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Circadian rhythms and glucocorticoids in a cell culture model of bipolar disorder

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Circadian Rhythms and Glucocorticoids in a Cell Culture Model of Bipolar Disorder

A thesis submitted in partial satisfaction of the requirements for the degree

Master of Science

in

Biology

by

Ryan Mitchell Darvish

Committee in charge:

Professor David K. Welsh Chair
Professor Susan Golden Co-Chair
Professor Andrew Huberman

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The Thesis of Ryan Mitchell Darvish is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

I would first and foremost like to dedicate this thesis to my parents Moez and Shahnaz and brother Davis who have filled my life with love and have provided me with the strength and motivation to follow my dreams.

I would also like to dedicate this thesis to Charles Thun whose psychology class introduced me to circadian rhythms. His course inspired me to pursue this field of study, providing me with a direction to focus my efforts; a decision that would change the course of my life.
Thank you Mr. Thun!

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ABSTRACT OF THE THESIS

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by

Ryan Mitchell Darvish

Master of Science in Biology

University of California, San Diego, 2011

Professor David K. Welsh, Chair

Professor Susan Golden, Co-Chair

Circadian rhythms are endogenous biological rhythms that oscillate on a 24-hour period. Dysfunction in the circadian system has been implicated in mood disorders, including bipolar disorder (BD). While genetics can explain 60-80% of the variance in expression of this disorder, 20-40% is unaccounted for and could be due to psychosocial factors such as stress. We hypothesized

that cells from patients with BD would be more susceptible to rhythm alterations than those of healthy controls when exposed to conditions modeling stress. In order to explore our hypothesis we employed dexamethasone, a synthetic version of the glucocorticoid hormones that are released in response to stress via the hypothalamic-pituitary-adrenal axis. We used qPCR to examine expression of genes indicating cellular stress and bioluminescent reporter assays of clock gene expression to examine circadian rhythm parameters of period, amplitude, and goodness-of-fit. We report that there are no differences in response to dexamethasone between the control and BD cells for period and amplitude. However, we found significant differences in goodness-of-fit, suggesting that glucocorticoid mediated stress could provoke symptoms of BD through a circadian clock mechanism.

INTRODUCTION

Circadian Rhythms 1.1

Circadian rhythms are endogenous, self-sustained, biological rhythms that cycle through an approximate twenty-four hour period (Welsh et al., 2010). The mechanisms that control these daily rhythms are highly precise and stable, compensating for a wide variety of perturbations. Thus, the circadian clock is remarkably stable and reliable at timekeeping under a variety of environmental conditions (Virshup et al., 2009).

In humans, at the physiological level, the circadian system is comprised of a central pacemaker designated the suprachiasmatic nucleus (SCN). The SCN is a paired structure located in the brain situated in the anteroventral hypothalamus on either side of the third ventricle, above the optic chiasm (Welsh et al., 2010). Studies of the SCN, including lesion and transplant experiments, have shown that this structure is the master circadian pacemaker in the mammalian system. In its absence the circadian behavioral and/or physiological rhythms are abolished (DeCoursey et al., 1989). This includes disruptions of oscillations of core body temperature or hormone secretion (Koenigsberg, et al., 2004). The SCN is comprised of approximately 20,000 neurons, each of which is an autonomous circadian oscillator (Welsh et al.,

1995), and these cells are synchronized to one another by various coupling mechanisms (Welsh et al., 2010 & Yamaguchi et al., 2003). As a result, the SCN generates persistent and stable rhythmic outputs even in the absence of environmental signals, allowing for circadian rhythms of behavioral and physiological processes to persist even under constant conditions. The principal input that modulates the mammalian circadian system is light exposure through specific photoreceptors within the eye, referred to as "intrinsically photosensitive retinal ganglion cells" (iPRGC's), which convey light information to the SCN via the retino-hypothalamic tract (Berson, 2003). As of late, studies of the cellular and molecular aspects of the circadian system have revealed insights into various disorders, suggesting that defects within the circadian system may be implicated in a range of clinical conditions, including psychiatric illnesses like bipolar disorder and major depression.

1.2 Circadian Clock Genes

At the cellular level, circadian clocks are comprised of a set of genes that interact within an oscillatory network. The core of this intracellular clock is a negative feedback loop in which the clock genes, *Period* (*PER*) and *Cryptochrome* (*CRY*), inhibit their own transcription. The positive regulatory elements of the clock *ARNTL* (*BMAL1*) and *CLOCK* (*CLK*) form heterodimers, activating transcription of the *PER* and *CRY* genes. Upon PER and CRY proteins forming their own complexes in the cell cytoplasm, they then

translocate to the nucleus, inhibiting the action of *BMAL1* and *CLOCK* (Figure 1). The cycle continues as inhibition gradually increases and then diminishes due to proteasomal degradation of the PER and CRY proteins, thereby re-initiating the cycle in a circadian pattern (Takahashi et al., 2008). Interestingly, nearly one-half of all mammalian genes are regulated in a circadian fashion in at least some tissues (Yan et al., 2008). Genes expressed in a circadian rhythm can be observed using a variety of methods, which include quantitative polymerase chain reaction (qPCR) or the use of viral vectors containing bioluminescent reporters transfected into cells to permit luminescent imaging of clock gene expression (Welsh et al., 2004).

1.3 Peripheral Cellular Oscillators

In addition to the SCN, recent work has revealed that circadian oscillators under the control of clock genes are present in a wide variety of cell types throughout the human body, from cardiac myocytes to liver hepatocytes and skin fibroblasts (Yagita et al., 2001). These peripheral cellular oscillators have been shown to contain clock genes whose expression is rhythmic (Yamazaki et al., 2000). In addition, the mechanisms behind their expression are similar to those in the SCN, a complex that is charged with the duty of coordinating the ensemble of peripheral cellular oscillators, aligning them with important environmental cues such as the light/dark cycle to facilitate biological processes. Specifically, cultured skin fibroblasts contain cell autonomous

circadian clocks whose phases persist even through cell divisions, and are able to maintain periods that oscillate in a circadian fashion corresponding to overall human circadian rhythms (Nagoshi et al., 2004 & Welsh et al., 2004).

1.4 Bipolar Disorder

Initially, links between mood disorders and the clock were drawn via clinical observations of behavior. This includes activities of BD patients at unusual times not synchronized with external environmental cues. In the more recent past, there have been associations between mood disorders and clock gene polymorphisms, which disrupt the circadian system, suggesting a shared etiology between circadian dysfunction and mood dysregulation. Of particular interest is bipolar disorder (BD). BD is a serious psychiatric illness that affects 1-3% of the population in the United States (National Institute of Mental Health, 2010). This disorder is characterized by two distinct mood phases, depression and mania. Mania is characterized by euphoric mood, having a great deal of energy, decreased need for sleep, and increased involvement pleasurable activities, which interfere with an individual's daily life (Diagnostic and Statistical Manual of Mental Disorders). Additionally, an individual typically experiences mania for only a limited period of time before switching to depression or normal mood (euthymia). Moreover, patients exhibit abnormalities in behaviors that are normally regulated in a circadian fashion, such as sleep/wake, appetite and peak cognitive performance.

Mood disorders are the 3rd leading cause of premature death and disability in the United States. Astonishingly, approximately 28% of BD patients attempt suicide, a rate that is 10-20 times higher than the general population. Bipolar disorder typically presents during adolescence through the mid-twenties, often in the context of psychosocial stressors (Schaffer et al., 2010). Furthermore, studies have provided a strong genetic basis for BD. In monozygotic twins, the concordance rate for BD is 60-80% (Craddock et al., 2001). Still, the discordance rates of 20-40% suggest that the genetic component may not be completely penetrant. This implies that genetic factors alone may be insufficient to cause BD illness and allow that other factors may be involved in the onset of BD. These elements could include gene alterations through epigenetic modifications of the genome and/or environmental factors such as psychosocial stressors (McGowan et al., 2008).

1.5 Treatment

As previously noted, the main modulator of the mammalian circadian system is exposure to light. The iPRGC's contain a special photopigment melanopsin, which is involved in non-visual responses to light (Berson, 2003) and the light information is then projected to the SCN via the retino-hypothalamic tract (Hattar et al., 2010 & Welsh et al., 2010). Bright light exposure during the subjective night can advance or delay rhythms by several hours. Also, light exposure has also been shown to affect mood, and has

proved to be an effective antidepressant treatment (Golden et al., 2005). In patients with depression, bright light therapy is often used in conjunction with drug treatment to help stabilize mood. Lithium, a pharmacological mood stabilizer has also been shown to alter circadian clock function, lengthening the period of free-running circadian clocks. It has been proposed that its circadian effect may contribute to its therapeutic action within the brain (Welsh et al., 1990), leading to mood stabilization.

1.6 The Hypothalamic Pituitary Adrenal Axis

Mood episodes are often preceded by a stressful life event, and stress has been hypothesized to play a role in the development of mood disorders. Some leading neuroscientific hypotheses of stress and mood dysfunction have focused on abnormalities in the hypothalamic-pituitary-adrenal axis (HPA axis) (Krishnan et al., 2008). The HPA axis modulates many of the human body's responses, including metabolic, immune, and others, in response to stressful stimuli. The activation of the HPA axis due to a stressor begins a signaling cascade which ultimately involves the release of cortisol, a glucocorticoid (GC) from the adrenal cortex (Nader et al., 2010) (Figure 2). GCs have effects upon mood, arousal, and cognition, typically promoting behavioral activation, vigilance, and increased attention. They are considered a primary mechanism in promoting the physiological stress response. In addition to GCs, catecholamines such as epinephrine and norepinephrine are also major

effectors of the stress response, working together with GCs in mediating the "fight or flight" response.

1.7 The Stress Response

The stress response is defined as a specific response by the body to a stimulus that disturbs or interferes with the normal physiological equilibrium of an organism. While the stress response is essential for surviving challenges to homeostasis, chronic induction of the stress response can prove to be detrimental. The importance of stress in psychiatric disorders such as BD is apparent from adoption studies of monozygotic twins reared separately from each other. Although the twins have identical genomes, they experience dissimilar life events such that one may ultimately display a bipolar disorder phenotype while the other may be unaffected (Mendlewicz et al., 1977).

In response to a physiological stressor, the HPA axis causes GCs to surge in the bloodstream, facilitating the biological stress response. GCs are endogenously synthesized steroid hormones secreted by the cortex of the adrenal glands. The release of GCs is regulated by a variety of mechanisms including diurnal or circadian rhythms in which the GCs are secreted into the bloodstream and reach their peak concentration early in subjective morning (Hibberd et al., 2000). GCs bind preferentially to two receptors: the mineralocorticoid receptor, which has a high affinity for GCs, and the

glucocorticoid receptor (GR), which has a 10-fold lower affinity (Nishi et al., 2007). Both of these receptors reside in the cytoplasm in the absence of GC. However, upon ligand binding, the receptor changes conformation and translocates to the nucleus, where it modulates the expression of approximately 10% of the genome via glucocorticoid response elements (GREs), which allow for the GC to bind a segment of DNA (Jauregui-Huerta et al, 2010). This action has been shown to be relatively slow. Labeled dexamethasone (Dex), a synthetic GC, is translocated into the nucleus in brains of adrenalectomized rats in about 60-90 min (DeKloet et al., 2007). Interestingly, reduced levels of GR have been detected in various brain regions of patients diagnosed with psychiatric illnesses compared to normal controls (Perlman et al., 2004). In these patients, diminished GR expression or function has been proposed as the causal factor for lack of feedback suppression of the GC hormone cortisol, which may be one explanation for increased HPA Axis activity and heightened sensitivity to stress; moreover, the GR has been shown to be the most important regulator of the HPA Axis (Webster et al., 2002).

Stressful experiences in life can be classified into two main categories: acute and chronic. Acute stress is characterized by rapid onset due to a stressor and has a short duration lasting only seconds to minutes. Physiological changes associated with acute stressors include activation of the

sympathetic branch of the autonomic nervous system, which mediates the “fight or flight” response mobilizing glucose stores, increasing heart rate, and diverting blood away from non-vital organs. On the other hand, chronic stress is distinguished by a persistent or repetitive stressor, whose duration is long term and can result in a multitude of ailments including: cardiovascular damage, impaired immune function, and of particular interest, mood disturbances and increased risk for development of a mood disorder (Sapolsky 1999). Additionally, it has been noted that stressful life events often precede a mood disorder, further building evidence linking the two (Spijker et al., 2009). Furthermore, multiple studies have shown elevated levels of the stress hormone cortisol in patients with mood disorders (Ellenbogen et al., 2004).

A leading hypothesis linking mood disorders to circadian rhythms is the Social Zeitgeber Theory (Grandin et al., 2006), which states that manic or depressive episodes are brought on by life stresses because they disrupt normal routines, leading to changes in biological rhythms. Despite the evidence correlating mood irregularities and circadian regulation, it still remains unclear whether mood disorders are caused by clock dysfunction, or by dysfunctional molecules or pathways that happen to impact both systems. It is known that clock genes have pleiotropic effects, and gene functions unrelated to those of circadian timing may account for effects on mood (Rosenwasser, 2010). Therefore, it is critical to define the precise nature of

clock dysfunction in mood disorders, which will provide insight into the mechanisms of mood disorders, and is a prerequisite to testing whether clock dysfunction is causal.

In addition to physiological stress, which occurs at the organismal level, we would also like to identify stress at a cellular level. We will use two genes implicated in cellular stress to measure the effects of GCs upon our cells. Stress-induced phosphoprotein-1 (*STIP1*) is an adaptor protein, part of the heat shock protein series (HSP), which is upregulated in response to a variety of cellular stresses. We will also examine the expression of BCL associated agonist of cell death (*BAD*), a pro-apoptotic marker, which is upregulated in response to a non-hospitable cellular environment. Stress is known to cause HSP cellular responses and ultimately apoptosis, which may be a mechanism of psychiatric illness in the context of stress.

1.8 Hippocampus

Work over many decades has shown that GCs are central to the deleterious effects of stress upon the hippocampus, a brain structure important for learning that is rich in corticosteroid receptors, specifically the GR (McEwen et al., 1986). The hippocampus has a regulatory influence on the HPA axis as well as belonging to the limbic system, which influences the endocrine system and the autonomic nervous system (McEwen et al., 1982).

The hippocampus is altered significantly in patients with mood disorders, e.g. hippocampal atrophy and decreased neurogenesis. Destruction of the hippocampus leads to hypersecretion of GCs under basal and stressed conditions (Sapolsky 1999). GCs endanger hippocampal neurons directly, rather than secondarily through the vast number of peripheral actions of GCs.

Stress and high levels of glucocorticoids over a period of weeks can induce dendritic atrophy and alter dendritic morphology. After months of GC exposure due to stressful conditions, GCs worsen hippocampal atrophy, which is associated with depression. GCs also suppress hippocampal neurogenesis and inhibit glucose uptake in the brain, and since neurons have little ability to store glucose, this could be one of the mechanisms of tissue endangerment (Jauregui-Huerta et al., 2010). Because of these features, GCs in the hippocampus compromise the ability of neurons to survive toxic insults, worsening the damage due to inflammation, which increases reactive oxygen species (ROS) (Sapolsky 2005). It is also important to note that the hippocampus highly expresses the GR, while the SCN does not, allowing the SCN to be relatively less sensitive to the clock resetting properties of GCs.

1.9 Peripheral Models of Psychiatric Illnesses

Studies of psychiatric illness are typically constrained by their inability to study brain specimens from patients. However, because clock genes are

expressed peripherally and can be studied using PCR and reporter genes, it may be possible to study the circadian clock system without using the brain. Skin fibroblasts can be obtained from patients diagnosed with BD and healthy controls, and cultured in vitro. An initial study employing skin biopsies from normal subjects and a lentiviral *Bmal1-luc* reporter found that rhythms in cultured fibroblasts are reproducible across different biopsies from the same subject, and correlate with behavioral differences in chronotype (morning-evening preference) (Brown et al., 2005). Using lentiviral vectors, we introduced a *Per2-luc* circadian reporter gene into fibroblast cells, and bioluminescence from the glowing cells was monitored by luminometry under conditions mimicking stressful conditions. This approach allows for the direct measurement of rhythmic clock gene expression. Combined with the precise control afforded by in vitro studies, this may be a good approach for examining circadian rhythms. Although they are not neurons, fibroblasts develop from the same germ cell lineage as neurons, originating in the ectodermal layer during embryogenesis. Since fibroblasts lack the complex signaling systems of neurons, they are a simplified clock system, and our study can focus directly on the circadian clock gene circuit.

In the studies that follow, we hypothesized that circadian clocks in patients diagnosed with BD are more sensitive to stress compared to healthy controls. In order to test this, we modeled stress using synthetic stress in

cultured fibroblasts and measured multiple parameters of circadian clock function (amplitude, period, and goodness-of-fit). We found that goodness-of-fit was different in Dex-treated cells from BD patients than in Dex-treated cells from normal controls, supporting the idea that GC-mediated stress could provoke symptoms in BD through a circadian clock mechanism.

MATERIALS AND METHODS

2.1 Bioinformatics Study

We examined data from 7 previous studies, one meta-analysis which identified approximately 10,000 rhythmic genes and 6 other studies that identified genes that contain glucocorticoid response elements (GREs) (Grenier et al., 2005, Ishmael et al., 2008, Muzikar,et al., 2009, Reddy et al., 2009, Schmidt et al., 2007, Tissing et al., 2007 & Yan et al., 2008). The data from these studies were organized to identify genes that were both rhythmic and GRE-containing. For our purposes, we only considered genes that were rhythmic in at least four tissue samples for comparison to genes containing GREs. Additionally, we identified rhythmic genes that contain GREs and are implicated in BD by comparing the data to data on genetic associations and human genome epidemiology obtained from the Huge Navigator database (http://hugenavigator.net/HuGENavigator/home.do).

2.2 Glucocorticoid Effects on Expression of Stress Associated Genes

Fibroblasts were grown to confluence in four separate culture dishes. 48 hours prior to qPCR analysis, a medium change was performed and cells were bathed in media with Dex (100 nM or 10 μM) or vehicle. After 48 hours, cells were then lysed and RNA was extracted using an RNeasy kit following manufacturers instructions (QIAGEN). Additionally, an acute time course was

performed in which cells were bathed in media containing Dex at 100 nM concentration for one hour prior to lysis and RNA extraction. RNA samples were quantified using a Thermo Scientific NanoDrop spectrophotometer and stored at -80 °C until cDNA synthesis. cDNA was synthesized using a high capacity cDNA synthesis kit from Applied Biosystems in which reverse transcription reactions were performed in a thermal cycler to make cDNA for qPCR according to manufacturer's instructions. cDNA was added to PCR master mix and all analyses were performed in duplicate. Samples were normalized to the housekeeping gene *GAPDH*. Primers were selected for genes that are expressed in fibroblasts. These genes were chosen because they are known not to be rhythmic, and are upregulated in response to cellular stress. TaqMan gene expression primers were purchased from Applied Biosystems and utilized to identify cellular stress in a Dexamethasone time course. The first primer, stress-induced-phosphoprotein 1 (*STIP1*), is a heat shock organizing protein and protein chaperone involved in the folding of the glucocorticoid receptor. The second primer, BCL2-associated-agonist of cell death (*BAD*) is a pro-apoptotic gene that is also implicated in psychiatric disorders such as schizophrenia. qPCR was carried out using standard procedures.

2.3 Cell Lines

Fibroblast cell lines were purchased from Coriell Cell Repositories-Coriell Institute for Medical Research, or were received from our collaborator Dr. Bruce Cohen at Harvard University. All Coriell fibroblasts come from outpatients recruited for family genetic studies clinically diagnosed as BD or appearing psychiatrically healthy (Breakefield et al., 1980). Cells from Dr. Cohen were obtained from clinically diagnosed BD inpatients and are age/sex/race matched with healthy controls.

2.4 Tissue Culture

Fibroblasts were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO 11995-065) containing high glucose, glutamine, and sodium pyruvate. The medium was supplemented with 10% Fetal Bovine Serum (FBS) (GIBCO 10082-139) as well as 25 U/ml Penicillin, 25 μg/ml Streptomycin (GIBCO 15140-122), 2mM glutamine (GIBCO 25030-081) and 1% Antibiotic-Antimycotic (GIBCO 15240-062). Fibroblasts were thawed from -80°C and plated into culture dishes and stored in a tissue culture incubator at 37°C equilibrated with 5% carbon dioxide. When cells had grown to confluence they were split into 35mm culture plates and again allowed to grow to confluence prior to bioluminescent recording. For luminescence recordings, cells were transferred to explant medium (EM) containing DMEM medium (GIBCO

12100-046), supplemented with 10mM HEPES buffer (GIBCO 15630-080), 1.2 g/L sodium bicarbonate (GIBCO 25080-094), 25 U/ml Penicillin, 25 μg/ml Streptomycin (GIBCO 15140-122), 2% B-27 (GIBCO 17504-044), 1mM Luciferin (BioSynth L-8220) and pH adjusted to 7.4 with NaOH. 35mm culture plates were then covered by circular coverslips and sealed with vacuum grease to prevent evaporation.

2.5 Reporter Construct

The Per2::luc reporter plasmid incorporates Per2 promoter sequence beginning 280bp upstream of the transcriptional start site. The promoter was cloned into the pLV156 basic vector, which contains an optimized firefly luciferase gene (luc) (Liu et al., 2007). Per2::luc was delivered to the cells via an HIV1 lentiviral vector. Media in the culture plate was aspirated and replaced with 1mL of fresh DMEM at the desired concentration of Dex or isoproterenol (iso). Virus mix (1:1 virus + PBS, 1:100 polybrene) was then added to the plate and incubated for 2 hours before adding an additional 1mL of growth media containing Dex or iso. The cells were incubated for 48 hours before bioluminescent imaging (Figure 3).

2.6 Drug Study

For our study, Dex was dissolved into 100% ethanol and diluted with growth medium to the desired concentration. In our experiment using

isoproterenol (iso), iso was dissolved in pure water and then mixed with growth medium to achieve the desired concentration. Acute stresses typically last on the order of seconds to minutes, whereas chronic stresses last hours to days, or longer. In order to model chronic stress, fibroblasts were pre-treated with Dex, iso, or vehicle for 48 hours prior to luminescent monitoring, and drug remained in the EM for the 4-5 days in which rhythms were measured. While this time period of days may not be considered long-term on the scale of life events, this approach made for a tractable model of stressful environmental conditions using a cell culture model.

2.7 Bioluminescence Recording and Data Analysis

The cells were synchronized by a medium change prior to recording. It has been observed that a media change, which is usually accompanied by a change in temperature, is sufficient for synchronizing peripheral cells such as fibroblasts (Welsh et al., 2004 & Liu et al., 2007). After the medium change to EM containing drug or vehicle, culture plates were sealed with vacuum grease to prevent evaporation and placed into a LumiCycle luminometer (Actimetrics, Inc.) which was kept in a tissue culture incubator at 35°C not equilibrated with carbon dioxide. Bioluminescence from each plate was recorded with a photomultiplier tube (PMT) for 70 seconds at intervals of 10 min. Recording of bioluminescence rhythms lasted for 5 days. Raw data (counts/sec) were plotted against time (days) in culture. For analysis of rhythms, the LumiCycle

Analysis program (Actimetrics, Inc.) was used and raw data were fitted (subtracting baseline) to a sine wave from which the period, amplitude, and goodness-of-fit (the proportion of variance in data accounted for by a fitted least squares sine wave) were determined using time points from 0.8 to 4.8 days. Due to high transient luminescence from the medium change, the first 0.8 days were excluded from rhythm analysis. All data for cells from both sources were analyzed separately and then combined because there were no significant differences between the cohorts.

RESULTS

3.1 Bioinformatics Study

In order to explore the plausibility of regulatory overlap between circadian clock genes and the glucocorticoid stress response system, we first conducted a bioinformatic survey of glucocorticoid response elements contained within rhythmic genes. We identified six studies that located GREs throughout the genome. Collectively, these identified approximately 615 genes with a GRE. Additionally, a study by Yan and colleagues was able to identify approximately 10,000 rhythmic genes of which 716 were rhythmic in 4 or more tissues. Comparing these two data sets allowed us to survey the overlap between these lists. We found that 114 rhythmic genes also contain a GRE (Table 1 & Figure 4). Five of these genes are core clock genes (*CRY2, CSNK1D, PER1, PER2, NR1D2*), hinting at possible mechanisms for the action of glucocorticoids upon circadian rhythms. Using the Huge Navigator database, we compared our set of 114 genes against a list of 724 genes previously implicated in BD. We identified 31 genes that are rhythmic, contain a GRE, and are implicated in BD. Taken together, these data suggest that there is a potential overlap between rhythmic clock genes, the glucocorticoid stress response system, and BD.

3.2 Interactions between Dexamethasone, Isoproterenol, and Circadian Rhythms

As a preliminary test of effects of Dex on circadian rhythms, we performed a dose response curve using five separate conditions (vehicle, 100 nM, 1 μM, 10 μM, 100 μM Dex). The dose response curve was performed on cells from a psychiatrically evaluated healthy control. We observed that Dex induced dose-dependent effects upon period and amplitude. Of the four doses of Dex, the most effective in eliciting period lengthening was 10 μM (Figures 5A & 6), which therefore was chosen as the concentration to mimic a stressful environment. Examining amplitude as another rhythm parameter, we observed that the most effective concentration of Dex in altering the amplitude of the rhythm was 1 μM. However, we also observed an increase in amplitude at 100 nM (Figures 5B & 6). We chose to use this lower concentration for our study due to the fact that it is closer to the physiologically relevant concentration of glucocorticoid released in response to a stressful event.

We also performed a dose response curve for the synthetic catecholamine isoproterenol. We again observed that the period length was lengthened most in comparison to baseline at 10 μM concentration (Figures 7a & 8). At any drug concentration, there was a suppression of amplitude (Figures 7B & 8). Although we observed an effect on rhythms with iso, there is a more substantial backing in the literature for interactions between

glucocorticoids and circadian rhythms. Additionally, in response to a stressor, catecholamines are released into the bloodstream for just seconds to minutes and are rapidly degraded, and therefore, are not part of the sustained stress response. Hence, we continued our assay of rhythm parameters using Dex.

3.3 Glucocorticoid Effects on Expression of Stress Associated Genes

Dex and other glucocorticoids cause cognitive impairments due to stress related changes in neuronal cell morphology, decreased neurogenesis, and cellular death (McEwen et al., 1995). Using the aforementioned primers for the genes *STIP1* and *BAD*, we conducted time course experiments in cells from patients diagnosed with BD or psychiatrically evaluated healthy controls. In the first experiment, we performed an acute time-course in which the fibroblasts were bathed in media containing Dex (100nM) for one hour prior to harvesting the RNA. We also performed a 48-hour time course simulating a chronic stressful environment at our two chosen concentrations of drug. After synthesizing cDNA, samples were prepared for qPCR and run in duplicate. Cycle threshold (CT) values ranged from 15.79-19.15 for our housekeeping gene *GAPDH*, 26.46-31.46 for *BAD,* and 20.46-23.70 for *STIP1*. Expression of *BAD* and *STIP1* was slightly higher at the high concentration of Dex (132% of vehicle and 128% of vehicle, respectively) but were not statistically significant, ($F_1 = 1.26$, $P < 0.29$ *BAD,* $F_1 = 0.86$, $P < 0.47$ for *STIP1*), only suggesting a trend to show that cells were indeed experiencing a stress (Figure 9A, 9B).

Within a cell line, *GAPDH* expression was constant regardless of treatment condition, indicating that the cells remained viable. Taken together, these data hint that the Dex treatment may be inducing an intracellular response to a perceived stress, but that this response may be relatively weak.

3.4 Bioluminescence Reporter Assay

We initially analyzed our data separately for our differently sourced cell cohorts (a Harvard cell group, as well as a Coriell cell group) and no significant differences were found between the groups. Therefore, data are being presented as a collective whole.

Under baseline conditions without drugs, we assessed period length, amplitude, and goodness-of-fit for our BD and control cell lines. We observed no statistically significant differences between the diagnosis groups (healthy control vs. BD) for any of the rhythm parameters we examined. Average period length for our healthy controls was 25.30 hours while cells from BD patients had an average period of 25.69 hours ($T(14) = 0.54, P > 0.6$) (Figure 10A). Average amplitude was 25.45 counts for the control group and 24.45 counts for BD group ($T(14) = 0.09, P>.93$) (Figure 10B), while the average goodness-of-fit of the rhythm was measured to be 77.36% for controls and 75.67% for BD ($T(14) = 0.34, P > 0.74$) (Figure 10C). An average trace for both diagnosis groups at baseline is shown (Figure 11).

In our experiments modeling the stress response using the synthetic glucocorticoid Dex, we also examined the same 3 rhythm parameters: period, amplitude, and goodness-of-fit. For the Harvard cohort (n = 4 healthy controls, n = 6 BD) we eliminated 2 cell lines from the healthy control group because they were arrhythmic. For our Coriell cell cohort (n = 3 healthy controls, n = 3 BD), we eliminated 1 cell line from the healthy controls because it too was arrhythmic. Examining all data from both diagnosis groups, we observed no difference in average period length from baseline at the lower concentration of Dex (100 nM) (Figure 12A) (all data reported are values subtracted from baseline). However, both diagnosis groups showed an approximate 1.75-hour lengthening of period at the higher concentration of Dex ($F_{1,14}$ = 14.97, P < 0.002) (Figure 12A). Examining the effect of Dex upon the amplitude of the rhythms, the lower concentration significantly increased the amplitude for both diagnosis groups (control amplitude 13.81 counts vs. BD amplitude 4.25 counts) in comparison to baseline and this result proved to be significant ($F_{1,14}$ = 5.98, P < 0.03) (Figure 12B). At the higher concentration of Dex, there was a dampening effect upon amplitude for both diagnosis groups (control amplitude -9.84 counts vs. BD amplitude -10.36 counts). Goodness-of-fit effects proved to be highly significant for diagnosis, for the drug, and for the interaction ($F_{1,14}$ = 6.57, P < 0.023; $F_{2,14}$ = 34.48, P < 0.0001; $F_{2,14}$ = 4.89, P < 0.015). This suggests that the BD cells and controls are inherently different in their stability when exposed to Dex (Figure 12C). Baseline (control = 77.36%, BD =

75.67%) and the low concentration of Dex (control = 77.13% BD = 82.23%) maintained a high stability rhythm, whereas at the high concentration of Dex (control = 11.02%, BD = 48.65%) rhythm stability dampened precipitously. Average traces are shown for healthy controls and BD (Figure 13). Traces from the LumiCycle (Figure 14) are to show as representative examples of goodness-of-fit for a healthy control and BD cell line under the different experimental conditions. We performed an extended chronic time-course experiment in which we were able to culture cells from a psychiatrically evaluated healthy control in media containing the high concentration of Dex (10 μM) for 30 days (Figure 15). However, there was a change in cell morphology associated with chronic exposure to GCs (McEwen et al., 1995). We also examined mean bioluminescence levels from our samples and observed no adverse effects due to Dex treatment (data not shown).

DISCUSSION

4.1 Bioinformatics

Fibroblast cells from patients diagnosed with BD or psychiatrically evaluated healthy controls are a suitable model for studying the effects of stress on the circadian clock and any potential differences in response between the two groups. The data presented here suggests that there is a potential for interaction between circadian rhythms, BD, and glucocorticoids, especially as we found a potential genetic basis for such an interaction. The identification of putative GRE sites within clock genes that can in principle interact with glucocorticoids illustrates the potential for stress and the release of glucocorticoids to alter clock function. With respect to mood disorders, several of these potentially stress-responsive clock genes have been implicated in BD. This interaction could possibly lead an individual to be more susceptible to stress, ultimately leading to disrupted circadian rhythms and a BD phenotype. Given more time to research additional data sets, we believe we would be able to obtain a more extensive list implying further regulatory overlap between circadian clock genes, the glucocorticoid stress response system, and BD.

4.2 Intracellular Stress Signals

A critical feature of our experimental model is that dexamethasone can be used to model chronic stress in fibroblast cell cultures. Our results were equivocal on this point. While cell culture models of stress have been previously developed using hippocampal neurons, fibroblasts have important differences. Skin fibroblasts are not as enriched in GR expression as are hippocampal neurons. Therefore, we used higher Dex concentrations than those typically used in neurons in order to test the limits of tolerance with respect to effects on circadian rhythms. In our analysis of intracellular stress signals, we observed a trend towards increased expression of cellular stress-related genes, but none of these differences were statistically significant, even though the PCR cycle threshold values for each gene suggest that the gene mRNAs were sufficiently abundant in the cells to measure. The similarity of GAPDH levels across dexamethasone concentrations suggests no major differences in cell viability as a result of increasing dexamethasone exposure. Therefore, it is possible that we selected genes that were not dexamethasone inducible, explaining our modest response in gene expression when cells were exposed to Dex. It remains possible that using additional stress induced genes in future studies could provide a better index of stress in response to Dex. During the course of our experiment, the cells are subject to a 48-hour pre-incubation of Dex and an additional approximate 96-hours of Dex exposure

during the luminometry portion of the experiment. We only measured stress gene expression at one 48-hour time point, so it remains possible that Dex induced gene expression changes would be more noticeable later in the time course.

4.3 Stress and Circadian Rhythms

Our hypothesis was that cells from patients diagnosed with BD would be more susceptible to alterations of their circadian rhythms than cells from psychiatrically evaluated healthy controls when exposed to conditions modeling the stress response. We began to work towards evaluating the hypothesis by employing Dex and observing if drug treatment would be able to elicit an alteration in circadian rhythms. We used two concentrations of Dex in order to more clearly separate our drug effects upon two rhythm parameters: period and amplitude. With Dex we were able to specifically alter the circadian clock in a concentration dependent manner. At the high concentration of Dex we were able to alter two rhythm parameters, period length and goodness-of-fit. At the lower concentration of Dex, we were able to alter amplitude.

In our assay we utilize amplitude as another quantifiable parameter of circadian rhythms. At the cell population level, amplitude is a measure of 3 main criteria: cell density, synchrony of the cells, and level of gene expression. In our experiments, cell density is unlikely to explain the differences observed

in amplitude since cells are plated from the same culture dish at equal concentrations. A known and well-used property of Dex is its ability to synchronize cells in circadian rhythm studies. This is known as “shocking” the cells, and realigns all the individual cell phases to a single one. Nevertheless, the cells may begin to drift out of phase from one another, thereby reducing average amplitude over an entire dish. Hence, cells may be less responsive to synchronizing effects at the high concentration of Dex due to receptor desensitization and not synchronize their phases as effectively as at the lower Dex concentration, therefore exhibiting a lower amplitude rhythm when measured in a cell population. To better understand amplitude, additional assays could be performed in which we would measure cell synchrony in single cell experiments in the absence or presence of Dex. Finally, the level of *Per2* and/or other clock gene expression could vary from sample to sample leading to the differences observed in amplitude. To better quantify gene expression we could perform qPCR, with or without Dex, to determine if the level of expression for a given gene is a factor in amplitude.

Examining rhythm parameters at baseline, we chose 0.5 as our cutoff for goodness-of-fit. All cell lines displaying a goodness-of-fit less than 0.5 were considered arrhythmic, leading us to throw out 1 control line from the Coriell cell cohort and 2 control lines from the Harvard cell cohort. This rate of arrhythmia was not significantly different between controls and BD ($p=0.07$).

We did not consider exclusion based at the highest concentration of Dex since a notable decrease in goodness-of-fit was observed for almost all cell lines. Additionally, we discovered differences in cells treated with Dex from patients diagnosed with BD as compared to healthy controls. Examining goodness-of-fit at the high concentration of Dex, there is a significant difference between the two groups. This may be due to coherence, which is the ability of the cells to stay in phase with one another. Cells from patients with BD may be able to maintain phases closer to one another and this could be potentially due to GR sensitivity in which receptor desensitization to Dex occurs in control cells more than in cells from patients with BD.

We also performed an assay in which we used the synthetic catecholamine isoproterenol. An area of further development would be to expand upon the experiments performed with Dex by using iso instead, examining stress mediated by the beta-adrenergic system and its interactions with circadian rhythms.

A marked characteristic of a circadian rhythm is a rhythm that is stable and able to compensate for a wide variety of perturbations and environmental changes with a relatively small effect upon rhythms. Hence, the effects seen in the presence of Dex could be buffered to a greater extent than otherwise expected and seem to be more so in cells from BD patients. The common assumption when examining BD in the context of circadian rhythms is that the

clock is broken. We may need to revise this notion and examine if the clock may in fact be more robust and therefore be less sensitive to outside inputs, potentially leading to circadian dysfunction.

FIGURES AND TABLE

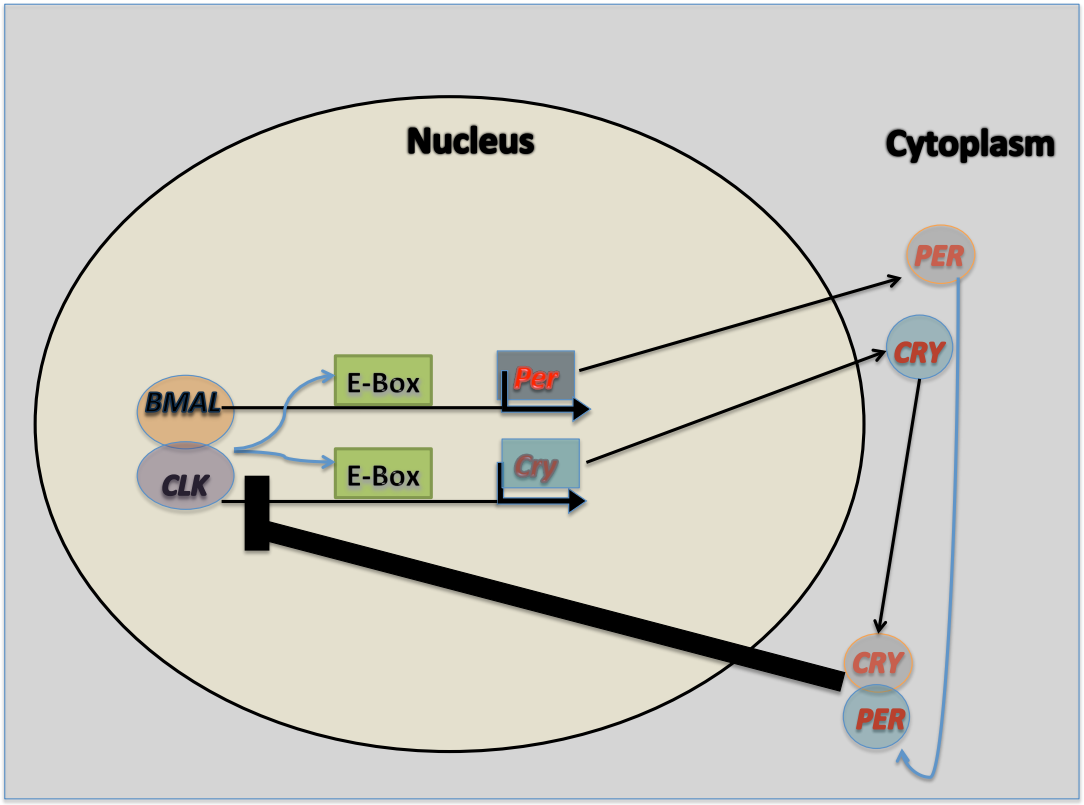


Figure 1. Mammalian Circadian Core Clock Genes. CLOCK/BMAL1 heterodimers bind to E-box elements to activate transcription of *Per, Cry*. PER/CRY heterodimers inhibit transcription of their own genes. As PER/CRY levels rise, inhibition gradually builds, and then declines due to ubiquitination and proteasomal degradation of PERs and CRYs, thereby relieving inhibition and re-initiating the cycle.

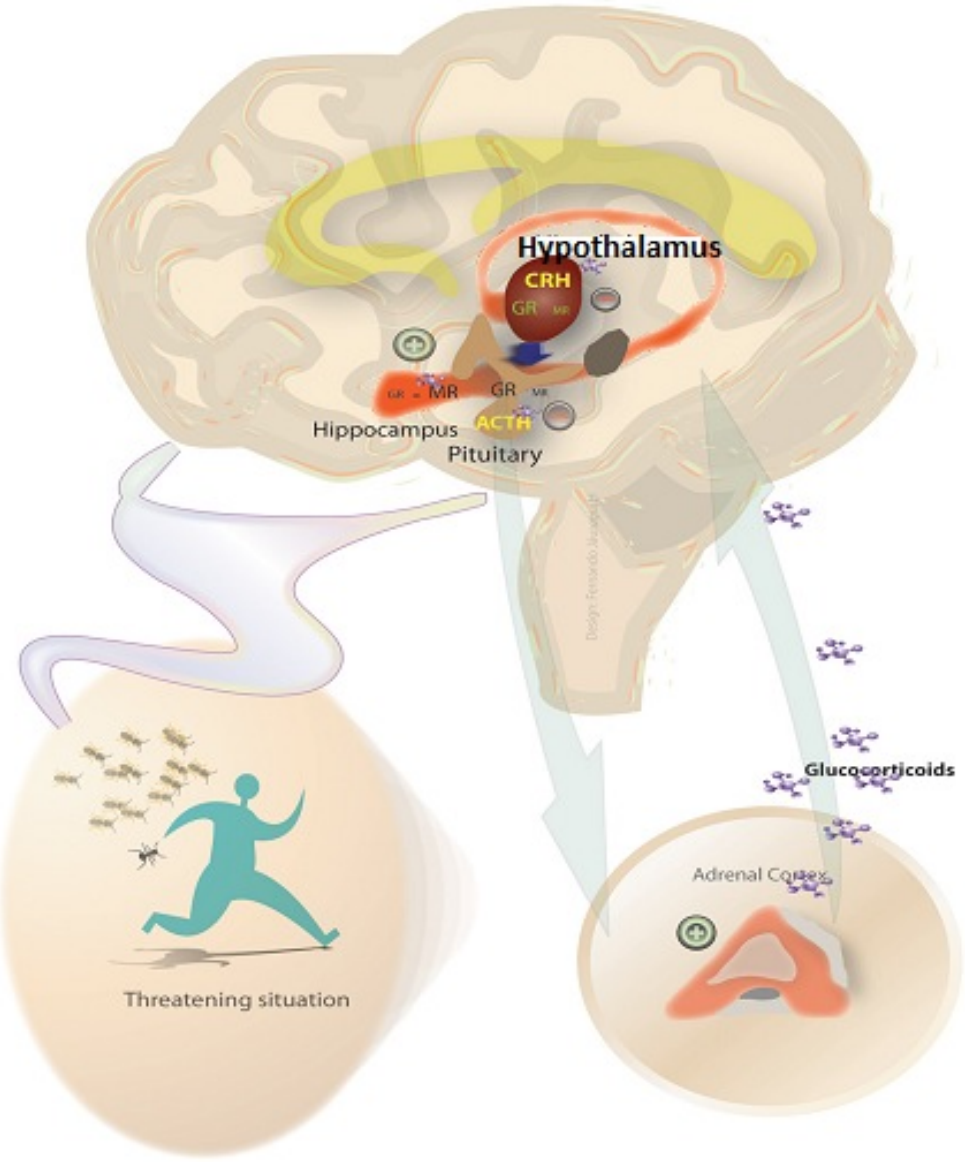


Figure 1. HPA axis function.
Neuroendocrinological diagram that shows how a perceived threat or stressor activates the Hypothalamic-Pituitary-Adrenal Axis. When the brain perceives stress, the hypothalamus releases corticotropin-releasing hormone (CRH), which triggers the release of adrenocorticotropic hormone (ACTH) from the pituitary gland. ACTH travels through the bloodstream and stimulates the cortex of adrenal glands to release glucocorticoids (GCs). Figure from Jauregui-Huerta (2010).

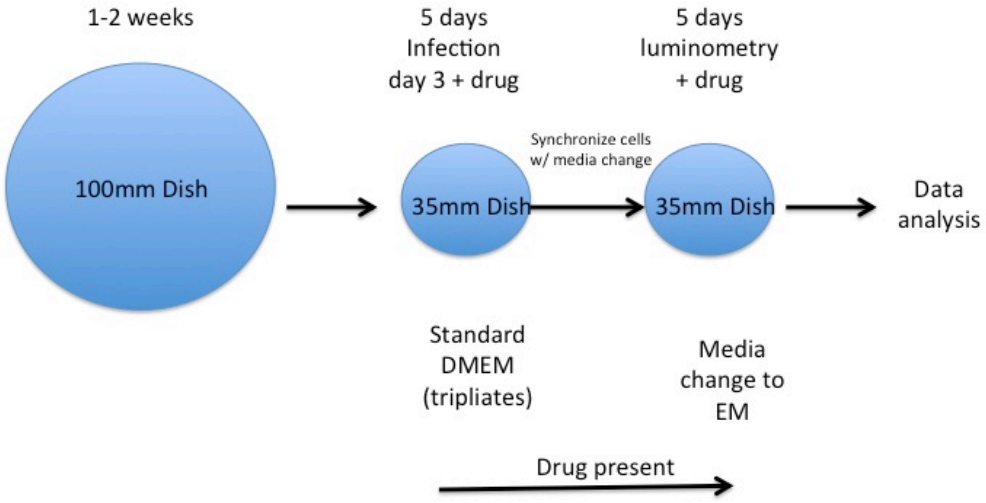


Figure 3. **Fibroblast Infection Protocol.** Illustration of events for each bioluminescent reporter assay. A confluent 100 mm dish of cells is split into three 35 mm dishes and allowed to grow to confluence in standard DMEM. Upon reaching confluence, medium is aspirated and replaced with fresh growth medium containing drug and virus. After incubating for 48 hours, medium is aspirated and replaced with explant medium containing drug. The plates are sealed and placed in the luminometer for 5 days. After 5 days plates are removed and data are analyzed.

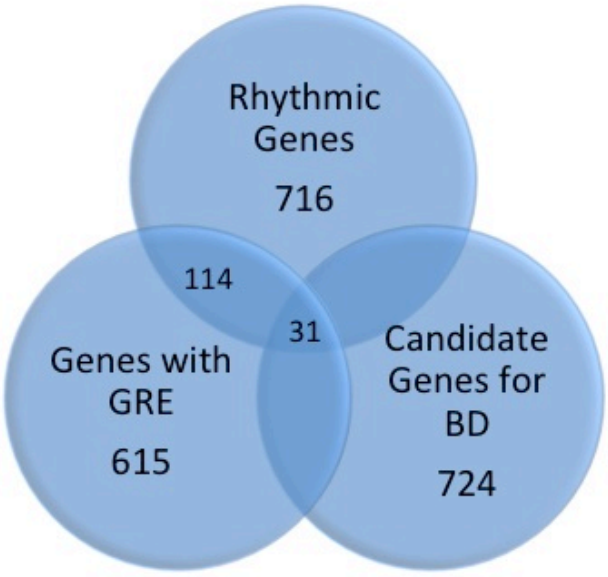


Figure 4. Bioinformatics. Venn diagram graphically illustrating the overlap between rhythmic genes and genes with GRE. Of the 114 overlapping genes, 31 of these are also candidate genes for BD.

Table 1. Genes identified in the bioinformatic assay. Of 716 rhythmic genes, 114 had a GRE, and of these 31 have been implicated in bipolar disorder.

Core Clock Genes with GRE (5)	Rhythmic Genes with GRE (114)			Rhythmic Genes with GRE implicated in BD (31)
CRY2	Acta2	GADD45A	PLK2	ADORA2A
CSNK1D	ACTB	GADD45B	PNRC1	BCR
PER1	ANGPTL4	HIST3H2A	Ppap2b	BDKRB2
PER2	Aqp1	Hmgcs1	PPP1R15B	BHLHB2
NR1D2	ARL4A	ID2	PRDX1	CCL2
	BCL2L11	IER3	PXN	CRY2
	BCL6	IER5	PYGB	CSNK1D
	BHLHB2	IRS2	RABGGTB	DBI
	Ccnd1	Itgb5	RASD1	DUSP6
	CDKN1C	KLF10	RBM3	FAM43A
	CEBPB	KLF6	RGS2	FKBP5
	CITED2	KLF9	RHOBTB1	FZD2
	CKB	Lasp1	RHOBTB2	HES1
	Cnn2	Lrig1	RhoC	LIF
	CPE	MAP2K3	RHOU	Mmp9
	CPEB4	Map3k6	RPL13	Net1
	CRY2	Marcks	RPL13a	NFIL3
	CSNK1D	MCL1	RRAD	Nlgn2
	Csrp2	METTL7A	SDC2	nos1
	CTGF	MID1IP1	SDPR	NR3C1
	CXCR7	MIDN	SEC14L2	NR4A1
	Dab2	MT1	SERTAD1	NR4A2
	DAD1	MYH9	SGK	Nxn
	DBI	Nedd4l	SLC22A5	PDE4B
	DDIT4	Net1	Slc6a6	PER1
	DSCR1	NFIL3	Smpdl3b	PER2
	EFNA1	Nfix	SOX4	Pkia
	Egr1	NFKBIA	SPON2	PTGER4
	Eif1a	NR1D2	Sqle	SDC2
	EIF2C2	NR3C1	STAT1	Slc1a3
	Ephx1	NR4A1	STOM	Slc6a9
	ERRFI1	NRARP	Tgfbr2	
	Fdps	PDK4	THBD	
	FGFR3	PER1	TSC22D3	
	Figf	PER2	Tubb5	
	FKBP5	PFKP	Vcam1	
	FTH1	PIM3	ZFAND5	
		PKP2	Plat	

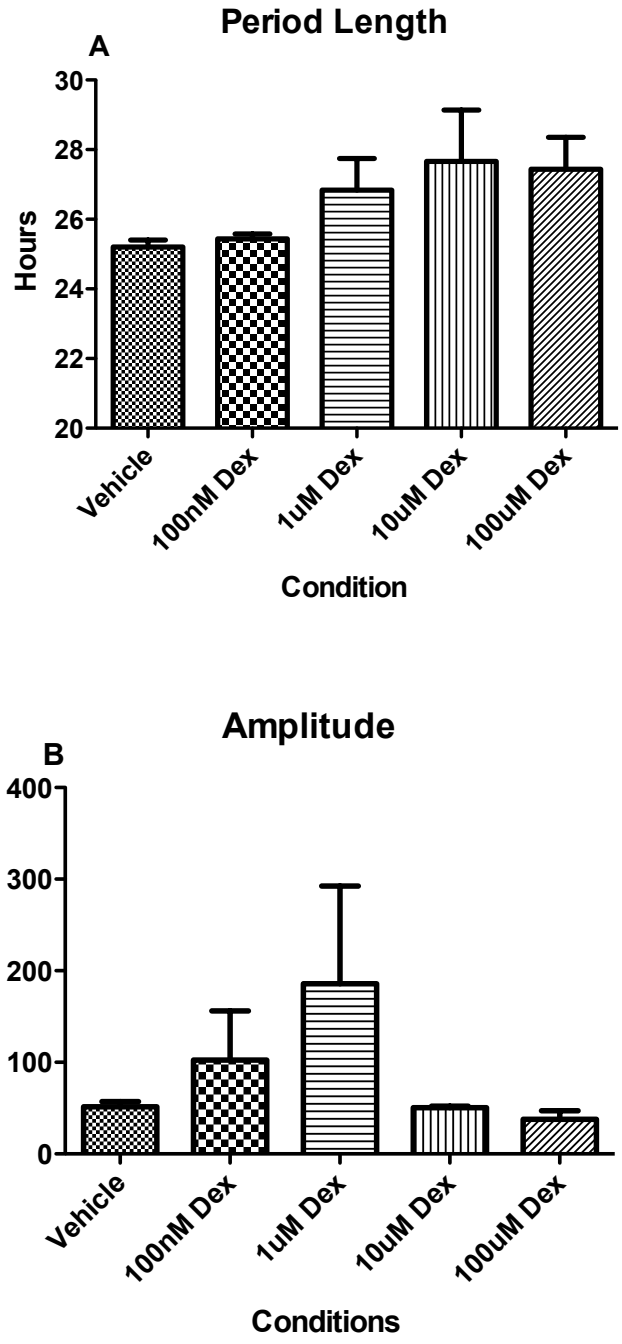


Figure 5. Dexamethasone Dose Response. A) Period length of cells treated with vehicle or Dex. Period length is 25.73 hours for vehicle and 27.33 hours for 10 μM. **B)** Amplitude of cells treated with vehicle or Dex. Amplitude is 51.42 counts for vehicle and 102.50 counts for 100 nM Dex. All values are averages of triplicate measurements.

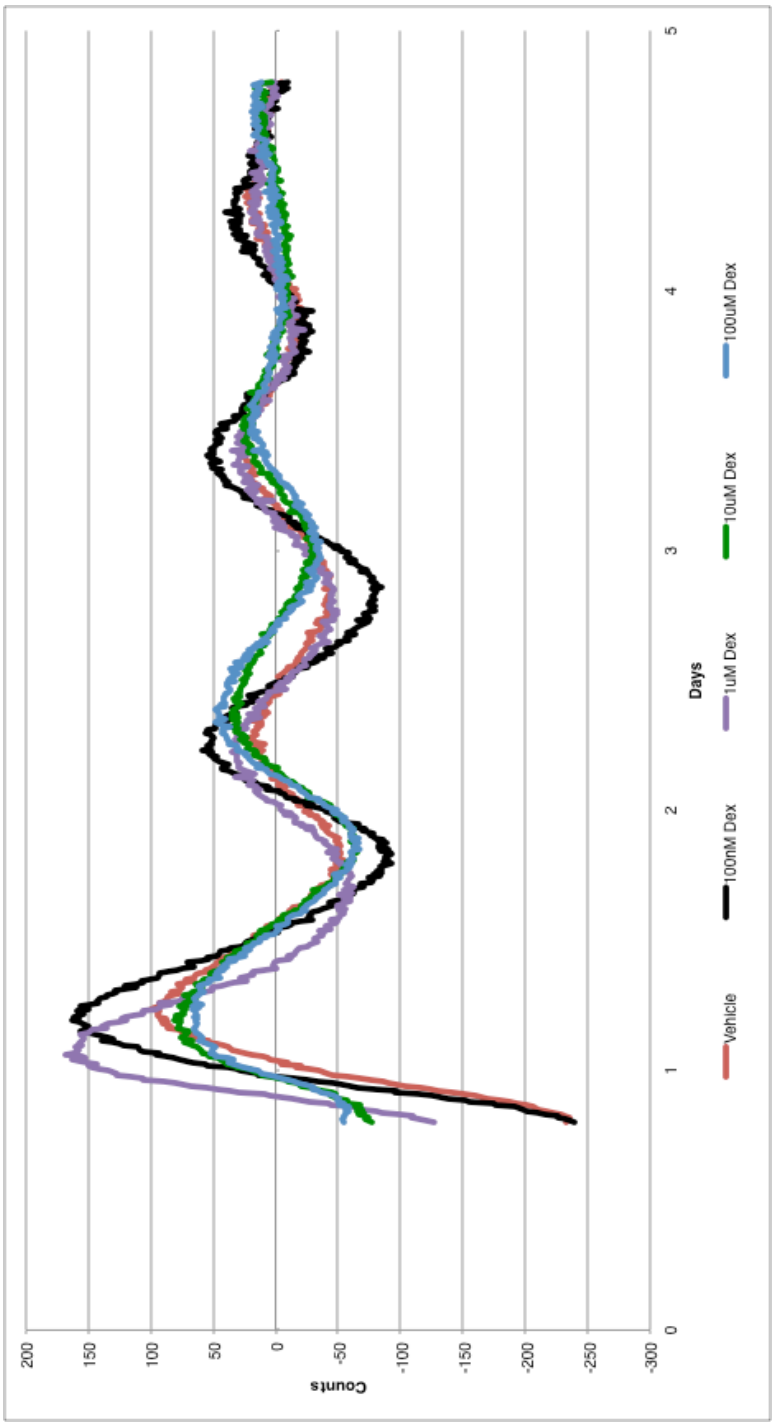


Figure 6. Dexamethasone Dose Response Traces. Average normalized Per2-luc bioluminescence traces of cells from the 5 conditions assayed for the dose response. Traces are best fit lines using sine waves and are averages of triplicate measurements.

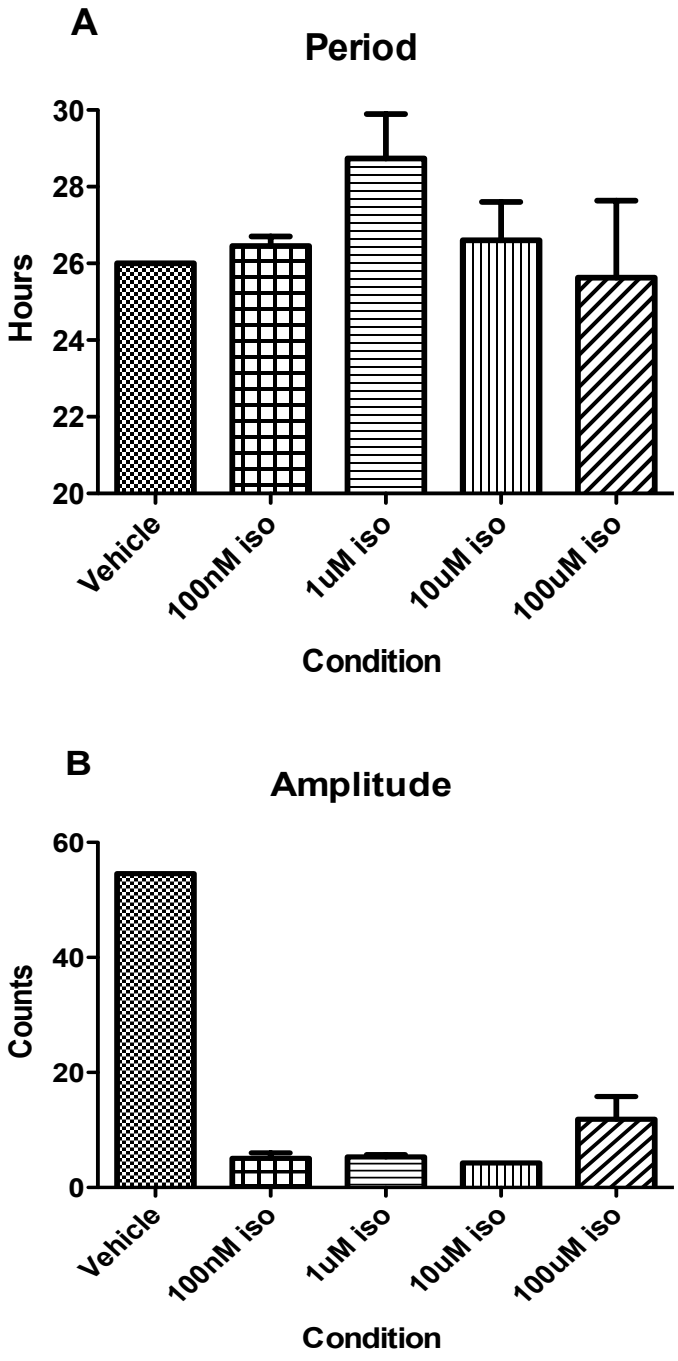


Figure 7. Isoproterenol Dose Response. A) Average period length of cells treated with vehicle and 4 different concentrations of isoproterenol. **B)** Average amplitude for cells for the 5 conditions used. All values are averages of triplicate measurements.

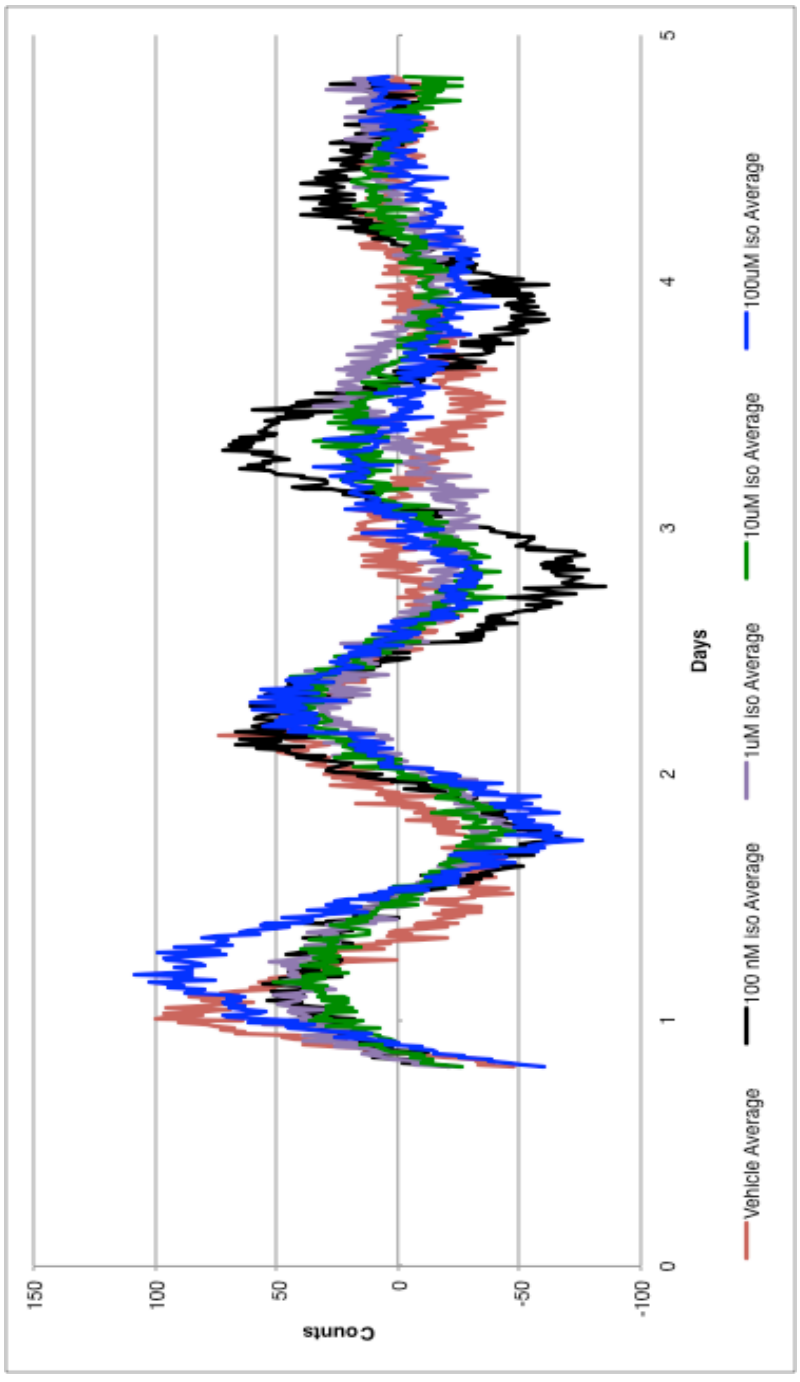


Figure 8. Isoproterenol Dose Response Curve. Average normalized Per2-luc bioluminescence traces from the 5 conditions assayed for the dose response. Traces are best fit sine waves and are averages of triplicates.

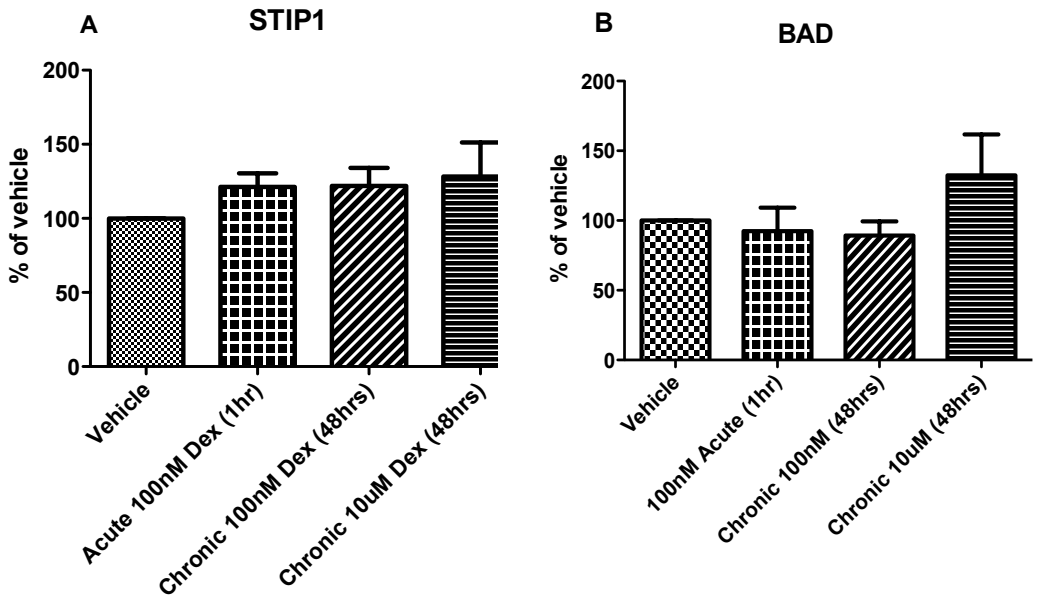


Figure 9. qPCR gene expression of stress-response genes. A) *STIP1* gene expression at low or high concentrations of Dex, modeling acute or chronic stress. **B)** *BAD* gene expression at low or high concentrations of Dex, modeling acute or chronic stress. All values shown are normalized to vehicle.

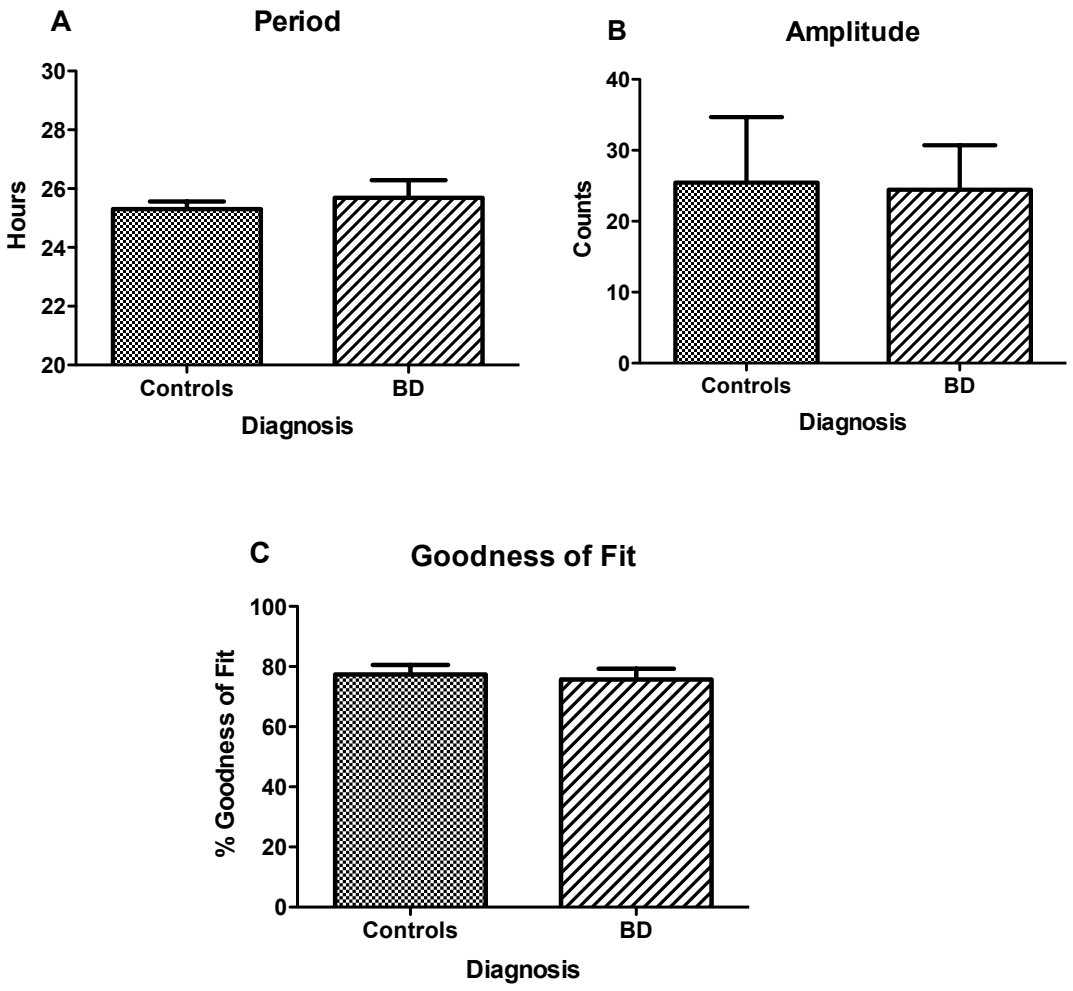


Fig 10. Rhythm Characteristics at Baseline. Values are an average of all subjects within a given diagnosis (n = 7 controls, n = 9 BD).

- **A)** Average period length for healthy controls = 25.30 hours vs. BD = 25.69 hours ($T(14) = 0.54$, $P > 0.6$).
- **B)** Amplitude for healthy controls = 25.45 hours vs. BD = 24.45 hours ($T(14) = 0.09$, $P > 0.93$).
- **C)** Goodness-of-fit for healthy controls = 77.36% vs. BD = 75.67% ($T(14) = 0.34$, $P > 0.74$).

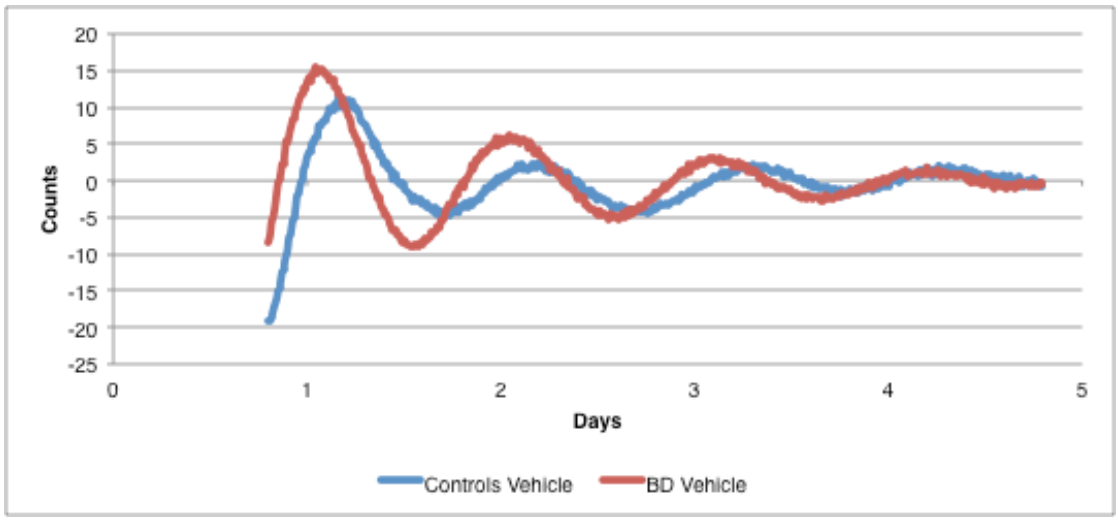


Figure 11. Average Traces At Baseline. Per2-luc bioluminescence rhythm traces from BD and control cells under baseline conditions. Traces are averages of all experiments, separated by diagnosis.

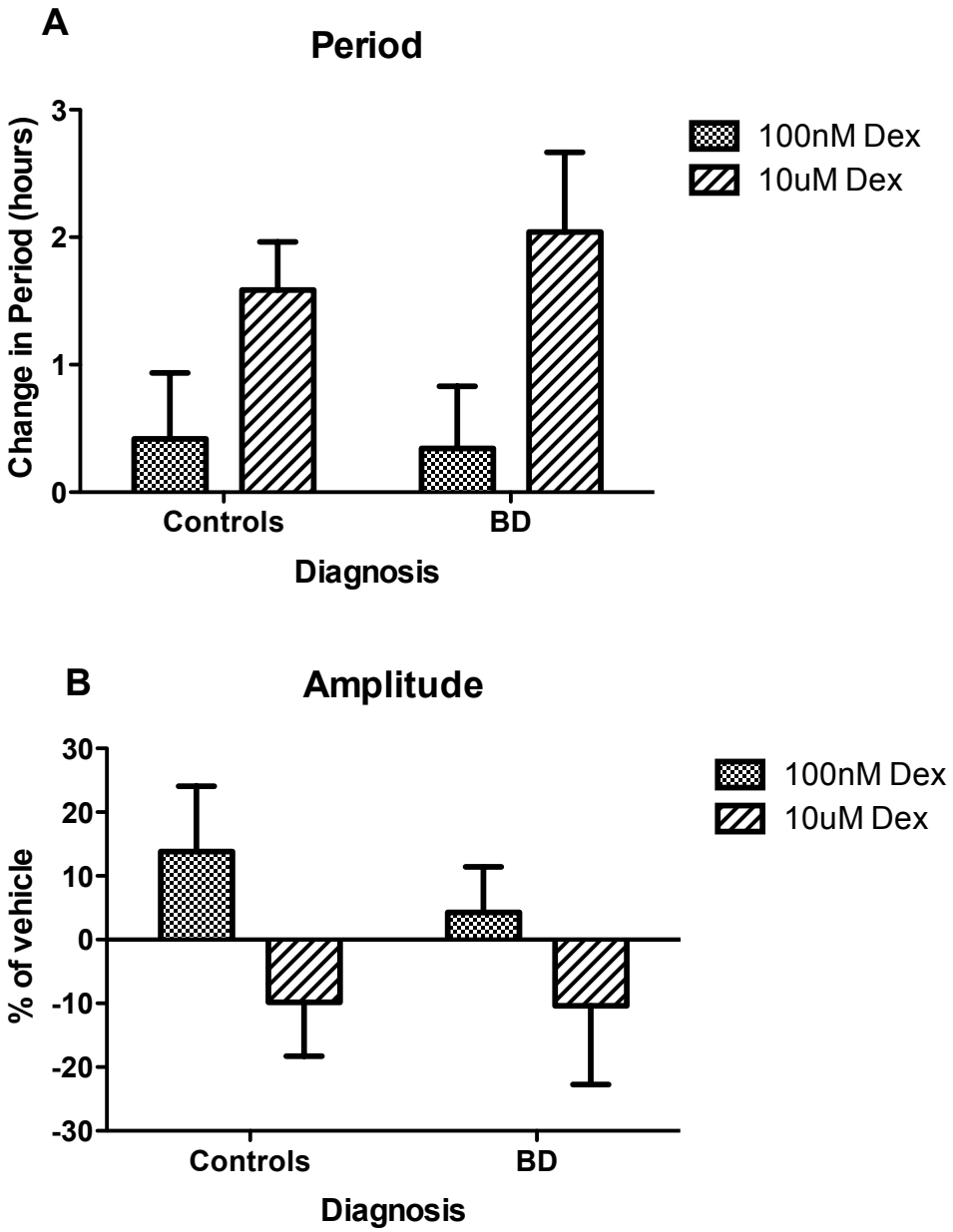


Figure 12. **Rhythm Characteristics with Dex.** Data are average values for all experiments for each condition, normalized to baseline. (2-way ANOVA)

- **A)** In 10 μM Dex, period lengthened by an average 1.75 hours relative to vehicle control ($F_{1,14}$ = 14.97, P < 0.002). No change at 100 nM in comparison to baseline.
- **B)** Amplitude (control amplitude 13.81% of vehicle control vs. BD amplitude 4.25%) ($F_{1,14}$ = 5.98, P < 0.03) control amplitude -9.84% vs. BD amplitude -10.36%).

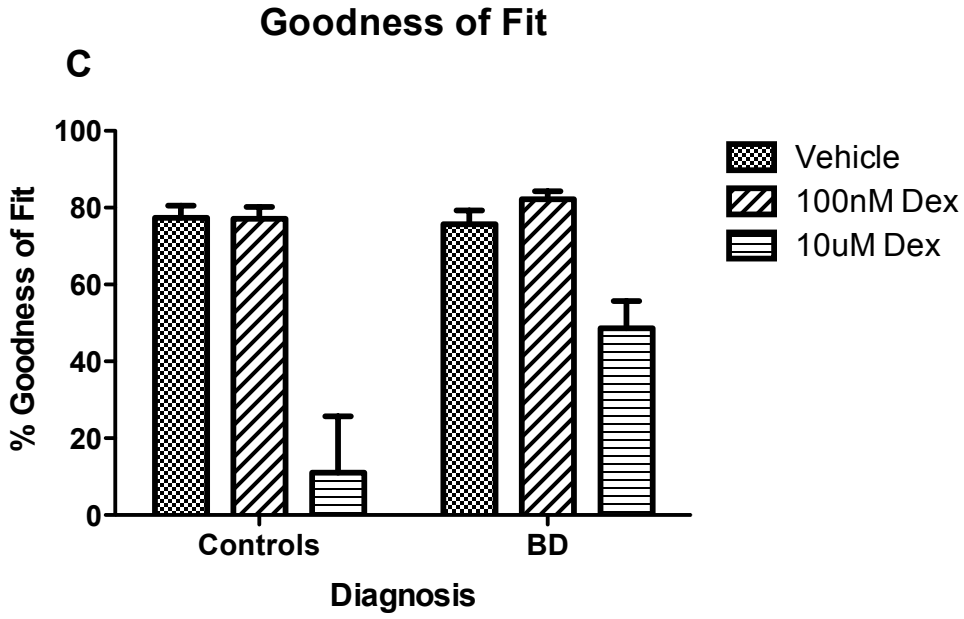
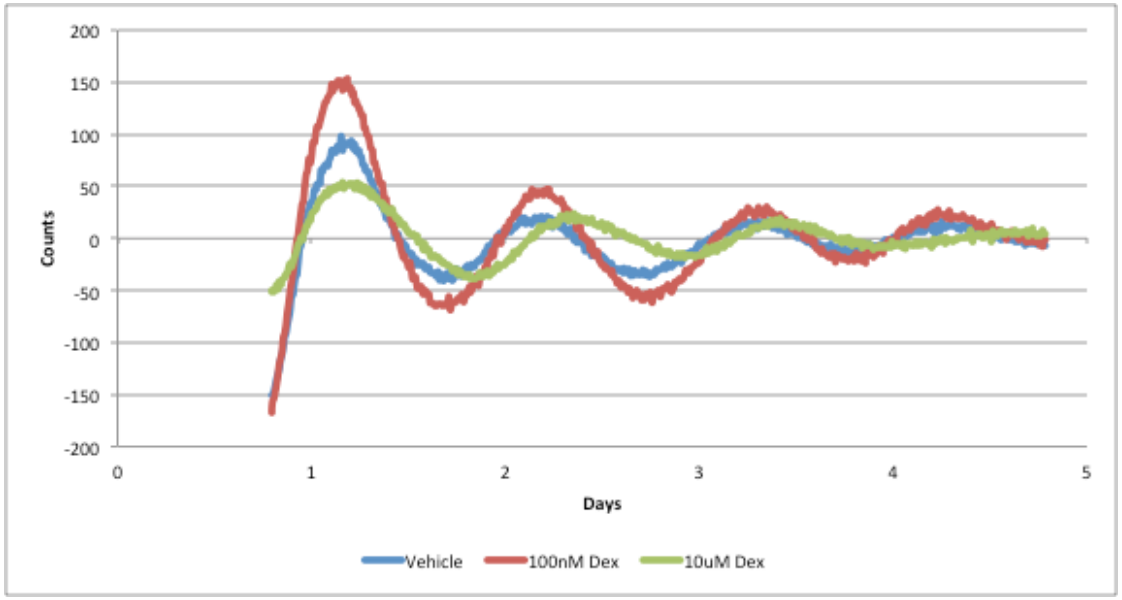


Figure 12. **Rhythm Characteristics with Dex (continued).**

A) Goodness-of-fit.
Diagnosis: ($F_{1,14}$ = 6.57, P < 0.023)
Drug: ($F_{2,14}$ = 34.48, P < 0.0001)
Interaction: ($F_{2,14}$ = 4.89, P < 0.015)
At baseline (control = 77.36% vs. BD= 75.67%) and 100nM Dex (control = 77.13% vs. BD = 82.23%) conditions, cells maintained a high stability rhythm (high goodness-of-fit) whereas at 10 μM Dex concentration (control = 11.02% vs. BD = 48.65%) goodness-of-fit decreased, but this effect was less pronounced for BD cells.

A)



B)

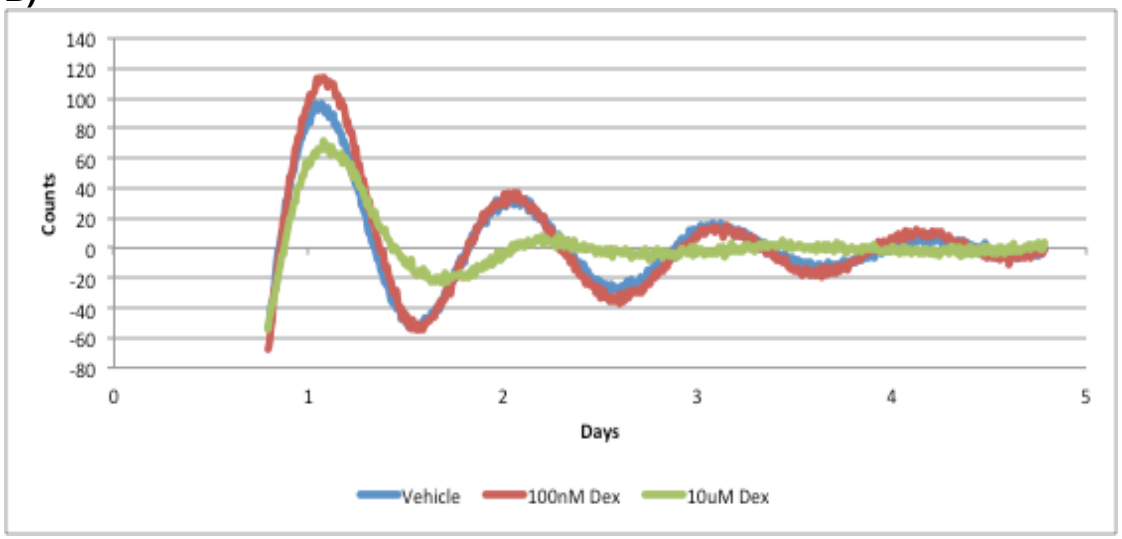


Figure 13. Average traces with Dex. Per2-luc bioluminescence rhythm traces from BD and control cells in the presence or absence of Dex.
A) Healthy controls (n=7)
B) BD (n=9)
Traces are average for all samples within a given diagnosis.

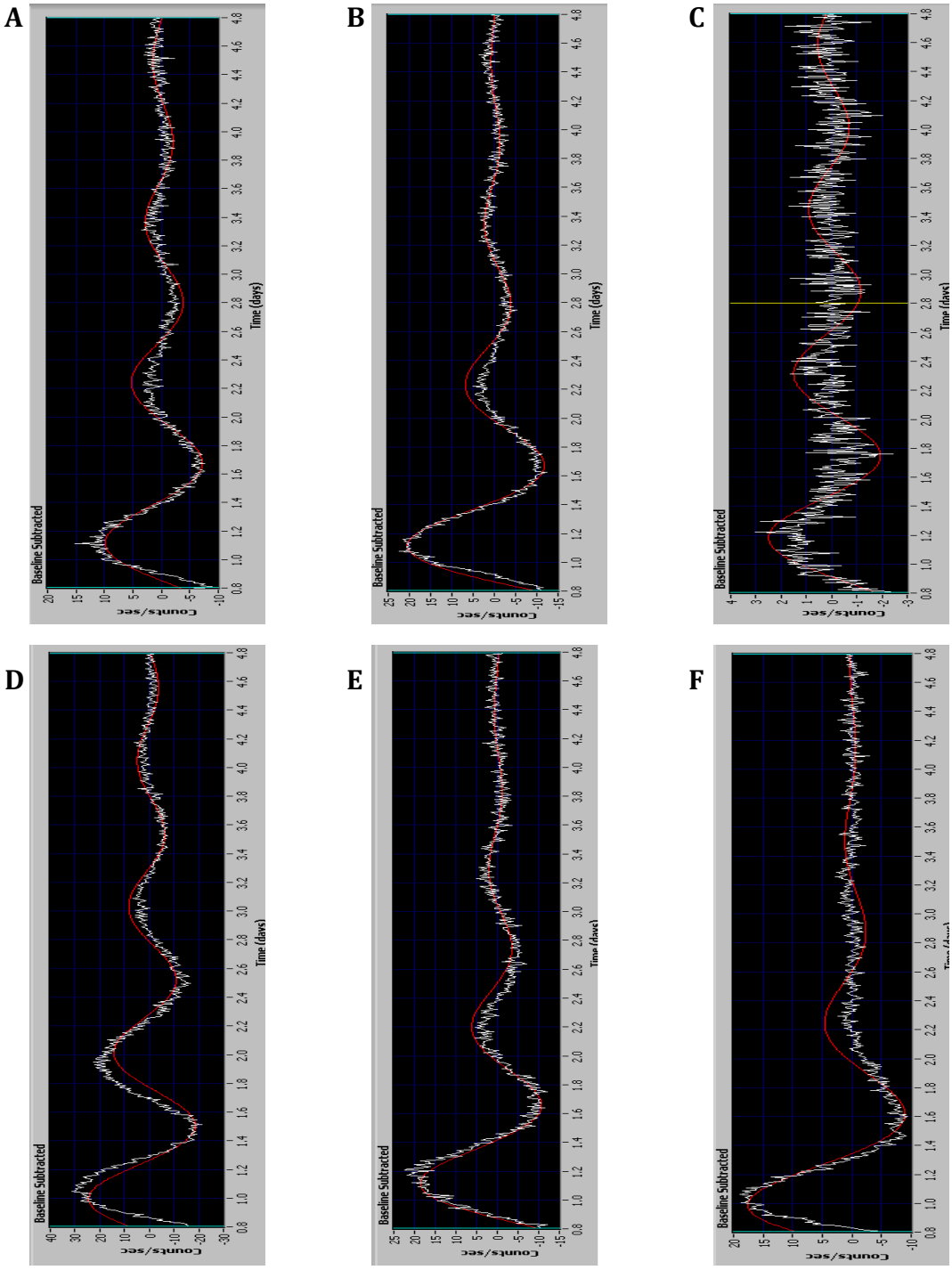


Figure 14. Lumicycle goodness-of-fit. Representative traces illustrating goodness-of-fit for healthy control cells (**A,B,C**) vs. BD cells (**D,E,F**). (**A,D** vehicle only **B,E** 100 nM Dex, **C,F** 10 μM) Cells from patients with BD maintained a higher goodness of fit compared to cells from healthy controls. Red line shows best-fit sine curve.

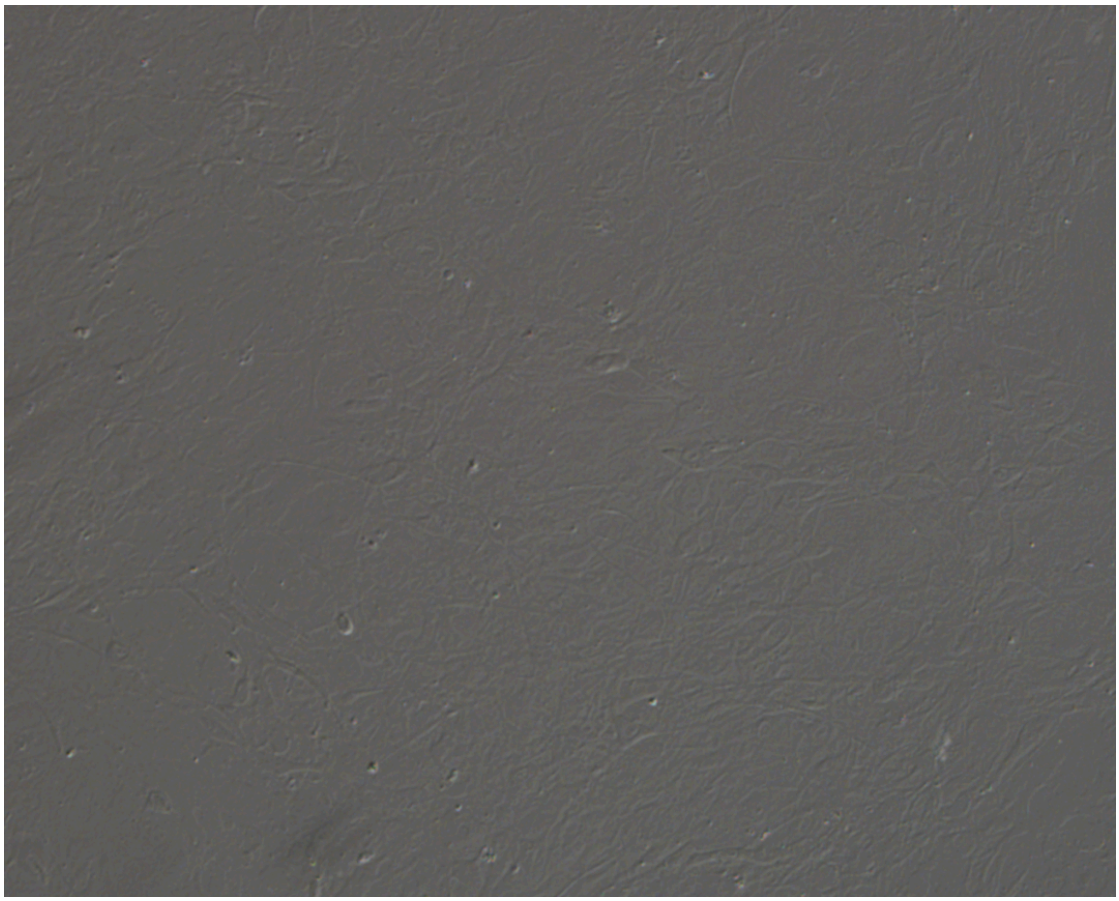


Figure 15. 30 Day Dex exposure. Fibroblast cells from a healthy control subject cultured in 10 μM Dex for 30 days. Changes in cell morphology are expected with extended exposure to GC.

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