Effects of Corticosterone and Stress on *Cry1* Expression in the Prefrontal Cortex of Male Rats, and Sex Differences in Stress-induced *Cry1* Expression in the Hypothalamic Suprachiasmatic and Paraventricular Nuclei of Male and Female Rats

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May 2016

Abstract

Promising evidence suggests that impaired circadian rhythms can lead to various mood disorders. Circadian rhythms are regulated by a master clock, the hypothalamic superchiasmatic nucleus (SCN), and its downstream circadian oscillators. Core clock genes compose the circadian clock through a system of feedback loops. A variety of inputs can modulate clock gene expression, such as glucocorticoid hormones, feeding cycles, temperature, and stress. In this study, I investigated the effects of acute stress on the expression of the core clock gene, *Cry1*, in the SCN, hypothalamic paraventricular nucleus (PVN), and prefrontal cortex (PFC) of rats maintained on a 12h/12h light-dark cycle. Stress has been shown to induce clock gene expression in various cell lines and tissues. In broad strokes the mechanism of stress induced clock gene expression is unknown. I hypothesized that glucocorticoid response elements (GRE's) may play a role in stress induced clock gene expression. When bound by the glucocorticoid hormone-glucocorticoid receptor dimer, GRE's promote transcription of genes. It has been shown that certain clock genes have GRE's in their promoter regions. Based on this, I presumed that acute stress could increase clock gene expression in brain regions containing a high density of GR's, if a functional GRE is present in the promoter region of the clock gene. Furthermore, I examined if there was a time of day effect of *Cry1* mRNA in all three regions of interest, since clock gene expression is variable throughout a 24-hour period. I also examined whether there was a sex effect in the SCN and PVN, since there are gender differences in the magnitude of the CORT response to acute stress.

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Introduction

Most organisms experience rhythms in their physiological systems on a daily basis. These fluctuations in biological activity are known as circadian rhythms. Circadian rhythms are the naturally occurring oscillations of physiological activity in a period of about 24 hours. These rhythms are regulated by a master clock, which in mammals is known as the suprachiasmatic nucleus (SCN) of the anterior hypothalamus.

The integrity of the SCN is necessary for synchronization of internal and external environments, as well as for synchronization of peripheral oscillators. Bilateral lesions of the SCN ablated circadian rhythms of drinking and locomotor activity in Sprague-Dawley rats (Stephan and Zucker, 1972). Lesions of the SCN also resulted in arrhythmicity of endocrine and body temperature rhythms, suggesting that this brain area is crucial for several circadian driven processes (Stephan and Zucker, 1972). While it is widely accepted that the SCN is in charge of coordinating circadian rhythms within the brain, the mechanisms of coordination are not well understood.

Circadian rhythms are the internal control system that allows an organism to function effectively in a 24-hour external environment. Rhythms in sleep/wake cycles, body temperature, blood pressure, and food intake rely on both the master clock and its downstream targets. Coordinated physiological rhythms depend on molecular oscillators known as clock genes. Clock genes are expressed in nearly all cells of the body and are expressed in an oscillatory fashion (Birky and Bray, 2014).

Rhythmic expression of core clock genes composes the circadian clock through a system of feedback loops. Circadian Locomotor Output Cycles Kaput Protein (CLOCK) and Brain and Muscle ARNT-like Protein 1 (BMAL) act as transcription factors that

heterodimerize and bind to the promoter regions of various genes through E-box sequences. Two important genes are *Period (Per)* and *Cryptochrome (Cry)*. Binding of the CLOCK-BMAL1 heterodimer to the promoter region of the *Per* and *Cry* genes activates their transcription. PER and CRY proteins are translated in the cytoplasm, and act through a negative feedback loop to inhibit the transcription of CLOCK and BMAL1 after translocation to the nucleus (McClung, 2007).

Core clock genes interact through a series of positive and negative feedback loops to create oscillations of gene expression. As mentioned earlier, the core clock genes include *Bmal1* and *Clock* of the positive regulatory arm, and the *Period* (*Per1, Per2,* and *Per3*) and *Cryptochrome* genes (*Cry1* and *Cry2*) of the negative regulatory arm (Reppert and Weaver, 2002). The expression of these core clock genes across a 24-hour period has been well characterized. *Bmal1* and *Per1/2* are in antiphase with each other, meaning highest and lowest expression of the two genes occurs in direct opposition. In the SCN, *Per1* and *Per2* have highest mRNA expression (peak) during the light phase, and have lowest expression (nadir) during the dark phase. In contrast, *Bmal1* mRNA peaks during the dark phase, and troughs during the light phase. In the PVN, *Per1/2* and *Bmal1* mRNA levels are in direct opposition with their expression in the SCN (Girotti et al., 2009). While the diurnal pattern of *Bmal1* and *Period* genes has been more extensively characterized, less is known about the rhythmicity of *Clock* and *Cryptochrome* genes. One study suggests that *Cry1* mRNA levels peak during the light phase around ZT8, and trough in the dark phase around ZT20 in the SCN (Miyamoto and Sancar, 1998). Zeitgeber time (ZT) refers to experimental time in chronobiology. ZT0 corresponds to the start of lights on, and ZT12 corresponds to the beginning of the dark period. There is little

evidence that *Clock* expression is rhythmic, suggesting *Clock* may be constitutively expressed.

Normal clock gene expression contributes to the regulation of an organism's circadian rhythm. For example, mice lacking functional *Bmal1* are not able to entrain to a light cycle (Bunger et al., 2000). Additionally, mice with a mutation in *Clock* exhibit reduced depression-like behavior, and reduced anxiety and sleep, which are behaviors common to the manic phase of bipolar disorder. In the same experiment, rescuing *Clock* in the ventral tegmental area of mutants normalizes behavior (Roybal et al., 2007). Further, knockdown of *Per1* and *Per2* in the nucleus accumbens (NAc) results in elevated anxiety-like behaviors in the elevated plus maze and open field-test in mice (Spencer et al., 2013).

In human studies, single nucleotide polymorphisms (SNPs) in clock genes are associated with a variety of mental disorders. A SNP is a change in one nucleotide in a sequence of DNA. One study examined a SNP in *Per1* in relation to stress-induced drinking behavior. Carrying the risk genotype, a SNP in the promoter region of the *Per1* gene, was associated with an increased risk for alcohol dependence in adults (Dong et al., 2011). SNPs in *Per2* and *Bmal1* have been connected with greater risk for developing seasonal affective disorder (SAD) (Partonen, 2007), while SNPs in *Clock* has been associated with increased bipolar episodes and more severe insomnia during treatment (Seretti et al., 2003 and Seretti et al., 2005). Dysfunction in the molecular clock has implications in various mood disorders, including major depressive disorder, bipolar disorder, and SAD (Bunney and Bunney, 2000; Mitterauer, 2000), and multiple sleep disorders including advanced sleep phase syndrome, delayed sleep phase syndrome, non

24-hour sleep-wake syndrome, and irregular sleep-wake pattern (Takahashi et al., 2008). Clearly, normal clock gene expression is essential for coordinated circadian rhythms.

Circadian rhythms occur naturally in the body, but are also affected by external cues such as light. Neurons from the retina project to the SCN through the retinohypothalamic tract, so that light entrains the SCN. This mechanism allows the SCN to synchronize oscillations to the solar day (Reppert and Weaver, 2002). Circadian clocks exist abundantly in downstream targets of the SCN, but are not directly regulated by light input. The SCN has limited projections to the central nervous system and peripheral tissues. The cellular clocks within organs that depend upon the SCN for rhythmic regulation are known as "slave" oscillators, and cannot maintain consistent 24-hour cycles without contribution from the SCN (Reppert and Weaver, 2002). The SCN controls the oscillation of downstream slave oscillators, which effectively controls cycles of physiology and behavior in many organisms (Reppert and Weaver, 2002). A variety of other inputs can modulate clock gene expression in extra-SCN regions, such as glucocorticoid hormones, feeding cycles, and body temperature (Saini et al., 2012).

Glucocorticoids are stress hormones that are normally secreted in a diurnal pattern. CORT, corticosterone in rodents and cortisol in humans, is the principle stress hormone that is produced through the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis controls levels of glucocorticoid hormones through a negative feedback system. When an organism is subjected to stress, activation of the parvocellular neurons of the paraventricular nucleus (PVN) of the hypothalamus causes release of corticotropin-releasing hormone (CRH). CRH is carried through the portal system to the anterior pituitary. CRH binds to CRH1 receptors in the anterior pituitary, which causes

corticotroph cells to release adrenocorticotropic hormone (ACTH) into the blood stream. ACTH acts on the adrenal cortex to stimulate release of CORT. CORT acts in a negative feedback fashion by inhibiting the hypothalamic PVN and pituitary gland. Without input from the pituitary gland, adrenal gland production of CORT is suppressed (Spiga et al., 2014).

Glucocorticoid receptors (GRs) are found throughout brain and peripheral tissues. Interestingly, GR's are absent in the SCN. Certain evidence suggests that glucocorticoid hormones could be an important entraining factor for extra-SCN brain regions, and may provide a mechanism for communication between the SCN and other brain regions. First, GR can translocate to the nucleus and bind DNA directly, making it a candidate for regulating clock gene expression (Rosenfeld et al., 1998). Treatment with a synthetic glucocorticoid, dexamethasone, quickly stimulates *Per1* mRNA expression in rat-1 fibroblasts, suggesting that glucocorticoids rapidly affect *Per1* gene expression (Balsolobre, 2000). Second, GR is expressed widely across peripheral cells, while remaining absent in the SCN. While the SCN remains unaltered by glucocorticoids, slave oscillators are receptive to phase resetting (Balsalobre, 2000). Thus, glucocorticoids could affect extra-SCN clocks, without affecting the SCN. Lastly, glucocorticoids are secreted in daily rhythms, suggesting they may play a role in synchronizing peripheral tissue downstream of the SCN (Balsalobre, 2000).

Steroid hormones, such as CORT, exert their effects on the body through binding of receptors. CORT binds to glucocorticoid receptors that are located in the cytoplasm. In the absence of CORT, the inactivated GR is bound to an oligomeric protein complex that includes two heat shock proteins (HSP90 and HSP70), as well as the FK506 binding

protein 51 (FKBP5). When CORT is present, it binds to the glucocorticoid receptor, causing dissociation of the protein complex (Guidotti et al., 2013). This allows the receptor to dimerize to another CORT-bound receptor. Once in a dimerized state, the hormone bound receptor translocates to the nucleus, where it can bind to a glucocorticoid response element (GRE) to activate transcription (Cox et al., 2012). GRE's are contained within promoters and enhancers of genes, and recruit additional transcription factors to alter transcriptional activity (Tronche et al., 1998). Interestingly, it has been shown that CORT and stress increase *Per1* expression in mice through a GRE in the *Per1* promoter (Yamamoto et al., 2005). Additionally, adults with a SNP in *Per1* exhibit altered *Per1* expression in response to stress-induced CORT. This further suggests that *Per1* is a target for CORT, through GR binding of a GRE in the *Per1* promoter (Dong et al., 2011). The presence of a GRE has also been discovered in the promoter of the *Per2* gene, and determined to be necessary for regulation of this gene by glucocorticoids (So et al., 2009); however, a conflicting study found that a functional GRE only exists in the *Per1* gene (Yamamoto et al., 2005). All of this evidence suggests that CORT can act as a regulator of certain clock genes through GR binding of GRE's in the promoter regions of these specific genes. Glucocorticoids may still indirectly affect clock genes that lack a GRE, through action of the "primary target gene products," or the genes that were primary targets of glucocorticoid action (So et al., 2009).

Stress has been show to modulate clock gene expression and may impair circadian rhythms through release of glucocorticoid hormones. CORT has a diurnal rhythm in which the peak occurs at the beginning of the active phase of an organism, and troughs during the inactive phase. In humans, the active phase is the onset of light, while the

active phase of rodents occurs at the onset of darkness (Chung et al., 2011). Interestingly, various mental disorders show unusual diurnal patterns of CORT, and interrupted sleep/wake patterns. Several studies have found that depressed patients have a hyperactive HPA axis, hypercortisolemia, and sleep disturbances (Arborelius et al., 1999; Buckley and Schatzberg, 2005). Furthermore, disruptions in diurnal rhythms of glucocorticoid secretion are common in many human mood disorders and diseases including metabolic syndrome, Cushing's syndrome, and chronic fatigue syndrome (Chung et al., 2011).

If CORT acts as an entrainment factor, then stress-induced release of CORT may alter rhythmicity of the molecular clock, due to increases outside normal peak release times, ZT0 for diurnal animals and ZT12 for nocturnal animals. Several studies have demonstrated that stress can alter the phase and amplitude of circadian rhythms, and affect clock gene expression (Segall and Amir, 2010). Balsalobre et al. (2000) found that glucocorticoid injection can alter the phase of the circadian clock in extra-SCN tissue. Mice injected with dexamethasone before or during normal peak expression of *Per1* mRNA, exhibited prolonged expression of *Per1* in the liver. Injections performed after peak expression of *Per1* mRNA stimulated an additional burst of *Per1* expression. Additionally, Takahashi et al. (2001) found that stress-induced CORT may be linked to stress-induced *Per1* expression in the PVN. Forced swimming and immobilization stressors both induced *Per1* expression in the PVN during the day, when levels are typically lowest. These stressors did not affect *Per1* expression in the SCN, presumably because of the absence of GR's. Further, Segall et al. (2006) performed an interesting experiment to observe CORT's role in control of PER2 rhythms in the bed nucleus of the

stria terminals and central nucleus of the amygdala, by removing the adrenal glands of rats. CORT replacement was administered through drinking water, which mirrors endogenous rhythms of CORT due to rat's preference for hydrating at the onset of their active phase, or through time-release pellets, which provide consistent levels of CORT. Adrenalectomy eliminated PER2 rhythmicity in both brain areas. Rhythms of PER2 were rescued when rats received CORT in their drinking water, but not when CORT was replaced through time-release pellets. This interesting result suggests the endogenous pattern of CORT secretion, and not just the presence of CORT, is necessary for rhythmic expression of PER2.

Due to their roles as master clock and head of the HPA axis, the SCN and PVN respectively, have been emphasized as regions of interest in studies involving the molecular clock. Additionally, support exists for the importance of glucocorticoid signaling in regulating circadian function in other brain regions, namely, the prefrontal cortex (PFC). The prefrontal cortex is the most developed area of the mammalian brain, and is associated with many higher order functions including cognitive behavior, processing emotional responses, and modulation of stress responses. Many of these PFC-regulated responses display rhythmic diurnal patterns (Perez-Cruz et al., 2009). Chun et al. (2015) found rhythmic expression of *Bmal1*, *Per1*, and *Per2* mRNA in various regions of the PFC. Unpublished data from our lab reveals stress-induced *Per1* expression in the PFC of both males and females. Further, specific subregions of the medial prefrontal cortex (mPFC) have been shown to modulate the HPA axis and stress-induced CORT activity (Herman et al., 2003). Sylvester et al. (2002) demonstrated an indirect connection from the SCN to the infralimbic cortex (ILC) of the mPFC through a relay involving the

paraventricular nucleus of the thalamus. Thus, the PFC is an essential region of interest in this study because of its important anatomical connections, role in stress modulation, and patterns of rhythmic clock gene expression.

In the past, the majority of studies in circadian research have utilized male subjects, while limited research has been performed on female subjects. Furthermore, few studies have compared differences in clock gene expression between the sexes. This assessment is necessary, because there are significant sex differences in the circadian systems of males and females (Baily and Silver, 2014). Also, the prevalence of certain diseases involving circadian dysfunction may be related to sex differences. Major depressive disorder, seasonal affective disorder, anxiety, and PTSD are more frequent in females than males, which emphasizes the importance of studying both sexes. To begin, sex differences exist within the master clock (Bailey and Silver, 2014). Robinson et al. (1986) found that the volume of the SCN was larger in males than females. Further, vasoactive intestinal polypeptide (VIP), which is synthesized by the SCN and transmits a circadian signal to regulate the surge of luteinizing hormone, has been found to have rhythmic mRNA expression in female rodents that is 12 hours out of phase compared with males. This may contribute to sexually differentiated physiology (Krajnak et al., 1998). Next, sex differences exist in circadian control of circulating hormone levels. Under basal conditions, the circadian peak of CORT and overall average levels are higher in female rats compared with males (Bailey and Silver, 2014). Further, there are sex differences in stress-induced CRH mRNA in the PVN and ACTH in the blood, with levels of both increasing more rapidly in females (Iwasaki-Sekino et al., 2009). Since few groups have specifically examined sex differences in clock gene expression in the brain,

one aim of this study sought to understand if differences do exist between male and female rats.

This study examined *Cry1* mRNA expression in the SCN, PVN, and various sub-regions of the PFC. *Cry1* is a component of the negative arm of the molecular clock. When *Cry1* mRNA is transcribed into protein, it interacts with PERIOD proteins. The PER-CRY1 protein complex acts as a negative regulator of clock gene expression, through transcriptional inhibition of CLOCK and BMAL1 (Reppert and Weaver, 2002). Whereas the activity of other core clock genes has been characterized extensively, less information has been published about *Cry1*, which contributes to its emphasis in this study.

Relatively new studies have highlighted *Cry1* as a "chemical energy sensor" that transmits nutritional information, such as glucose availability, to extra-SCN clocks (Lamia et al., 2009). Metabolic signals affect *Cry1* expression in peripheral tissue, which suggests that *Cry1* may contribute to metabolic entrainment of extra-SCN clocks. Specifically, Lamia et al. (2009) found that the adenosine monophosphate (AMP)-activated protein kinase (AMPK), which regulates metabolic signals, phosphorylates and degrades CRY1 protein in mouse liver. Since metabolic signals are highly circadian controlled, Lamia et al. (2011) further investigated cryptochrome regulation of glucocorticoid receptors, because nuclear hormone receptors are widely accepted as regulators of metabolism. They found that *Cry1* does interact with GR, by repressing the ability of GR to activate a GRE containing gene. Further, a dexamethasone test revealed that treatment with a synthetic glucocorticoid activated over 200 more genes in cells lacking *Cry1/2* than in controls, which suggests that *Cry1/2* are important for inhibition

of some genes that rely on nuclear localization of GR for expression (Lamia et al., 2011). In addition, this study revealed that CRY1 interacts with a GRE in the promoter of phosphoenolpyruvate carboxykinase 1 (Pck1), an enzyme important for control of the metabolic pathway that generates glucose (Lamia et al., 2011). This study provides a series of experiments that demonstrate that CRY1 interacts with GR to repress GR-dependent transcription. Nonetheless, there is a lack of information on how stress may affect *Cry1* expression in extra-SCN tissue through glucocorticoid signaling.

The diurnal circadian pattern of *Cry1* expression is not as concrete as other clock genes such as *Per1*, *Per2*, and *Bmal1*. While Miyamoto and Sancar (1998) demonstrated that peak *Cry1* mRNA levels occur in the light phase in the SCN, Lamia et al. (2009) proposed a model that suggests *Cry1* mRNA levels are highest during the dark phase in peripheral clocks, due to reduced AMPK activity. The antiphasic relationship of peak clock gene expression between the SCN and extra-SCN clocks is also present in *Per1*, *Per2*, and *Bmal1* (Girotti et al., 2009). Increased CRY1 protein should hypothetically result in lower expression of genes of the positive arm, *Clock* and *Bmal1*. Accordingly, *Bmal1* displays lowest expression during the dark phase outside of the SCN, which supports the hypothesis that peak expression of *Cry1* occurs at night in peripheral clocks (Girotti et al., 2009).

Experimental Design

I chose to investigate the clock gene *Cry1* in three brain areas, utilizing two different experiments. The first experiment was a 2x2x2 factorial design that examined the effect of stress-induced CORT on *Cry1* expression in the PFC. To determine the

potential effects of CORT, half of the rats had their adrenal glands removed. Adrenalectomy surgery abolishes endogenous circulating CORT. Additionally, half of the rats were subjected to restraint stress. The last independent variable was time of day, either ZT4 or ZT16. I hypothesized that *Cry1* expression within the PFC would increase when a rat was subjected to acute stress if the rat's adrenal glands were intact. While there is not evidence that confirms the existence or absence of a GRE in the promoter of *Cry1*, I proposed that *Cry1* might be stress reactive because of its shared role with *Per1* in the negative regulatory arm of the molecular clock. Also, there is evidence that glucocorticoids can rapidly induce *Cry1* in a cell line (Lamia et al., 2011). If the *Cry1* gene contains a GRE, the mechanism of induction would involve an increase in endogenous CORT after acute stress, which would bind to GR, and trigger its nuclear localization and binding to the GRE of *Cry1*. This would ultimately increase expression of *Cry1* in the PFC. If the promoter region of *Cry1* does not contain a GRE, expression would remain unaffected. Additionally, I hypothesized that *Cry1* expression would be higher at ZT16, during the rats active phase. This premise is based on previous research that suggests *Cry1* is highest during the dark phase in extra-SCN clocks (Lamia et al., 2009).

The second experiment was a 2x2x2 factorial design that investigated sex differences in *Cry1* expression in the SCN and PVN of rats after acute stress. Time of day, ZT4 vs. ZT16, was also an important variable. For this experiment, my hypothesis was that acute stress would induce *Cry1* expression in the PVN, but not the SCN. GR's are relatively absent in the SCN; hence stress-induced CORT should not have an effect on the SCN. This is also based on the presumption that *Cry1* has a promoter containing a

GRE. If a GRE does not exist, there should not be differences in expression between the stress groups. However, this does not rule out sex or time of day differences in *Cry1* expression. In rodents, there are sex differences in the magnitude of CORT response to acute stress, where females show elevated CORT levels compared to males (Bale and Epperson, 2015). This indicates that females exhibit a greater physiological response to short term stress. Therefore, I hypothesized that females would have higher *Cry1* expression after acute stress, presumably through the actions of elevated CORT. This should only occur in the PVN, since CORT cannot exert its effects in the SCN. As in the first experiment, I hypothesized that *Cry1* expression would be higher at ZT16 in peripheral clocks. Due to the common antiphasic relationship between the SCN and extra-SCN clocks, I hypothesized that *Cry1* expression would be higher at ZT4 in the SCN.

My overall objective was to investigate the expression of a core clock gene, *Cry1*, as it is a key component of the molecular clock. Utilizing brain tissue from two experiments allowed me to test how *Cry1* expression is modulated by stress, sex, and time of day, and how the presence of endogenous CORT may alter its expression. The PFC was a region of interest because of its role in stress modulation. The SCN and PVN were included in this investigation due to strong support that they exhibit diurnal expression of clock genes, and their respective roles as master clock and head of the HPA axis. While this research observes molecular changes as a result of psychological stress using an in vivo model, these findings may provide a better understanding of mental health on a larger scale.

Materials and Methods

Study Site and Contribution

All research was conducted in the Spencer Neuroendocrinology Lab at the University of Colorado in Boulder, CO. It should be noted that the experimental procedures and tissue collection were performed prior to my admittance into the lab. My role was to run the in situ hybridization, densitometry, and analyses for *Cry1* mRNA on the brain tissue.

Subjects

Subjects were three-month old Sprague-Dawley rats acquired from Harlan Laboratories (Indianapolis, IN). Male rats weighed 300-350 g across both experiments, and female rats weighed 200-250 g. The rats were age matched in both experiments. Rats were pair housed and evenly distributed among four different rooms that were temperature and humidity controlled and allowed two weeks to acclimate to the novel environment and their new light:dark cycle. Full access to water and food was available ad libitum. Rats were maintained on a 12:12 hour light:dark cycle in both experiments; however, the time of lights-on varied across experiments (06:00-19:00, experiment one; 05:00-22:45, experiment two). The Institutional Animal Care and Use Committee at the University of Colorado at Boulder approved the ethical use of animals in this experiment. Procedures for the ethical treatment of animals were performed following standards from the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (DHHS Publication No. [NIH] 80-23, revised 2010 eighth edition).

Experiment 1: Effects of Adrenalectomy and Stress on Cry1 mRNA expression in the PFC of Male Rats

Male rats were maintained on a 12:12 h light:dark cycle with half of the rats experiencing lights on at 06:00 h or 08:00 h, and the other half of rats on a reverse cycle with lights on at 17:00 h or 19:00 h. After sufficient time for re-entrainment, reverse light:dark cycle has been shown to have no adverse effect on circadian function, and allows for rats to all be sacrificed at the same clock time. After a two-week acclimation period, adrenalectomy surgery (ADX) was performed on half of the rats. The other half of the rats underwent a control surgery (SHAM), in which all surgical conditions were the same, except adrenal glands remained intact. This manipulation was performed to observe the role of endogenous CORT in stress-induced clock gene expression. After one week of recovery from surgery, rats were challenged with thirty minutes of restraint stress. The restraint stress procedure involves placing rats in Plexiglas cylindrical restraint tubes (20.7 cm X 7.0cm X 8.0 cm) to restrict movement, which stimulates acute psychological stress. Half of rats experienced restraint stress for 30 min before being sacrificed, while the group that was not stressed was sacrificed immediately after being removed from their home cages. Rats were sacrificed at either ZT4 or ZT16.

Experiment 2: Stress-induced Cry1 mRNA expression in the SCN and PVN of Male and Female Rats

Male and female rats were maintained on a 12:12 h light: dark cycle with half of rats experiencing lights on at 0500 h or 0545 h, and the other half of rats on a reverse cycle with lights on at 2200 h or 2245 h. Half of the rats experienced restraint stress in

Plexiglas cylindrical restraint tubes (20.7 cm X 7.0cm X 8.0 cm for males, 18.2 cm X 5.9cm X 7.5cm for females) for 30 min before decapitation. The group that was not stressed was sacrificed immediately after removal from their home cages. Rats were sacrificed at either ZT4 or ZT16. Post-mortem vaginal smears were performed to assess estrous cycle stage in female rats at time of death.

Tissue Collection

For both experiments, rats in the stressed group were taken from restraint tubes and immediately sacrificed through rapid decapitation using a guillotine at either ZT4 or ZT16. Control rats were removed directly from their home cages and sacrificed through rapid decapitation. Brains were extracted and flash frozen in isopentane maintained at a temperature between -20°C and -30° C using dry ice. Once frozen, brains were wrapped in aluminum foil and stored in a -70° C freezer.

Processing brains

Using a cryostat (Leica CM 1850), coronal brain slices 12μm thick were cut at the level of the mPFC (2.2 to 3.2 mm anterior to Bregma), SCN (1.3 to 1.4 mm posterior to Bregma), and PVN (1.8 to 1.88 mm posterior to Bregma) using the Paxinos and Watson Rat Brain Atlas (fourth edition, 1998). Brain slices were thaw mounted on Colorfrost Plus microscope slides and stored at -70° C until use with in situ hybridization.

In Situ Hybridization

Day 1

In situ hybridization was performed to analyze *Cry1* mRNA in the SCN, PVN, and PFC. Protocols previously described in Ginsberg et al. (2003) and Girotti et al. (2009) were followed for this two-day gene expression assay, with slight modifications. A ^{35}S radiolabeled *Cry1* riboprobe was constructed in house, complementary to *Cry1* mRNA. The *Cry1* cDNA primer that was used to make the riboprobe was composed of a forward primer (GCAGTGGTGGCGGAAACTGTC) and reverse primer (TTGGGGCCAACACTCTGTGCG) ranging from nucleotides 2113 to 2278. Radiolabeled riboprobes bind to the mRNA of interest to show relative amounts of gene expression. The ^{35}S radiolabeled riboprobe was made through the combination of ^{35}S-UTP, 5x transcription buffer, T7 RNA polymerase, GCA nucleotide components, 0.1M dithiothreitol (DTT), RNASE inhibitor, and linearized Cry1 DNA using a series of 37° C incubations and further separated through a G50/50 sephadex column.

Colorfrost Plus microscope slides mounted with brain slices were removed from storage in -70° C and fixed in 4% paraformaldehyde (PFA) solution for 15 minutes, washed 3X in 2x saline-sodium citrate buffer (SSC), and then placed in a mixed solution of triethanolamine (TEA) and acetic anhydride. Next, slides were washed in distilled water, dehydrated in increasing concentrations of ethanol, and allowed to air dry. The *Cry1* radiolabeled riboprobe was mixed with hybridization buffer, and approximately 1,500,000 counts of radioactivity (65 µl/slide) were pipetted onto each slide before being coverslipped. Slides were placed in plastic trays lined with filter paper, and covered with a 1:1 formamide:distilled water mixture. Trays were wrapped 3X in saran wrap and incubated overnight in a 54° C oven.

Day 2

Plastic trays were removed from the oven and opened. Coverslips were floated off from slides using 2x SSC, and washed twice more in 2x SSC. Once all coverslips were floated off, slides were incubated in 200 RNAseA (cat No. R5503; Sigma, St. Louis, MO) for one hour at 37° C. After incubation, slides were washed in decreasing concentrations of SSC and incubated in 0.1x SSC for one hour at 65° C. Lastly, slides were dehydrated in increasing concentrations of ethanol and allowed to air dry before exposure to x-ray film for 2-4 weeks.

Densitometry

X-ray film was developed using an auto developer (Konica Minolta Medical and Graphic, Inc.) in a dark room, producing autoradiographs. Autoradiographs are the brain images produced by radioactive decay of the ^{35}S isotope. Autoradiographs allow for relatively quantitative analysis of gene expression across brain regions of interest (ROI). Once the film was captured and saved, images were opened in ImageJ64 software (NIH). Using the Paxinos and Watson Rat Brain Atlas (fourth edition, 1998), ROI's were determined based on anatomical landmarks. ROI's were outlined and the mean gray values were converted into uncalibrated optical densities. Densitometry of autoradiographs allows for quantification of mRNA, through calculating the density of pixels. Optical densities were averaged for each animal, and then averaged into treatment groups.

Statistical analysis

Statistical tests were conducted using the Statistical Package for Social Sciences (SPSS, Mac version 21, 2012), by 2-way analysis of variance (ANOVA). Univariate analysis was used to determine if there were significant main effects of stress (no stress vs. stress), ADX (sham vs. ADX), and time of day (ZT4 vs. ZT16) in the PFC, and stress (no stress vs. stress), sex (males vs. females) and time of day (ZT4 vs. ZT16) in the SCN and PVN, or any significant interactions between these variables. Any significant F-values ($p < 0.05$) were further examined using post hoc analysis, specifically Fisher's least significant difference (FLSD).

Results

Experiment 1: Time of day effect in various subregions of the PFC was modulated by adrenal status. Stress had no effect on *Cry1* mRNA in the PFC.

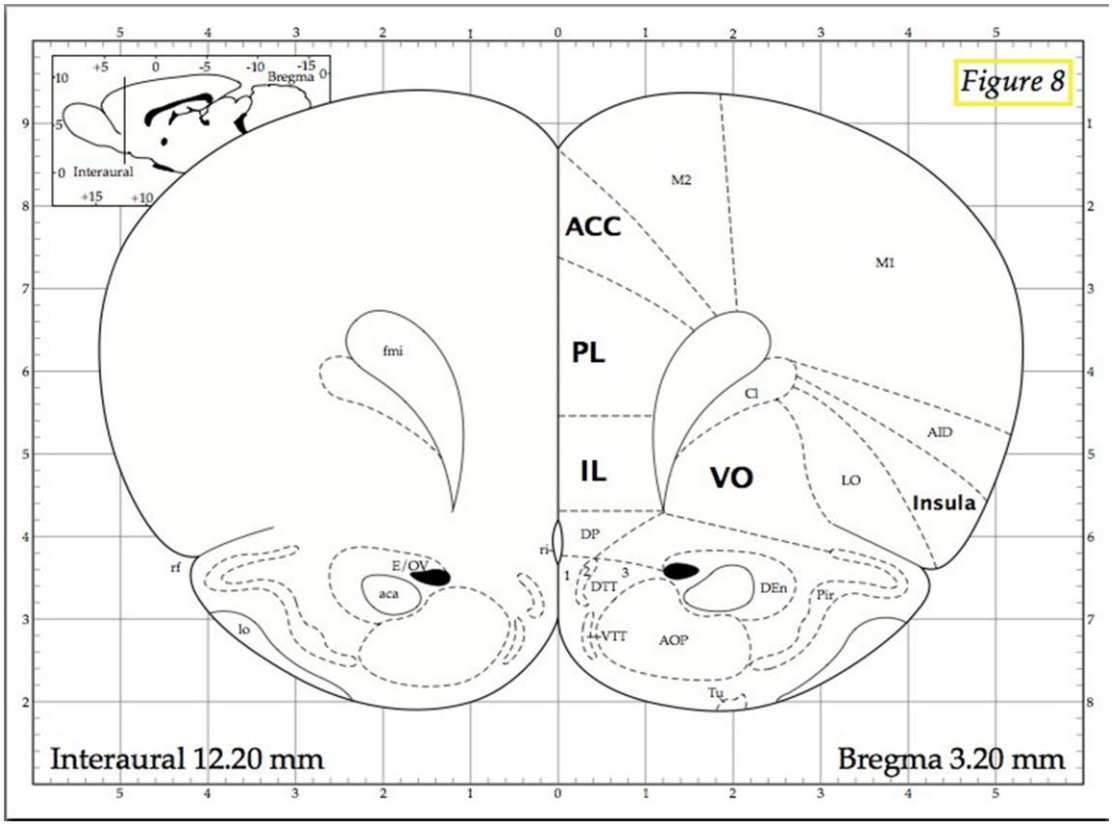

Figure 1. *Paxinos and Franklin* Rat Brain Atlas image highlights of the anterior cingulate cortex (ACC), prelimbic cortex (PL), infralimbic cortex (IL), and ventral orbital (VO) subregions of the PFC, and the rostral agranular insula (insula).

Anterior Cingulate Cortex (ACC)

There was a significant time of day effect for *Cry1* mRNA in the anterior cingulate subregion of the PFC ($F_{(1,42)} = 16.919$; $p<0.001$), and a significant interaction between time of day and adrenal status ($F_{(1,42)} = 8.177$; $p=.007$). FLSD post hoc test

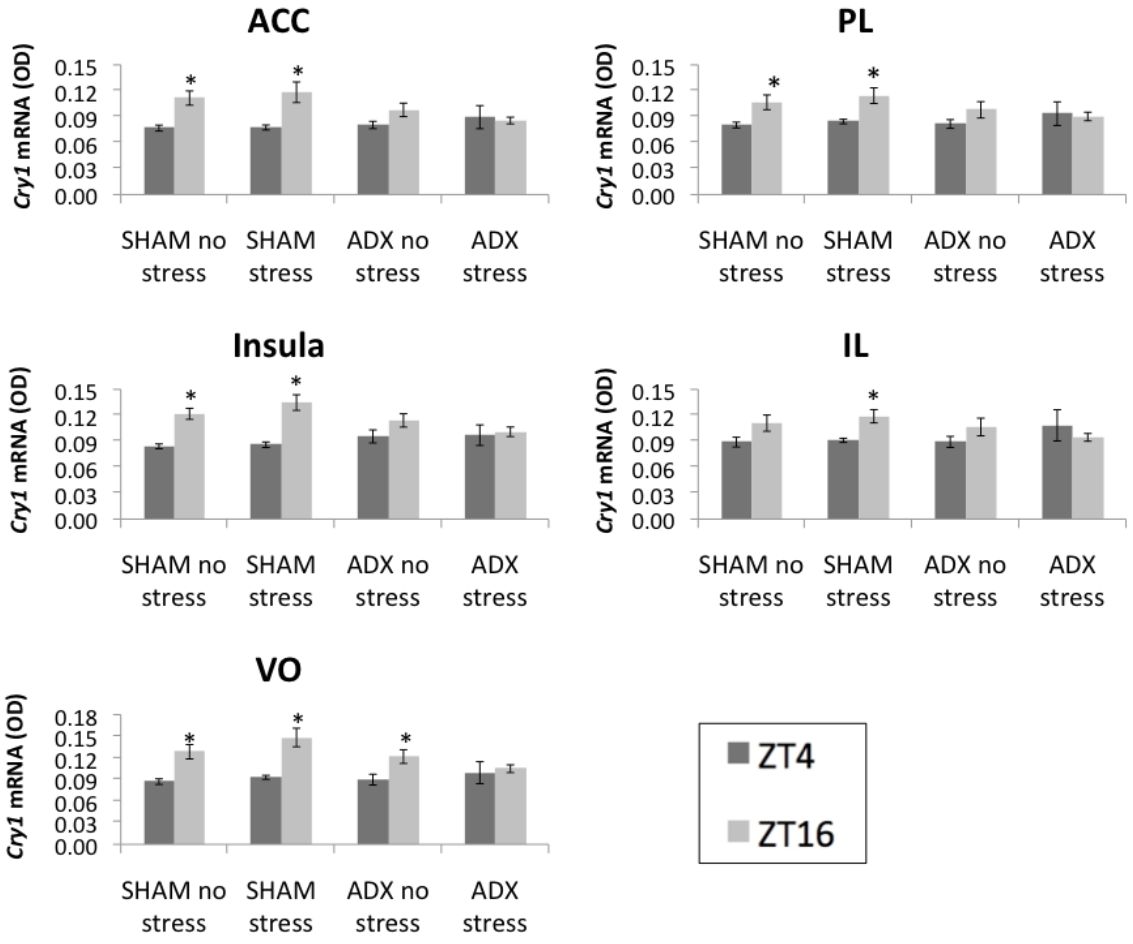

Figure 2. *Cry1* mRNA expression in various subregions of the PFC in male rats represented by time of day. Significances determined through Fisher's LSD post-hoc test. *p<0.05 represents significant statistical difference.

revealed that the main effect was only present in adrenal intact rats (SHAM), but not in adrenalectomized rats (ADX). *Cry1* mRNA was higher at ZT16 than ZT4 in SHAM rats not subjected to stress (p=0.003), and SHAM rats subjected to stress as shown in Figure 2. Stress had no effect on *Cry1* mRNA in the ACC (Figure 3). Although there was not a significant main effect of adrenal status, FLSD post hoc analysis showed that in stressed

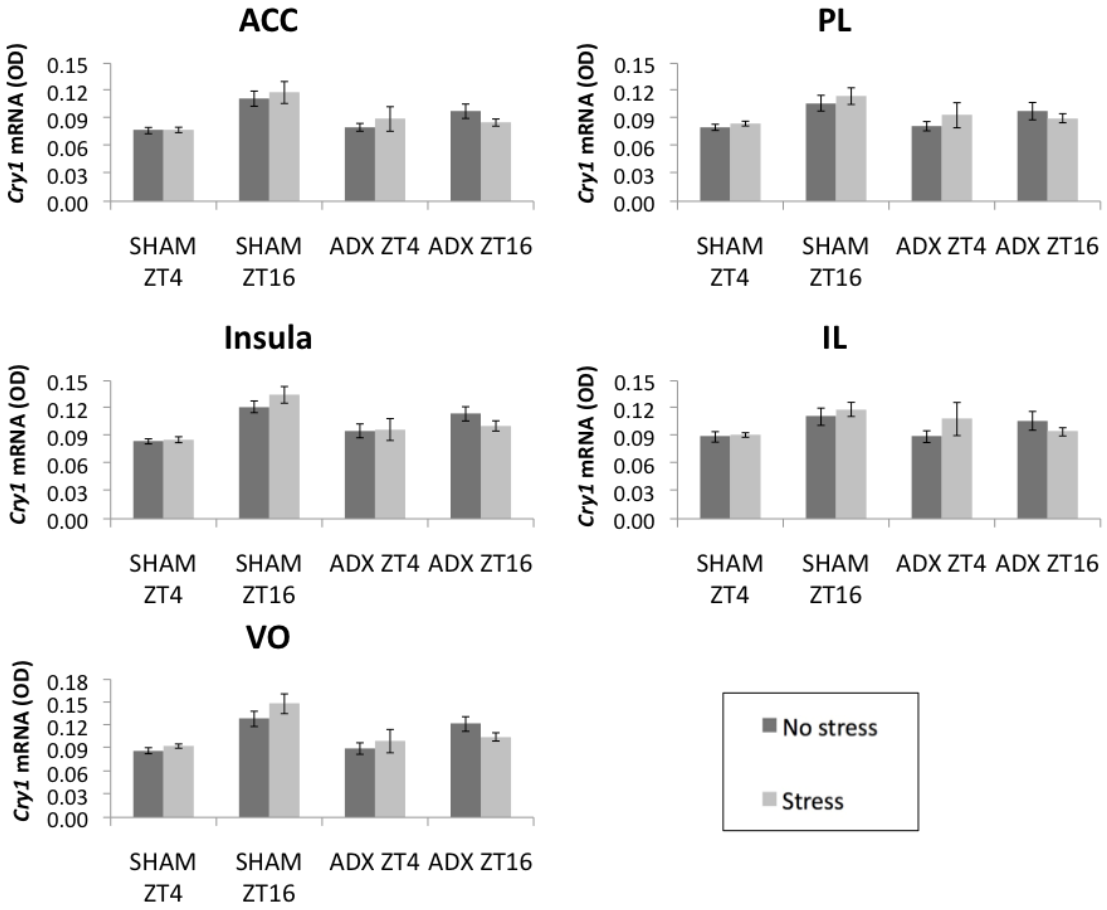

Figure 3. *Cry1* mRNA expression in various subregions of the PFC in male rats represented by stress. Significances determined through Fisher's LSD post-hoc test. *p<0.05 represents significant statistical difference.

conditions at ZT16, SHAM rats had higher *Cry1* mRNA than ADX rats (p=0.005) in the ACC (Figure 4).

Prelimbic Subregion of the PFC

There was a significant time of day effect for *Cry1* mRNA in the prelimbic subregion of the PFC ($F_{(1,42)} = 9.861$; p=0.003), and an almost significant interaction between time of day and adrenal status (p=.058). FLSD post hoc test revealed that the

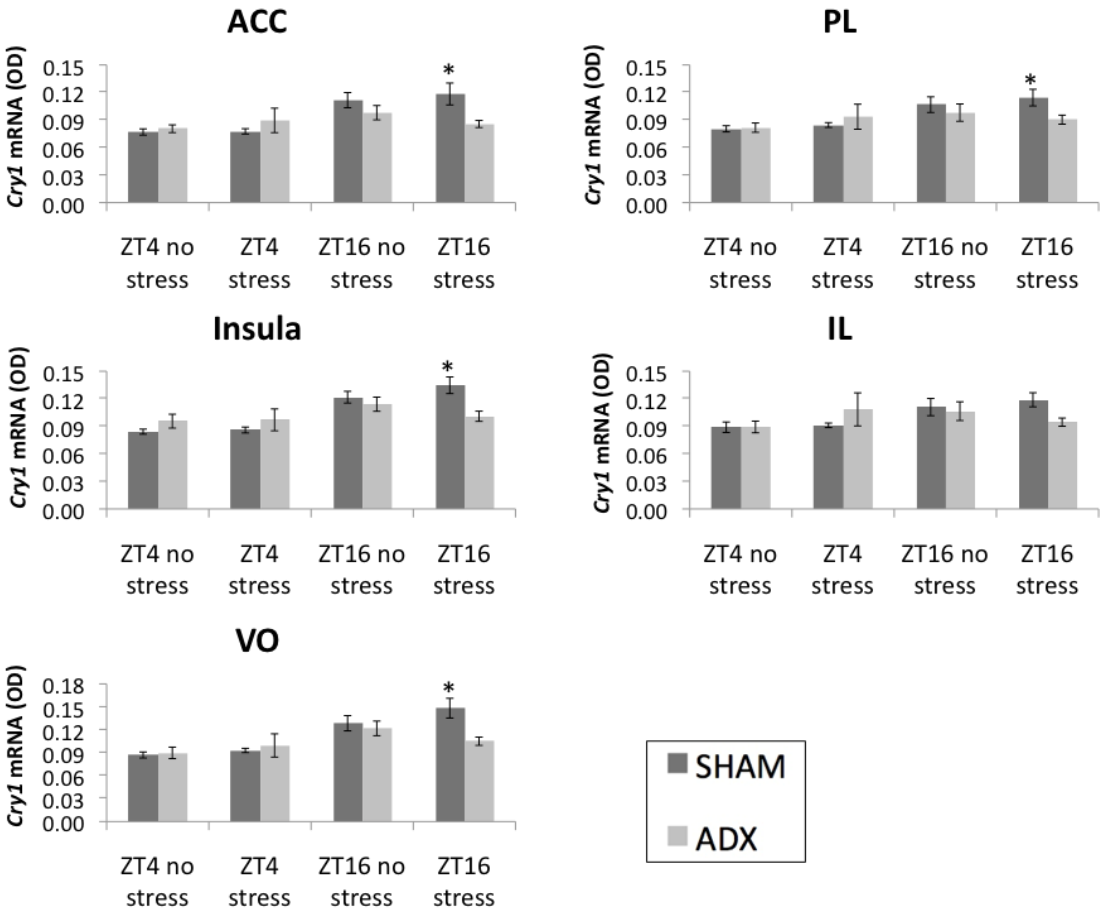

Figure 4. *Cry1* mRNA expression in various subregions of the PFC in male rats represented by adrenal status. Significances determined through Fisher's LSD post-hoc test. *p<0.05 represents significant statistical difference.

main effect was only present in SHAM rats, but not in adrenalectomized rats. Additionally, *Cry1* mRNA was higher at ZT16 than ZT4 in unstressed SHAM rats (p=0.024), and stressed SHAM rats (p=0.011) as shown in Figure 2. Stress had no effect on *Cry1* mRNA in the prelimbic subregion of the PFC (Figure 3). Although there was not a significant main effect of adrenal status, post hoc analysis showed that in stressed conditions at ZT16, SHAM rats had higher *Cry1* mRNA than ADX rats (p=0.038) in the PL (Figure 4).

Rostral Agranular Insula

There was a significant time of day effect for *Cry1* mRNA in the rostral agranular insula ($F_{(1,41)} = 26.806$; $p<0.001$), and a significant interaction between time of day and adrenal status ($F_{(1,41)} = 9.283$; $p=0.004$). Post hoc analysis revealed that the main effect was only present in SHAM rats, but not in adrenalectomized rats. *Cry1* mRNA was higher at ZT16 than ZT4 in unstressed SHAM rats ($p=0.001$), and stressed SHAM rats ($p<0.001$) as shown in Figure 2. Stress had no effect on *Cry1* mRNA in the rostral agranular insula (Figure 3). Although there was not a significant main effect of adrenal status, post hoc analysis showed that in stressed conditions at ZT16, SHAM rats had higher *Cry1* mRNA than ADX rats ($p=0.004$) in the insula (Figure 4).

Infralimbic Subregion of the PFC

There was a significant time of day effect for *Cry1* mRNA in the infralimbic subregion of the PFC ($F_{(1,42)} = 4.246$, $p=0.046$). Post hoc analysis revealed that the main effect was only present in stressed SHAM rats, but not in any other experimental group. *Cry1* mRNA was higher at ZT16 than ZT4 in stressed SHAM rats ($p=0.04$), represented in Figure 2. Stress and adrenal status had no effect on *Cry1* mRNA in the infralimbic subregion of the PFC (Figures 3 and 4).

Ventral Orbital Subregion of the PFC

There was a significant time of day effect for *Cry1* mRNA in the ventral orbital subregion of the PFC ($F_{(1,42)} = 26.983$; $p<0.001$) and a significant interaction between time of day and adrenal status ($F_{(1,42)} =5.268$; $p=.027$). Post hoc analysis revealed that the

main effect was present in all experimental groups besides stressed adrenalectomized rats. *Cry1* mRNA was higher at ZT16 than ZT4 in unstressed SHAM rats (p=0.003), stressed SHAM rats (p<0.001), and unstressed ADX rats (p=0.012) represented in Figure 2. Stress had no effect on *Cry1* mRNA in the ventral orbital subregion of the PFC (Figure 3). Although there was not a significant main effect of adrenal status, post hoc analysis showed that in stressed conditions at ZT16, SHAM rats had higher *Cry1* mRNA than ADX rats (p=0.002) in the VO (Figure 4).

Experiment 2: No time of day effect of *Cry1* mRNA in the SCN or PVN. Sex and stress had no effect on *Cry1* expression in the SCN or PVN.

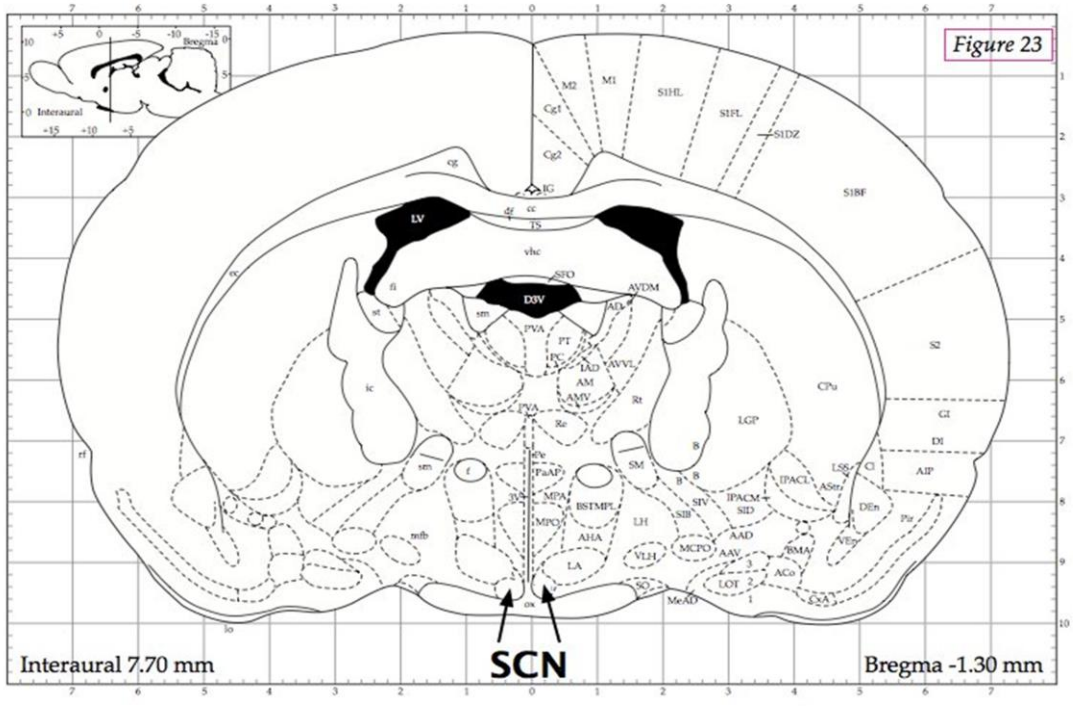

Figure 5. *Paxinos and Franklin* Rat Brain Atlas image highlights of the Suprachiasmatic Nucleus of the Hypothalamus (SCN).

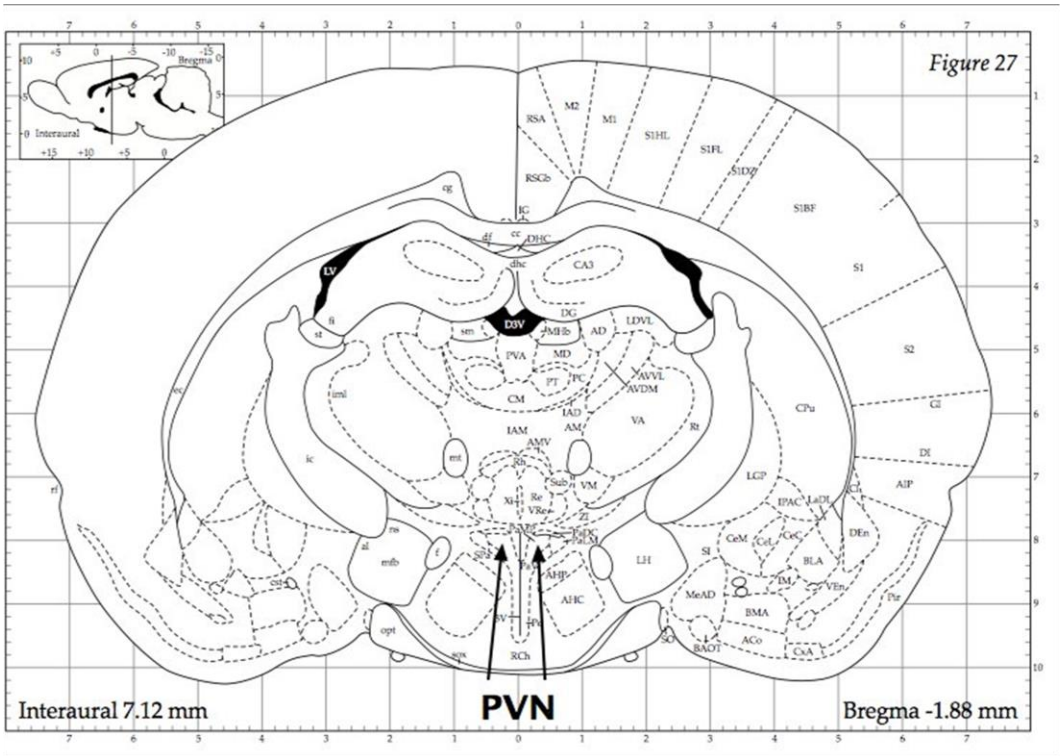

Figure 6. *Paxinos and Franklin* Rat Brain Atlas image highlights of the Paraventricular Nucleus of the Hypothalamus (PVN).

No Main Effects in the Suprachiasmatic Nucleus of the Hypothalamus

Two-way ANOVA revealed that there were no significant main effects of sex (Figure 7), stress (Figure 8), or time (Figure 9) in the SCN for *Cry1* mRNA.

No Main Effects in the Paraventricular Nucleus of the Hypothalamus

Two-way ANOVA revealed that there were no significant main effects of sex (Figure 7), stress (Figure 8), or time (Figure 9) in the PVN for *Cry1* mRNA.

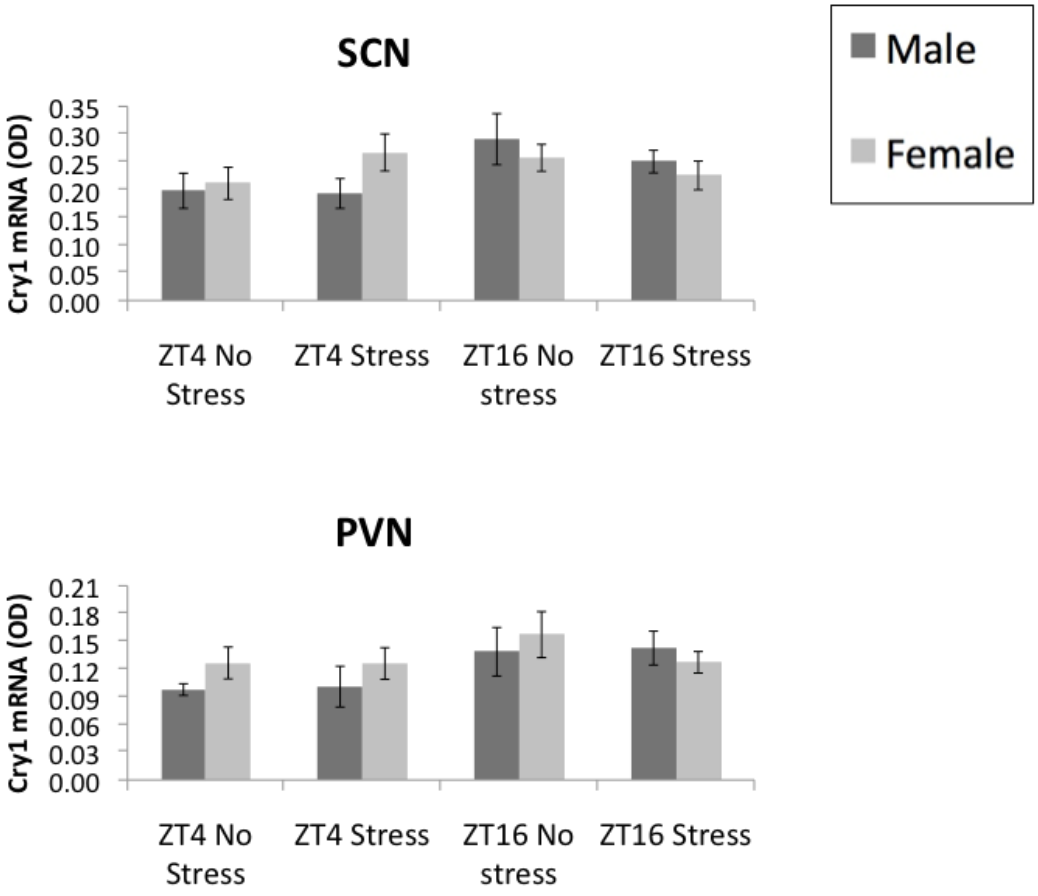

Figure 7. *Cry1* mRNA expression in suprachiasmatic and paraventricular nuclei of the hypothalamus represented by sex. Significances determined through Fisher's LSD post-hoc test. *$p<0.05$ represents significant statistical difference.

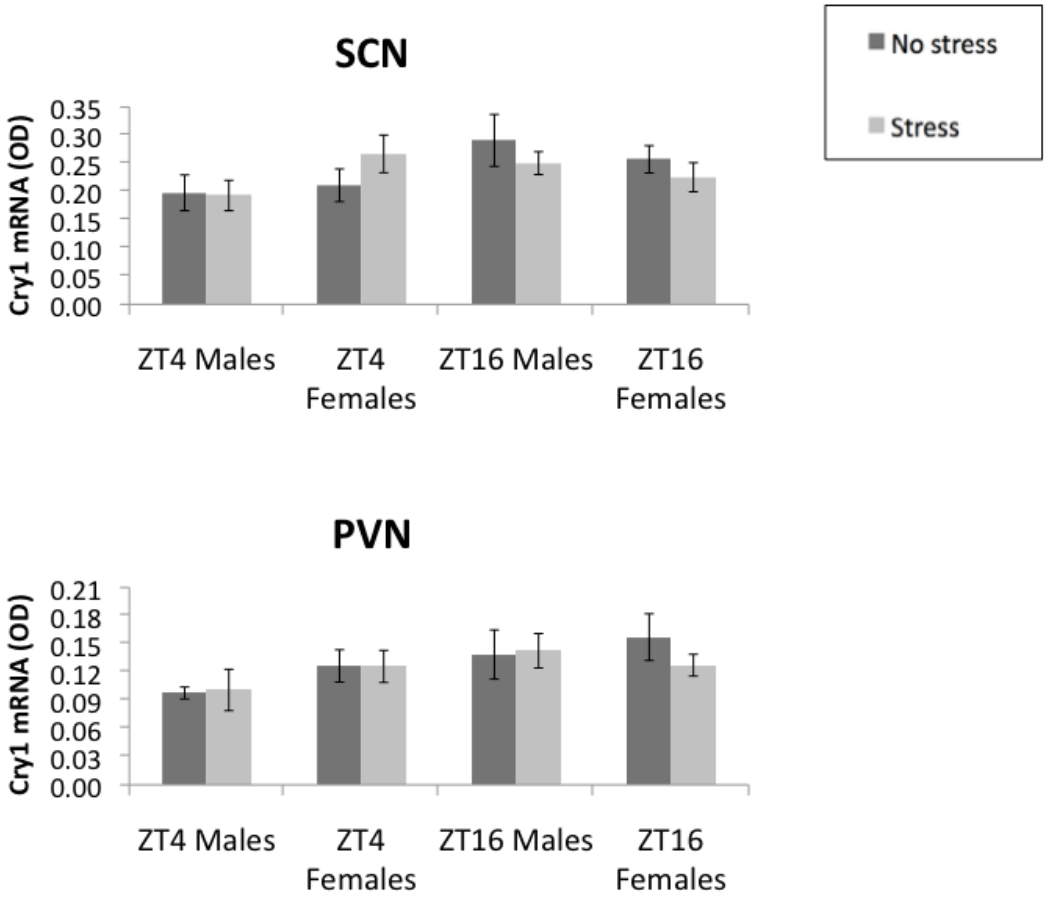

Figure 8. *Cry1* mRNA expression in suprachiasmatic and paraventricular nuclei of the hypothalamus represented by stress. Significances determined through Fisher's LSD post-hoc test. *$p<0.05$ represents significant statistical difference.

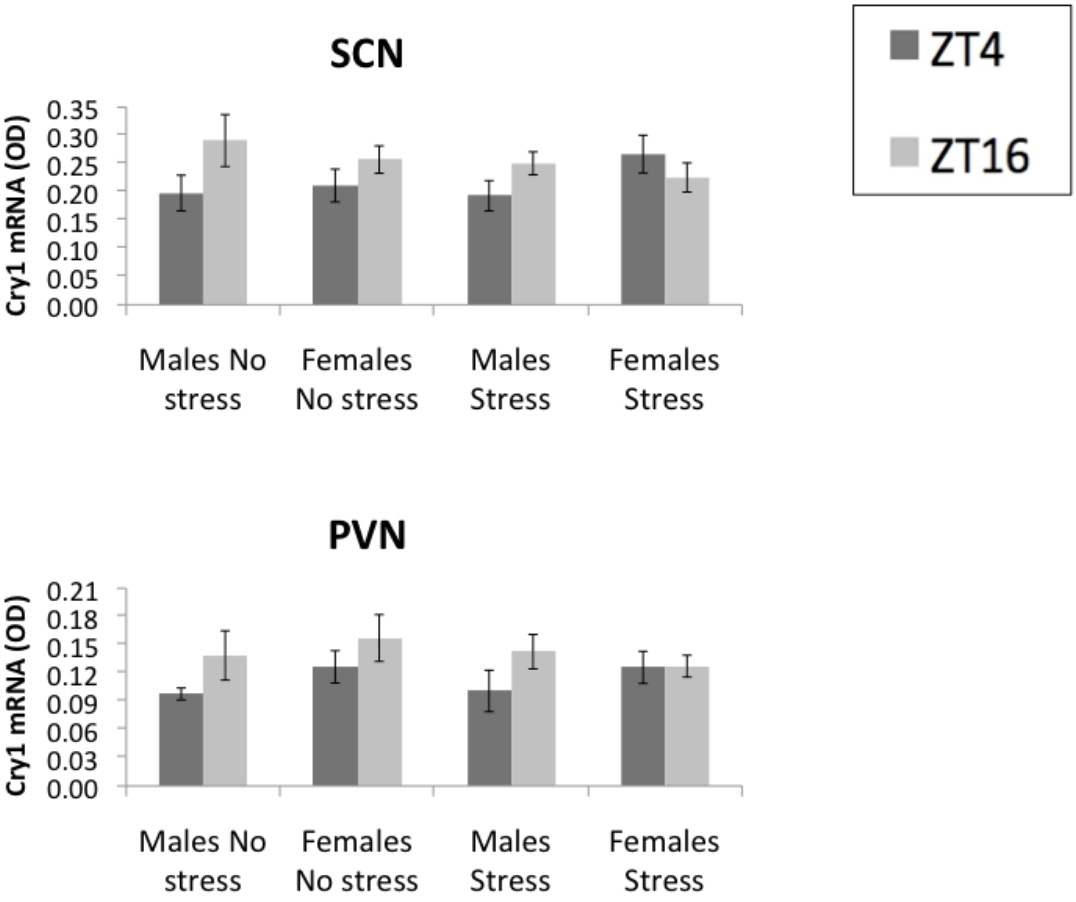

Figure 9. *Cry1* mRNA expression in suprachiasmatic and paraventricular nuclei of the hypothalamus represented by time of day. Significances determined through Fisher's LSD post-hoc test. *$p<0.05$ represents significant statistical difference.

Discussion

Circadian research has long since established that the SCN is the master clock within the brain that is responsible for coordinating circadian rhythms. More recently it was discovered that there are clocks in virtually all cells and organs of our bodies. The concept of how the SCN communicates with extra-SCN clocks is not well understood due to the limited direct projections from the SCN to other areas of the brain and body. CORT may provide a mechanism for communication between SCN and extra-SCN clocks and serve as an entrainment factor for peripheral tissue. There is reason to believe that CORT may play a role in synchronizing peripheral clocks due to its daily rhythmic secretion pattern and ability to affect extra-SCN clocks without affecting the SCN. Additionally, stress-induced CORT has been shown to rapidly induce the core clock gene, *Per1* (Takahashi et al., 2001). This led to the basis of experiment 1, in which I observed the effect of CORT and stress on *Cry1* mRNA in the PFC of male rats at two time points. There was a time of day effect of *Cry1* mRNA expression in the PFC and these findings suggest this effect is modulated by adrenal status. Stress had no effect on *Cry1* mRNA in the PFC. Since there are significant sex differences in the circadian systems of males and females, experiment 2 investigated sex differences in *Cry1* expression in the SCN and PVN of rats after acute stress at two time points. There were no significant effects of sex, time, or stress in the SCN or PVN.

Cry1 mRNA expression in various subgregions of the PFC

As hypothesized, there was a significant main effect of time for *Cry1* mRNA in the anterior cingulate cortex (ACC), prelimbic cortex (PL), rostral agranular insula (insula), infralimbic cortex (IL), and ventral orbital subregion (VO) of the PFC, where

expression of *Cry1* was higher at ZT16 than ZT4. This correlates to previous findings that suggest *Cry1* mRNA is highest during the dark phase in extra-SCN clocks (Lamia et al., 2009). I hypothesized that *Cry1* mRNA would be higher at ZT16 outside the SCN because this expression profile mimics *Per1*. Since *Bmal1* and *Per1/2* have antiphasic expression patterns, I proposed that *Cry1* should be more similar to *Per1* as they are both negative components of the molecular clock. Notably, the time of day difference in *Cry1* mRNA was only present in adrenal intact rats in the ACC, PL, IL and insula. While it is evident that *Cry1* mRNA is expressed higher during the dark phase, I am unable to conclude that peak expression occurs at ZT16 and trough expression occurs at ZT4, because this study is limited by having only two time points. However, I can conclude that *Cry1* expression is higher during the dark phase in the PFC.

There was not a significant main effect of adrenal status of *Cry1* mRNA in the PFC; however, there was a significant interaction with time of day in the ACC, insula, and VO (the interaction was almost significant in the PL). *Cry1* mRNA was only significantly higher during the dark phase in adrenal intact (SHAM) rats. The removal of endogenous CORT (ADX) abolished this effect. There are two theories that may explain this pattern. First, the presence of circadian CORT may be necessary for normal rhythmicity of *Cry1* mRNA in these brain regions. Thus, while SHAM animals have rhythmic *Cry1* expression, ADX animals exhibit a blunted rhythm attributed to the lack of endogenous CORT. The second explanation suggests that the absence of endogenous CORT may alter the circadian phase of *Cry1* expression. Woodruff et al. (2016) demonstrated that adrenalectomy surgery alters the pattern of clock gene expression in the PFC. The absence of circulating CORT did not abolish the diurnal pattern of *Per 1/2*;

however, it altered the phase of expression. Peak *Per1* mRNA exhibited a phase advance, while the peak of *Per2* mRNA was phase delayed. Therefore, it is reasonable to propose that CORT is necessary for the normal diurnal pattern of *Cry1*, and removal of endogenous CORT pushes around the circadian phase. Thus, the peak and trough of *Cry1* mRNA would not be detected through the time points of ZT4 and ZT16 in adrenalectomized rats.

While the VO was very similar to the ACC, PL, and insula, unstressed ADX animals also showed significantly higher expression of *Cry1* at ZT16. This may indicate that adrenal status does not modulate time of day differences in the VO. Further, an anomaly occurred in the IL where *Cry1* expression was not significantly higher at ZT16 in unstressed SHAM animals. Although this data did not quite reach significance, it was very similar to data from other subregions of the PFC. I would need to replicate this experiment to determine if there is a true absence of a time of day effect in unstressed SHAM animals in the IL.

While I hypothesized that *Cry1* expression would increase when a rat was subjected to acute stress if the rat's adrenal glands were intact, this experiment revealed that stress had no effect on *Cry1* mRNA in any subregion of the PFC. This hypothesis was attributed to the fact that restraint stress should lead to increased activation of the HPA axis, and higher production of CORT. CORT would bind to GR, and if a GRE were present in the *Cry1* gene, GR would bind to the GRE. This would ultimately increase expression of *Cry1* mRNA in the PFC. I proposed that this effect would only occur in SHAM animals due to the lack of CORT in ADX animals. However, there were no effects of stress across the treatment groups. While stress-induced CORT has been shown

to rapidly induce *Per1* expression (Takahashi et al., 2001), stress has not been shown to affect the other core clock genes. Unpublished data from our lab also confirms that stress does not affect the expression of *Bmal1* or *Per2* mRNA, while it rapidly induces *Per1* expression. This suggests that *Per1* is the only core clock gene that is reactive to stress. It is likely that *Per1* is selectivity induced by stress because of the presence of a GRE in its promoter region (Yamamoto et al., 2005). While evidence suggests that GRE's may be present in *Bmal1* and *Per2*, *Per1* appears to be the only clock gene that possesses a functional GRE (Cheon et al., 2013).

Although there was not a significant 3-way interaction between time of day, adrenal status, and stress, post hoc analysis revealed that stressed SHAM rats at ZT16 had greater *Cry1* induction compared to stressed ADX rats at ZT16 in all regions of the PFC except the IL (although it was almost significant, p=0.070). Thus far, the results have been interpreted based on hormonal activity. However, this interesting phenomenon may be explained through a neural pathway. ZT16 corresponds to the time when most physiological activity occurs in rats, and high neural activity is likely occurring at ZT16 when animals are stressed. Increased neural activation could induce *Cry1* expression through activation of transcription factors that interact with CORT. Lee et al. (2010) found that the repression of the transcription factor cAMP response element-binding protein (CREB) led to a significant decrease in *Per1* and *Per2* expression. Further, an interaction exists between the glucocorticoid receptor and CREB (Imai et al., 1992). This phenomenon in the data may occur through a pathway where increased neural activation elevates CREB levels, which interacts with GR and/or CORT to increase *Cry1* expression. Since CORT is a necessary component of this proposed pathway, SHAM rats

would express significantly higher *Cry1* expression than animals lacking endogenous CORT in conditions that were otherwise the same. The lack of a significant effect in the IL may be due to neural differences across regions of the PFC.

Cry1 mRNA expression in the SCN

The peak and trough expression patterns of *Bmal1* and *Per1/2* have been well characterized in the SCN, and determined to be in direct opposition with their respective expression patterns in extra-SCN tissues. This led me to hypothesize that while *Cry1* mRNA would be higher at ZT16 in extra-SCN tissue, expression would be higher at ZT4 in the SCN. However, the data revealed that there was not a significant main effect of time of *Cry1* mRNA in the SCN. Little characterization of the *Cry1* expression pattern in the SCN exists; however, Miyamoto and Sancar (1998) performed an in situ hybridization experiment that revealed *Cry1* mRNA levels across seven time points. Through quantitative analysis, they determined that highest *Cry1* mRNA expression occurred at ZT8, and lowest expression occurred around ZT20. Therefore, having only two time points, ZT4 and ZT16, was a significant limitation of this study. The time points were not selected at periods that would capture peak and trough expression of *Cry1* mRNA, and ZT4 and ZT16 were likely periods when a difference in expression was less detectable. Thus, limited temporal resolution is a probable explanation for the lack of a time of day effect of *Cry1* expression in the SCN.

As hypothesized, acute stress did not induce *Cry1* expression in the SCN. This is consistent with previous findings from our lab that confirm that the SCN is resistant to stress. This is due to the absence of GR's in the SCN. There is evidence that GR's exist in

the SCN during the first few weeks of life; however, an irreversible decrease occurs that results in the absence of GR in the SCN of a mature organism (Rosenfeld et al., 1988). Without GR, CORT is unable to exert its effects on the SCN. Evolutionarily this makes sense, because the integrity of the SCN is paramount for regulation of circadian rhythms in an organism. The SCN synchronizes the internal environment with external cues such as light, and influences circadian clocks in peripheral tissue throughout the body. If the master clock were susceptible to short term stressors, which are unavoidable across the life span, the consequences would be detrimental to an organism. Therefore, the absence of GR appears to be a protective feature of the SCN, allowing an organism to maintain synchronized circadian rhythms under conditions of acute stress.

Gender differences exist in the circadian systems and stress responsivity of males and females, which explains why I chose to investigate sex differences in this study of *Cry1*. Female rodents have higher basal levels of circulating CORT and exhibit an elevated CORT response to acute stressors compared with males (Beiko et al., 2004). Higher levels of CORT, such as what is present in female rats whether stressed or not, should theoretically result in increased induction of *Cry1*. However, the absence of GR in the SCN should prevent CORT from exerting its actions in response to stress. As hypothesized, there was no main effect of sex for *Cry1* mRNA in the SCN. While females had higher levels of basal CORT and elevated levels of CORT induced by acute stress (unpublished observations, Chun et al.) the actions of CORT would not have an affect on the SCN.

Cry1 mRNA expression in the PVN

There has yet to be a study that examines the diurnal pattern of *Cry1* mRNA in the PVN. I hypothesized that *Cry1* expression would be higher at ZT16 compared to ZT4 in the PVN, since it is an extra-SCN clock. The data revealed that there was not a significant main effect of time of day for *Cry1* mRNA in the PVN. Once again, this can be explained because of the limitation of having only two time points in this study. Additionally, Lamia et al. (2009) provided a figure that revealed relative amplitudes of *Cry1* across 20 hours through QPCR analysis in mouse liver. The highest amplitude of *Cry1* occurred around ZT18, while *Cry1* expression troughed during the light phase. Similar to the SCN, it is likely that ZT4 and ZT16 were periods when a difference in *Cry1* expression was undetectable. Therefore, limited temporal resolution accounts for the lack of a time of day effect of *Cry1* mRNA in the PVN.

The PVN consists of cell bodies in the hypothalamus that are activated when an organism is subjected to stress. Through a pathway that involves the anterior pituitary gland, CORT is produced from the adrenal glands in response to stress. GR's are abundant in the PVN, which was the basis for my hypothesis that acute stress would induce *Cry1* expression in the PVN. Restraint stress should lead to increased activation of the HPA axis, and an elevated CORT response. CORT would bind to GR in the PVN, and if a GRE were present in the *Cry1* gene, GR would activate the GRE. This would ultimately increase expression of *Cry1*. However, there were no effects of stress for *Cry1* mRNA in the PVN. Similar to the PFC, stress has only been shown to rapidly induce *Per1* expression in extra-SCN tissue. While the core clock genes interact through a series

of positive and negative feedback loops to maintain a functional molecular clock, *Per1* appears to be the only component that is directly affected by stress. This is likely due to the presence of a functional GRE in the promoter region of *Per1*. Rather than affecting each clock gene individually, stress may exert its effects on circadian rhythms at the molecular level through specific action on *Per1*. Despite the fact that stress does not rapidly induce *Cry1*, it may affect *Cry1* and other components of the molecular clock indirectly through rapid induction of *Per1*. Further, stress may induce expression of *Cry1* after a greater time lag. If *Cry1* is reactive to stress, but not rapidly, this study would not detect stress induced *Cry1* because expression was only observed after 30 min.

I hypothesized that female rats would show higher *Cry1* expression after acute stress, due to the presence of higher basal CORT levels and increased CORT in response to stress. While I proposed that this difference should not exist in the SCN because CORT is unable to exert its effects without the presence of GR, I assumed that there would be a significant difference in the PVN, where GR is abundant. However, the data revealed that there was not a significant effect of sex on *Cry1* expression in the PVN. This did not come as a complete surprise, since there are not sex differences in *Per1*, *Per2*, or *Bmal1* expression in the PVN (Chun et al., 2015). While sex differences do exist in the circadian systems of males and females, these differences do not occur at the molecular clock level in the PVN.

Conclusion

Clock genes compose the circadian framework found throughout the brain and virtually all cells of the body through oscillations of gene expression. Circadian

regulation allows an organism to remain in sync with the external world. In order to understand how organisms function across a 24-hr day, it is imperative to study how various factors influence clock gene expression. For this study, I chose to examine *Cry1*, as it is a core component of the molecular clock. Across subregions of the PFC, *Cry1* expression was higher at ZT16 than ZT4. The presence of endogenous CORT was necessary to see this effect, suggesting a role for CORT in regulating the diurnal pattern of *Cry1* expression. Further, the data suggests that *Cry1* could be controlled through a neural pathway that involves CORT. The lack of a time of day effect in the SCN and PVN is likely due to the limitation of having only two time points in this study, while the lack of a sex effect confirmed that there are not sex differences in core clock gene expression between males and females in either of these brain regions.

Although this data suggests that CORT is necessary for the diurnal rhythm of *Cry1* in the PFC, it does not promote rapid induction of *Cry1* in the PFC, SCN, or PVN after stress. Across all regions of interest, there were no significant effects of stress on *Cry1* expression. Therefore, *Per1* appears to be the only component of the molecular clock that is directly affected by stress. This is likely due to the presence of a functional GRE in the promoter region of this gene. This is advantageous when viewed through an evolutionary perspective. If every component of the molecular clock were susceptible to acute stress, there would be severe desynchronization of physiological and behavioral rhythms in an organism after exposure to short-term stressors. Instead, acute stress may only have a direct impact on the molecular clock through its actions on *Per1*. Stress-induced *Per1* could then have downstream effects on other components of the molecular clock, which would eventually lead to circadian dysregulation. Overall, this study may

facilitate a better understanding of the intrinsic molecular clock, which has beneficial implications to those suffering from various mood and psychiatric disorders that involve disrupted circadian rhythms.

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