transient transfection, confluent plates of GT1-7 cells were washed with 1X phosphate buffered saline (PBS), trypsinized, counted with a hemocytometer, and 1.5 x 10^5 cells were plated into each well of a 24-well plate. Into each well 500-1000 μL additional 10% FBS-DMEM was then added.

3.2 Transient Transfection

Transient transfection occurred approximately 24 hours after plating. All wells were transfected with 0.1 μg RSV-ß-galactosidase, and sets of triplicate wells were plated with 0.4 μg promoter plasmids and 0.5 μg clock overexpression plasmids. The transfection agent, FuGENE 6 (Roche), was used according to manufacture's protocols.

3.3 Treatments

Wells on the 24-well plate containing GT1-7 cells were transfected with either a GnRH-luc promoter plasmid or pGL3, and select wells were also transfected with clock overexpression plasmids or the control, pcDNA3.1. Our ultimate goal through this experimental design was to be able to determine if clock expression proteins alter levels of GnRH gene expression by directly binding to GnRH promoter-enhancer regions, or even indirectly affecting GnRH expression, by using different combinations of clock expression proteins and analyzing different promoter constructs. RSV-ß-gal was used in

these experiments as a control for variation in cell number among wells. One set of triplicates for each promoter construct was transfected with only with RSV-ß-gal. The minimal promoter plasmid control serves as a baseline measure of promoter activity, against which to measure any changes in protein expression that occur when clock overexpression plasmids were co-transfected. The vector in which all of the clock overexpression plasmids were housed (pcDNA3.1) was also used as a transfection control. The vector control was included to verify that any quantified changes in protein expression were due to the addition of clock proteins and their action on the promoter causing transcriptional changes and not just the addition of any plasmid DNA. The promoterless luciferase pGL3 was used to control for any changes in basal level of luciferase expression that may occur by addition of any plasmid DNA. Because the pGL3 vector is promoterless, any changes in luciferase expression observed are due to the non-specific actions of overexpression of clock gene DNA, and not via binding to GnRH promoter sequences. The *m-Per*1 promoter, on which action of clock proteins has been well documented, was used in early experiments to assure the validity of the actions of the NPAS2, BMAL1, and CRY1 clock proteins in subsequent experiments.

Each set of treatments consisted of a triplicate set of all treatments and controls. This experiment protocol was replicated six times. A diagram of the design of the experiment is shown in **Figure 2**.

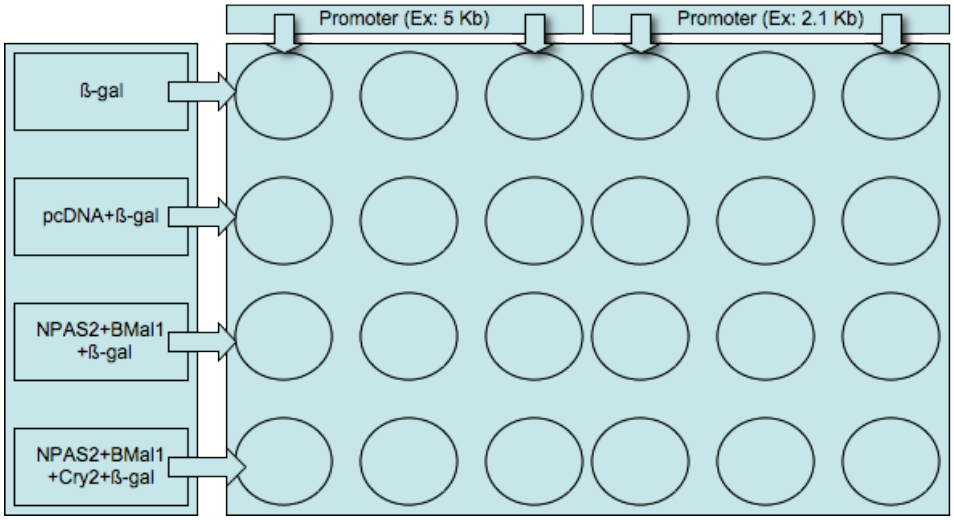

Figure 2: Example of experimental design of transient transfection of 24-well plate

3.4 Harvesting

GT1-7 cells were harvested 24, 30, or 36 hours after transfection. Transfection was not performed at the same time period for each sample because of one of the original goals of this research: to determine whether GnRH secretion displayed circadian oscillation. However, as the data gathered from these points of harvest at 24, 30, and 36 showed similar patterns of luciferase expression for all treatments, data from all time points were included in final calculations. Lysate from cells was obtained after a 1X PBS wash followed by administration of 60 µg lysis buffer per well, containing 0.2 M potassium phosphate buffer at pH 7.8 and 1% Triton X-100. The mixture was shaken for five minutes on a plate shaker to lyse the cells. Twenty µL of lysate from each well was added to a corresponding well on a 96-well luminometer plate. Luciferase activity was measured by adding 100 µL of a luciferin substrate solution composed of Tris-HCL,

$MgSO_4$, H_20, adenosine triphosphate (ATP) as an energy source for the reaction, and purified firefly luciferin. This mixture was added via injectors within a microplate luminometer. The chemical reaction of the luciferin substrate binding to the luciferase enzyme results in light emission, and the degree of intensity of light emission was detected by a CCD photomultiplier tube and measured in the arbitrary luciferase units (ALU) by the microplate luminometer. The ß-gal activity present in the lysate was also measured by the luminometer with a Galacto-light Plus assay kit (Tropix) using manufacturer protocols. *Met*-luc activity was measured using a luminometer in conjunction with addition of an *M. Longa* luciferase substrate and the accompanying manufacturer protocol provided by the Ready to Glow Secreted Luciferase Reporter System (Clonetech Laboratories, Inc.).

3.5 Data analysis

Luciferin and ß-gal assays gave raw data in ALU for luciferase and ß-gal expression, respectively. Three data analysis processes were needed to completely normalize these values and achieve statistically viable data. In the first, luciferase values were divided by ß-gal values for each corresponding well. This removed variability of expression values due to differences in cell count among wells. In the second, averages for each triplicate set were calculated and then the average of treatment triplicates of the 5-kb, 2.1-kb and 520-bp promoter was divided by the average of pGL3 levels with the corresponding treatment. This normalized the data to account for any basal levels of luciferase expression associated with the treatments of clock overexpression plasmids. Finally, a second normalization, one to pcDNA, was performed. The pGL3-normalized

value of pcDNA for each promoter construct was divided by that of the corresponding promoter with no treatment. This calculated number obtained for each promoter construct was set as “one.” The pGL3-normalized values for the two treatments (NPAS2+BMAL1 and NPAS2+BMAL1+CRY1) were then divided by the arbitrary “one” for their corresponding promoter construct. The values achieved from these final calculations accounted for any decrease in luciferase expression due to the addition of the expression plasmid vector DNA.

4. RESULTS

Our results show that overexpression of core clock genes *NPAS2*, *Bmal1*, and *Cry1* affected *GnRH* transcription on all of the promoter constructs tested.

The *Npas2*-, *Bmal1*-, and *Cry1*-expressing plasmids used in all experiments were found to produce expected results when overexpressed with *mPer1*-luc. During the time these experiments were being performed there were technical difficulties with the ß-gal assay due to problems with the minimal thymidine kinase (tk)-□gal reporter, and thus for these experiments, only ALU values could be obtained. The trends in this data nonetheless reflect those which one would expect with treatment of these clock overexpression plasmids. Results of the two trials (three triplicates each) performed using the *mPer*-1 promoter co-transfected with nothing, pcDNA, NPAS2+BMAL1, and NPAS2+BMAL1+CRY1 are shown in **Table 1** and **Figure 3**.

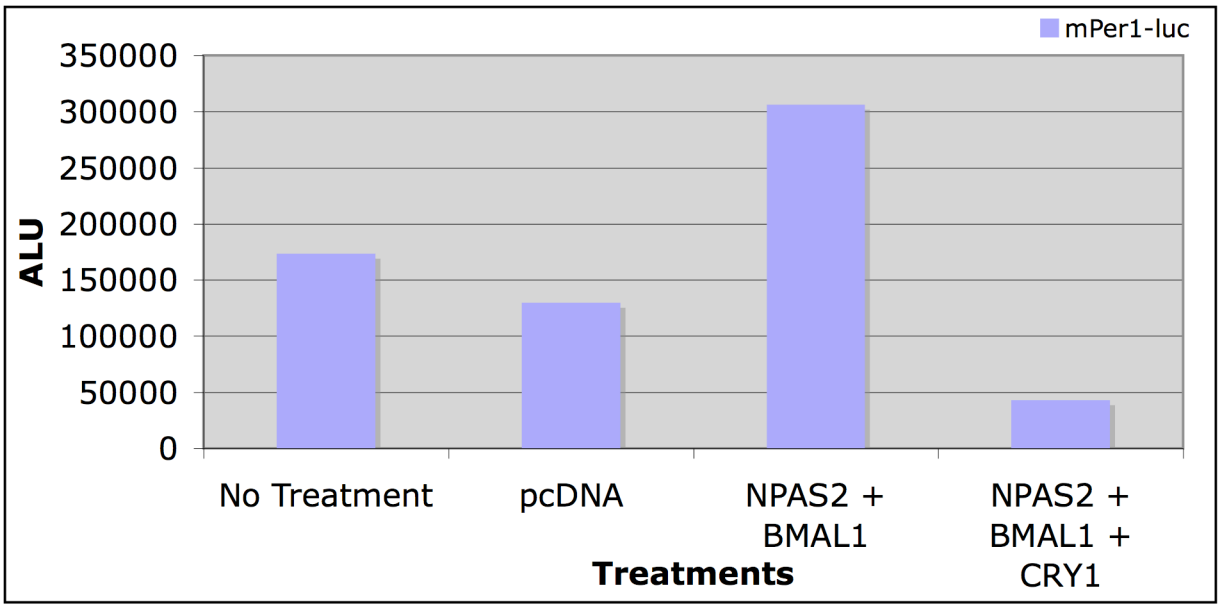

Figure 3: Averages of luciferase expression in ALU for *mPer*-1 promoter

	Trial 1			Trial 2			AVERAGE
No Treatment	221101	276677	35636	160282	227846	120130	**173,612**
pcDNA	167032	199704	217045	80932	62895	52878	**130,081**
NPAS2 + BMAL1	401262	428234	68732	333999	412202	193059	**306,248**
NPAS2 + BMAL1 + CRY1	55769	52535	55785	30509	30518	33055	**43,029**

Table 1: Averages of luciferase expression in ALU (numerical) for *mPer*-1 promoter

Following confirmation of the effectiveness of NPAS2/BMAL1 and CRY1 overexpression in GT1-7 cells on this control reporter, I began to examine the effects of core clock gene overexpression on GnRH transcriptional activation. The three promoter constructs I used (5-kb, 2.1-kb, and 520-bp-regions) show incremental decreases in GnRH expression regardless of treatment. This can be observed in **Figure 4**, in which the values of protein expression have been normalized to pGL3 activity, and in the corresponding **Table 2**, which shows these data in numerical form. The 520-bp promoter exhibits by far the smallest amount of GnRH expression. In the samples not co-transfected with any clock expression plasmids, the 5.1-kb promoter construct exhibits ~96% more promoter activity as measured by luciferase expression than that seen with the 520-bp promoter construct, but only ~12% more promoter activity than that of the 2.1-kb promoter construct.

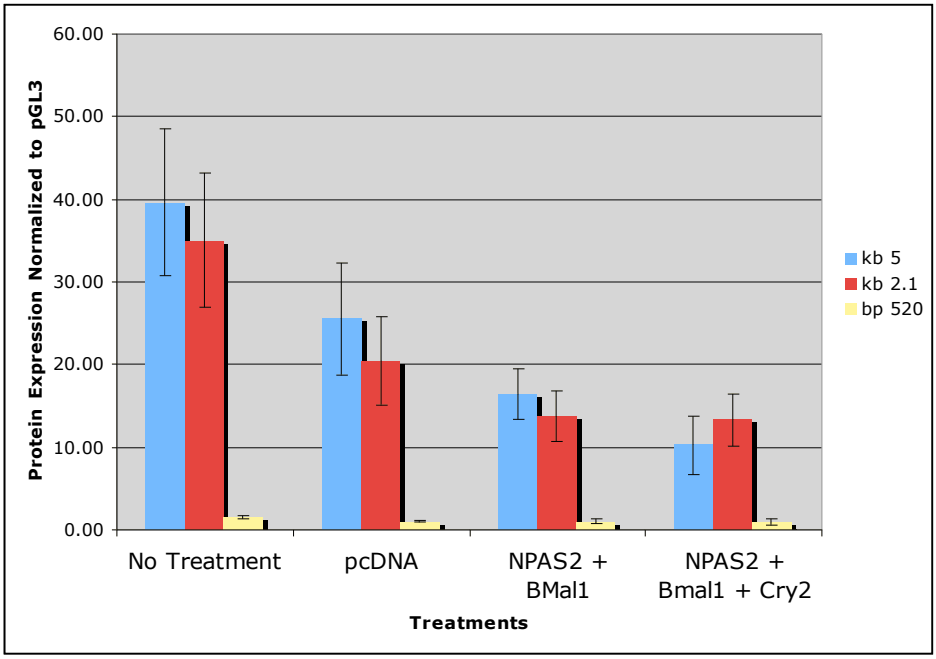

Figure 4: Averages of protein expression normalized to pGL3 for all promoter constructs (graph)

Promoter Size	No Treatment	pcDNA	NPAS2 + BMAL1	NPAS2 + BMAL1 + CRY1
5-kb	39.62	25.56	16.41	10.23
2.1-kb	35.03	20.48	13.77	13.35
520-bp	1.55	1.05	1.04	0.97

Table 2: Averages of protein expression normalized to pGL3 for all promoter constructs (numerical)

To ultimately show if and how clock genes or clock-controlled genes regulate changes in GnRH expression, one must analyze the final normalized data, that which is normalized to pcDNA. The data in this format shows the most clearly observable changes in levels of expression **Figure 5** and **Table 3** shows the average of the final normalized data for all promoter constructs in both numerical and graph form, and **Figures 6, 7, and 8** shows the same data for individual promoter constructs.

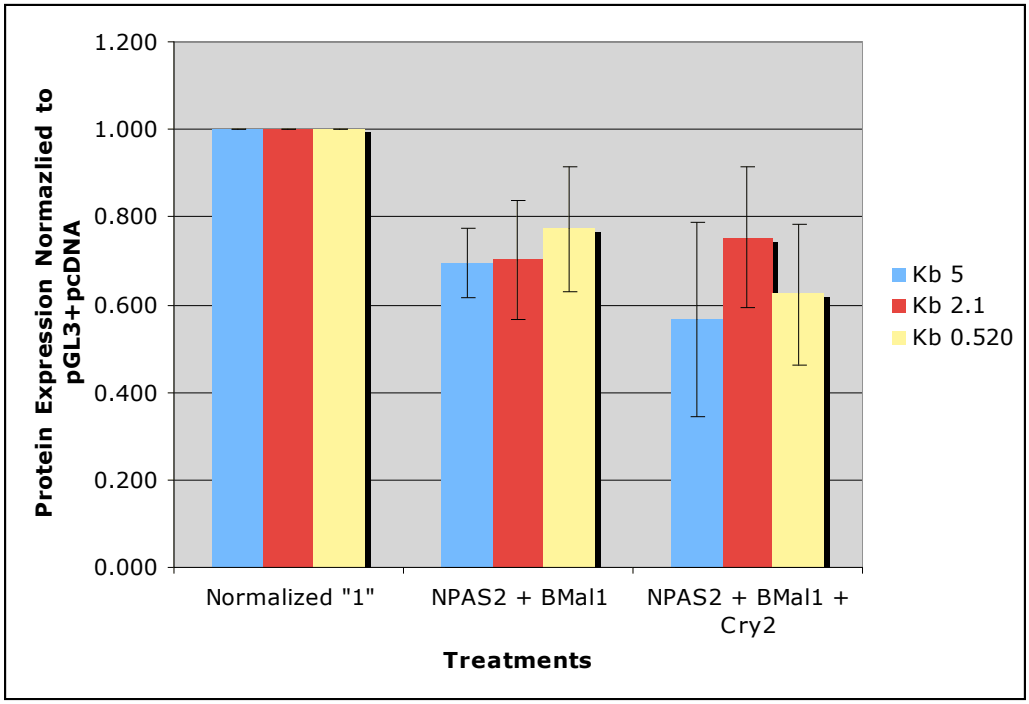

Figure 5: Averages of protein expression normalized to pGL3 and pcDNA for all promoters (graph)

Promoter Size	No Treatment	NPAS2 + BMal1	NPAS2 + BMAL1 + CRY1
5-kb	1.000	0.695	0.567
2.1-kb	1.000	0.701	0.753
520-bp	1.000	0.772	0.623

Table 3: Averages of protein expression normalized to pGL3 and pcDNA for all promoters (numerical)

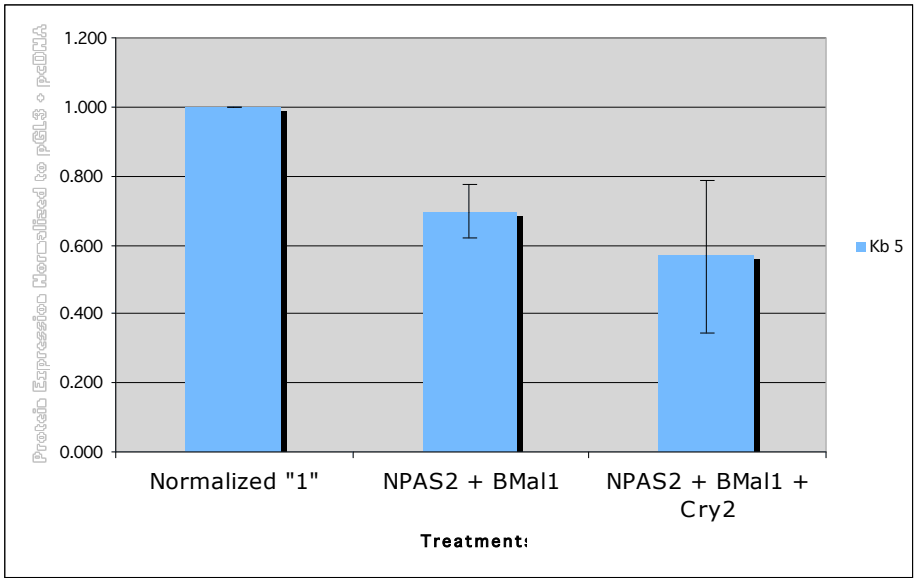

Figure 6: Averages of protein expression normalized to pGL3 and pcDNA for 5-kb promoter

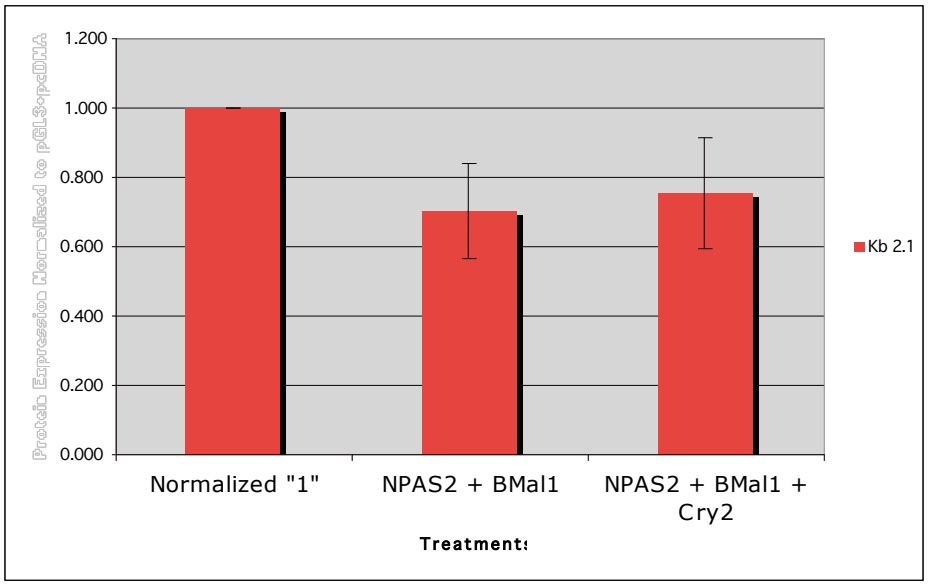

Figure 7: Averages of protein expression normalized to pGL3 and pcDNA for 2.1-kb promoter

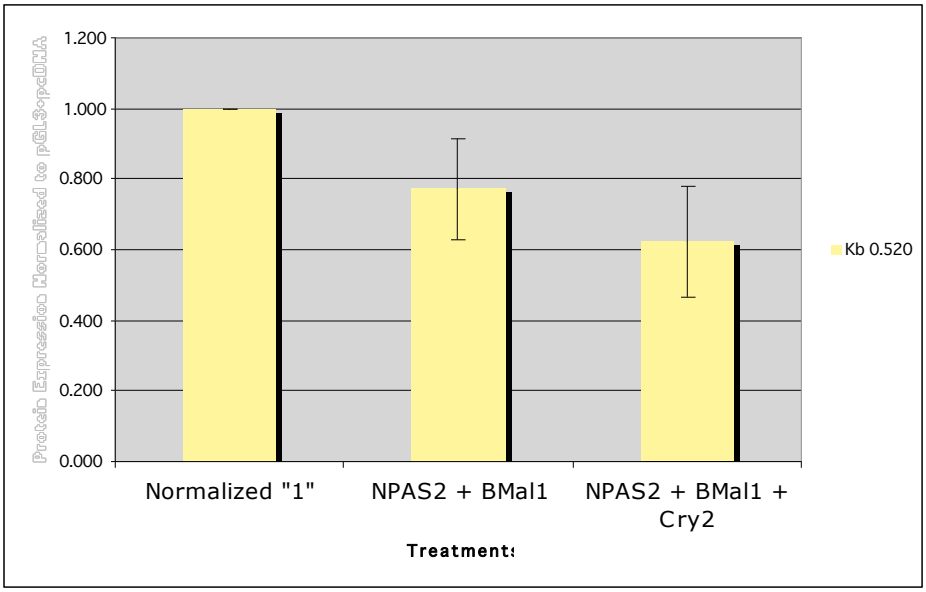

Figure 8: Averages of protein expression normalized to pGL3 and pcDNA for 520-bp promoter

In all of the promoter constructs, overexpression of NPAS2 and BMAL1 results in a decrease in GnRH promoter activation. Over-expression of NPAS2 and BMAL1 leads to a an average ~30% decrease in luciferase expression on the 5.1 and 2.1-kb promoter constructs, while overexpression of these clock components on the 520-bp promoter construct shows an average 23% decrease. CRY1, when overexpressed in addition with NPAS2 and BMAL1, does not give conclusive data because of the large standard error of the mean values. It seems to elicit a further decrease in luciferase expression in 5-kb and 520-bp promoter constructs, but this not validated by my data. While the averages do show trends in the data, smaller errors of the mean would make conclusions from these results more viable.

5. DISCUSSION

Statistical significance of the data was calculated via a one way analysis of variance test followed by Bonferroni's multiple comparison pos-hoc test. None of my data was found to be statistically evident due to its wide range. However, certain trends were shown in the average, and the following discussion examines these trends.

Because of the decrease in expression using all GnRH promoter constructs when NPAS2 and BMAL1 are overexpressed, one can conclude these clock genes have an indirect regulatory function on GnRH gene expression. Many previous studies have shown that the binding of the heterodimer NPAS2/BMAL1, similar to CLOCK/BMAL1, functions to increase transcriptional activity of clock-controlled genes (15). The inhibitory rather than stimulatory effect observed in this research would suggest that, following overexpression of NPAS2 and BMAL1, GT1-7 cells then transcribe certain factors that bind to a repressor site on the GnRH promoter/enhancer.

Although the average luciferase expression of the 520-bp promoter construct transfected with NPAS2 + BMAL1 drops about 7% more than that of the 5 and 2.1-kb constructs, the standard error of mean bars negate this difference in luciferase expression. Therefore, luciferase expression for all three promoter constructs transfected with NPAS2 + BMAL1 plasmids exhibits relatively the same amount of decrease. From the conclusion, I would hypothesize that the repressor site for the factor(s) transcribed by the binding of NPAS2/BMAL1 heterodimer is downstream of the 520-bp region of the GnRH promoter.

There are also multiple enhancer sites in the GnRH promoter/enhancer region that regulate basal levels of transcription The decrease of 11% from the 5-kb promoter to the 2.1-kb promoter shows that not as many enhancer sequences are located in this ~3 kb region in between these two constructs as there is in the ~1.58 kb between the 520- bp and 2.1-kb regions, as a decrease of ~83% in GnRH expression occurs between the two regions.

The overexpression of CRY1 in addition to NPAS2 and BMAl1 does not yield conclusive evidence, but our data suggests transfection of CRY1 could possibly cause further transcriptional inhibition by the binding of another repressor site on the promoter. As discussed before, the relatively large standard error of the mean values allow only suggestions to be made according the trend of the averages.

Looking at the pGL3-normalized data in **Figure 4** and **Table 2**, one can observe that the addition of the clock protein home vector, pcDNA caused a decrease in transcription. This shows that the transfection of any DNA, and not just clock genes, will cause a decrease in transcription on the GnRH promoter. I attempted to troubleshoot the recurring problem by molar titrating the pcDNA plasmid with clock overexpression plasmids. In this procedure, I calculated, using the mass of each base pair, how much mass of each plasmid I should use. This resulted in the addition of a greater mass of pcDNA, with fewer total base pairs, as compared with the clock overexpression plasmids, with more total base pairs. However, my results continued to show a decrease in luciferase transcription upon pcDNA transfection. Determining the cause of this transcriptional inhibition would require further troubleshooting.

This study would be greatly aided by further analysis of the GnRH promoter/enhancer to determine exact enhancer and repressor locations and sequences. One method to do this would be to perform an electromobility shift assay (EMSA) on select sequences of the promoter incubated with GT1-7 nuclear extracts. Radiolabeled sequence-specific DNA probes would then be used to detect DNA sequences of shifted bands followed by the addition of specific antibodies that bind clock proteins and potential clock controlled repressor and promoter transcription factors present in the nuclear extracts.

The full-length GnRH-*Met*-luc reporter has yielded evidence that luciferase is being secreted into media, as shown in **Figure 9**. The figure shows a luciferase assay taken from the media of the same well for every time point showing trends of normalized luciferase values of various treatments on the GnRH-*Met*-luc reporter. The graph shows an increase in luciferase expression in media measured from the same wells measured at different time points.

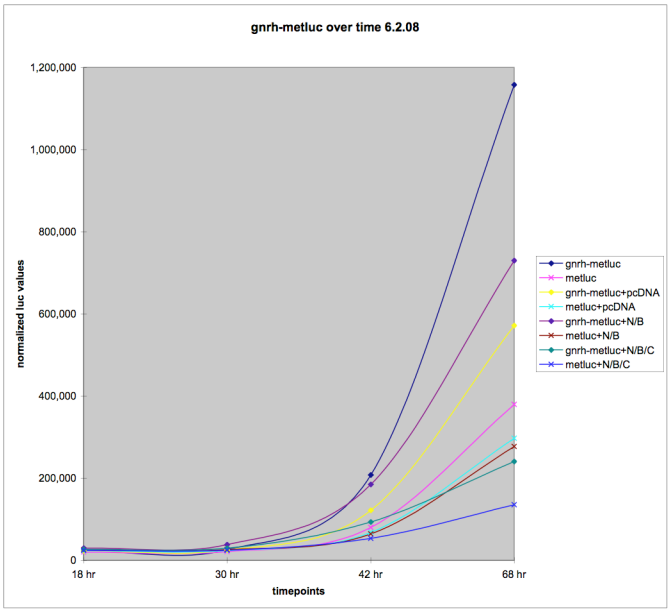

Figure 9: GnRH-*Met*-luc normalized luciferase expression

The process described above of cloning the *Met*-luc sequence into the GnRH promoter has also been previously performed on the BMAL1 promoter, RORE, with success. Figures 10, 11, and 12 show secretion of luciferase from RORE-*Met*-luc over time. **Figure 10** shows that the secretion of RORE-*Met*-luc increases over time, validating that the RORE is driving the *Met*-luc reporter and that secreted luciferase can be easily detected in the media. Luciferase secreion was measured over time via a cell perifusion, in which GT1-7 cells stably expressing RORE-*Met*-luc and adhered to beads were loaded into perifusion columns and perfiused over time. Media was collected from the perifused GT1-7 cells every ten minutes. **Figures 11 and 12** show secretion of RORE-*Met*-luc in perifusion. Figure 11 is a close up of a section at the end of the first graph and it suggests an oscillatory circadian secretion pattern, as noted by the surge and subsequent ebb in luciferase secretion.

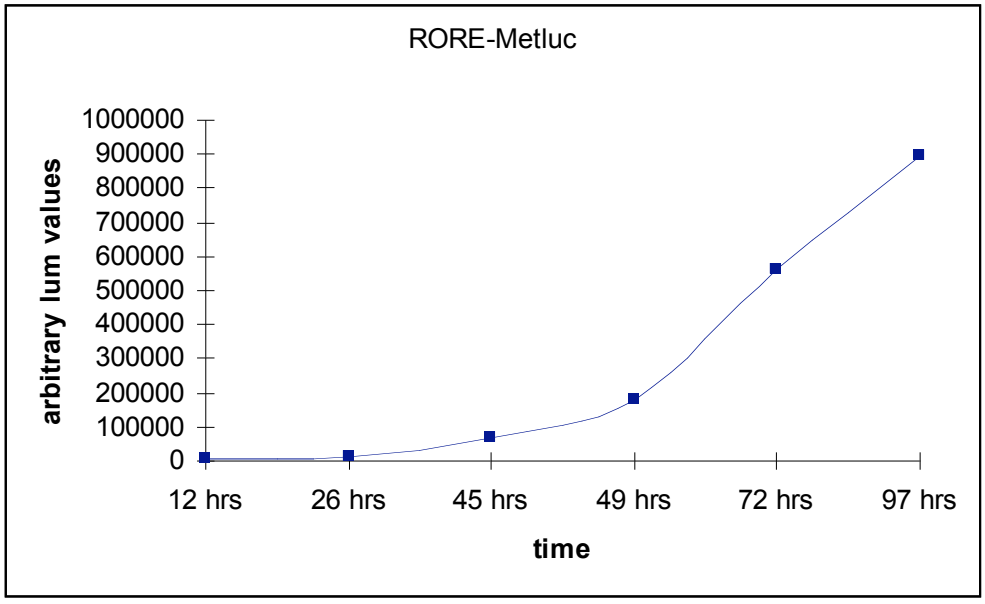

Figure 10: Secretion of luciferase from GT1-7 cells transfected with RORE-*Met*-luc

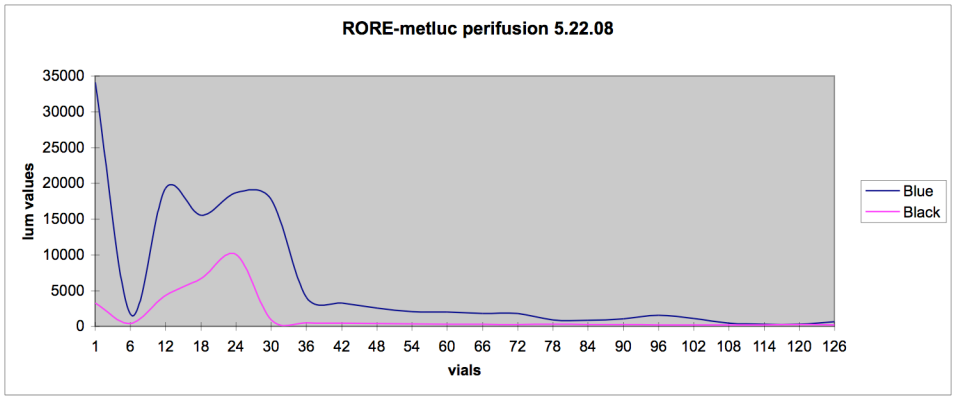

Figure 11: Secretion of RORE-*Met*-luc over time via perifusion

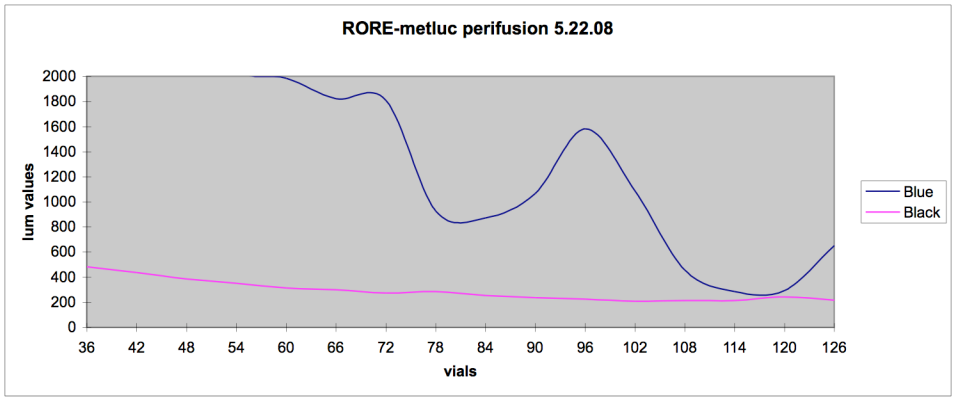

Figure 12: Secretion of RORE-*Met*-luc over time via perifusion (close up of last half of figure 10)

To determine if the GnRH promoter exhibits secretion in circadian patterns, perifusion of the full GnRH-*Met*-luc promoter will be necessary. Also, development and perifusion of various truncated GnRH-*Met*-luc promoters would give a better understanding of which areas of the GnRH promoter clock proteins or clock-controlled proteins are binding that cause GnRH promoter to transcribe protein in a circadian fashion. This methodology allows for a far superior investigation into circadian oscillations in promoter activity, since the same population of cells can be monitored over

multiple days without having to lyse multiple plates and correlate timed expression levels from lysates of different cells.

A continuation of study of the circadian clock's involvement in regulating the expression and secretion of GnRH using the aforementioned methods is important for human health. This neurohormone is a requirement for human fertility, making knowledge of its molecular mechanisms an inherent necessity in the advancement of our understanding of fertility and associated disorders.

6. BIBLIOGRAPHY

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