The Effect of Stress, Time of Day and Adrenal Hormones on Bmal1, Per2 and C-Fos Expression in the Rat Hippocampus and Amygdala

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The Effect of Stress, Time of Day and Adrenal Hormones on Bmal1, Per2 and C-Fos Expression in the Rat Hippocampus and Amygdala

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Abstract

The term circadian rhythm refers to the behavioral and physiological changes an organism exhibits during an approximately 24-hour period. Plants, animals and fungi all maintain circadian rhythms, by part, with a molecular clock system. The molecular clock consists of a positive and negative feedback loop that alter gene transcription and translation. BMAL1 and CLOCK make up the positive transcription arm that stimulates the production of two classes of genes, period (Per 1-3) and cryptochrome (Cry 1-2). PER and Cry protein make up the negative feedback loop of the molecular clock and inhibit their own transcription in the nucleus.

The molecular clock system must be reset, or entrained, on a daily basis. The steroid hormone corticosterone (CORT) is thought to be the hormonal signal that entrains peripheral molecular clock systems. CORT is released when stressors activate the hypothalamic pituitary adrenal (HPA) axis.

This study examines the role stress, endogenous CORT and time of day play in the expression of Per2, Bmal1 and c-Fos in rat hippocampus and amygdala. C-fos was chosen as a positive control to ensure that cells were generally responsive to stress. Acute restraint stress was used to model mild psychological stress in rats. Endogenous CORT was manipulated using sham and adrenalectomy (ADX) surgeries. Tissue samples were taken at ZT4 and ZT16 and analyzed via insitu hybridization to measure relative mRNA levels.

There was a robust stress effect seen with c-Fos expression in all brain regions examined except for the superior portion of the dentate gyrus. Per2 shows a significant time of day effect in CA1, CA3 and BLA. CEA showed an interesting time of day by endogenous CORT status interaction. These data show that Per2 and Bmal1 are not rapidly induced by stress like the clock gene Per1. Future studies should analyze data at supplementary time points and alter the type of stressor utilized to see if those variables effects Per2 and Bmal1 expression.
Introduction

Throughout the course of a day, the environment displays various predictable changes. For example, the sun’s location always changes from east to west as the day progresses. Temperatures typically change from relatively low in the evening and early morning to warmer in the afternoon. These are just two pieces of evidence to support the notion that the Earth displays a rhythmic pattern of activity over the course of 24 hours.

Humans, and most other organisms, also display a rhythmic pattern of changes in activity on a daily basis. The term circadian rhythm refers to the behavioral and physiological changes that the body exhibits during an approximately 24 hour period\textsuperscript{1–3}. These rhythmic changes affect virtually every organ system, with peaks and troughs of activity at predictable times each day (Figure 1). These changes help synchronize biological processes that must happen in tandem, as well as help prepare the body for daily activities as mediated by their environment\textsuperscript{2}.

The most well recognized example of a circadian rhythm is the oscillation of daily sleep patterns. Sleep is in part regulated by melatonin, a hormone secreted from the pineal gland.
This endocrine organ is believed to be under tonic inhibition during an organism’s wakeful hours. Regulation of melatonin circulation depends on a brain region called suprachiasmatic nucleus of the hypothalamus (SCN). When the retina senses light in the environment, it sends an electrical signal to SCN via the retino-hypothalamic tract (RHT). SCN then signals to inhibit the pineal gland and its release of melatonin. Therefore, during the night, the pineal gland is relieved of SCN-mediated tonic inhibition. Circulation of melatonin has far reaching effects that prepare the entire biological system for rest.

In this example, and many other instances, light serves as an entrainment factor for establishing a biological rhythm. Entrainment occurs when physiological or behavioral processes coincide with a period of environmental cues. At the beginning of every 24-hour cycle, all biological clocks must be entrained for proper functionality. Entrainment occurs in the SCN at the beginning of every circadian phase. Light:dark cues from the environment are arguably the most potent zeitgebers, or “time givers”, which are exogenous cues that have the potential to entrain, regulate or disrupt circadian rhythms.

Time of day is also a zeitgeber that has potent effects on circadian rhythms. Zeitgeber time (ZT) is a method of measuring time that is particularly salient for examinations of circadian rhythms. On a typical 12 hour light:dark cycle, ZT0 marks the beginning of the light phase and ZT12 is the time of lights off. Nocturnal animals, such as rodents, start their active phase at ZT12.
Circadian Rhythms: SCN & Peripheral Clocks

SCN is often referred to as the “master pacemaker” because it is the region of the brain that is responsible for entraining all biological clock systems. SCN contains approximately 20,000 timekeeping neurons, each of which is sensitive to external zeitgebers. These cells have an oscillatory pattern of neurotransmitter release that mediates their timekeeping abilities. SCN has direct neuronal interactions with the retina, via RHT, which allow it to rapidly and directly integrate salient photic signals (Figure 2).

Peripheral clocks, also called peripheral oscillators, are timekeeping cells that exist in regions of the body other than SCN. These timekeeping cells have similar molecular machinery when compared to SCN neurons. They also mediate the physiological changes that are under circadian control (Figure 1). Like SCN, peripheral clocks must be entrained at the start of every circadian cycle.
A multitude of experimental findings support the notion that SCN is the master biological clock. First, as previously stated, SCN receives direct innervation from the retina. This is an attractive quality in the candidate for the master clock because it allows SCN to readily, and rapidly, integrate photic cues. Also, SCN is the only tissue type that can sustain circadian rhythms in culture for up to 32 days. In contrast, peripheral clock systems lose their periodicity after a few rounds of reproductive cycling. Next, lesioning the SCN eliminates the expression of all circadian rhythms throughout the body. These rhythms are normalized following SCN transplantation. Finally, there is an absence of glucocorticoid receptors (GCRs) in SCN. This is important because the substance that binds to GCS, corticosterone (CORT), is the chemical messenger that entrains all peripheral clocks (more on this later). Lack of GCRs in SCN implies that this hypothalamic region is not responsive to the effects of CORT that is secreted in a non-circadian fashion.

**Circadian Rhythms: The Molecular Clock**
The molecular clock system consists of a positive and negative feedback loop that alters both transcription and translation of clock genes (Figure 3). The positive feedback arm consists of the transcription factors CLOCK and BMAL1\textsuperscript{13,14}. These two proteins form a heterodimer in the nucleus of the time keeping cell and bind to the E box to initiate transcription of two classes of clock genes, Period 1-3 (Per 1-3) and cryptochrome 1-2 (Cry 1-2)\textsuperscript{15}. After a period of time, PER and CRY proteins will reach maximum levels, then they begin to form homodimers and heterodimers. These complexes are then phosphorylated, tagging them for translocation into the nucleus\textsuperscript{14,15}. In the nucleus, PER and CRY proteins directly interact with BMAL1 in order to stop transcription and translation of more PER and CRY proteins. This oscillatory cycle occurs once a day both in the SCN and in peripheral tissues, however, the phase and amplitude of these oscillations varies by region\textsuperscript{14}.

**Stress, Glucocorticoids & Training Peripheral Clocks**

In this modern, fast paced world, psychological stress is a common experience. There is no completely accurate definition of stress but most people can describe the physiological changes their body exhibits when experiencing it. Shaking hands, increased heart rate and sweating are just a few of the stress responses that are mediated by the sympathetic nervous system. These phenomena are supported by complementary actions of the hypothalamic pituitary (HPA) axis (Figure 4). The HPA axis displays a circadian rhythm of activation that is mediated by both the master clock in the SCN and peripheral clocks associated with the adrenal cortex\textsuperscript{16}.
External stress is identified by a network of stress-reactive neurocircuits that include components of the limbic system and key brainstem nuclei. Once the stressor is identified by this neurosystem, local projections are sent to stimulate the paraventricular nucleus of the hypothalamus (PVN). PVN then releases corticotrophin releasing factor (CRF) to the pituitary gland. The pituitary releases adrenocorticotropic hormone (ACTH), which stimulates the adrenal cortex to produce and secrete the steroid hormones CORT in rats.

After activation of the HPA axis, CORT enters general circulation and it ultimately binds to cytoplasmic GCRs. The circadian oscillation of CORT concentration in the blood coupled with the ubiquitous expression of GCRs makes CORT an attractive candidate for a molecular signal to entrain circadian rhythms in individual clock keeping cells. The lack of GCRs in the SCN makes the master clock insensitive to the downstream consequences of its circadian control of CORT secretion. It also means that this brain region can retain its circadian pattern of activity when CORT levels are altered by stress, which has been confirmed.

Activation of GCRs leads to varied, tissue specific events such as metabolism of carbohydrates, proteins and lipids to generate energy to combat exogenous stress. GCRs also have a mechanism for potentially regulating...
transcription of Per2 and a variety of other genes because the glucocorticoid response 
elements (GREs) are present in the promoter region of these genes. Activated GCRs can bind to 
GREs and mediate transcription of downstream genes. However, the presence of a GRE does 
not ensure that the gene can be regulated by glucocorticoids.

Hippocampus and amygdala are both brain regions that are highly responsive to 
stress in humans and rodents. In laboratory settings, different types of complex stressors that 
humans experience can be modeled in rodents. The cold-water task, which is meant to induce 
intense psychological stress, has been shown to rapidly increase Per1 expression in the 
hippocampus of rats. This finding was not true when restraint stress was utilized. Restraint 
stress directly activates the HPA axis and is therefore used as an established means of 
modeling acute psychological stress. This suggests that altering the type of stress administered 
to the rats also leads to modifications in different underlying cellular processes.

In this study, restraint stress was used in order to analyze the effect of mild stress on 
clock gene expression. Per2 and Bmal1 were chosen as genes of interest because most studies 
on stress effects of period gene expression focus on Per1.

Circadian Rhythms & Stress: Mood Disorders

Circadian disruption has been classified as both a cause and symptom of many mood 
disorders including depression and bipolar disorder. For example, in depressed humans, many 
physiological traits that are typically under circadian control have disrupted daily rhythms. 
Oscillations in body temperature, plasma cortisol concentration, blood pressure and melatonin
rhythms have all been reported in patients with depression. Interestingly, antidepressant administration restores these rhythms.

Healthy individuals may also have symptoms due to dysregulation of circadian rhythms. Shift workers who reverse their light:dark cycle are more prone to developing mood disorders and a slew of other pathologies. They also exhibit difficulties concentrating and problems with memory consolidation.

The role of circadian rhythm dysregulation in depression is an attractive concept for three reasons: (i) monoamine neurotransmitters (NTs) display predictable oscillations in secretion throughout the day, implying that they are under circadian control, (ii) BMAL1 & PER2 influence the degradation pathway and receptor expression for monoamine neurons in the nucleus accumbens, (iii) administration of antidepressants normalizes behavioral and physiological rhythms.

All monoamine NTs (dopamine, serotonin, norepinephrine) display strong circadian rhythms in their extracellular concentration patterns, which is likely due to their association with clock proteins. PER2 and BMAL1 have been identified as agents that alter the transcription of monoamine oxidase A (MAOA), the gene which produces MAOA protein, an enzyme essential for the degradation of dopamine. This may be one mechanism in which the molecular clock mediates rhythmic concentrations of these NTs.

Circadian Rhythms in the Hippocampus & Amygdala: Memory Consolidation

Amgydala and hippocampus are coupled brain regions that are believed to be important for memory induction, consolidation and storage. Amygdala is especially important for
maintenance of emotional memories. In hippocampus, the MAPK pathway is vital for the consolidation of memories, regardless of emotional state\textsuperscript{25}. c-AMP and adenylyl cyclase (AC) are two components that are vital for the functionality of the MAPK pathway. Both of these substances also display a strong circadian rhythm in concentration. SCN lesions reduce contextual memory and obliterate the oscillation of cAMP and AC which suggests that these processes may be mediated by peripheral clocks entrained by SCN\textsuperscript{26}.

Activation of the MAPK pathway leads to increased memory consolidation (Figure 5)\textsuperscript{25}.

BMAL1 knockout mice have been shown to have overall decreased MAPK signaling and an eradication of the typical phasic oscillations of cAMP and MAPK\textsuperscript{25}. These findings imply that Bmal1 is an important factor for maintaining the processes underlying memory consolidation.
GCs have also been implicated in memory processes in humans\textsuperscript{27}. Studies have shown that administration of cortisol during early periods of sleep attenuated memory processes that were hippocampus dependent\textsuperscript{28}. Similarly, synthetic activation of GCRs also reduced memory on contextual tasks\textsuperscript{28}.

**Current Study**

**Genes of Interest: Per2, BMAL1 & c-Fos**

*Per 2*: Circadian rhythms in hippocampus and amygdala are closely coupled with the sympathetic nervous system and HPA axis. Analyzing how Per2 expression is altered in stressful contexts is one of the first stepping-stones in discovering how stress, mood, memory and circadian rhythms all interact with one another. Examining Per2 in a stressful context may help shine light on possible cellular mechanisms that mediate various forms of circadian dysregulation. If there is a linkage between stress and dysregulation of Per2, future studies can focus on how to regulate this component of the molecular clock in order to see if it induces therapeutic effects.

There have been no studies that examined the effects of stress on Per2 expression. Studies have shown that Per1 can be rapidly induced under stressful conditions. For this reason, it is predicted that Per2 expression will be stress and endogenous CORT sensitive in each region of interest.

*Bmal1*: Bmal1 was chosen as a gene of interested because it is involved in the positive transcription loop in the molecular clock. Expression patterns are typically antiphasic of Per2, due to the fact that these two clock genes participate in complementary
transcriptional/translational activities (Figure 3). We predict that Bmal1 expression will display patterns of oscillation that are in opposite phase of Per2.

**C-Fos:** C-Fos was chosen as a positive control because it can be utilized as a biomarker for cellular activity. Detectable levels of c-Fos would indicate that the cell was generally reactive to stress. C-Fos also displays a circadian pattern of activation; with levels being highest during the animals wake cycle. For this reason, c-Fos expression in the hippocampus and amygdala should display higher levels at ZT16 than ZT4. Stress will also prompt a rapid induction of c-Fos.

**Time of Day & Adrenal Hormones**

Tissue samples were taken at ZT4 and ZT16. These time points are 4 hours past the start of the light or dark phase respectively. The four hours were used as an acclimation period. The status of adrenal hormones was altered surgically. Animals either received an adrenalectomy (ADX) or sham surgery. Removal of the adrenal gland also removes endogenous CORT. This manipulation allows us to examine if the stress effects observed are CORT mediated.

**Regions of Interest (ROIs)**

The ventral tegmental area (VTA) is a brain region that is important for learning and memory as well as mood regulation. VTA sends direct dopaminergic projections to hippocampus and amygdala. Rodents that possess a Per2 mutation displayed greater
depressive symptoms in the forced swim task. These animals also presented with elevated levels of dopamine in VTA\textsuperscript{32}. Studies have also shown that acute stress can lead to a decrease in long-term potentiation, a phenomenon vital for learning and memory functions, in VTA\textsuperscript{33}. Because both hippocampus and amygdala receive direct innervation from VTA, they are brain regions that are heavily implicated in the generation of depressive mood in humans who have been exposed to acute stress\textsuperscript{34} and in animals that have received acute or prolonged stress\textsuperscript{35}.

Few studies have analyzed levels of Per2 and Bmal1 expression under stressful conditions in hippocampus and amygdala. This study is an attempt to contribute more data on mood and memory structures and their molecular clock’s reactivity to stress. In hippocampus we expect to see stress induced c-Fos expression in all regions\textsuperscript{32}. No studies have examined the role stress plays in Per2 expression. If Per2 has a similar physiology as Per1, it should have stress-induced expression in both the hippocampus and amygdala.
Methods

Subjects

The subjects were commercially obtained male Sprague Dawley rats aged 3 months (Harlan Laboratories; Harlan, Indianapolis, IN). Rats were divided evenly into four different light/dark rooms as described in supplementary figure 1. Food and water were available ad libitum. After arrival at the facility, rats were given 2 weeks to acclimatize to their new environment. The acclimation period is vital for ensuring that any displayed stress effects are due to the experimental treatment rather than a reaction to a novel environment. Animals were ethically treated and their use in this experiment was approved by the University of Colorado’s Institutional Animal Care and Use Committee.

Experimental Design

![Figure 6: Summary of treatment groups. (A) displays the variables that were manipulated (B) describes the resulting 8 treatment groups]
This study utilizes a 2x2x2 factorial design. Subjects were placed in treatment groups based on time of day (ZT4 or ZT16), adrenal status (ADX or SHAM) and the presence of absence of restraint stress (Figure 6).

**Surgery**

After the 2 week acclimation period, rats were given either adrenalectomies (ADX) or control (SHAM) surgeries. Both treatment groups received bilateral peritoneum incisions in order to expose the adrenal glands. In the ADX group, subjects’ adrenal glands were located and removed using intestinal tissue forceps. The SHAM group followed the same procedure without removal of the adrenal glands. Both treatment groups were given one week to recover from surgery before test day. Trunk blood was then analyzed to ensure the absence of CORT in ADX animals.

The purpose of the surgery was to examine the role endogenous CORT plays in the expression of the aforementioned clock genes. In ADX subjects, no endogenous CORT is present while in the SHAM subjects, CORT is still being produced in a predictable manner.

**Restraint Stress**

In order to assess the role of stress in clock gene expression, half of the rats experienced restraint stress one week after surgery. 25 rats were kept in plastic restraint tubes acutely that allow for minimal movement for 30 min (diameter: 6.3 cm, length: 16cm). This type of stress has been shown to increase CORT in adrenal intact rats, which is a biomarker for stress effects.
Tissue Preparation

Immediately after restraint stress, decapitation took place at either ZT4 or ZT16. ZT16 subjects were sacrificed underneath a red light to reduce light induced clock gene expression\textsuperscript{36,37}. Control rats were kept in their home cage until decapitation, whereas stressed rats were placed in restraint tubes 30 min before sacrifice. Brains were extracted and flash frozen in isopentane that was cooled with dry ice to between -20\degree C and -30 \degree C. Frozen brains were then wrapped in aluminum foil and kept in a -80\degree C freezer.

12\mu M coronal sections were obtained using a cryostat (Leica CM 1850) at the level of the hippocampus/amygdala (Figure 7). Tissue sections were thaw mounted onto Colorfrost Plus microscope slides (Plus Slides, VMR) and stored at -70\degree C until subsequent use.

Insitu Hybridization & Densitometry

A two day insitu hybridization was conducted as previously described\textsuperscript{36,37}. First, riboprobes for Per2, Bmal1, and c-Fos were generated in house following an established protocol\textsuperscript{38}.

On the first day of the insitu hybridization, slides were pulled from storage (-70\degree C) and fixed in 4\% paraformaldehyde. Afterward, slides were placed in standard sodium citrate buffer (SSC) followed by 0.1M triethanolamine (TEA) spiked with 0.25M acetic anhydride. The slides were subsequently dehydrated in progressively more concentrated ethanol baths. Simultaneously, a radiolabeled riboprobe was generated using 35S. The riboprobe was
incubated in a water bath for two hours at 37°C. Afterward; the probe was separated using a G50/50 sephadex column. The riboprobe was added to hybridization buffer and 98 μl of the solution was used to coverslip the slides. Finished slides were put into a cassette with a 50% formamide at 54°C.

On the second day of the insitu hybridization, cover slips were floated off of the slides in 2X SSC bath. Slides were then incubated in a 37°C water bath with RNase (cat No. R5503; Sigma, St. Louis, MO) in order to degrade any remaining unhybridized mRNA. Slides were then dehydrated in ethanol and placed on film to develop for 3 weeks. Films processed into digitalized images using Northern Light lightbox model B95 Imagining Res Inc., St. Catharines, Ontario Canada) as described with Chun et. al (2015).

To analyze the photo product of the insitu hybridization, the mean optical density of each slide was measured using ImageJ64(NIH). ImageJ allows for freehand definition of regions of interests (ROIs). For this analysis, the superior and inferior portions of the dentate gyrus of the hippocampus as well as the media, basolateral and central amygdala were examined bilaterally. Each subject had 8 different brain slices from the previously prepared coronal slices. Results from each slide were then averaged to find the mean optical density for each subject. The Paxinos and Watson Brain Atlas (1998) was used as a reference to locate ROIs.
Statistical Analysis

Statistical Package for Social Science (SPSS) conducted a three-way analysis of variance (ANOVA) to determine if there was a main effect of stress, time and adrenal status on per-2, Bmal1 and c-Fos expression. For significant main effects (p < 0.05), a Fisher's Least Squares Difference post-hoc test was performed to explore pairwise comparisons of interest.\(^{36}\)
Results

The results from the ANOVA analysis of each gene within each ROI are summarized in Table 1 (page 35). The significant main effects and interactions found for each gene are described below.

**Per2**

![Figure 8: Relative optical density reflecting per2 expression in the hippocampus. Post hoc analysis revealed # significant ZT effect within groups that share the same stress and ZT conditions. Fisher’s LSD test was used to establish significance in all cohorts (p<0.05)](image-url)
There was no significant effect of acute stress on Per2 mRNA expression in any ROI (Figure 8, Figure 9, Table 1). There were, however, time of day differences in CA1, CA3 and BLA, with greater levels of Per2 mRNA at ZT16 rather than ZT4. Interestingly, there was a significant adrenal status by time of day interaction in CEA. In contrast to the other brain regions, in CEA, Per2 mRNA levels of sham rats were lower at ZT16 than ZT4, but this time of day pattern was reversed in ADX rats.

**Figure 9: relative optical density reflecting per2 expression in the amygdala.** Post hoc analysis revealed # significant ZT effect within groups that share the same stress and ZT conditions. † significant adrenal status effect within same stress and ZT conditions. Fisher’s LSD test was used to establish significance in all cohorts (p<0.05)
Figure 10: relative expression of BMAL in CA1, CA3 and dentate gyrus. Post hoc analysis # significant ZT effect within groups that share the same stress and ZT conditions. Fisher’s LSD test was used to establish significance in all cohorts (p<0.05)
There was no stress effect observed in any ROI. Time of day differences persisted in CA1, CA3 and BLA with ZT4 displaying more Bmal1 expression when compared to ZT16. This pattern is anti-phasic to patterns seen in Per2, which is to be expected given their roles in the molecular clock system. A significant adrenal effect was detected in SupraDG (Fig 10 and 11, Table 1) with SHAM animals having higher expression than those that received ADX.

**Figure 11: relative expression of BMAL in basolateral amygdala, central amygdala and medial amygdala.** There is a significant main effect of ZT in the basolateral amygdala. Fisher’s LSD test was used to establish significance in all cohorts (p<0.05)
Figure 12: relative optical density reflecting c-fos expression in the following hippocampal nuclei: CA1, CA3, superior portion of the dentate gyrus and the inferior portion of the dentate gyrus. * significant stress effect for subjects that share the same ZT and adrenal status. # significant ZT effect within groups that share the same stress and ZT conditions. † adrenal status comparison within same stress and ZT conditions. Fisher’s LSD test was used to establish significance in all cohorts (p<0.05)
In contrast to Bmal1 and Per2, there was a strong stress effect on c-Fos expression in all ROIs except for SupraDG. Stressful conditions consistently increased c-Fos levels. There was also a main effect of time for each ROI. In CA3, InfraDG and CEA there was an adrenal status effect (Figure 12, Figure 13, Table 1).

**Figure 13**: relative optical density reflecting c-fos expression in basolateral amygdala, central amygdala and medial amygdala. Post hoc analysis revealed * significant stress effect for subjects that share the same ZT and adrenal status; # significant ZT effect within groups that share the same stress and ZT conditions. Fisher’s LSD test was used to establish significance (p<0.05)
Discussion

In contrast to previous studies examining Per1\textsuperscript{21}, there was no stress induction of Per2 or Bmal1 expression in any of the ROIs. However, two novel findings emerged from this study: First, a significant interaction of time of day and adrenal status was observed in Per2 induction in CEA. Second, an ADX effect was found for Bmal1 expression in SupraDG.

Per2

Per2 was expected to lose rhythmic expression in the amygdala following ADX\textsuperscript{35}, however, that trend was not directly observed. There was a main effect of time in CA1, CA3 and BLA. These rhythms are consisted with previously published research with adrenal intact animals\textsuperscript{36}

Although insignificant, there was a general trend of ZT4 having higher expression than ZT16, however this trend was reversed in sham animals in CEA. This result is consistent with other experiments that show Per2 mRNA and PER2 protein levels display opposite rhythms than BLA and hippocampus\textsuperscript{35,36} Interestingly, we found that this time of day difference had the opposite pattern in ADX rats, resulting in a significant time by adrenal status interaction.

Future studies should examine the role different types of stressors play in Per2 expression. Restraint stress activates the HPA axis, making it a good candidate to study mild stress. While mild stress did not alter Per2 expression, different stress models may yield dissimilar results. A good starting point would be analyzing prolonged and repeated stress because these conditions have been shown to modulate many processes that are under circadian control\textsuperscript{39}. 
BMAL1

Stress had no effect on BMAL1 expression in hippocampus or amygdala. The results show a significant time of day effect in CA1 and CA3 for Per2 with expression being higher at ZT16. Bmal1 displayed higher expression at ZT4 in both of these brain regions as well as BLA. The molecular clock model suggests that Per2 expression should be anti-phasic of Bmal1, which is consistent with these data. This may be due to the time points selected. Additional time points could possibly see oscillatory pattern of Bmal1 expression.

Bmal1 also showed a significant ADX effect in SupraDG. This may have to do with the regions importance in memory consolidation. Bmal1 has the ability to alter MAPK functionality via circadian entrained processes. CORT is the signal that is used by SCN to entrain the MAPK pathway. The effects of CORT can be modulated by Bmal1 via acetylation of GCRs. For these reasons, Bmal1 may be less responsive to the influence of stress induced CORT that is out of sync with its typical rhythms. This dampened response could help explain why some memory processes are disrupted with stress while others remain unaltered.
c-Fos

c-Fos was the most reactive of the genes analyzed. It showed the greatest stress effects in amygdala and hippocampus (except for supraDG). Previous studies have established c-Fos as a marker for cellular activity. The robust stress response could be an indication that the restraint stress elicited physiological changes that are consistent with activation of the HPA axis.

There was also a trend for c-Fos expression to be higher at ZT16. This is consistent with other studies that show higher c-Fos expression during the active phase of animals. In all, these data show that c-Fos was a reliable positive control for this study. These findings are also consistent with behavioral studies. Rapid c-Fos induction has been seen in the hippocampus after swim stress and restraint stress in mice (Figure 15). Future studies should have animals that were restraint stressed and analyze their c-Fos mRNA levels minutes and hours after stress has stopped to see how long the stress effect is maintained.

Conclusion
The purpose of this study is to examine the role stress, endogenous CORT and time of day play in the expression of Per2, BMAL1 and c-Fos in hippocampus and amygdala. These data will allow us to understand how stress influences the functionality of the molecular clock system in these extra SCN regions. Stress was measured using restraint stress and endogenous CORT was altered using adrenalectomy. Per2 and Bmal1 were chosen as the clock genes of interest because few studies have examined the role stress and adrenal status play on their expression in hippocampus and amygdala\textsuperscript{36,37}. c-Fos was used as a positive control to confirm stress reactive brain regions. In situ hybridization analyzed the relative amount of mRNA from each gene of interest.

Acute stress had no effect on clock gene expression, however, time of day effects were seen in CA1, CA3 and BLA for Per2 and Bmal1. These differences in expression likely display the circadian cycling of these mRNA transcripts. This is a heavy implication to make when only two time points are utilized, so future studies should examine the role of acute stress at additional time points.

Taken together, these data pave the way for new research. Further studies should be conducted on Per2 to see if its oscillatory pattern is fully maintained at different time points when exposed to stress. BMAL1 expression should be analyzed under different stress conditions to see if it can induce expression in some contexts rather than others, like Per1. C-Fos data can be used as a basis of comparison for future studies as well.
References

37. Woodruff, E. R. *et al.* Adrenal-dependent diurnal modulation of conditioned fear


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<th>ROIs</th>
<th>Time</th>
<th>Adrenal Status</th>
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<tbody>
<tr>
<td>Per2</td>
<td>CA1</td>
<td>F(1,28) = 14.1***</td>
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Table 1: F statistics for all ROIs and treatment conditions. ** p < 0.05, *** p < 0.001
Supplementary Figure 1: 50 Sprague Dawley rats were pair-housed in four different rooms. Rooms one and two had a standard 12:12 light dark cycle starting with lights on at 06:00 in room one and 08:00 in room two. In rooms three and four, rats were housed in a similar fashion except they were on a reverse 12:12 light/dark cycle. In room 3, lights turned on at 17:00 while in room four, the lights turned on at 19:00.