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Effect of Corticosterone on Clock Gene Expression in the Dorsomedial Hypothalamus, in Rats

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Abstract

Many individuals may view stress as an emotion experienced by many; I, myself, was once one of those individuals. In actuality, stress is also a response composed of a series of complex events beginning with the hypothalamo-pituitary-adrenal (HPA) axis. It turns out that components of the HPA axis can be altered by input from hypothalamic nuclei such as the suprachiasmatic nucleus, SCN, and the dorsomedial hypothalamus, DMH, and external environmental cues (such as stress and photoperiod). Through connections between each other and the paraventricular nucleus (PVN-head of HPA axis), the DMH and the SCN can influence the release of corticosterone (CORT-final product of the HPA axis), in rats. In addition to mediating the body's stress response, we believe that CORT has the ability, through cues from the SCN, and possibly the DMH, to synchronize an individual's circadian rhythm throughout the body during unstressed conditions. CORT potentially accomplishes this through interaction with core clock genes (*per1* and *bmal1*), the molecular basis for an individual's circadian rhythm, in certain brain regions, including the PVN and DMH.

CORT levels are also associated with depressive disorder. A number of studies have shown that glucocorticoid receptors (GRs) do not function normally in depressed patients and that some antidepressants can directly enhance GR expression as a form of treatment (17). Moreover, depression is associated with features of disrupted circadian function, including dysregulation of CORT secretion, altered appetite time-course and impaired sleep patterns (2). Hyperactivity of the HPA axis with an enlarged pituitary and adrenal gland and consequently higher concentrations of CORT are all consistent findings in depressed patients (17). All these are evidence for the idea that the dorsomedial hypothalamus, the paraventricular nucleus, the suprachiasmatic nucleus, normal diurnal CORT secretion, GRs, and clock genes are important molecular targets to consider for the treatment of depressive disorders.

In this study, we have manipulated CORT levels in order to determine whether circadian and/or stress effects on PVN and DMH *per1* and *bmal1* gene expression were dependent on CORT. Interestingly, we found that both *per1* and *bmal1* mRNA diurnal expression appear to be dependent on the tonic rhythmic release of CORT in both the DMH and PVN, but not the SCN. We also found that CORT seems to be necessary for acute stress to exert its effects on *per1* mRNA expression in the PVN and DMH, and not SCN. *Bmal1* expression is not influenced by acute-stress-induced CORT in any of the brain regions. These results suggest the importance of CORT as a hormone important in the synchronization of an individual's circadian rhythm, as well as a mediator of stress-induced disruption of circadian rhythms.

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Introduction:

Millions of individuals experience psychological stress on a daily basis. Whether human stress stems from a college exam, a competitive game, or a relationship, it is a complex physiological and psychological state that is the product of a well-developed and well-regulated physiological system. It is widely known that the stress system utilizes the hypothalamo-pituitary-adrenal (HPA) axis to exercise its effects. The HPA axis is regulated by several brain regions, including the suprachiasmatic nucleus (SCN), the paraventricular nucleus (PVN), and the dorsomedial hypothalamus (DMH). All of these are major focuses of this thesis and will be discussed in detail. Although an acute stressor itself is probably not sufficient enough in causing disorders, determining the molecular processes by which these brain regions are involved in the stress response is critical in understanding a wide range of mood and depressive disorders, including major depressive disorder, bipolar disorder, and seasonal affective disorder (2, 17).

Stress Response: HPA Axis

The response to stress is a complex phenomenon composed of highly coordinated events within the body. An individual experiences stress on both a psychological and physiological level. It is important to distinguish between the two. Psychological stress encompasses the emotional side of stress, which we, as humans, can often relate to. Physiological stress is defined as the measured component of stress potentially observed through hormone concentrations and gene expression throughout the body and the brain. In rodent studies, restraint stress is often used to simulate a psychological stressor (5) and blood and tissue are then used to analyze the physiological stress response.

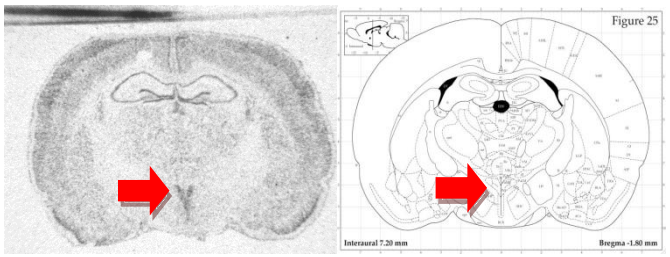

Figure 1. Both images are coronal sections of a rat at the level of the PVN. Image on left is a section with *per 1* mRNA after 30 minutes of restraint stress at ZT16. Image on the right is a representation of a rat brain slice from a rat atlas. Red arrows on both images point to PVN.

As an example of how the stress response is represented both psychologically and physiologically, let's examine the series of events that transpire as an animal is being hunted by a predator. Initially, upon realization of the life-threatening situation at hand, the animal begins to experience emotions such as fear and anxiety. Within seconds, the sympathetic nervous system overrides the parasympathetic nervous system. As a result, release of catecholamines (epinephrine and norepinephrine) leads to a rise in heart rate and blood pressure, and the pancreas releases glucagon into the blood stream (22). Catecholamines cause vasoconstriction involved in organ systems of the body not essential to escaping or fighting the impending threat, such as the digestive system. As a results, a greater percentage of blood flows to the skeletal muscles, allowing the animal to flee from the enemy (21). All this disruption in homeostasis induced by the sympathetic nervous system response provides the animal with an improved chance for survival (23). The other response simultaneously stimulated with the sympathetic response is the activation of the HPA axis.

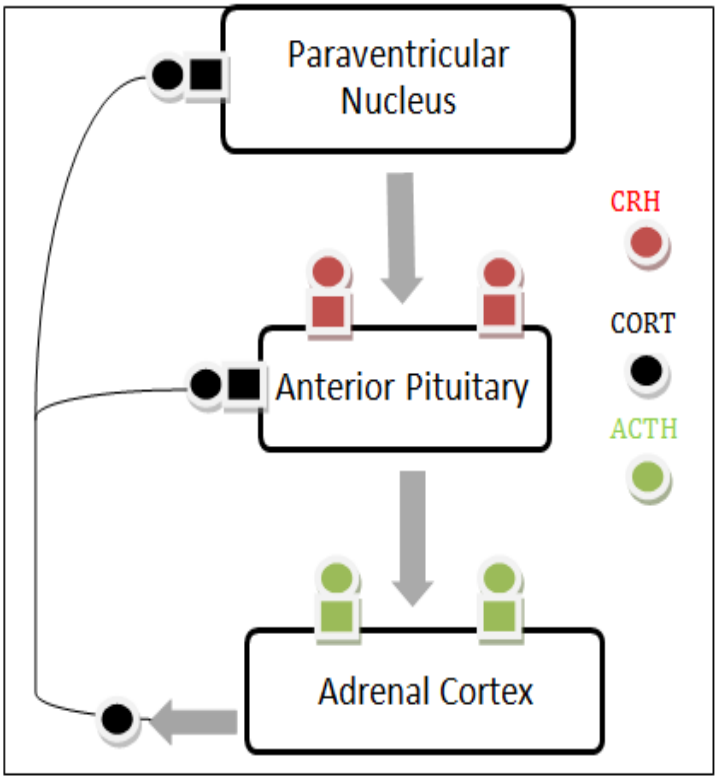

Figure 2. Simple model of HPA axis to demonstrate glucocorticoid negative feedback on anterior pituitary and PVN.

Intricate stress-related neural interactions between a variety of brain regions and the hypothalamus trigger excitation of neurons in the PVN to activate the HPA axis. The hypothalamus is one of the key integrative centers of the brain with a wide range of functions. It is composed of many distinct nuclei, one of which is the PVN (1). Anatomically, the PVN is a bilateral nucleus surrounding the dorsal portion of the third ventricle (Figure 1). There are two different subregions within the PVN, the parvocellular and magnocellular regions. The parvocellular region of the PVN is the component involved with the HPA axis. Corticotropin-releasing-hormone (CRH) neurons

located within the medial parvocellular region of the PVN releases CRH into the primary capillary plexus of the hypothalamo-hypophyseal portal system. This portal system carries the CRH to the anterior pituitary, which is densely populated with CRH1 receptors (1). In response to CRH binding to CRH1 receptors, endocrine cells in the anterior pituitary release adrenocorticotropic hormone (ACTH) into the blood stream. ACTH then acts on the adrenal cortex leading to the release of glucocorticoids (CORT, cortisol in humans and corticosterone in rats) into the blood stream. Following release from the adrenal cortex, CORT then provides negative feedback onto the PVN and the anterior pituitary by binding to glucocorticoid receptors (GRs) within the cells of these regions, resulting in reduced CRH and ACTH production. Under normal circumstances, this negative feedback reduces the amount of stress-induced CORT the adrenal gland produces and restores basal levels of CORT (Figure 2).

It is important to note that the HPA axis is involved with a wide variety of other brain functions, in addition to the stress response, including CORT effects on neuronal survival, neurogenesis, maintenance of alertness, and modulation of sleep (1). HPA axis activity, specifically CORT, has a diurnal rhythm which suggests that CORT may play a significant role in reacting to environmental circadian entrainment signals (such as light) and thereby serve as a regulator of peripheral tissue circadian synchronization to the environment (9). An entrainment factor, such as light, synchronizes the organism to its environment. Thus, stress-induced disruptions in CORT's diurnal rhythm could potentially lead to desynchronization of regions throughout the body and brain. As a result, it may not be surprising to see that abnormalities in HPA axis function are prominent in psychiatric disorders, which are marked by symptoms of circadian dysregulation (1, 18). In fact, major depressive and bipolar patients show disruptive CORT diurnal rhythms and hypersecretion of CORT in addition to a disruption in sleep/wake patterns (4). Moreover, an acute stressor can induce a depressive episode in major depressive patients and a flip from mania to depression in bipolar patients (4). Consequently, study of the acute stress effects of CORT on circadian function throughout the brain may help to uncover the underlying mechanisms by which stress can induce or exacerbate these psychiatric disorders.

CORT: Circadian rhythm and introduction to entrainment of clock genes

Before discussing the effects CORT has on clock gene expression in the SCN, PVN, and DMH and on circadian synchronization, it is important to understand mechanistically how CORT influences these and many other regions of the body. CORT is an endogenous steroid hormone capable of crossing the plasma membrane of a cell and binding to glucocorticoid receptors (GRs), which are present throughout almost the entire body. GRs reside in the cytoplasm of a cell while unbound by glucocorticoids. When CORT binds to the cytoplasmic GR receptor, this GR complex translocates into the nucleus where it can dimerize with another CORT-bound GR. This GR-homodimer acts as a transcription factor by binding to the responsive elements within the promoter region of certain genes, thereby initiating or inhibiting transcription. Many genes throughout the body contain the glucocorticoid response element (GRE) within their promoter region, and thus CORT is capable of affecting many different biological processes. For this reason, we, along with many other researchers, are exploring the molecular basis by which this one hormone can contribute to such a diverse array of biological processes (31).

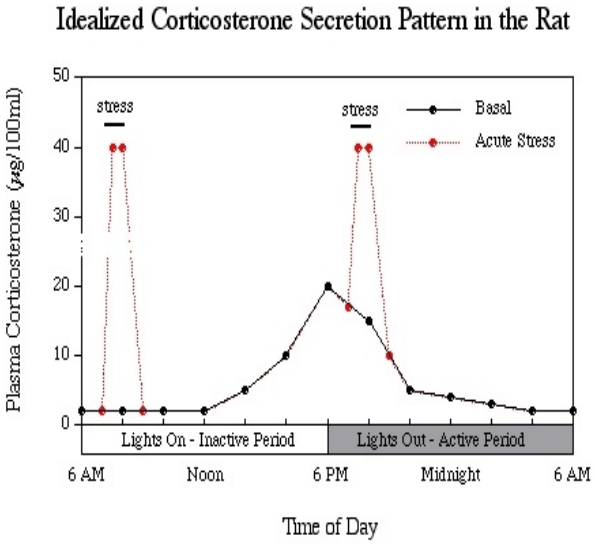

Figure 3. Diurnal secretion of CORT in a normal rat throughout a 24 hour cycle. Stress induced plasma CORT levels peak at the same level, regardless of time of day. Magnitude of change in CORT is much smaller at ZT12 (6PM) than at ZT 4 (9AM). (Spencer Lab Unpublished Figure)

CORT's secretion from the adrenal cortex has two distinct patterns: a strong circadian rhythm under non-stress-related situations and a clear increase with stress. Under normal (i.e., no stress) conditions, CORT concentrations peak at the onset of an animal's active phase. For example, in nocturnal animals, such as rats, CORT concentrations typically peak immediately upon onset of the dark phase (Figure 3). In chronobiological terms, CORT peaks at zeitgeber time (ZT) 12 for nocturnal animals on a 12:12 light:dark cycle. Zeitgebers are exogenous environmental factors, such as light, that

can entrain the circadian rhythm (any biological process that displays a 24-hour rhythm) of an animal. Zeitgeber time, or ZT, is a standardized 24-hour circadian cycle referencing the dominant entrainment factor, light. ZT0 refers to the time of lights-on; ZT4 refers to 4 hours following lights-on, and ZT12 refers to 12hours following lights-on. We utilize these terms as an accepted convention for convenient comparison across research studies.

CORT is also dynamically released upon the onset of a stressor. While the absolute magnitude of circulating CORT levles following a stressful situation is similar throughout the day, CORT's relative increase varies based on time of day (15). The relative increase of stress-induced CORT from baseline is much greater at ZT4 compared to ZT16. This phenomenon is due to a "ceiling effect" that is a consequence of the maximum adrenal CORT production rate, and is illustrated in Figure 3. It is also important to note that stress-induced release of CORT demonstrates a rapid onset and turn-off, while also demonstrating significantly higher concentrations of CORT than CORT's circadian peak at ZT12 under unstressed conditions.

Since we know CORT's diurnal rhythm and that GRs are expressed in nearly every cell of the body, it makes sense that glucocorticoids may play a significant role in the synchronization of the body to the environment (such as photoperiod) (9). Circadian clocks, or internal molecular oscillators that adhere to a 24-hour rhythm, are found not only centrally within many brain regions (such as the PVN and DMH), but also in the peripheral body, including the liver, lungs, spleen, adrenal glands, and uterus. Synchronization between central nervous system clocks and peripheral clocks, in addition to synchronization of cellular clocks within different brain regions, impacts circadian timing, physiology, and behavior (6). Disruptions in circadian rhythm and synchronization of body and brain clocks via the stress-induced disruptions of CORT may be linked to the psychiatric disorders listed earlier. However there is very little evidence to date implicating stress in affecting circadian clocks and altering the circadian functioning of an organism.

Clock Genes: Role of SCN; Interaction between per 1 and bmal 1; Connection with CORT

As a means to coordinate the peripheral and central clocks, virtually all animals have a master clock of the brain and body, the suprachiasmatic nucleus (SCN), which accomplishes this task in part by receiving environmental light impulses. The SCN synchronizes an individual's circadian rhythm with the light:dark cycle by light input from the environment through the retina via the retinohypothalamic pathway (28). Anatomically, the SCN is a bilateral nucleus of the hypothalamus located at the bottom of the third ventricle and rostral to the PVN (Figure 4).

A functional SCN is essential in the synchronization of all the peripheral oscillators to each other and the environment's photoperiod (9). In addition, it aids in controlling the diurnal secretion of glucocorticoids (9). Together, these facts support the idea that the SCN utilizes CORT as a possible form of communication for full-body synchronization. The SCN exhibits neural control of diurnal CORT release via activation of the HPA axis and indirect autonomic innervation of the adrenal gland (9). Even though the SCN is known to modulate diurnal glucocorticoid secretion, it is actually one of the few regions of the brain not known to express any GRs. Consequently, any stressful stimuli that increase CORT levels do not influence gene expression in the SCN.

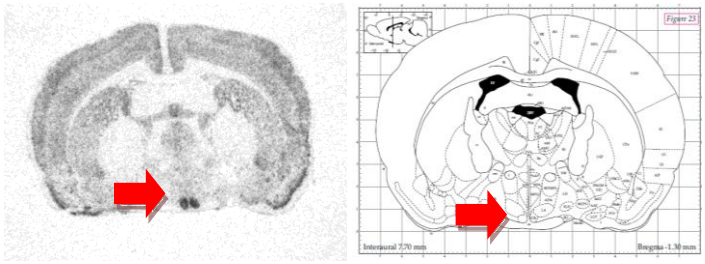

Figure 4. Both images are coronal sections of a rat at the level of the SCN. Image on left is a section with *per 1* mRNA after 30 minutes of restraint stress at ZT4. Image on the right is a representation of a rat brain slice from a rat atlas. The red arrows clearly point out the location of the SCN on both images.

In addition to the SCN, molecular oscillators known as clock genes are involved in the regulation of an individual's circadian rhythm (4). Since the discovery of clock genes, it has been made clear that these molecular oscillators are found within virtually all cells in the body (similar to GRs), including non-SCN brain regions and peripheral tissues such as the pancreas, thymus, and lungs (22, 24). Clock genes are self-autonomous molecular oscillators that sustain a 24-hour rhythm in their expression. There are two groups of clock genes that researchers have come to recognize: core and non-essential clock genes. Core clock genes are genes whose protein products are necessary in the maintenance and regulation of the circadian rhythm

(14). These include *brain and muscle aryl hydrocarbon receptor nuclear translocator* (*bmal1*), the period genes (*per1, per2, and per3*), the cryptochrome genes (*cry1* and *cry2*), and *Circadian Locomotor Output Cycles Kaput* (*CLOCK*). The transcription of the period and cry genes initiates when CLOCK and BMAL1 proteins form a heterocomplex that binds to the E-box of the *per1* and *cry* promoters. The translated PER1 protein then dimerizes with CRY which in turn inhibits the CLOCK-BMAL1 heterocomplex, halting further production of *per1 and cry* mRNA (30). Figure 5 provides a diagram of the interaction between these molecular proteins in *per1* expression. Due to this oscillator mechanism, *bmal1* and *clock* are referred to as the positive arm of the molecular clock, and the period and cry genes are referred to as the negative arm of the molecular clock.

In addition to the core clock genes, two known nuclear receptors, REVERB-α (and its family member REVERB-β) and RORα (also including β and γ isoforms) also play an important role in the transcription of *bmal1* (11). The REVERB family represses *bmal1* transcription, and the ROR family activates *bmal1* transcription. As a result, transcription of *bmal1* is a result of the competition between these two receptor families. These families of nuclear receptors are significant components of the molecular circadian clock (11). However, these genes are considered non-essential; knocking out these clock genes does not ablate the circadian rhythm of the core clock genes. However, it is important to note that while the rhythms are not ablated, knocking out *Reverb-α* in animal models alters the circadian rhythm, with an increase in *bmal1* and *clock* expression. Furthermore, *Rorα* and *Rorβ* knockout mice display aberrant circadian rhythm, marked by a blunted expression of *bmal1* (26).

As previously mentioned, CLOCK-BMAL1 dimer binds to an E-box located within *per1*'s promoter region. Additionally, there is also a cAMP-responsive element (CRE) within the *per1* promoter region, which binds CREB protein in response to light input received from the SCN to initiate *per1* transcription (30). A final important component of the promoter region of *per 1* is the glucocorticoid response element, or GRE. Glucocorticoids bind to GRs to become a transcription factor for the transcription of *per1* by binding the GRE, suggesting that diurnal and/or stress-induced CORT can induce *per1* gene expression in brain regions containing GRs, such as the PVN and the DMH. Abnormalities in CORT secretion could accordingly disrupt the body's circadian rhythm over time (18, 30).

Unlike the SCN, which lacks GRs, there are documented non-SCN regions of the brain that contain GRs where CORT can influence clock gene expression; these brain regions include the central nucleus of the amygdala, the bed nucleus of the stria terminalis (22), the PVN, and the dorsomedial hypothalamus (DMH) (24). It is commonly believed that a large

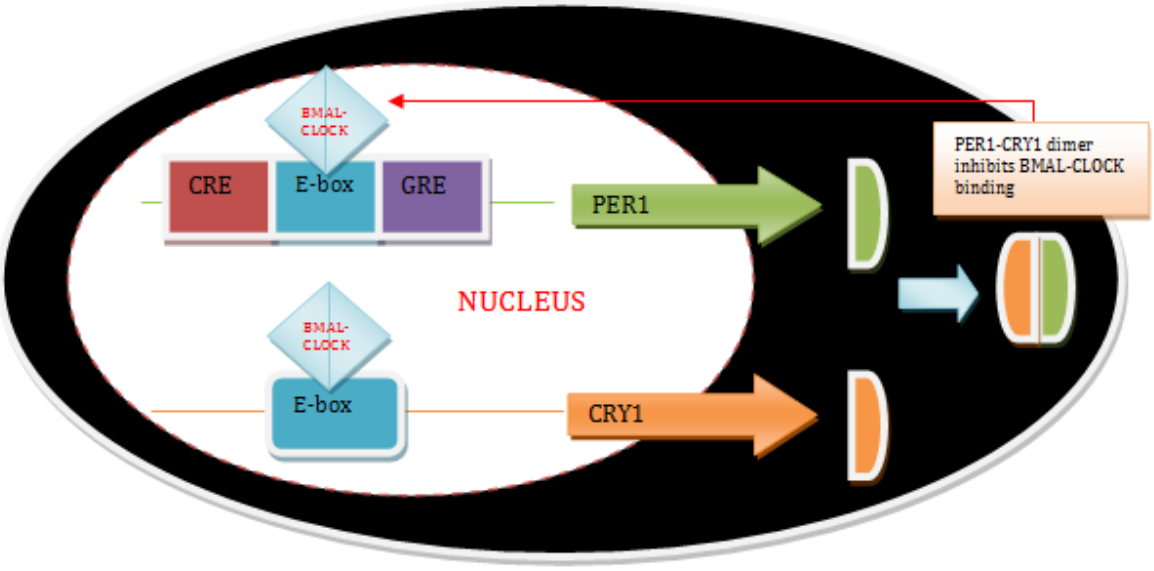

Figure 5. Diagram outlining the regulation of *per1* transcription. This shows BMAL-CLOCK heterodimer binding to E-box of *cry1* and *per1* initiating transcription, and eventually translation of protein. All transcription takes place in nucleus of cell.

portion of hormonal secretions and bodily functions are regulated by clock gene expression (21). However, with the most recent data implicating CORT's ability to influence clock gene expression, it is in our interest to further characterize how CORT may actually regulate clock gene expression.

For the remainder of this paper, we will focus on two of the core clock genes: *bmal1* and *per1*. The expressions of these genes are in anti-phase with each other, meaning that as one gene peaks in expression, the other is at its trough in expression (9). *Per1* expression is known to have a diurnal rhythm with a peak at about ZT4 and trough at ZT16 in the SCN in rats. Expression of *bmal1* in the SCN peaks at ZT16 and troughs around ZT4. This anti-phasic relationship between *per1* and *bmal1* is possibly due to their oscillatory characteristic by which *bmal1* acts as the positive arm of the molecular clock while per1 acts as the negative arm. It is also important to note that per1 expression within the PVN is anti-phasic to *per1* expression within the SCN; similarly, *bmal1* expression in the PVN is

anti-phasic to *bmal1* expression in the SCN (when *per1* peaks in SCN, *per1* troughs in PVN) (9).

Clearly, these core clock genes play a significant role in the regulation of an individual's circadian rhythm. However, the diurnal expression of these clock genes can be tampered with, which could have negative effects on an individual's circadian rhythm. For example, abnormalities or shifts in the circadian rhythm are associated with specific types of psychiatric disorders, including seasonal affective disorder (SAD) (2), bipolar disorder, and major depression (17). In fact, studies have shown that a single nucleotide polymorphism (mutation) in CLOCK, a core clock gene, is associated with bipolar disorder; because this mutation is not linked to major depressive disorder, we have reason to believe it is involved in the mania-hypomania phenotypes of bipolar disorder (26, 24). In turn, single nucleotide polymorphisms in CRY1 demonstrate a significant association with both major depressive disorder and bipolar disorder (26). With this increasing classification of consequences in polymorphic clock genes, new antidepressant drugs could potentially emerge that could target and correct abnormalities in the circadian timing system (13). Clock genes such as per1 and bmal1 have strong interactions with CLOCK and CRY and are consequently important to characterize the behavioral and physiological consequences of their disruptions.

Obviously, maintaining a normal diurnal rhythm of core clock genes is important in avoiding disorder. What if rhythmic CORT is necessary in maintaining that normal diurnal rhythm; what would happen to that rhythm if there were a stress-induced disturbance in CORT? Research within and outside our lab has shown that CORT can indeed entrain and influence clock gene expression in non-SCN regions. Specifically, the Spencer lab is interested in characterizing exactly how circadian variations in CORT and stress-induced CORT can influence clock gene expression in extra-SCN brain regions (SCN is not influenced by any such changes in CORT due to its lack of GRs). From unpublished data in our lab, we have found that whether or not stress induces a significant change in clock gene expression is highly dependent on the specific brain region involved and the specific clock gene under investigation. The exact mechanism by which endogenous CORT accomplishes the entrainment of clock genes, such as *per 1* and *bmal*, is still unknown. Furthermore, whether altered clock gene expression due to stress-induced changes in CORT causes

disorder is also unknown. Exploring the effects that acute psychological stress has on clock gene expression in various brain regions gives us an insight into how circadian rhythms regulate all aspects of the body, and how stress may disrupt that normal process.

Dorsomedial Hypothalamus: Importance and basis for research focus

While the importance of normal clock gene expression is becoming clearer, very few labs have examined clock gene expression and its regulation in brain regions other than the SCN. Because it is the master clock of the body, it makes sense that many studies have researched clock gene expression in the SCN. Also, because the PVN is the head of the HPA axis, investigating it is another obvious choice. However, why we decided to feature the DMH in this study, the final hypothalamic region of interest, may not be as apparent.

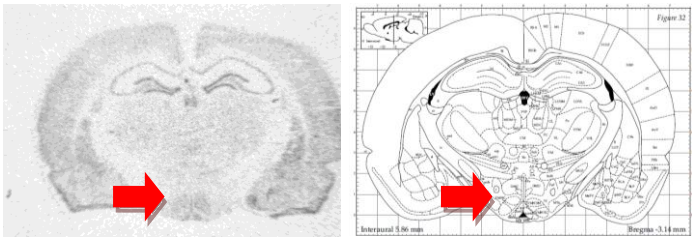

Figure 6. Both images are coronal sections of a rat at the level of the DMH. Image on left is a section with *per 1* mRNA after 30 minutes of restraint stress at ZT4. Image on the right is a representation of a rat brain slice from a rat atlas. The red arrows point to the location of the DMH on both images.

Anatomically, the DMH is caudal to both the SCN and PVN. Similar to these structures, it is a hypothalamic nucleus located on either side of the third ventricle. Figure 6 provides a visual representation of the relative location of the DMH. The DMH has been acknowledged as a putative food-entrainable oscillator, meaning food availability can entrain the molecular clock of this region. This region and the SCN have been classified as key players in the coordination of physiological responses to feeding cues and the light:dark cycle, respectively (9). Due to a dual importance of physiological regulation shown by these two regions, it makes sense that SCN neurons have a significant number of projections to the DMH (21), and vice versa (10). Studies have shown that this close connection between the two regions is partly due to the DMH's role as a food-entrainable oscillator (9). Via inhibitory GABA neurons, the DMH can inhibit neuronal activity in the SCN, as measured by c-fos, according to an organism's food availability (10). This interaction between the SCN and DMH is significant to this project because it suggests the DMH's importance for the regulation of the body's circadian rhythm.

In conjunction with being a well-known food entrainable oscillator and having significant neuronal connections to the SCN, the DMH has been shown to have influences on the HPA axis and the sympathetic nervous system, both important elements of the stress response. Regarding the sympathetic response, research has shown that microinfusion of muscimol, a $GABA_A$ receptor agonist, into the DMH eliminates the stress-induced increase of arterial pressure and heart rate, or tachycardia (7). Conversely, blockade of $GABA_A$ receptors via bicuculline methiodide (BMI), in the DMH also promotes increases in heart rate and blood pressure. This implies that activity of some neurons within the DMH plays a critical role in the sympathetic cardiac stimulation seen in stress and anxiety.

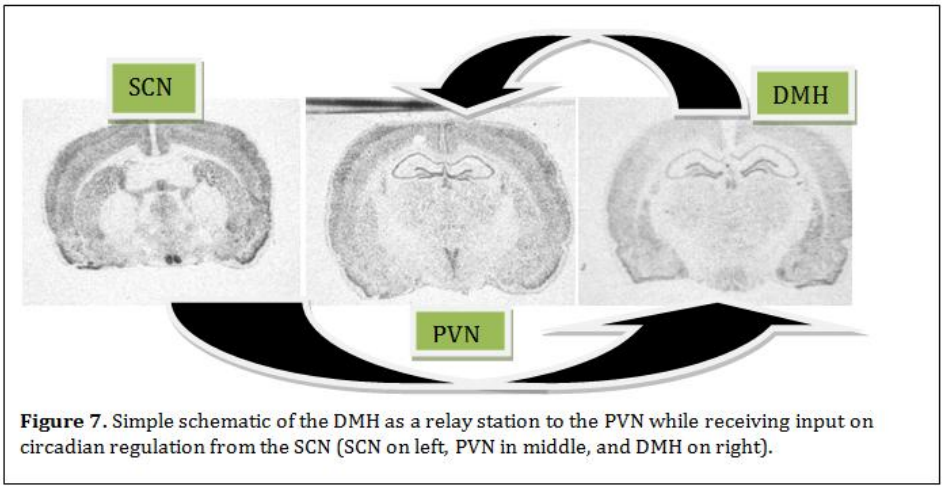

Figure 7. Simple schematic of the DMH as a relay station to the PVN while receiving input on circadian regulation from the SCN (SCN on left, PVN in middle, and DMH on right).

Concerning the HPA axis, there is evidence for the DMH being a major relay center to the PVN for circadian rhythm influences from the SCN (Figure 7). In fact, lesioning the DMH disrupts the diurnal secretion of CORT (12). Moreover, research shows that the DMH is involved in the activation of the HPA axis by emotional and exteroceptive forms of stress via glutamate-mediated excitatory input to neurons in the PVN (7). Microinfusion of the $GABA_A$ receptor antagonist, BMI, into the DMH, but not the PVN, increases plasma ACTH and CORT in rats. A $GABA_A$ receptor antagonist selectively inhibits $GABA_A$ neurons, which are inhibitory in nature, thus leading to an overall increase in PVN neural activity, thus increase in ACTH and CORT. As a result, the PVN and DMH could possibly be a part of a hypothalamic circuit that coordinates the physiological, behavioral, and neuroendocrine responses associated with stress (12). If CORT really is an important factor in the synchronization of an individual's circadian rhythm, clock gene expression in the DMH is an important area of research that needs to be investigated.

In conjunction with this overwhelming evidence of the importance of the DMH in the stress response, a collection of research has also shown that the DMH is a major influencing factor on an enormous array of behavioral circadian rhythms (3). Some of these behaviors include wakefulness, feeding, and locomotor activity, which are behaviors that can be disrupted by CORT and are associated with symptoms of major depressive disorder. We believe that disturbances in circadian rhythms have a strong impact on these symptoms experienced by depressive patients, and that disruptions in clock gene expression in the DMH may play an important role.

Experiment: Reasoning and brief description

There is a substantial amount of evidence that suggests CORT's importance as a possible synchronizer of the organism to the environment. However, there are very few labs, outside our own, that have directly investigated this potential. For this experiment, we looked at the effects of circadian variations in basal and stress-induced CORT on clock gene expression in the DMH, PVN, and SCN using rats as an animal model. Specifically, we chose to investigate the clock genes *bmal1* and *per1*. Eventually, it will be beneficial to analyze all core clock genes within the DMH, but due to time constraints, we chose *per1* and *bmal1* as the most logical choices for this study. We decided on *per1* because we have unpublished data in our lab demonstrating *per1* expression in the DMH. In addition, our lab also has data that show that CORT affects *per1* mRNA expression in the PVN. We chose *bmal1* because this gene is in anti-phase with *per1.*

Because the irregular expression of CORT (possibly stress-induced) and the desynchronization of an individual's circadian rhythm appear to be strongly associated with previously discussed disorders, we attempted to disrupt the circadian rhythm, and therefore *bmal1* and *per1* mRNA rhythmic expression in rat models by eliminating endogenous CORT and/or exposing rats to an acute psychological stressor. We eliminated CORT by performing an adrenalectomy surgery on half of the rats (by removing the adrenal glands, rats cannot synthesize and release CORT into their blood stream). If endogenous CORT is necessary for the normal diurnal rhythm of *per1* and *bmal* within any of these brain regions, we expected that we would see a clear difference in the diurnal expression of these genes between ADX no stress and SHAM no stress groups. If endogenous CORT is

necessary for acute stress to exert its effects on *per1* and *bmal1* expression in these brain regions, we expected to see a blunted stress effect reflected in clock gene mRNA expression in the ADX stress group compared to the SHAM stress group. If this holds true, it furthers the idea that CORT can act as an entraining factor for clock gene expression.

Our first hypothesis was that *bmal1* expression within all three brain regions would remain unaffected by any CORT manipulations due to the absence of a GRE in its promoter region. We also hypothesized that CORT would be necessary for both the diurnal expression of *per1* and stress-induced expression of *per1* in the DMH and PVN, but not the SCN. (The SCN does not have GRs, so we did not expect any changes in clock gene expression in the SCN due to any manipulations of CORT). Because the DMH may be a potential circadian relay station to the PVN, we expected these two regions to have similar *per1* and *bmal1* diurnal expression, in addition to similar effects from the two different CORT manipulations. Whether ADX or stress effects on *bmal1* and *per1 expression* in the PVN are secondary to alterations in the DMH would be difficult to infer from this experiment.

Furthermore, unpublished data in our lab demonstrate the ability of an acute psychological stressor to alter clock gene expression in many brain regions. We believe that this stress-induced disruption in clock gene expression has the potential to desynchronize an individual's circadian rhythm over time, which is commonly observed in patients with certain psychiatric disorders, including seasonal affective disorder (SAD), major depressive disorder, and bipolar disorder (1, 4, 27). Clearly, the exact means by which stress potentially contributes to the desynchronization of an individual's circadian rhythm and acts as an indicator or exacerbator for these psychiatric disorders is not well understood and needs to be investigated further. Thus, our study explored the foundational building blocks of this gap in scientific knowledge by investigating clock gene expression in three hypothalamic nuclei (SCN, PVN, and DMH), which all have strong interactions with the physiological aspect of the body's stress response.

There has not been a study characterizing how endogenous and stress-induced CORT influences clock gene mRNA expression in the DMH, and whether CORT is necessary to alter this expression. Our results bring about unanswered questions that lead us closer in understanding the importance of CORT in the circadian regulation of the SCN, PVN, and

DMH, and consequently the synchronization of the whole body to the SCN's molecular clock.

Methods:

Subjects

Subjects were 48 two-to-three-month-old male Sprague-Dawley rats, obtained from a commercial vendor (Harlan Laboratories), and weighing between 260 g and 316 g two weeks after arrival at our animal housing facility. Half of the rats were put on a normal 12:12 h light:dark schedule (lights ON at 6:00 am, lights OFF at 6:00 pm), and the remaining rats were put on a 12:12 h reverse light:dark schedule (lights OFF at 6:00 am, lights ON at 6:00 pm), for test day and experimental manipulation convenience. The rats were then randomly distributed among four different rooms. Then, the rats were allowed to acclimate to their new housing conditions for two weeks. This was to ensure that the results from the rats in the control group and ADX groups were not attributed to the stress involved with the new environment, which can alter CORT release. Furthermore, our lab has shown (unpublished data) that a rat's behavioral and physiological circadian rhythms fully adapt within 2 weeks to a reversed light:dark cycle. All rats were pair-housed in polycarbonate tubs (47 cm x 23 cm x 20 cm) with full access to water and food ad libitum. All care, handling, and use of animals followed ethical guidelines posed by the University of Colorado Institutional Animal Care and Use Committee and adhered to the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Surgery: SHAM vs. ADX

The experiment used a 2x2x2 factorial design; the independent variables were the time of day (ZT4 or ZT16), the presence or absence of endogenous CORT (SHAM or ADX), and whether the rats underwent restraint stress or not (n=6-7 per treatment group, N=50). Two weeks after the rats arrived, we performed an adrenalectomy surgery (ADX) on half of the rats (ADX group). These rats were given bilateral dorsolateral incisions while under halothane anesthetization and had their adrenal glands removed. The remaining half of the rats underwent a control (SHAM) surgery, where rats remained adrenal-intact, but still

endured a sham surgery in which all surgical procedures matched those of the ADX group except the adrenals were left in place. This resulted in two glucocorticoid status test groups. The SHAM group served as a comparison to the ADX group to ensure surgical stress was not a confounding factor in our results. All rats were administered 0.33 mL of Baytril antibiotic intramuscularly and 1mL of saline subcutaneously immediately following surgery. Rats were allowed one week to recover from surgery before being tested. During this time, ADX rats were given a 0.9% saline solution as their primary source of drinking water in order to compensate for their inability to retain ions and water as a result of the removal of endogenous aldosterone.

Test day: Restraint Stress

To simulate psychological stress, the rats receiving stress were placed in a cylindrical Plexiglas tube (23.5 cm long, with a diameter of 7 cm, with several air holes) for 30 minutes to restrict the rat's movement without causing any physical harm or pain (5). This restraint procedure occurred at ZT3.5 or ZT15.5. Immediately following restraint, the rats were sacrificed by rapid decapitation. The rats in the No Stress group were left in their home cage and sacrificed at ZT4 or ZT16. On test day, half of the rats were sacrificed at ZT4 and the remaining half at ZT16. These times were chosen based on previous experiments showing that both *bmal1* and *per1* mRNA peak and trough during these times in both the SCN and PVN (9).

Tissue and Blood Collection

Rats were taken either directly from their home cages or from the restrainers and immediately decapitated with a guillotine. Trunk blood was collected into EDTA-coated test tubes from Becton-Dickinson (Franklin Lakes, New Jersey, USA) and immediately placed on ice. These blood samples were then spun down in the centrifuge to separate plasma from blood cells for 10 minutes at 4,000 rpm at 4°C, and stored for the ACTH radioimmunoassay in 125-µL aliquots and for the CORT ELISA in 25-µL aliquots. They were kept at -80°C until use.

In addition to collecting trunk blood samples, we also extracted pituitary glands and brains. All pituitaries were collected into the cap of a 1.7 ml polypropylene tube filled with M1 embedding matrix. Upon extraction of the brains, we used dry-ice chilled isopentane (between -20°C and -30°C) to flash freeze the brains, which were then wrapped in aluminum foil and stored in a -80°C freezer. Using a cryostat, the brains were then sliced into 12-μm thick coronal sections onto electrostatically charged plus slides, which were also stored in the -80°C freezer to be used later for in situ hybridization.

ACTH Radioimmunoassay

Plasma ACTH was measured (pg/ml) in duplicate (125-μl plasma) using a radioimmunoassay (RIA). This RIA procedure is adapted from a well-established protocol (16) and modified by the Spencer lab. ^{125}I radiolabeled ACTH was obtained from PerkinElmer Inc. (Santa Clara, CA, USA); primary ACTH rabbit antiserum (Rb7, final dilution 1:40,000) was donated by Dr. Robert England (University of Minnesota). The intra-assay coefficient of variability was 6.6%.

CORT ELISA

Plasma CORT was measured (ng/ml) in duplicate (100 μl plasma) using an ELISA kit (Arbor Assays, Ann Arbor, MI) according to the kit's instructions. 20 μL of plasma in 980 μL of buffer was then heat-inactivated at 65°C for 60 minutes. The intra-assay coefficient of variability for the assay was 0.7% and 2.8% for quality control samples containing low or moderate levels of CORT, respectively.

In situ Hybridization and Image Analysis

Using a cryostat, 12 μm coronal sections of the rat brain were obtained at the levels of the SCN (bregma~-1.35 mm), PVN (bregma~-1.80 mm), and DMH (bregma~ -3.00 mm). Sections were mounted on electrostatically charged slides (plus slides, VWR), and stored at -80°C.

A two-day in situ hybridization procedure was used for the analysis of *bmal1* and *per1* mRNA in the SCN, PVN, and DMH. We followed the protocol previously described in

Girotti et al. (9), with some provisions that should be noted. Slides were fixed in 4% PFA (paraformaldehyde), washed in 2X SSC (saline-sodium citrate buffer) washes, and then treated with triethanolamine (TEA). We added acetic anhydride to the TEA, which is thought to be important for decreasing background signal and strengthening specific signal by inactivating RNase. Following the TEA/acetic anhydride treatment, we dehydrated the slides via graded ethanol washes (50%, 75%, 95%, 95%, 100%, and 100%). Once air-dried, slides were cover-slipped with 65µl of radioactive hybridization buffer (with 1.5e6 cpm of radioactivity per slide). The hybridization buffer contains a riboprobe made earlier in the day through a series of incubations in a 37°C water bath and further separation via a column using G50/50 sephadex. Both bmal1 and per1 plasmids were generated in house from rat cortex total RNA using the RT-PCR method (9). Transcription-buffer, DNASE, T7 (per1 RNA polymerase) and T3 (bmal1 RNA polymerase) polymerases, and GCA nucleotide components were all obtained from PROMEGA (Madison, WI, USA). 0.1M DTT (Dithiothreitol) and RNASE Inhibitor were obtained from Invitrogen (Canada). All these ingredients were used to make the S_{35}-labeled riboprobe.

Radioactive slides were placed on film for 3 weeks before being developed by an auto-developer (Konica Minolta Medical and Graphic, Inc.). All brain images on film were captured using Scion Image (Scion Corporation). Images from previous capture were analyzed further at the levels of the SCN, PVN, and DMH for *per1* and *bmal1* mRNA expression utilizing ImageJ, a program designed to measure the optical density of specific brain regions guided by the user. Four sections at each level of the SCN, PVN, and DMH were analyzed per brain. The location of these individual regions of interest was determined using a rat brain atlas (Paxinos and Watson, 1998). For analysis of these regions using ImageJ, we took a rectangular outline of the entire brain region of interest.

Statistical Analysis

All statistical tests were performed using the SPSS statistical analysis program 10.5 (Chicago, IL, USA) for Macintosh operating system. The data were first analyzed using univariate analysis to determine whether there was an effect of stress, adrenalectomy, time of day, or the interaction between each of the independent variables. If there was a

significant F-value ($p < 0.05$), Fisher's least significant difference (LSD) post-hoc test was performed in order to examine pair-wise comparisons of interest. Groups were considered to be significantly different if the post-hoc test had a p-value < 0.05.

Results:

ACTH RIA Assay

ACTH profiles in rats with ADX and/or stress manipulations have been well established. Thus, ACTH data were collected in this experiment primarily to demonstrate the effectiveness of the experimental manipulations and hence the validity of the other data collected. (The ACTH data were expressed as pg/ml, Figure 8). A logit transformation was used to interpolate the data from a standard curve; the final statistical analysis was run on non-normalized ACTH values.

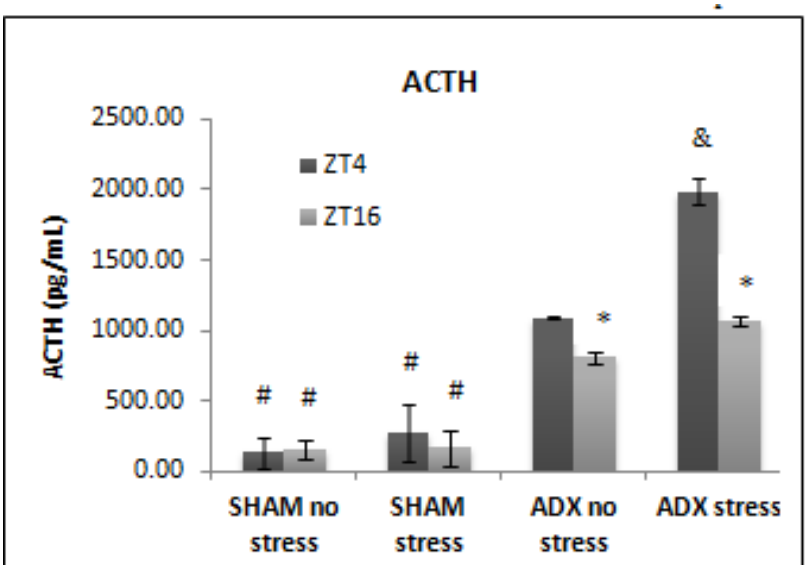

Figure 8. ACTH data from experiment. #Significant difference between SHAM and ADX cohorts at the same time of day and same stress effect. *Significant difference between ZT time within the same stress and adrenalectomy effect group. &Significant difference between stress and no stress groups within the same ZT time and ADX effect. All significances were established through Fisher's LSD post-hoc test ($p<.05$).

There were a clear ZT effect ($F_{1,42} = 19.809$, $p<0.05$), a very strong adrenal status effect ($F_{1,42} = 231.283$, $p<0.05$), and a stress effect ($F_{1,42} = 24.433$, $p<0.05$). In addition, there were significant interactions between ZT and adrenal status ($F_{1,42} = 14.198$, $p<0.05$), ZT and stress condition ($F_{1,42} = 7.509$, $p<0.05$), and adrenal status and stress condition ($F_{1,42} = 8.854$, $p<0.05$). Fisher's LSD post-hoc test showed significant difference between all SHAM and ADX groups (comparison between groups at the same time of day and with the same stress effect) ($p<.05$). It also revealed a significant time of day difference within the ADX groups

with the same stress effect ($p<.05$). Finally, there was a significant stress effect in the ADX stress group compared to the ADX no stress group at ZT4 ($p<.05$).

CORT ELISA Assay

Similar to ACTH levels in rats with ADX and/or stress manipulations, CORT response to these changes is also well established. The CORT data we collected validate the effectiveness of the acute stress and adrenalectomy effects on the HPA axis. (The CORT data are expressed as ng/ml; Figure 9 – note that ADX groups are not displayed because they had undetectable levels of CORT, as expected).

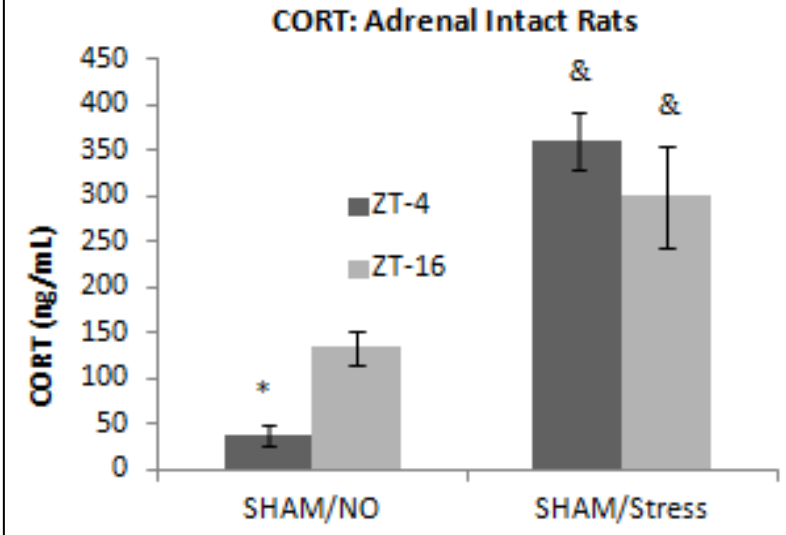

Figure 9. CORT data for SHAM treatment groups. . *Significant difference between ZT times within the same stress effect group. &Significant difference between stress and no stress groups within the same ZT time. All significances were established through Fisher's LSD post-hoc test ($p<.05$).

There was a clear adrenal status effect ($F_{1,42}$ = 329.892, $p<0.05$) and a stress effect ($F_{1,42}$ = 59.294, $p<0.05$). Fisher's LSD post-hoc test revealed a significant ZT time difference between the SHAM no stress cohorts ($p < 0.05$). This test also showed a clear stress effect between both no stress groups and stress groups at the same ZT time (ZT4 or ZT16) ($p<.05$). In addition, there was a significant two-way interaction between ZT and stress condition ($F_{1,42}$ = 9.534, $p<0.05$) and adrenal status and stress ($F_{1,42}$ = 63.369, $p<0.05$), and a three-way interaction between ZT, adrenal status, and stress status ($F_{1,42}$ = 9.077, $p<0.05$).

In Situ Hybridization Image Analysis: per1 mRNA in SCN and PVN

Data collected from the in situ hybridization testing for *per1* mRNA expression in the SCN and PVN is represented in Figures 10 and 11, respectively. Similar to the CORT and ACTH results, the *per1* expression in the SCN and PVN validates that in the SHAM no stress group, *per1* expression was high at ZT4 in the SCN and at ZT16 in the PVN, and lower at ZT16 in the SCN and ZT4 in the PVN. In addition, our results show that stress did in fact cause an increase in *per1* expression in the PVN but not the SCN. Also, there was a clear adrenalectomy effect in the PVN, indicated by a blunted stress effect and blunted time of day effect in ADX rats (Figure 11).

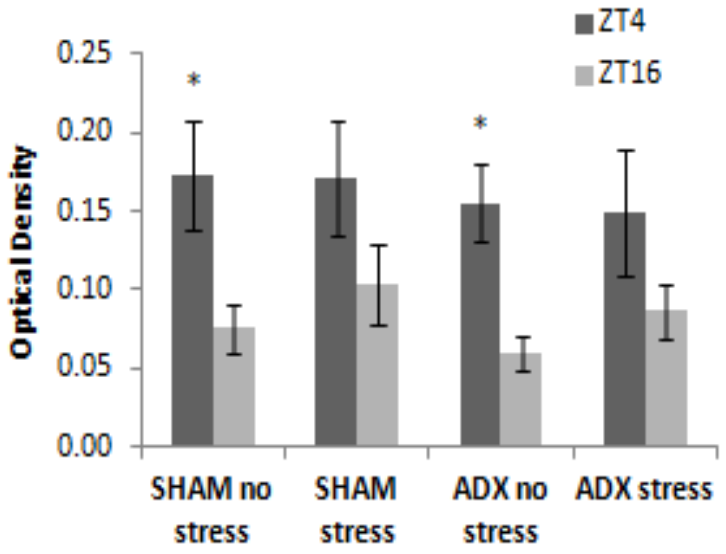

Figure 10. *Per1* data from in situ hybridization. *Significant difference between ZT time within the same stress and adrenalectomy effect group. All significances were established through Fisher's LSD post-hoc test ($p<.05$).

In the SCN, there was a significant ZT effect ($F_{1,41} = 19.567$, $p<0.05$). All other effects and interactions were non-significant. The Fisher's LSD post-hoc test revealed significant ZT time differences between SHAM no stress groups and between ADX no stress groups ($p<.05$).

In the PVN, there was a significant stress effect ($F_{1,40}$ = 10.360, p<0.05). Also worth noting was a nearly significant ZT effect ($F_{1,40}$= 3.764, p=0.059), trending towards having higher *per1* expression at ZT16. Finally, there was a significant interaction between adrenal status and stress status ($F_{1,40}$ = 4.328, p<0.05). There was no significant post hoc time of day difference in sham no stress rats, contrary to previous unpublished data in our lab. However, there was a noticeable trend between the ZT times, as seen in Figure 11. Fisher's LSD post-hoc test revealed a significant stress in the SHAM groups (p<.05). In addition, the tests demonstrated a significant adrenalectomy effect between ZT4 SHAM stress rats and ZT4 ADX stress rats (p<.05).

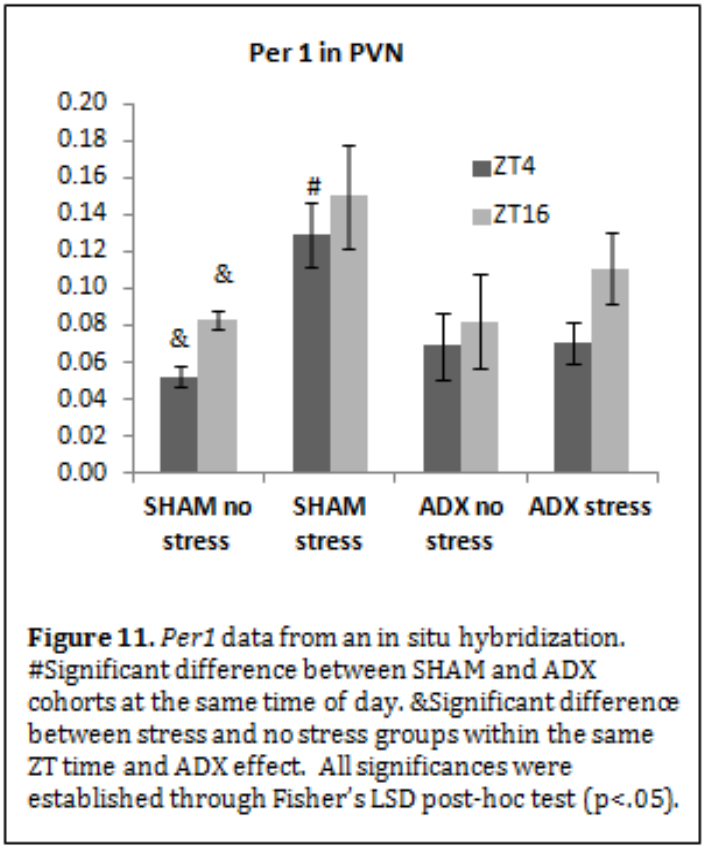

Figure 11. *Per1* data from an in situ hybridization. #Significant difference between SHAM and ADX cohorts at the same time of day. &Significant difference between stress and no stress groups within the same ZT time and ADX effect. All significances were established through Fisher's LSD post-hoc test (p<.05).

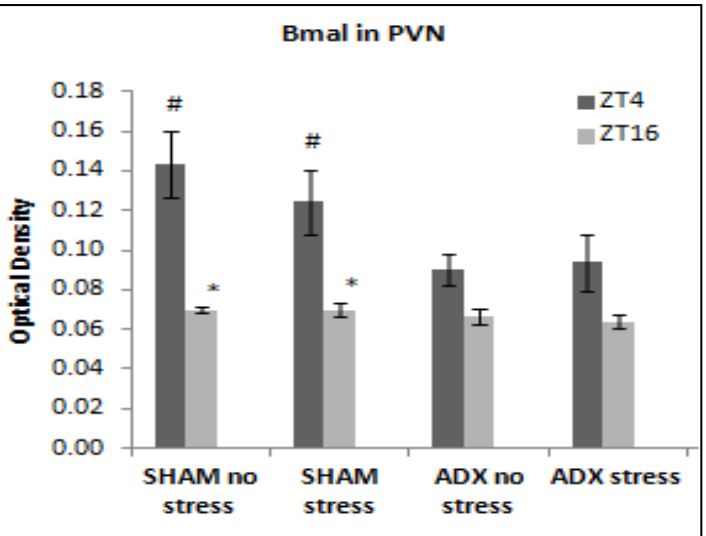

Figure 13. *Bmal1* data from in situ hybridization. #Significant difference between SHAM and ADX cohorts at the same time of day. *Significant difference between ZT time within the same stress and adrenalectomy effect group. All significances were established through Fisher's LSD post-hoc test (p<.05).

In Situ Hybridization Image Analysis: bmal1 mRNA in SCN and PVN

Bmal1 mRNA expression in the SCN and PVN is shown in Figure 12 and 13, respectively. Similar to the results for *per1* expression in the PVN and SCN, *bmal1* expression in the SCN and PVN validates that *bmal1* was high at ZT16 in the SCN and at ZT4 in the PVN. It is also

worth noting the presence of a clear ADX trend between SHAM no stress and stress groups at ZT4 and ADX no stress and stress groups at ZT4 in the PVN.

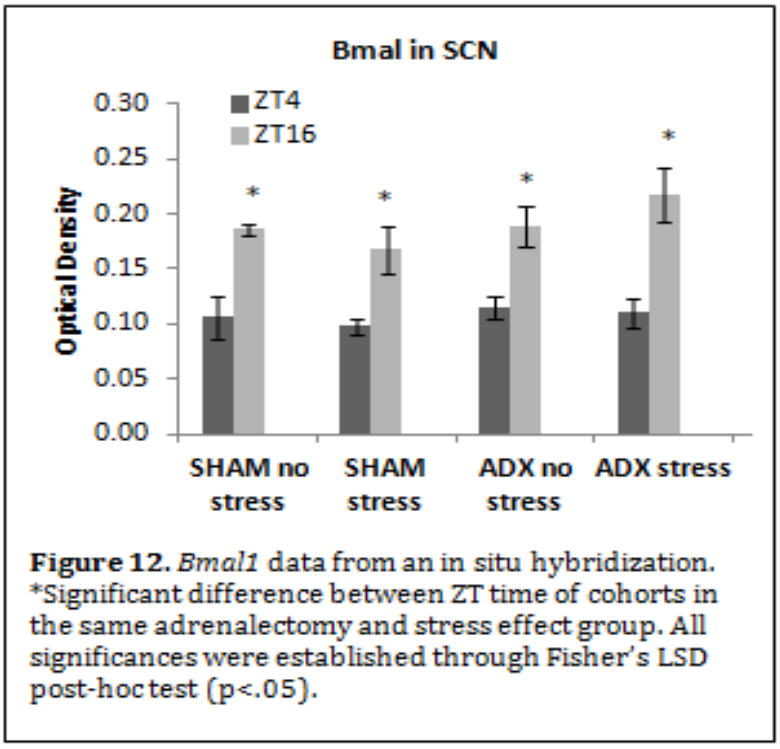

Figure 12. *Bmal1* data from an in situ hybridization. *Significant difference between ZT time of cohorts in the same adrenalectomy and stress effect group. All significances were established through Fisher's LSD post-hoc test (p<.05).

In the SCN, there was a significant ZT effect ($F_{1,42}$ = 50.597, p<0.05). Specifically, the Fisher's LSD post-hoc tests revealed a clear significant difference between all ZT4 and ZT16 groups (p<.05). All other effects and interactions were non-significant (Figure 12).

In the PVN, there was a significant ZT effect ($F_{1,39}$ = 36.534, p<0.05) and adrenal status effect ($F_{1,39}$ = 9.581, p<0.05). Post-hoc tests revealed a significant adrenal status difference between ZT4 no stress rats and between ZT4 stress rats (p<.05). In addition, tests showed a significant ZT difference between both SHAM no stress groups and between SHAM stress groups (p>.05). Finally, there was a significant interaction between adrenal status and ZT time ($F_{1,39}$ = 6.170, p<0.05) (Figure 13).

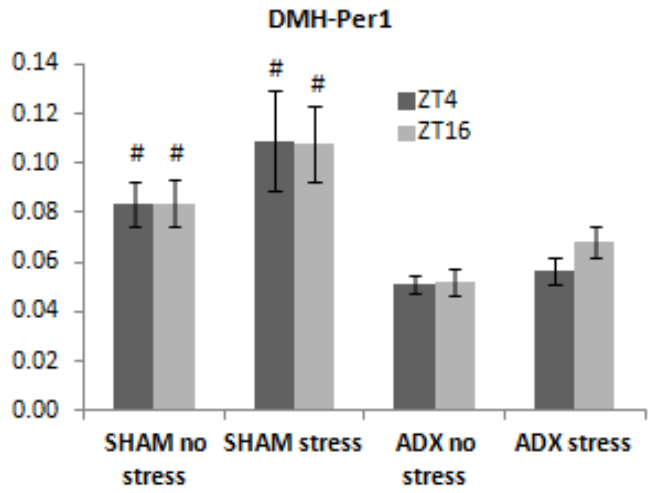

Figure 14. *Per1* data from in situ hybridization. #Significant difference between SHAM and ADX cohorts at the same time of day. All significances were established through Fisher's LSD post-hoc test (p<.05).

In Situ Hybridization Image Analysis: per1 and bmal1 in DMH

In contrast to the SCN and PVN, there was no ZT effect on *per1* expression in the DMH. *Per1 mRNA* in the DMH, however (Figure 14) exhibited a clear ADX effect ($F_{1,42}$ = 30.870, p<0.05) and a stress effect ($F_{1,42}$ = 6.689,

p<0.05). Fisher's LSD post-hoc tests revealed significant adrenal status differences between all groups of the same ZT time and stress condition (p<.05). There were no significant interactions between the treatment factors.

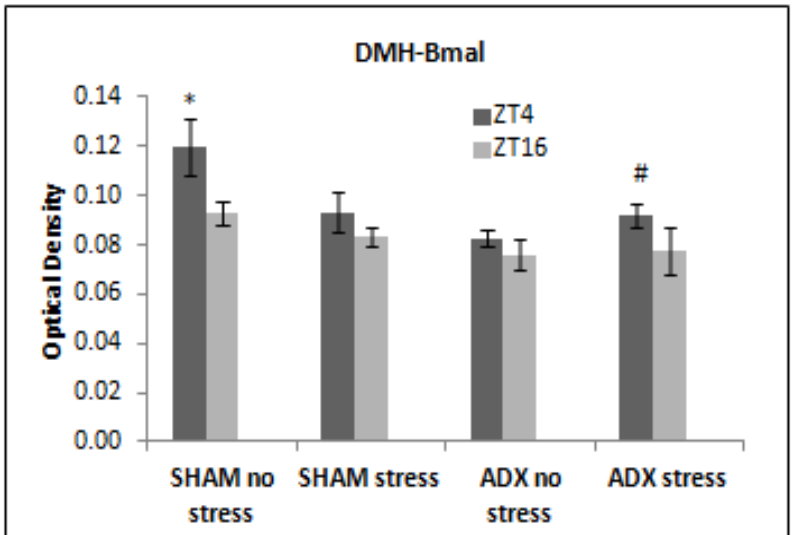

Figure 15. *Bmal1* data from in situ hybridization. #Significant difference between SHAM and ADX cohorts at the same time of day. *Significant difference between ZT time within the same stress and adrenalectomy effect group. All significances were established through Fisher's LSD post-hoc test (p<.05).

Bmal1 expression in the DMH exhibited a ZT effect ($F_{1,42}$ = 8.301, p<0.05) and an adrenal status effect ($F_{1,42}$ = 9.461, p<0.05). Post-hoc tests showed a significant ZT time difference between both SHAM no stress groups (p<.05). Tests also revealed a significant adrenal status effect between ZT4 SHAM stress and ZT4 ADX stress (p<.05). In addition, there was a significant interaction between adrenal status and stress effect ($F_{1,42}$ =5.523, p<0.05).

Discussion:

As an attempt to further characterize clock gene expression in regions of the brain outside of the SCN, we looked at *bmal1* and *per1* mRNA expression in the DMH and PVN, in addition to the SCN. We also wanted to examine how stress-induced and endogenous CORT may play a role in the circadian rhythm of clock genes in these specific brain regions. To understand how changes in an individual's homeostasis while experiencing acute stress can influence *bmal1* and *per1* mRNA expression in the DMH, PVN, and SCN, we manipulated CORT levels in order to determine whether circadian or stress effects on extra-SCN clock gene expression were dependent on CORT. Thus, we investigated whether or not endogenous CORT is necessary for acute restraint stress to induce changes in *bmal1* and *per1* mRNA expression in the DMH, SCN, and PVN, and whether the diurnal CORT rhythm is necessary for the rhythmic expression of these genes.

Based on our findings, endogenous CORT is indeed necessary for acute restraint stress to induce changes in *per1* mRNA expression, but not *bmal1* mRNA expression in the DMH and PVN. The lack of effect in *bmal1* expression was possibly due to its lack of a glucocorticoid response element (GRE) in its promoter region, which *per1* has. In the SCN, neither stress nor adrenal status influenced *bmal1* or *per1* expression due to the absence of GRs. The trends in the ACTH and CORT data were consistent with previous findings (16), with a clear increase in CORT with restraint stress in SHAM rats and low levels of ACTH in SHAM groups due to glucocorticoid negative feedback. ADX rats did not have detectable levels of CORT, and did have abnormally high levels of ACTH due to the absence of glucocorticoid negative feedback. This is also consistent with previous findings (16). Because these results report well-known trends in the literature, we can place more confidence in the new clock gene findings in this experiment.

ACTH/CORT Data

The ACTH levels collected in various treatment groups matched what is established in the literature (8). There were significantly lower levels of ACTH in the SHAM groups compared to the ADX groups regardless of time of day. This phenomenon can be explained by the absence of tonic presence of CORT in ADX groups. In the absence of CORT, there is no glucocorticoid negative feedback inhibition of the PVN or the anterior pituitary, thereby leading to over production of ACTH, and consequently, higher levels of ACTH in the plasma. There was also a very clear activation of the HPA axis in the rats that experienced 30 minutes of restraint stress in ADX groups. Because SHAM groups have CORT that participate in glucocorticoid negative feedback, thereby controlling ACTH levels, we did not see as large a difference between SHAM no stress and SHAM stress treatment groups. Also, ACTH generally peaks about 15 minutes after the onset of stress due to the initiation of glucocorticoid negative feedback (16). Because we restrained rats for 30 minutes instead of 15 minutes, we may not have seen the peak of stress-induced ACTH levels. What is especially interesting is the difference in the stress-induced ACTH between ZT4 and ZT16 in the ADX groups (Figure 8). At ZT4, there was clearly a larger difference in ACTH between the ADX no stress and ADX stress treatment groups than at ZT16. We also saw this phenomenon in the SHAM treatment groups (Figure 16). We hypothesize that this

difference in magnitude could be attributed to the fact that at ZT4 these rats were in their sleep state. Consequently, they experienced more stress during the restraint procedure and therefore a more intense activation of the HPA axis leading to higher levels of ACTH. This would differ from rats stressed at ZT16 when the rats are in their awake and alert state. To further validate this hypothesis, we could conduct further tests on the experimental tissue and perform an in situ hybridization for *c-fos*. *C-fos* is an immediate early gene (a gene activated transiently and rapidly in response to a wide range of cellular stimuli, including stress) (23). By examining *c-fos* not only at the level of the PVN, but in other brain regions, we would be able to determine whether the time of day differences seen in the ACTH results are due to the differential magnitude of the central response to stress that varies with time of day.

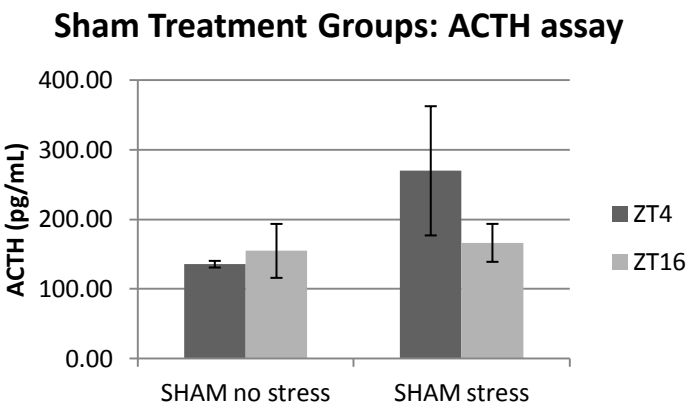

Figure 16. Blow up version of the SHAM treatment groups from Figure 8 to better view the differences between each group. Clear difference in magnitude between the stress response at ZT4 and ZT16.

Similar to the ACTH levels collected, the CORT results also showed ADX and stress effects observed in the literature (8). The SHAM treatment groups are the only groups graphed in Figure 9 because all the ADX rats had levels of CORT undetectable by the assay due to surgical removal of their adrenals. There was also an evident stress effect at both ZT4 and ZT16, indicating proper and expected activation of the HPA axis, validating our experiment. We also saw a difference in magnitude of the stress response between ZT4 and ZT16, where ZT4 exhibits a magnitude greater than ZT16. This concurs with experimental expectations, as explained in the introduction.

Per 1 mRNA expression in the SCN

Per1 has been known to have a glucocorticoid response element (GRE) within its promoter, leading us to believe that CORT has the potential to influence *per1* expression (30). When we looked at *per1* mRNA expression in the SCN, we did not see a significant ADX or stress effect. This is consistent with unpublished findings in our lab. The SCN is one of the few regions of the body that does not have any GRs (28), meaning that CORT is not known to influence gene expression in the SCN. This is an important characteristic of the SCN. If stress could influence clock gene expression in the master clock of the body, the physiological effects would be detrimental to the body. Synchronization of the body to the light-dark cycle could be compromised during a psychological stressor because stress could then be a second circadian disruptive factor for the molecular clocks. Fortunately, this is not the case; neither the tonic absence of CORT's diurnal rhythm nor the stress-induced elevation of CORT influences the SCN. Therefore, we saw a lack of a significant ADX or stress effect on *per1* mRNA expression in the SCN and a clear, significant time of day difference. *Per1* naturally has a diurnal rhythm that peaks at ZT4 and troughs at ZT16 in the SCN (9). Our results followed this trend, further validating our analysis and experimental procedure.

Per1 mRNA expression in the PVN

The PVN has a dense population of GR receptors (16) in addition to being the head of the HPA axis. Consequently, we would expect both an ADX and a stress effect on *per1* mRNA expression in the PVN. Looking at the SHAM no stress treatment group, *per1* mRNA expression in the PVN was higher at ZT16 than at ZT4, which is congruent with previous research and further validates our findings. This is in anti-phase with *per1* expression in the SCN. It is not known exactly what dictates the regional differences in clock gene expression throughout the central nervous system. However, we can suspect that, because the PVN receives photic cues from the SCN and non photic (feeding, arousal, and general metabolic condition) cues from the DMH (8), the phase difference between the SCN and PVN may be the product of PVN integration of both these cues.

Comparing both the SHAM treatment groups, there was a clear stress effect on *per1* mRNA expression, indicating an acute stressor can induce *per1*. In the ADX stress groups,

there was a significantly blunted stress effect. Without CORT, the final hormone of the HPA axis, the body cannot experience the physiological component of stress to the same magnitude as in the presence of CORT. Consequently, *per1* mRNA expression during a psychological stressor in the absence of CORT is also blunted. In response to stress, *per1* acts like an immediate early gene where its expression is rapidly induced upon stressor onset. This is likely due to the GRE within *per1*'s promoter region. In conclusion, endogenous CORT is necessary for acute restraint stress to exert its effects on *per1* mRNA expression in the PVN.

Furthermore, comparing the ADX and SHAM groups gives insight to whether or not the tonic diurnal expression of CORT is necessary for normal rhythmic *per1* expression in the PVN. When comparing the SHAM no stress groups to the ADX no stress groups, the levels of *per1* were significantly different. In the ADX group, there was not a clear time of day difference. We can attribute this absence of difference in time of day to the chronic absence of tonic CORT. There are two possible explanations for this difference. First, in the absence of CORT, the diurnal rhythm of *per1* in the PVN is abolished meaning that, through an unknown mechanism, CORT drives the rhythmic expression of *per1*. We can hypothesize that CORT successfully achieves this by acting on *per1*'s GRE. This would support the idea that the SCN controls the diurnal CORT rhythm and utilizes clock genes as one form of communication between regions of the brain and body for circadian synchronization. Thus, without the presence of that communication, clock gene expression in that specific brain region is eliminated. The other explanation stems from unpublished results from our lab looking at *per1* expression in the prefrontal cortex. These results, which looked at *per1* expression at four different ZTs, showed a phase shift in the diurnal rhythm of *per1* (instead of peaking near ZT16 in the prefrontal cortex, expression peaked at ZT12). This could potentially explain why we did not see a significant difference between ZT4 and ZT16 in the ADX no stress treatment group. The *per1* rhythm could have potentially shifted to a point at which *per1* levels are equal at ZT4 and ZT16 thereby masking the diurnal rhythm. Therefore, the tonic expression of CORT is necessary for normal *per1* expression in the PVN. To further investigate whether CORT eliminates the diurnal rhythm of *per1* or if CORT shifts the rhythm of *per1* expression, we would need to conduct another experiment with parameters exactly the same as mentioned in this study,

except instead of stressing and sacrificing the rats at only two time points (ZT4 and ZT16), we would use six different time points equally spaced over the 24 h period.

Bmal1 mRNA expression in the SCN

Similar to *per1 mRNA* expression in the SCN, *bmal1* mRNA expression in the SCN is not reliant on the presence of endogenous CORT. Again this is due to the absence of GR receptors in the SCN. There was a clear time of day difference between ZT4 and ZT16, consistent with previous findings. In the SCN, *bmal1* was higher at ZT4 then at ZT16 (8). This was in perfect anti-phase with *per1* expression in the SCN.

Bmal1 mRNA expression in the PVN

Bmal1 does not have a GRE in its promoter region. Therefore, we would not expect stress-induced CORT to have an effect on *bmal1* expression in the PVN, or any other brain region. This holds true among the SHAM groups, where *bmal1* mRNA expression in the PVN was higher at ZT4 than at ZT16 regardless of stress effect (8). The absence of a stress effect in the SHAM groups supports the notion that an acute stressor (dynamic increase of CORT) has no effect on bmal1 mRNA expression.

Surprisingly, even though there was no stress effect on *bmal1* expression, in both SHAM and ADX groups, there seemed to be an ADX effect, where peak bmal1 expression was blunted, but not completely ablated. For some undefined reason, the chronic diurnal rhythm of CORT appears to be necessary for the robust rhythmic expression of *bmal1* in the PVN. In ADX rats, the chronic elimination of endogenous CORT either blunts the rhythmicity of *bmal1* in the PVN or shifts its diurnal rhythm completely, similar to *per1*. Either of these explanations is possible, but further experimentation must be conducted to determine which actually occurs. A possible explanation for why the chronic elimination of endogenous CORT, not stress-induced CORT, affects *bmal1* expression could be due to the effects this elimination has on *per1* expression. Through some undefined phenomenon, the chronic absence of CORT might influence *bmal1* through interactions with the GRE on the promoter region of *per1*. We expected to see stress effects on *bmal1* in the PVN to be similar to those in the SCN, where CORT has no effect on clock gene expression due to the lack of a GRE in *bmal1*. The fact that the tonic presence of CORT may actually be necessary

in PVN *bmal1* expression may be a reflection of the hypothesis that CORT acts as an important entrainment factor to non-SCN brain regions. This may be why the robust daily peak in basal CORT levels occurs and is necessary. CORT could potentially be the synchronizing entrainment factor for numerous brain regions and peripheral organs throughout the body.

Per1 and bmal1 mRNA expression in the DMH

The DMH has proved to be an exceptionally interesting region of the brain. It has strong, direct interactions with both the PVN and SCN, so we hypothesized that its clock gene diurnal rhythm would follow the pattern of either the PVN or the SCN. If the DMH did indeed follow one of these regions' clock gene expressions, we would have seen a very clear difference between ZT4 and ZT16. Unexpectedly, this was not the case. *Per1* mRNA expression in the DMH was not significantly different at ZT4 and ZT16. This does not necessarily indicate a lack of rhythm in *per1* expression, but that is still a possibility that we cannot rule out. In fact, a research paper analyzing PER1 protein expression at four different time points in the DMH observed a lack of rhythm (28). However, it is important to note that there is a difference in magnitude between protein and mRNA expression. An alternative reason as to why our data demonstrated equivalent expression of *per1* in the DMH at ZT4 and ZT16 is that we could have chosen two time points where the expression of *per1* is the same; the peak and trough could be at other time points. Again, examination of rats at more time points over the course of the day would resolve this question of whether or not *per1 mRNA* has a robust diurnal rhythm. A lack of a rhythm could also indicate that another core clock gene has a more dominant role in controlling the molecular clock in the DMH. Some regions of the brain, such as the anterior pituitary (8), have undetectable *per1* oscillations, but have robust *per2* and *bmal1* oscillations while still having a functional clock mechanism. It could be possible, that the DMH contains the same molecular clock mechanism as the anterior pituitary where *bmal1* and *per2*, not *per1,* are the dominant core clock genes. Our results show a possible time of day effect in *bmal1* expression sham no stress rats. This needs to be replicated for confirmation. Performing an in situ hybridization for *per2* on the remaining experimental tissue could potentially answer this question.

Another important characteristic of *per1* and *bmal1* expression in the DMH was that, in the absence of endogenous CORT, *per1* expression was decreased in both ADX no stress and ADX stress groups. However, neither stress-induced nor endogenous CORT seems to affect *bmal1* expression, consistent with our original hypothesis. *Per1* expression in the ADX treatment groups was significantly lower than the SHAM treatment groups. Whether this was due to the shift in *per1*'s diurnal rhythm, the abatement of this rhythm, or even the absence of a *per1* diurnal rhythm entirely in the DMH depends on results in future experiments. Regardless, these results further support our hypothesis that endogenous CORT appears to be an important modulatory factor for *per1* expression in the DMH.

Conclusion:

Characterizing how CORT can influence clock gene expression in various brain regions is an important area of research. Analyzing the consequences of CORT disturbances during acute stress on an individual's physiological health could be important in understanding the progression of many psychiatric disorders. Even though CORT does not influence clock gene expression in the SCN, we have shown that it can exert its effects on the PVN's and DMH's molecular clocks, suggesting a potential role of CORT as an individual's hormonal circadian synchronizer. Through the DMH, the SCN can influence circadian functioning of the PVN, the head of the HPA axis, reinforcing the importance of the DMH in maintaining a healthy molecular clock. Our findings are consistent with previous findings indicating CORT's negligible effects on clock gene expression in the SCN. Our results also demonstrate a clear effect of tonic and stress-induced CORT on *per1* expression in both the PVN and DMH. There was no significant stress effect in *bmal1* expression in either the PVN or SCN. However, there was a significant effect on *bmal1* expression in the PVN, not DMH, in the tonic absence of CORT. Taken together, our results support the idea of CORT as an important entrainment factor for *per1* and *bmal1* expression in the PVN and *per1* expression in the DMH.

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