Clock genes, acute stress, and the role of glucocorticoids in male and female rat brain.

by

Lauren Elizabeth Chun

B.S., University of Michigan, 2009

M.A., University of Colorado, 2013

A thesis submitted to the Faculty of the Graduate School of the

University of Colorado in partial fulfillment of the requirement for the

degree of Doctor of Philosophy

Department of Psychology & Neuroscience

2017

This thesis entitled:
Clock genes, acute stress, and the role of glucocorticoids in male and female rat brain
written by Lauren Elizabeth Chun
has been approved for the Department of Pyschology & Neuroscience

_____
Robert L. Spencer, PhD

_____
Serge Campeau, PhD

_____
Ryan K. Bachtell, PhD

_____
Pei-San Tsai, PhD

_____
Sona Dimidjian, PhD

Date_____

The final copy of this thesis has been examined by the signatories, and we
Find that both the content and the form meet acceptable presentation standards
Of scholarly work in the above mentioned discipline

IACUC protocol # ____1403.02_____

ABSTRACT

Chun, Lauren Elizabeth (PhD, Psychology & Neuroscience)

Clock genes, acute stress, and the role of glucocorticoids in male and female rat brain

Thesis directed by Professor Robert L. Spencer

In order to optimize its survival, an organism needs to be synchronized to its environment. Thus, properly entrained circadian rhythms are critical for normal health and behavior, as many mood disorders are associated with disrupted circadian rhythms. Circadian rhythms are controlled by the oscillating expression of core clock genes, including *Per1, Per2,* and *Bmal1.* While this molecular clock is well-established in the body's master clock, the hypothalamic suprachiasmatic nucleus (SCN), there is limited characterization of oscillating molecular clocks in extra-SCN brain regions. Furthermore, there are sex differences in circadian rhythms. Thus, the first study characterized the ubiquitous existence of oscillating molecular clocks (*Per1, Per2,* and *Bmal1* mRNA; in situ hybridization) in the prefrontal cortex, amygdala, hippocampus, and the hypothalamic paraventricular nucleus (PVN) in male and female rats. It has been proposed that the SCN is able to entrain these extra-SCN molecular clocks through the diurnal rhythm of glucocorticoids (CORT). Adrenalectomy disrupts clock gene expression in the PFC. If a timely peak in diurnal CORT is necessary for normal clock gene rhythm, then untimely stress-induced peaks in CORT may disrupt clock gene expression. There are sex differences in stress. Thus, it was examined if 30 minutes of acute restraint stress could alter clock gene expression in the PFC and PVN of male and female rats, whether this effect was dependent on the presence of CORT, and if this effect could be modulated by estradiol in female rats. Acute stress selectively and rapidly induces *Per1* mRNA (in situ hybridization) in the PVN and PFC of male and female rats. There were no sex differences, despite greater stress-induced CORT in females. Furthermore, stress-induced *Per1* mRNA was largely

independent of CORT in both males and females, as adrenalectomy had limited effects. Additionally, in females, estradiol had no effect, either acutely or permissively, on stress-induced *Per1* mRNA. The selectivity of *Per1* induction suggests that *Per1* may act as an incident detector for the molecular clock to salient cues in the environment in order for the organism to adapt to changes in its environment.

DEDICATION

To my parents, Olivia and Robert Chun, for all of their unconditional love and support.

ACKNOWLEDGEMENTS

This work would not have been possible without the vision, encouragement, and mentorship of Dr. Robert Spencer. Bob has taught me what it really means to be a scientist: to let curiosity lead the way, to have child-like wonder and enthusiasm for what one's curiosity has found, to approach difficulties with unfailing tenacity, and to discover with precision, meticulousness, collaboration, and independence. I owe all of this to you. Thank you so much for the opportunity and the wonderful mentorship.

This work also would not be possible without the amazing help from the Spencer Lab, in particular: Elizabeth Woodruff, Laura Hinds, Jenny Christensen, Sarah Morton, and Angela Tomczik. To all past and present members of the Spencer Lab – thank you for all the learning, the fun, and the support (both inside and outside of the lab). And to the other amazing undergrads I've had the opportunity to work with: Nick Varra, Blair Swearingen, Serena Sweet, Aaron Neal, and Jeanelle France – it was truly inspiring working with you all. If I could have only been as put together and on top of my game as you guys were during undergrad, I could have ruled the world by now.

I would not be here if it weren't for all of those along the way who have gotten me interested in science in the first place and fostered my love for research. Thank you for stoking the flame: Dr. Jeff Charney for starting it all, Dr. Terry Robinson whose Drugs of Abuse class put me down the road of Neuroscience, the Becker Lab (Melissa Luma, Brandon Luma, Jen Cummings, and Brooke Davis) where I first fell in love with research, the Berridge Lab (Jocelyn Richard), and the Katz Lab (Gigi Tanda, Madda Mereu, Steve Kohut, Jon Slezak, Takato Hiranita, Betty, and Dawn).

I would not be where I am today without my parents, Olivia and Robert Chun. Thank you so much for all of your sacrifices and support throughout my life. Your deepest desire to see your daughters obtain the best possible college education, in large part fueled by your lack of resources to get the same education yourselves, impressed upon me how much of a privilege it is to have an education. I would not be where I am now without all the wonderful opportunities I've had, thanks to your sacrifice and hard work. I feel incredibly lucky for these opportunities and to have you as my parents. Thank you for always making our happiness a priority and for whole-heartedly supporting me in my dream to get a PhD in Neuroscience. To my sisters, Jessica and Nicole, thank you for being the best siblings anyone could have asked for. You guys are my best friends and have had my back since Day 1.

I would also like to acknowledge the unwavering support of my friends. I would not have made it through grad school without these amazing people. Thank you for all the laughs, the climbing adventures, the philosophical discussions, the fun, the silliness, and the lessons on life, love, and how to crush rocks. Thanks for always having my back (both figuratively and literally): Danyelle McNeary, Jacob Stanley, April Goebl, Franziska Schweiger, Rachel Thayer, and Lee Miller.

Finally, to my cat Pickles. His cute fluffy face never fails to make me smile.

TABLE OF CONTENTS

TABLES

FIGURES

CHAPTER I: INTRODUCTION

The importance of circadian rhythms

Circadian rhythms are fluctuations in behavior and physiology that occur throughout a 24 hour period. They are behavioral and physiological representations of an internal timing system. The most obvious circadian rhythm is the sleep/wake cycle. However, there are circadian rhythms in a multitude of behaviors and physiology, such as hormone secretion (e.g., melatonin, glucocorticoids), cardiovascular efficiency, cognition, body temperature, and blood pressure. These circadian rhythms are tightly regulated so that the phase of circadian rhythms occur at the appropriate time in order to synchronize the organism to its environment. Proper entrainment of an organism to its environment optimizes its survival. The most potent entrainment factor that synchronizes an organism to its environment is light. Importantly, all circadian rhythms do not have to be synchronized with each other; the phase of circadian rhythms are optimized for the function of the tissue (e.g., the timing of optimal liver functioning may differ from the timing of body temperature) (Reppert & Weaver, 2002).

There is a strong association between properly entrained circadian rhythms and the health of an organism. Many mood disorders, such as major depressive disorder (MDD), anxiety disorders, and post-traumatic stress disorder (PTSD), are associated with disruptions in a variety of circadian rhythms, including sleep/wake cycles and a blunted diurnal rhythm of glucocorticoid (CORT) release (Johansson et al., 2002; Lamont et al., 2009; Partonen et al., 2007). Additionally, Li et al. (2013) have found that genes that have a circadian rhythm are less synchronized to the environment in post-mortem brains of people who had MDD, compared to the post-mortem brains of healthy controls. Thus, there is clearly a link between disrupted circadian rhythms and mood disorders. It has also been found that shift workers, who work at night during their inactive phase, had a greater chance of developing depression, compared to

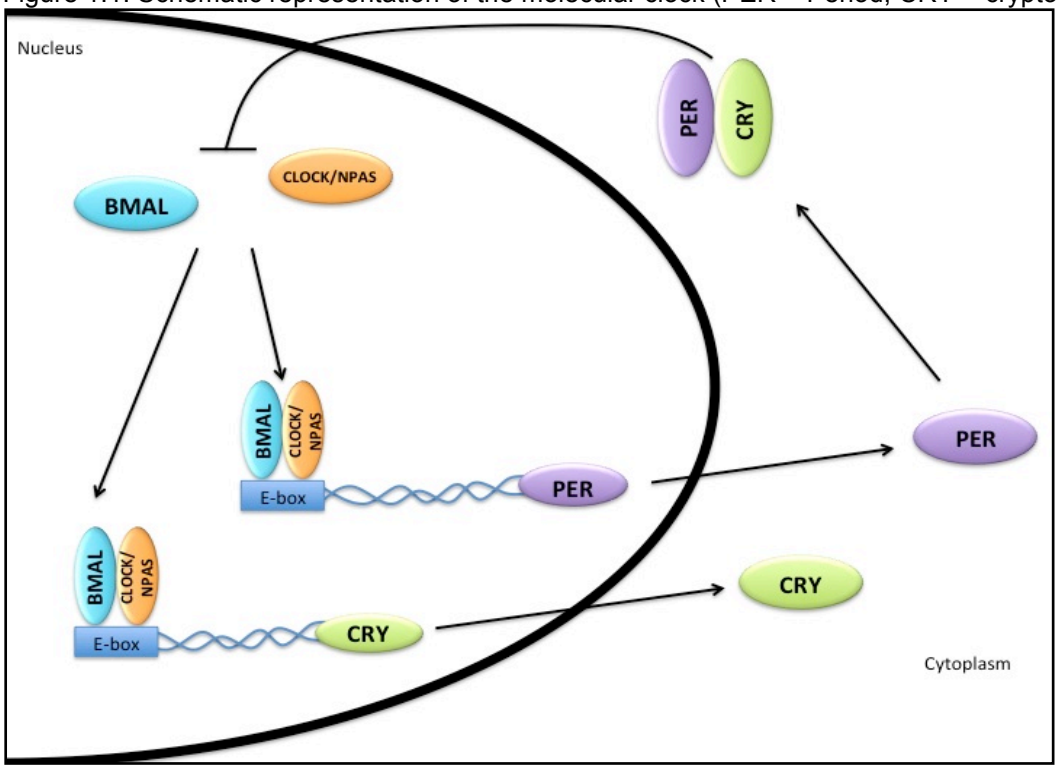
people who work during the active phase in the daytime (Caruso, 2014; Lee et al., 2015; Ma et al., 2015; Togo et al., 2017). These data highlight how detrimental the desynchronization of one's circadian rhythms from their environment is on healthy, normal mood behaviors.

Circadian rhythms can be characterized based on three main parameters. *Period* describes the duration of the rhythm. Because most organisms' biological rhythms are entrained to the light:dark cycle, circadian rhythms have a period of 24 hours. Circadian is Latin for "around a day". The peak, or highest point in a rhythm, is called the *acrophase*. The *trough* or *nadir* is when the rhythm is at its lowest expression. Additionally, the robustness of the rhythm can be described in terms of *amplitude*, which is the difference between the nadir and acrophase of the rhythm. The greater the amplitude of a rhythm, the more robust the rhythm is.

The molecular clock underlies circadian rhythms

Underlying all circadian rhythms is an oscillating molecular clock. The molecular clock consists of core clock genes that can be grouped into two different components. The positive component of the molecular clock includes Bmal1 and Clock or Npas2 (Clock and Npas2 can substitute for each other (DeBruyne et al., 2007; Landgraf et al., 2016)). Bmal1 and Clock/Npas2 form dimers that bind to the E-box, an enhancer within the promoter region of the clock genes that comprise the negative component of the molecular clock, the Period genes (Per1, Per2, Per3) and the Cryptochrome genes (Cry1, Cry2). The Per and Cry transcripts are translated into protein products in the cytoplasm, which are translocated back to the nucleus to interfere with the dimerization of BMAL1 and CLOCK/NPAS2. Thus, PER and CRY are able to negatively regulate their own transcription [Figure 1.1]. This transcription/translation loop approximates 24 hours (Hastings, 2004; Reppert & Weaver, 2002; Takahashi et al., 2008).

Figure 1.1. Schematic representation of the molecular clock (PER = Period; CRY = cryptomchrome).



In addition to the core clock genes, there are other factors that regulate the molecular clock. In the cytoplasm, PER and CRY proteins undergo post-translational modifications. Their protein products are phosphorylated by Casein kinase 1 ε and δ (CK1ε and CK1δ), which functions to tag these proteins for degradation via ubiquitination, or for translocation into the nucleus (Lee et al., 2001; Reppert & Weaver, 2002). CK1ε is an enzyme that is critical to establishing a 24 hour period for the molecular clock, as mutations to CK1ε result in a shortened period (Lowrey & Takahashi, 2004) and sleep phase disorders in humans (Gallego & Virshup, 2007; Toh et al., 2001). Other post-translational modifications of the core clock proteins include mitogen-activated protein kinase (MAPK), which phosphorylates BMAL1 in order to decrease its ability to act as a transcription factor (Sanada et al., 2002). Additionally, glycogen synthase kinase 3 (GSK3), can phosphorylate PER2 in order to regulate its translocation into the nucleus (Martinek et al., 2001). Thus, the molecular clock is heavily regulated by a variety of kinases in

order to modify its robustness, period, and phase (Gallego & Virshup, 2007; Reppert & Weaver, 2002).

There is also an accessory loop to the molecular clock that primarily regulates the expression of Bmal1. REV-ERBα can repress Bmal1 transcription by binding to the retinoid-related orphan receptor (ROR) response element (RORE) within the promoter region of Bmal1. REV-ERBα is in-phase with the negative component of the molecular clock (Per, Cry), so while *Bmal1* transcription decreases, *Per1,2* and *Cry1,2* transcripts rise. PER and CRY, upon translocating back into the nucleus of the cell, inhibit *Rev-erb*α, relieving its inhibitory actions and causing activation of *Bmal1* transcription. Thus, Rev-erbα serves to add robustness to the rhythmic expression of Bmal1 (Preitner et al., 2002; Ueda et al., 2002). An additional part of this accessory loop is retinoic acid orphan receptor α (RORα), which functions to increase the expression of *Bmal1* transcription (Duez & Staels, 2008).

The master clock of the body: the suprachiasmatic nucleus of the hypothalamus

The molecular clock is well-established in the body's master clock, the suprachiasmatic nucleus of the hypothalamus (SCN) (Dunlap, 1999; Hastings, 2004; Reppert & Weaver, 2002; Shearman, 2000; Takahashi et al., 2008). The SCN is a bilateral nucleus located in the anterior hypothalamus, immediately dorsal to the optic chiasm. This nucleus entrains the organism to its environment and coordinates all circadian rhythms throughout the body. Lesioning the SCN results in arrhythmic behaviors, such as locomotor activity and diurnal hormone secretion (Moore & Eichler, 1972; Stephan & Zucker, 1972). Furthermore, implanting SCN cells into an animal dictates the animal's subsequent circadian rhythm phase (Lehman et al., 1987; Pando et al., 2002; Ralph et al., 1990), emphasizing the ability of the SCN to dictate circadian rhythms. The SCN receives direct light input from the environment. Light enters the retina and this information is sent through the retinohypothalamic tract (RHT) directly to the SCN, where glutamate is released (Morin, 2013; Reppert & Weaver, 2002; Sollars & Pickard, 2015).

Glutamate binds to both NMDA and AMPA receptors, causing an influx of calcium. Calcium initiates the activation of a variety of kinases (calcium/calmodulin-dependent protein kinase (CaMK), protein kinase A (PKA), and mitogen-activated protein kinase (MAPK)), which then phosphorylate CREB (Carlezon et al., 2005; Welsh et al., 2010). pCREB acts as a transcription factor and binds to cAMP response elements (CRE) within the promoter regions of Per1 and Per2 to induce their expression. This is the mechanism by which the molecular clocks within the SCN are able to tell time (Welsh et al., 2010). Light is detected by melanopsin receptors in the retina; however, it is still unknown if melanopsin is the sole detector of light (Sollars & Pickard, 2015). In addition to the RHT, the SCN also receives afferent input from the intergeniculate leaf (IGL) via the geniculohypothalamic tract, and from serotinergic input from the raphe nucleus (RN). Both the IGL and RN also receive light input from the RHT (Morin, 2013; Reppert & Weaver, 2002; Sollars & Pickard, 2015). Thus, SCN afferents are almost exclusively dependent on light input from the environment.

The SCN can be structurally divided into two different sections: the ventrolateral section, which contains the majority of the vasoactive intestinal polypeptide (VIP) neurons and where most of the retinal input terminates, and the dorsomedial section, which contains the majority of vasopressin (AVP) neurons (Sollars & Pickard, 2015). Other neuromodulators found within the SCN include gastrin-releasing peptide, substance P, somatostatin, calbindin, calretinin, enkephalin, neurotensin, and cholecystokinin (Morin, 2013; Sollars & Pickard, 2015). The vast majority of neurons within the SCN are GABAergic. GABA is critical in coordinating rhythms within the SCN, and aiding in phase shifts within the SCN (Liu & Reppert, 2000). VIP is another peptide that is critical in the intercellular synchronization of the SCN. Lack of VIP or its receptor, VPAC2, leads to loss of circadian rhythms in behavior and loss of rhythmic clock gene expression within the SCN (Colwell et al., 2003; Cutler et al., 2003; Harmar et al., 2002; Hastings & Herzog, 2004).

The SCN has very limited direct efferent projections. The SCN directly projects to the medial preoptic area (MPOA). This brain region critically projects to the paraventricular nucleus of the thalamus (PVT), which then goes on to project to the infralimbic subregion of the medial prefrontal cortex (Sylvester et al., 2002). The SCN also projects directly to the retrochiasmatic area, which projects to the ventromedial nucleus of the hypothalamus. Perhaps the most major efferent projection of the SCN is to the subparaventricular zone (SPZ) of the hypothalamus, which projects to the DMH; the DMH projects to other key areas of the brain, including the paraventricular nucleus of the hypothalamus (PVN). Lesions to the SPZ results in decreased amplitude or disrupted rhythm of sleep/wake, body temperature, locomotor activity, and neural activity of extra-SCN brain regions (Sollars & Pickard, 2015). While there is potential for many multi-synaptic efferent projections from the SCN, it is overall limited.

Molecular clocks exist outside of the SCN

The molecular clock, which is defined as having rhythmic expression of both the positive and negative components, has been well-established in the SCN (Dunlap, 1999; Hastings & Herzog, 2004; Reppert & Weaver, 2002; Shearman, 2000; Takahashi et al., 2008). More recently, studies have begun to examine rhythmic clock gene expression in extra-SCN brain tissue (e.g, bed nucleus of the stria terminalis, nucleus accumbens, hippocampus, lateral septum, amygdala, hypothalamic nuclei, striatum, caudate putamen, cortex) (Amir et al., 2004; Ángeles-Castellanos et al., 2007; Feillet et al., 2008; Girotti et al., 2009; Guilding & Piggins, 2007; Harbour et al., 2014; Jilg et al., 2009; Lamont et al., 2005; Masubuchi et al., 2000; Perrin et al., 2006; Rath et al., 2012; Reick et al., 2001), as well as peripheral tissue (e.g., liver, lung, skeletal muscle, heart, stomach, spleen, kidney) (Yamamoto et al., 2004; Yamazaki et al., 2000). However, the existing literature mostly examines either the positive component or the negative component, never the entire molecular clock. There are only a few instances where both the positive and negative core clock genes have been characterized (Girotti et al., 2009;

Harbour et al., 2014; Jilg et al., 2009; Masubuchi et al., 2000; Rath et al., 2012; Reick et al., 2001), thus making it difficult to determine with certainty how extensive the molecular clock exists in extra-SCN tissue.

The molecular clock within the SCN is considered to be autonomous, meaning that it is able to stay rhythmic without input from other sources. This has been displayed ex vivo in cell culture, where SCN cells have oscillating expression of clock genes for ~32 days (Yamazaki et al., 2000). This differs from rhythmic clock gene expression found in extra-SCN brain regions and peripheral tissue, where rhythmic clock gene expression is considered to be either semi-autonomous or slave oscillators and lose rhythmic expression after a few cycles (Yamazaki et al., 2000). Unlike the master clock, clock gene expression in extra-SCN tissues is dependent on input from the SCN in order to stay rhythmic. SCN-dependent input to extra-SCN clocks has been demonstrated by lesioning the SCN, which results in arrhythmic Per1 or Per2 expression in cerebral cortex and peripheral tissue (Sakamoto et al., 1998; Wakamatsu et al., 2001).

Properly timed rhythmic expression of clock genes is necessary for normal health and behavior. In rodent students, rats that received knockdown of Bmal1 or knockdown of both Per1 and Per2 resulted in behavioral arrhythmicity, as determined by aberrant and arrhythmic locomotor activity (Bae et al., 2001; Bunger et al., 2000). These studies also emphasize the importance of both the positive and negative components of the molecular clock in normal circadian behavior. Disrupted Per1 and Per2 expression in the nucleus accumbens, a brain region implicated in reward and motivation, is linked with anxiety-like behavior in mice (Spencer et al., 2012). ClockΔ19 mutant mice exhibit manic-like behaviors and have been proposed as a model for bipolar disorder (McClung et al., 2005; Mukherjee et al., 2010; Roybal et al., 2007). These rodent studies are corroborated by research in humans that have identified mutations in *Clock, Npas2, Per1, Per2, and Bmal1* genes in a variety of mental illnesses, including major depressive disorder, anxiety, post-traumatic stress disorder, schizophrenia, autism, bipolar disorder, and seasonal affective disorder (Bunney et al., 2014; Bunney & Bunney, 2000; Etain et

al., 2011; Johansson et al., 2002; Lamont et al., 2009; Landgraf et al., 2014; Li et al., 2013; McCarthy & Welsh, 2012; McClung, 2007; Partonen et al., 2007). These studies, in both rodents and humans, exemplify the importance of functional clock genes in maintaining normal health and behavior.

Entrainment of extra-SCN clocks by glucocorticoids

Rhythmic expression of clock genes in extra-SCN brain regions and in peripheral tissue is dependent on input from the SCN, as exemplified by the fact that if the SCN is lesioned, extra-SCN clock gene expression becomes arrhythmic (Sakamoto et al., 1998; Wakamatsu et al., 2001). However, the SCN has limited efferent projections and considering the expanse of extra-SCN molecular clocks, multisynaptic connections cannot account for all SCN input to extra-SCN molecular clocks. Thus, glucocorticoids (CORT; corticosterone in rodents, cortisol in humans) have been proposed as an ideal candidate (Balsalobre et al., 2000) to serve as an entrainment factor between the master clock and extra-SCN molecular clocks.

CORT is the final output of the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis allows an organism to adapt to challenges in their environment. This is because CORT is a hormone with vast functions, including muscle, bone, glucose, and lipid metabolism, immune function, vascular tone, growth and development, and important central nervous system functions (Herman et al., 2003). The head of the HPA axis is the paraventricular nucleus of the hypothalamus (PVN). This bilateral nucleus sits at the dorsal portion around the third ventricle in the anterior hypothalamus. The PVN receives both direct and indirect input from other areas in the brain, including the limbic system structures (prefrontal cortex (PFC), hippocampus (HPC), amygdala(AMYG)). Many of these inputs are integrated at the level of the bed nucleus of the stria terminalis (BNST) (Herman et al., 2003). If enough input activates the PVN, it releases corticotropin releasing hormone (CRH) into the median eminence to reach the anterior pituitary, where CRH binds to CRH receptors. This causes the release of adrenocorticotropic hormone

(ACTH) from the anterior pituitary into the blood stream. ACTH travels to the adrenal glands where it binds to its receptor, the MC2 receptors. This activates the adrenal cortex to produce and release CORT into the general blood stream. CORT is a steroid hormone derived from cholesterol and can readily pass through the phospholipid bilayer of cells, where glucocorticoid receptors (GR) are found intracellularly. CORT-bound GRs dimerize, translocate to the nucleus, and act as a transcription factor at glucocorticoid response elements (GREs) to alter the transcription of genes. GRs are ubiquitously expressed, with the SCN as a notable exception (Balsalobre et al., 2000; Rosenfeld et al., 1993; Rosenfeld et al., 1988), which explains the diverse functions of CORT.

The ubiquitous nature of GRs makes CORT an ideal candidate for acting as the entrainment factor between SCN and extra-SCN molecular clock coordination. Additionally, CORT has a diurnal rhythm that is controlled by the SCN (Moore & Eichler, 1972; Szafarczyk et al., 1983). Its acrophase is at the onset of the organism's active phase (dark phase in nocturnal laboratory rats, light phase in diurnal animals such as humans). This SCN-controlled diurnal peak in CORT may be the signal by which the SCN is communicating to extra-SCN clocks. GR binding sequences (GBS) have been found in the promoter regions of Per1 and Per2 (Cheon et al., 2013; Reddy et al., 2007; So et al., 2009; Yamamoto et al., 2005). While the functionality of these GSBs in the promoter region of Per2 are less established, it has been shown that administration of CORT can acutely upregulate *Per1* gene expression in rat-1 fibroblast cells (Balsalobre et al., 2000), human peripheral blood mononuclear cells (Burioka et al., 2005) and in hippocampal tissue ex vivo (Conway-Campbell et al., 2010). CORT or GR agonist treatment ex vivo also can induce rhythmic expression of many of the core clock genes, including *Per1, Per2,* and *Bmal1* (Balsalobre et al., 2000; Reddy et al., 2007; So et al., 2009). Additionally, it has been found that removal of endogenous (and therefore diurnal) CORT via adrenalectomy (ADX) results in phase-shifted or arrhythmic clock gene expression in the PFC (Woodruff et al., 2015) and the oval nucleus of the BNST (Amir et al., 2004). Importantly, Woodruff et al. (2016)

has shown that when giving ADX rats a daily replacement CORT injection when animals' diurnal CORT normally occurs, normal clock gene rhythmicity in the PFC is restored. Daily CORT injections given antiphasic to the normal diurnal CORT acrophase disrupts clock gene expression even more. Thus, diurnal CORT is necessary for rhythmic expression of extra-SCN molecular clocks.

The effect of stress on clock gene expression

If an appropriately timed peak in diurnal CORT under basal conditions is necessary for the rhythmic expression of clock genes, what would happen to clock gene expression if there are untimely, stress-induced peaks of CORT? A few studies have shown that acute stress can rapidly induce the expression of *Per1* mRNA in the PVN (Takahashi et al., 2001) and PER1 protein in the PVN, dorsomedial nucleus of the hypothalamus, and the piriform cortex (Al-Safadi et al., 2014). Additional studies have also determined that chronic stress can affect rhythmic clock gene expression by altering the phase or disrupting the amplitude. Unpredictable chronic stress increases the amplitude of rhythmic Per2:luciferase expression in the nucleus accumbens, but decreases Per2:luciferase rhythm in the SCN (Logan et al., 2015). Additionally, three consecutive days of 2 hours of restraint stress induced a phase-shift in both *Per1* and *Per2* mRNA in peripheral tissues, hippocampus, and cortex of rats (Tahara et al., 2015). However, Kinoshita et al. (2012) did not find an effect of chronic restraint stress on PER2 protein expression in the HPC or PFC, but found blunted PER1 rhythm in the SCN. Other studies using chronic stress paradigms have also found other changes in rhythmic clock gene expression. However, exact results varied. Notably, many of these studies used light pulses or changes in the light:dark schedule as a stressor; thus, these results must be considered with caution (Calabrese et al., 2016; Christiansen et al., 2016; Jiang et al., 2013; 2011; Takahashi et al., 2013). These studies collectively suggest that stress – both acute and chronic – affect clock

gene expression. Thus, it would be critical to further characterize stress-induced clock gene expression and possible underlying mechanisms.

The importance of sex differences

Women have greater prevalence of mood disorders that are associated with both disruptions in circadian rhythms and stress (e.g., major depressive disorder (MDD), anxiety disorders, post-traumatic stress disorder (PTSD)) compared to men (Kessler, 2003). Thus, it is important to incorporate female subjects into studies examining stress, circadian rhythms, or both. This is further supported by the fact that there are sex differences in both circadian rhythms (Bailey & Silver, 2014), as well as in stress reactivity with a particular emphasis on stress-induced HPA axis activation (Babb et al., 2014; Handa et al., 1994; Iwasaki-Sekino et al., 2009; Kitay, 1961; Viau & Meaney, 1991). The interaction between stress and circadian rhythms may also contribute to the greater prevalence of MDD and anxiety disorders in women compared to men.

Rodents have an estrous cycle that lasts 4-5 days. There are four stages in the estrous cycle, with each stage about 1-2 days longs. The estrous cycle begins with the development of the follicles in the ovary. This follicular phase consists of two stages: metestrus, followed by diestrus. Steroidogenesis occurs during the follicular phase, resulting in slowly rising estrogen levels. The next stage is the periovulatory phase, or proestrus, which prepares the body for ovulation. Proestrus is tightly regulated in regards to timing, only lasts for approximately 12 hours, and occurs in the animal's active phase. During proestrus, estrogen levels peak followed 4-6 hours by peaks in progesterone levels. The final stage is estrus, which is the day that ovulation occurs and is the stage of greatest sexual receptivity, though at this point, estradiol levels have significantly declined (Becker et al., 2004).

There are sex differences in a variety of different aspects of circadian rhythms. In rats, males have a larger SCN, more axospinal synapse, and post-synaptic density materials in the

SCN, compared to females. However in humans, women have a larger SCN compared to men. Furthermore, there is a vast distribution of estrogen receptors and androgen receptors in the retina, in afferents to the SCN, and in efferents from the SCN, leaving the potential for gonadal hormones to influence inputs to and outputs from the SCN, that can subsequently influence entrainment of circadian rhythms. Furthermore, in human studies, there are sex differences in sleep, where women sleep more but worse than males, are more susceptible to insomnia, and react worse to sleep deprivation. These differences begin during adolescence and end at menopause, which suggests that gonadal hormones may have an activational role on sleep (Bailey & Silver, 2014). In addition to sex differences in circadian rhythms, there is evidence for a direct role of gonadal hormones on basal clock gene expression. In the uterus, *Per1* mRNA is only rhythmic during proestrus but not during other stages of the estrous cycle (Nakamura et al., 2010). Additionally, administration of either estradiol or progesterone can induce *Per1* and *Per2* expression in reproductive tissue of female rats (He et al., 2007; Nakamura et al., 2010). In the brain, rhythmic expression of PER2 protein varies in amplitude depending on the stage of the estrous cycle in the oval nucleus of the BNST and in the central amygdala (CEA) (Perrin et al., 2006). Thus, gonadal hormones may play an important role in clock gene expression, which may underlie the sex difference seen in circadian rhythms.

There are robust sex differences in response to stress. In rodents, it is well-established that females have greater HPA axis activation (greater ACTH and CORT release) in response to an acute stressor, compared to males (Babb et al., 2014; Handa et al., 1994; Heinsbroek et al., 1991; Iwasaki-Sekino et al., 2009; Kitay, 1961; Rivier, 1999; Seale et al., 2004; Viau & Meaney, 1991). There is evidence to suggest that females have decreased glucocorticoid negative feedback, resulting in greater and prolonged CORT in response to an acute stressor (Bland et al., 2005; Burgess & Handa, 1992; Heinsbroek et al., 1991; Viau & Meaney, 1991; Weiser & Handa, 2011). The greater stress-induced CORT seen in females is believed to be due to the

activational effects of estradiol, as the sex difference effect is pronounced in females with higher estradiol levels (Carey et al., 1995; Goel et al., 2014; Iwasaki-Sekino et al., 2009; Rivier, 1999).

In addition to sex differences in stress-induced activation of HPA axis, there are reported sex differences in stress-induced neuronal activity, as measured by CREB or the immediate early gene *c-Fos*. C-Fos is rapidly induced and is considered a marker for neuronal activation; pCREB is a transcription factor for c-Fos (Sheng & Greenberg, 1990). However, sex differences in stress-induced *c-Fos* expression are less consistent, as *c-Fos* gene expression differs based on stressor used and brain region examined (Figueiredo et al., 2002; Ter Horst et al., 2009; Sterrenburg et al., 2011). Some studies have found that females have greater *c-Fos* expression in response to stress in the oval nucleus of the BNST (Sterrenburg et al., 2011). Other studies have found the opposite, where males and females in diestrus have greater *c-Fos* expression in the HPC and cortex compared to female rats in proestrus (Figueiredo et al., 2002). Additionally, Ter Horst et al. (2009) have found that stress actually decreases *c-Fos* expression in females, but increases *c-Fos* expression in male rat brains. Other studies still find no sex difference of stress-induced *c-Fos* expression in the PVN, medial amgydala, and ventral lateral septum (Sterrenburg et al., 2011). Interestingly, Bland et al. (2005) did find a time course difference in stress-induced *c-Fos* expression, where 30 minutes after stressor onset, males have greater *c-Fos* expression compared to females. However, at 60 and 90 minutes after stressor onset, male *c-Fos* levels returned to baseline, while female *c-Fos* levels remained elevated. Many of these inconsistences may have to do with the stressor used, duration between stressor onset and time of sacrifice, and brain region examined. Further studies are needed to get a clearer understanding of sex differences in stress-induced *c-Fos* expression.

Brain regions involved in mood disorders

The primary brain regions examined in the subsequent studies are the PVN and the prefrontal cortex (PFC). The PVN was chosen as a region of interest due to its critical role as

head of the HPA axis. The PFC was chosen due to its critical role in executive functioning, emotional control, working memory, learning of extinction of conditioned fear, attention, and stress reactivity (Arnsten & Rubia, 2012; Goldman-Rakic, 1996; Robbins, 1996; Vertes, 2003). Furthermore, dysfunction of the PFC is implicated in a number of mood disorders, in particular PTSD, depression, and anxiety (Baxter et al., 1989; Drevets et al., 1997; Elliott et al., 1997; Mayberg et al., 1999; Radkovsky et al., 2014). Also, the PFC plays an important role in glucocorticoid negative feedback, where it provides inhibitory influence over the HPA axis, which aids in shutting off of the stress response (Figueiredo et al., 2002; Herman et al., 2003).

The PFC has several subregions which were examined: the anterior cingulate cortex (AC), the prelimbic medial prefrontal cortex (PL), the infralimbic medial prefrontal cortex (IL), and the ventral orbital cortex (VO). The PL and IL comprise the medial PFC (mPFC), which is of particular interest due to the mPFC's predominant role in stress and mood disorders. The hippocampus (HPC; subregions include the CA1, CA3, superior DG (supraDG), and inferior DG (infraDG)) and the amygdala (AMYG; subregions: basolateral (BLA), central medial (CEA), and medial (MEA)) were also examined in a few of the studies described herein. These brain regions were chosen because of their role in the limbic system, emotion, learning and memory, and stress responsivity (Conrad, 2008; Herman et al., 2003; Joëls et al., 2013; Lebow & Chen, 2016). Similar to the PFC, the dysfunction of these brain regions has been implicated in the cause/symptom of mood disorders (Bremner et al., 2000; Drevets et al., 2008a; Lebow & Chen, 2016; Videbech & Ravnkilde, 2004).

Objectives

The studies herein sought to fully characterize extra-SCN molecular clocks in the SCN, PVN, PFC, HPC, and AMYG. This included examining clock gene expression from both the positive (*Bmal1* mRNA) and negative (*Per1, Per2* mRNA) components of the molecular clock, which many previous studies are lacking. Furthermore, no study to date has directly compared

male and female rats despite evidence for the role of gonadal hormones on clock gene expression. Thus, Chapter 2 examined whether there was an oscillating molecular clock in these brain regions of male and female rats. Chapter 3 determined whether acute restraint stress could rapidly (within 30 minutes) alter the expression of clock genes in the PFC and PVN of male and female rats. It has yet to be determined if stress-induced clock gene expression occurs in the PFC, and there has yet to be a study that directly compares stress-induced clock gene expression in male versus female rats. A subsequent study in Chapter 3 examined in male rats if stress-induced clock gene expression in the PFC and PVN was dependent on CORT. Chapter 4 sought to extend the work to determine if stress-induced *Per1* mRNA in female rats was dependent on CORT, and if estradiol can modulate this effect either acutely or permissively. In this study, in addition to the PFC and PVN, stress-induced *Per1* mRNA was also examined in the HPC. The studies herein are critical for understanding the interactions between stress, clock genes, and gonadal hormones, in brain regions critical to the expression of normal mental health.

CHAPTER II: VARIATIONS IN PHASE AND AMPLITUDE OF RHYTHMIC CLOCK GENE EXPRESSION ACROSS THE PREFRONTAL CORTEX, HIPPOCAMPUS, AMYGDALA, AND HYPOTHALAMIC PARAVENTRICULAR AND SUPRACHIASMATIC NUCLEI OF MALE AND FEMALE RATS

ABSTRACT

The molecular circadian clock is a self-regulating transcription/translation cycle of positive (*Bmal1*, *Clock/Npas2*) and negative (*Per1,2,3, Cry1,2*) regulatory components. While the molecular clock has been well-characterized in the body's master circadian pacemaker, the hypothalamic suprachiasmatic nucleus (SCN), only a few studies have examined both the positive and negative clock components in extra-SCN brain tissue. Furthermore, there has yet to be a direct comparison of male and female clock gene expression in the brain. This comparison is warranted, as there are sex differences in circadian functioning and disorders associated with disrupted clock gene expression. This study examined basal clock gene expression (*Per1, Per2, Bmal1* mRNA) in the SCN, prefrontal cortex (PFC), rostral agranular insula, hypothalamic paraventricular nucleus (PVN), amygdala, and hippocampus of male and female rats at 4-h intervals throughout a 12:12h light:dark cycle. There was a significant rhythm of *Per1, Per2,* and *Bmal1* in the SCN, PFC, insula, PVN, subregions of the hippocampus, and amygdala with a 24-h period, suggesting the importance of an oscillating molecular clock in extra-SCN brain regions. There were three distinct clock gene expression profiles across the brain regions, indicative of diversity amongst brain clocks. Although generally the clock gene expression profiles were similar between male and female rats, there were some sex differences in the robustness of clock gene expression (e.g., females had less robust rhythms in the medial PFC, more robust rhythms in the hippocampus, and a greater mesor in the medial amygdala). Furthermore, females with a regular estrous cycle had attenuated aggregate rhythms in clock gene expression in the PFC compared to non-cycling females. This suggests that gonadal hormones may modulate the expression of the molecular clock.

INTRODUCTION

The existence and characteristics of intrinsic molecular circadian clocks in brain regions outside of the body's master circadian pacemaker, the suprachiasmatic nucleus of the hypothalamus (SCN), require further exploration, as these intrinsic clocks, if operational, may contribute to the normal functioning of these extra-SCN brain regions. Furthermore, there are sex differences in circadian rhythms (Bailey & Silver, 2014), yet there has been virtually no direct comparison of basal clock gene expression at the mRNA level in the brains of male and female subjects. The approximately 24-hour rhythmic expression of clock genes in the SCN comprises a molecular clock that is necessary for the expression of circadian rhythms throughout the body (Reppert & Weaver, 2002; Takahashi et al., 2008). This molecular clock consists of counter-regulatory and oscillatory transcription/translation interactions between positive (*Bmal1, Clock* or *Npas2*) and negative (*Per1, Per2, Per3, Cry1, Cry2*) clock gene components (Darlington et al., 1998; Dunlap, 1999; Gekakis, 1998; Reppert & Weaver, 2002). Expression of both the positive and negative clock gene components are thought to comprise a functional molecular clock, as genetic knockdown of either the positive or negative components results in behavioral arrhythmicity in rodents (Bae et al., 2001; Bunger et al., 2000).

The SCN exhibits rhythmic expression of *Bmal1*, *Per1*, *Per2*, *Cry1*, and *Cry2* mRNA with a genetically determined period that approximates 24-h (Bae et al., 2001). These genes have been found to be rhythmically expressed in many other mammalian brain and peripheral tissues, raising the prospect that extra-SCN clock gene expression may directly contribute to neural function throughout the brain (Abe et al., 2002; Sakamoto et al., 1998). In rodents, rhythmic clock gene expression has been found in a variety of peripheral tissues, and their rhythmic expression contributes to the optimal functioning of these tissues (Balsalobre et al., 2000; Yamamoto et al., 2004; Yamazaki et al., 2000). Rhythmic clock gene expression has also been observed in some extra-SCN brain regions (Amir et al., 2004; Ángeles-Castellanos et al., 2007; Feillet et al., 2008a; Girotti et al., 2009; Guilding & Piggins, 2007; Lamont et al., 2005;

Masubuchi et al., 2000; Perrin et al., 2006; Rath et al., 2012; Rath et al., 2014; Reick, 2001; Yamamoto et al., 2004). Most reports of clock gene expression in the brain have provided limited insight into the nature of extra-SCN clocks because those reports focused on only one or a few clock genes and brain regions (Guilding & Piggins, 2007). Only more recently has both the positive (*Bmal1*) and negative (*Per1/Per2*) components of the molecular clock been well-examined within a few extra-SCN brain regions (PVN, central amygdala, hippocampus, neocortex, and cerebellum) (Girotti et al., 2009; Harbour et al., 2014; Jilg et al., 2009; Rath et al., 2012). Thus, it is not possible to discern from the extant literature whether there is evidence for oscillatory expression of positive and negative regulatory clock components within most brain regions, whether intrinsic clock gene expression is ubiquitous throughout the brain, and whether the expression rhythms (phase relationships and amplitude) are similar across brain regions.

Normal clock gene expression has been implicated in overall mental health (Bunney et al., 2014; Bunney & Bunney, 2000; Etain et al., 2011; Johansson et al., 2002; Lamont et al., 2009; J. Z. Li et al., 2013; McCarthy & Welsh, 2012; McClung, 2007; Partonen et al., 2007). In mice, mutation or knockdown of *Clock* or *Per1/Per2* are associated with manic-like behavior and increased anxiety (Dzirasa et al., 2011; Mukherjee et al., 2010; Spencer et al., 2012). The prevalence of mood disorders associated with clock gene disruption (e.g., depression, anxiety, and post-traumatic stress disorder) is greater in women compared to men. There also appears to be a role for gonadal hormones in circadian function (Bailey & Silver, 2014; Thomas & Armstrong, 1989) and clock gene expression in female rodents (He et al., 2007; Nakamura et al., 2010; 2005; Nakamura et al., 2001; Smith et al., 2010). However, there has yet to be a study to directly compare in males and females the expression of positive and negative clock genes.

Consequently, the objective of our first experiment was to directly compare in male and female rats clock gene expression of both the positive (*Bmal1*) and negative (*Per1, Per2*) components of the molecular clock in brain regions important in the regulation of emotion,

mood, and stress responsivity (prefrontal cortex (PFC), rostral agranular insula, amygdala, and hippocampus). In a second experiment we examined whether clock gene expression profiles varied in female rats depending on the presence or absence of estrous cycle cyclicity. For comparison purposes we also examined clock gene mRNA expression in the SCN and PVN, brain regions we previously demonstrated have robust rhythmic expression of each of these clock genes in male rats (Girotti et al., 2009). The dysfunction of the medial PFC (mPFC), hippocampus, and amygdala are implicated in the same mood and anxiety disorders associated with disrupted clock gene expression (Adhikari, 2014; Del Casale et al., 2013; Drevets et al., 2008b; Koenigs & Grafman, 2009; Mayberg et al., 2005). The mPFC, amygdala, and hippocampus are also critical components of fear conditioning and extinction of fear conditioning, both of which exhibit diurnal fluctuations in behavioral expression (Chaudhury & Colwell, 2002; Eckel-Mahan et al., 2008; Smarr et al., 2014; Valentinuzzi et al., 2001; Woodruff et al., 2015b). Characterization of basal clock gene expression in these brain regions may contribute to a better understanding of circadian function in mental health.

MATERIALS AND METHODS

Animals

Sprague Dawley rats (Harlan, Indianapolis, IN), aged approximately 2.5 months, were used in both experiments. Rats were allowed 2 weeks to acclimate to the facility, cage mates, and light:dark cycle. Half of the rats were maintained on a normal 12:12 h light:dark cycle (lights on at 0500 h or 0530 h) and the other half were maintained on a reverse 12:12 h light:dark cycle (lights on at 1700 h or 1730 h). Rats were divided evenly between four individual rooms that were temperature and humidity-controlled. Rats were pair-housed and had free access to food and water. Procedures for the ethical treatment of the animals were conducted in accordance to the guidelines found within the *Guide for the Care and Use of Laboratory Animals* (DHHS

Publication No. (NIH) 80-23, revised 2010 8th edition) and were approved by the University of Colorado's Institutional Animal Care and Use Committee.

Experiment 1: Basal clock gene expression throughout the brain of male and female rats

In order to assess basal clock gene expression, two cohorts of rats were used, with sex counterbalanced across cohorts (total of 48 males and 49 females; 8-9 rats of each sex at each time point). Females and males were age-matched and housed in separate rooms. Female estrous cycle was not tracked for this first experiment. Thus, all female data from this experiment were pooled regardless of estrous cycle stage.

Experiment 2: Basal clock gene expression throughout the brain of normally cycling versus non-cycling female rats

74 female rats were used (12-13 rats at each time point). Estrous cycle phase was assessed by daily vaginal lavage for 2 weeks. The rats on the reverse light:dark schedule were lavaged daily at zeitgeber time 1 (ZT1 – ZT refers to the number of hours after the light phase onset), and the rats on the normal light:dark schedule were lavaged daily at ZT8. Lavages began 2 days after arrival and after the light:dark schedule was set. For lavage, a blunt tip glass eye dropper filled with ~0.5mL of sterile saline was inserted into the vagina. To gently wash off vaginal cells for examination, the saline was expelled into the vagina two to three times before being extracted up by the eye dropper. A drop of the sample was placed on concave microscope slides and immediately examined under 40x light microscopy to assess estrous cycle stage. Rats were considered cyclers if they had at least three different estrous cycle stages within the last five days before sacrifice (Goldman et al., 2007; Marcondes et al., 2002).

Tissue Collection

For both experiments, rats were removed from their home cage and immediately decapitated at six evenly spaced times across the 24 hour day (ZT0, 4, 8, 12, 16, 20). Procedures were performed under red light conditions for rats killed during the dark phase (ZT12, 16, 20). Trunk blood was collected into EDTA-coated tubes and centrifuged at 4,000 rpms for 10 minutes at 4°C. Plasma was then aliquoted and stored at -70°C until subsequent use. Brains were extracted and flash frozen in -25 ± 5°C isopentane chilled with dry ice. Brains were then cut (12 µm thick coronal sections) with a cryostat (Leica CM 1850) at the level of the mPFC (Bregma ~ 2.2-3.2 mm anterior to Bregma), SCN (Bregma ~ 1.3 to 1.4 mm posterior to Bregma), PVN (Bregma ~ 1.8 to 1.88 mm posterior to Bregma), and hippocampus/amygdala (Bregma ~ 2.5 to 2.8 mm posterior to Bregma) according to Paxinos & Watson (4^{th} edition). Brain slices were thaw-mounted onto Colorfrost Plus microscope slides, and stored at -70°C until subsequent use.

Corticosterone (CORT) Hormone Assays

Plasma samples were assayed in duplicate using an ELISA kit according to the manufacturer's instructions (Cat #ADI-901-097 Enzo Life Sciences, Plymouth Meeting, PA or Cat #K014-H1 Arbor Assays, Ann Arbor, MI). Sensitivity for CORT was 18.6 pg/mL (Enzo Life Sciences) or 27.0 pg/mL (Arbor Assays) according to the manufacturer. Plasma was diluted 1:50 in assay buffer and heat inactivated for 60 minutes at 65°C in order to denature corticosteroid binding globulin. Intra-assay coefficient of variation averaged 8.9%, and the inter-assay coefficient of variation was 12.8%.

In situ Hybridization

In situ hybridization for *Per1*, *Per2*, and *Bmal1* mRNA was performed as previously described (Ginsberg et al., 2003; Girotti et al., 2009) with slight modifications as follows. Hybridization was performed in a 50% formamide humidified atmosphere at 54°C for 16-18

hours. Slides were then treated with RNase A (Cat #R5503, Sigma, St. Louis, MO) at 37°C for an hour, washed in decreasing concentrations of standard saline citrate solution (SSC), incubated in 0.1X SSC at 65°C for an hour, then dehydrated through a series of ethanol washes. Dried slides were then exposed to X-ray film for 2-4 weeks, after which films were digitized by use of Northern Light lightbox model B95 (Imaging Res. Inc, St. Catharines, Ontario, CAN), a Sony CCD video camera model XC-ST70 fitted with a Navitar 7000 zoom lens (Rochester, NY) connected to a LG3-01 frame grabber (Scion Corp., Frederick, MD) inside a Dell Dimension 500, and captured with Scion Image beta rel. 4.0.2.

Densitometry

Digitized brain images were analyzed using ImageJ64 (NIH) to quantify the mean optical density (OD) of regions of interest (ROI). ROIs were hand drawn using visible anatomical landmarks with aid of the Paxinos & Watson Brain Atlas. An experimenter blind to the treatment group assignments generated ROIs for both hemispheres. PFC and rostral agranular insula (insula) measures were based on 6 coronal slices per brain, and all other measures were based on 4 coronal slices per brain. The mean gray values of all ROIs were converted into uncalibrated ODs using ImageJ (NIH). Densitometry was performed on films in which the gray level signal fell within the linear range of the gray level to OD relationship. These ODs were then averaged to get the mean OD for each animal. The mean OD for each rat was normalized by converting the mean OD values to a percent of mean value for the male ZT0 group in Experiment 1, or a percent of the mean value for the overall ZT0 group for Experiment 2.

Statistical Analysis

Data were analyzed by two-way analysis of variance (ANOVA) in order to assess clock gene variation across time of day and sex in Experiment 1, or clock gene variation across time of day and estrous cyclicity in Experiment 2. Rats in Experiment 2 were divided into non-cyclers or cyclers, rather than stage of estrous cycle, in order to increase power of analysis due to a limited number of subjects in each estrous stage at each ZT. For significant main effects or

interactions, Fischer's least significant difference (FLSD) post-hoc test was used to assess significant sex or cyclicity differences at a specific time of day. P-values < 0.05 were considered to indicate significant differences between groups. Statistical Package for Social Sciences (SPSS, Mac version 21, 2012) was used for ANOVA and post-hoc analysis. For most clock gene measures in most brain regions there was minimal, but occasional missing data due to the absence within that assay of tissue sections for a particular brain that included the appropriate region of interest. Those missing data are reflected in the small variations in denominator degrees of freedom for the ANOVA analyses. Due to technical difficulties, there were not enough tissue sections to measure *Per2* gene expression for the first cohort of rats in Experiment 1 at the level of the SCN. Consequently, that measure is based on group sizes of 4-5 rats. All line graphs showing relative clock gene expression over the course of the day have double-plotted ZT0/ZT24 data for aid in visualization of the underlying rhythm.

Data with a significant time of day effect were further analyzed using least-squares rhythmometry method with ChronoLab 3.0.3 (Mojón et al., 1992) and separated out by male or female (Experiment 1), and by cycling or non-cycling (Experiment 2). Data were tested for fit to a cosine curve constrained to a 24-h period and considered to have a significant 24-h period if the p-value was < 0.05. In cases where there was a significant 24-h period, estimates of amplitude and acrophase, along with their corresponding 95% confidence intervals (CIs), were generated. Amplitude refers to the distance from the MESOR (rhythm adjusted mean) to the peak of the rhythm, and is reported in units of normalized percent OD values in each experiment. Acrophase, the phase angle where the peak of the fitted curve falls, was converted to ZT time.

RESULTS

Experiment 1: Basal clock gene expression throughout the brain of male and female rats

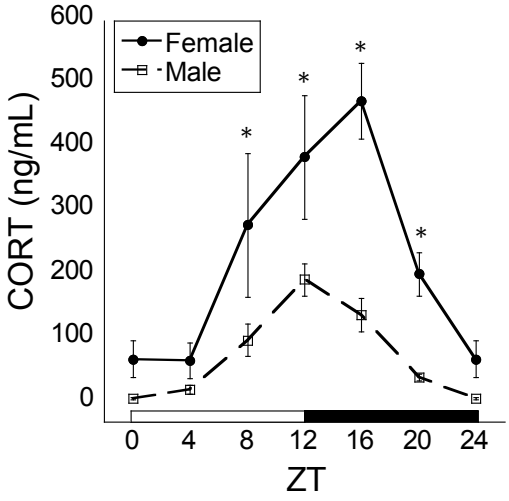
Table 2.1. Experiment 1: Statistical analysis of basal *Per1*, *Per2*, and *Bmal1* mRNA throughout the brain. Two-way ANOVA results for effect of time of day (ZT), sex, and their interaction, *p < 0.05, **p < 0.001. Cosinor analysis test for 24 h rhythmicity was analyzed separately for each sex (x = rhythmic, p < 0.05). SCN = suprachiasmatic nucleus, PVN = paraventricular nucleus of the hypothalamus, AC = anterior cingulate cortex, PL = prelimbic cortex, IL = infralimbic cortex, VO = ventral orbital cortex, Insula = rostral agranular insular cortex, CA1 (of hippocampus), CA3 (of hippocampus), supraDG = superior blade of the dentate gyrus, infraDG = inferior blade of the dentate gyrus, CEA = central amygdala, BLA = basolateral amygdala, MEA = medial amygdala.

					Cosinor Analysis (x = p<0.05)	
		ZT	Sex	Sex × ZT	Male	Female
Per1	SCN	$F_{(5,82)} = 20.9^{**}$	$F_{(1,82)} = 1.7$	$F_{(5,82)} = 0.0$	x	x
	PVN	$F_{(5,84)} = 12.8^{**}$	$F_{(1,84)} = 0.0$	$F_{(5,84)} = 0.4$	x	x
	AC	$F_{(5,84)} = 7.7^{**}$	$F_{(1,84)} = 0.5$	$F_{(5,84)} = 0.5$	x	x
	PL	$F_{(5,84)} = 3.0^{*}$	$F_{(1,84)} = 0.0$	$F_{(5,84)} = 0.4$	x	
	IL	$F_{(5,82)} = 3.0^{*}$	$F_{(1,82)} = 0.0$	$F_{(5,82)} = 0.9$	x	
	VO	$F_{(5,84)} = 11.5^{**}$	$F_{(1,84)} = 0.4$	$F_{(5,84)} = 0.7$	x	x
	Insula	$F_{(5,84)} = 8.9^{**}$	$F_{(1,84)} = 0.0$	$F_{(5,84)} = 0.5$	x	x
	CA1	$F_{(5,85)} = 4.5^{*}$	$F_{(1,85)} = 0.1$	$F_{(5,85)} = 0.7$		x
	CA3	$F_{(5,85)} = 4.2^{*}$	$F_{(1,85)} = 0.9$	$F_{(5,85)} = 1.2$	0.07	x
	supraDG	$F_{(5,85)} = 2.0$	$F_{(1,85)} = 0.4$	$F_{(5,85)} = 0.8$		
	infraDG	$F_{(5,85)} = 1.2$	$F_{(1,85)} = 0.4$	$F_{(5,85)} = 1.2$		
	CEA	$F_{(5,85)} = 5.1^{**}$	$F_{(1,85)} = 0.3$	$F_{(5,85)} = 1.5$		
	BLA	$F_{(5,85)} = 6.7^{**}$	$F_{(1,85)} = 0.1$	$F_{(5,85)} = 2.2$	x	x
	MEA	$F_{(5,85)} = 2.9^{*}$	$F_{(1,85)} = 0.1$	$F_{(5,85)} = 0.2$		
Bmal	SCN	$F_{(5,82)} = 22.1^{**}$	$F_{(1,82)} = 0.0$	$F_{(5,82)} = 0.6$	x	x
	PVN	$F_{(5,83)} = 32.2^{**}$	$F_{(1,83)} = 0.2$	$F_{(5,83)} = 1.5$	x	x
	AC	$F_{(5,83)} = 10.5^{**}$	$F_{(1,83)} = 0.0$	$F_{(5,83)} = 1.8$	x	x
	PL	$F_{(5,83)} = 8.3^{**}$	$F_{(1,83)} = 0.2$	$F_{(5,83)} = 1.0$	x	x
	IL	$F_{(5,83)} = 5.8^{**}$	$F_{(1,83)} = 0.1$	$F_{(5,83)} = 1.1$	x	x
	VO	$F_{(5,83)} = 10.2^{**}$	$F_{(1,83)} = 0.0$	$F_{(5,83)} = 0.6$	x	x
	Insula	$F_{(5,83)} = 13.4^{**}$	$F_{(1,83)} = 0.0$	$F_{(5,83)} = 1.0$	x	x
	CA1	$F_{(5,85)} = 3.4^{*}$	$F_{(1,85)} = 0.0$	$F_{(5,85)} = 0.8$	0.05	x
	CA3	$F_{(5,85)} = 3.2^{*}$	$F_{(1,85)} = 0.9$	$F_{(5,85)} = 0.4$	0.07	x
	supraDG	$F_{(5,85)} = 1.9$	$F_{(1,85)} = 0.9$	$F_{(5,85)} = 0.7$		
	infraDG	$F_{(5,85)} = 1.8$	$F_{(1,85)} = 0.2$	$F_{(5,85)} = 0.7$		
	CEA	$F_{(5,85)} = 9.5^{**}$	$F_{(1,85)} = 12.2^{*}$	$F_{(5,85)} = 1.4$	x	x
	BLA	$F_{(5,85)} = 7.6^{**}$	$F_{(1,85)} = 1.5$	$F_{(5,85)} = 0.9$	x	x
	MEA	$F_{(5,85)} = 7.0^{**}$	$F_{(1,85)} = 20.7^{*}$	$F_{(5,85)} = 0.5$	x	x
Per2	SCN	$F_{(5,36)} = 23.4^{**}$	$F_{(1,36)} = 2.4$	$F_{(5,36)} = 1.1$	x	x
	PVN	$F_{(5,78)} = 14.3^{**}$	$F_{(1,78)} = 0.4$	$F_{(5,78)} = 0.8$	x	x
	AC	$F_{(5,84)} = 12.0^{**}$	$F_{(1,84)} = 0.3$	$F_{(5,84)} = 1.3$	x	x
	PL	$F_{(5,84)} = 6.7^{**}$	$F_{(1,84)} = 0.1$	$F_{(5,84)} = 1.3$	x	x
	IL	$F_{(5,81)} = 4.0^{*}$	$F_{(1,81)} = 0.0$	$F_{(5,81)} = 1.0$	x	
	VO	$F_{(5,84)} = 17.9^{**}$	$F_{(1,84)} = 0.3$	$F_{(5,84)} = 1.0$	x	x
	Insula	$F_{(5,84)} = 18.0^{**}$	$F_{(1,84)} = 0.0$	$F_{(5,84)} = 1.3$	x	x
	CA1	$F_{(5,85)} = 10.6^{**}$	$F_{(1,85)} = 0.1$	$F_{(5,85)} = 1.2$	x	x
	CA3	$F_{(5,85)} = 11.0^{**}$	$F_{(1,85)} = 0.0$	$F_{(5,85)} = 1.7$	x	x
	supraDG	$F_{(5,85)} = 3.3^{*}$	$F_{(1,85)} = 0.1$	$F_{(5,85)} = 1.2$		x
	infraDG	$F_{(5,85)} = 1.7$	$F_{(1,85)} = 0.2$	$F_{(5,85)} = 0.8$		
	CEA	$F_{(5,85)} = 5.1^{**}$	$F_{(1,85)} = 0.9$	$F_{(5,85)} = 0.5$	0.06	x
	BLA	$F_{(5,85)} = 7.9^{**}$	$F_{(1,85)} = 0.1$	$F_{(5,85)} = 2.1$	x	x
	MEA	$F_{(5,85)} = 2.8^{*}$	$F_{(1,85)} = 0.5$	$F_{(5,85)} = 0.7$		
CORT		$F_{(5,85)} = 10.2^{**}$	$F_{(1,85)} = 29.4^{*}$	$F_{(5,85)} = 2.0$	x	x

CORT hormone levels

There was a robust diurnal rhythm of basal CORT levels, with an acrophase centered around the onset of the dark phase (male acrophase = 12.7±0.9, 95% confidence interval (CI); female acrophase = 13.8±1.5, 95% CI) [Fig 2.1]. Two-way ANOVA found significant main effects of ZT and sex. Post-hoc tests (FLSD) revealed that females had greater levels of CORT compared to males at ZT8-20 (i.e., during all times except the diurnal nadir) [Table 2.1, Fig 2.1].

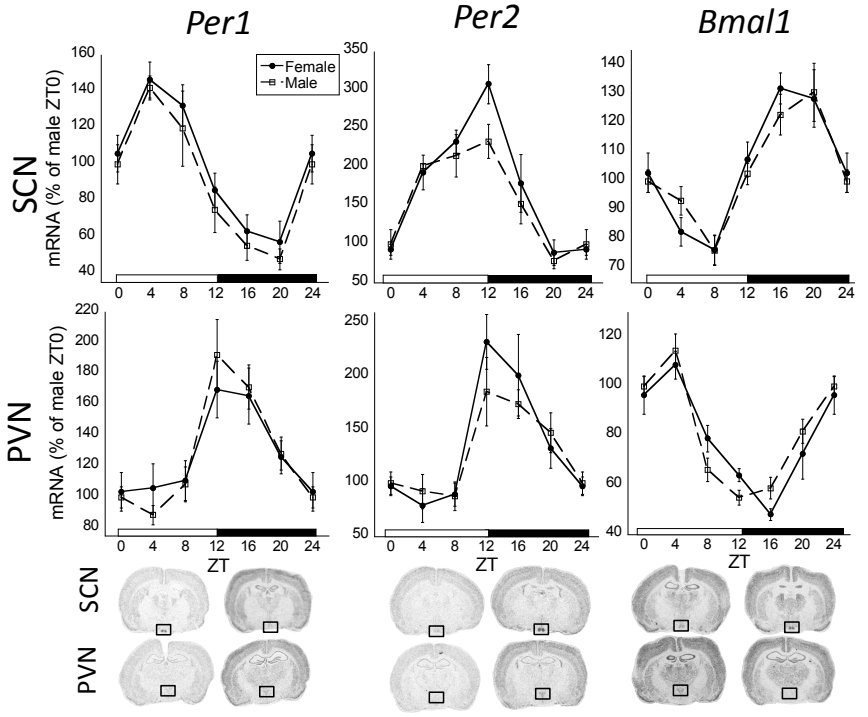
Figure 2.1. Basal levels of plasma CORT hormones in male and female rats. There is a significant ZT effect in CORT levels (two-way ANOVA, $p < 0.05$). Males and females also had a significant rhythm of CORT as determined by cosinor analysis ($p < 0.05$). There was also a significant sex effect where females had greater CORT than males at all times except the diurnal nadir (Fischer's LSD, *$p < 0.05$).



Clock Gene Analysis – Sex Comparison of Time of Day (ZT) and 24-h Rhythmic Expression

As expected, the SCN had robust, 24-h rhythmic *Per1*, *Per2*, and *Bmal1* mRNA expression (significant main effect of ZT; significant cosinor analysis) [Table 2.1, Fig 2.2].

Figure 2.2. A) 24-h rhythmic expression of *Per1*, *Per2*, and *Bmal1* mRNA in the SCN and PVN. There was an overall significant ZT effect (two-way ANOVA, $p < 0.05$) and 24-h rhythm (cosinor analysis, $p < 0.05$) for all clock genes in both the SCN and PVN of both males and females (n=8 for each sex and ZT time, except n = 4 for SCN *Per2* mRNA). Female profiles are denoted by filled circles connected by a solid line; male profiles are denoted by open squares connected by a dashed line. B) Representative autoradiographic images were taken from the diurnal peak and trough of each clock gene's rhythmic expression in the SCN and PVN (brain regions of interest denoted within black rectangle).



There was no main effect of sex or sex by ZT interaction for *Per1*, *Per2*, and *Bmal1* mRNA in the SCN. There were also no sex differences in acrophase or amplitude for all clock genes examined (cosinor analysis). There was also robust 24-h rhythmic *Per1*, *Per2*, and *Bmal1* mRNA expression for both sexes in the PVN [Fig 2.2], anterior cingulate cortex (AC), ventral orbital cortex (VO), rostral agranular insula (insula) [Fig 2.3], and basal lateral amygdala (BLA) [Fig 2.5] [Table 2.1].

Figure 2.3. 24-h rhythmic expression of *Per1*, *Per2*, and *Bmal1* mRNA in the anterior cingulate (AC), prelimbic (PL), infralimbic (IL), and ventral orbital (VO) subregions of the prefrontal cortex, as well as the rostral agranular insula. There was an overall significant ZT effect for all clock genes across all subregions of the PFC and insula (two-way ANOVA, $p < 0.05$; n=8 for each sex and ZT time). Although there was a significant 24-h rhythm of each clock gene for males in all brain regions (cosinor analysis, $p < 0.05$), females failed to have a significant rhythm for *Per1* in the PL and IL subregions, and for *Per2* in the IL. Female profiles are denoted by filled circles connected by a solid line; male profiles are denoted by open squares connected by a dashed line.

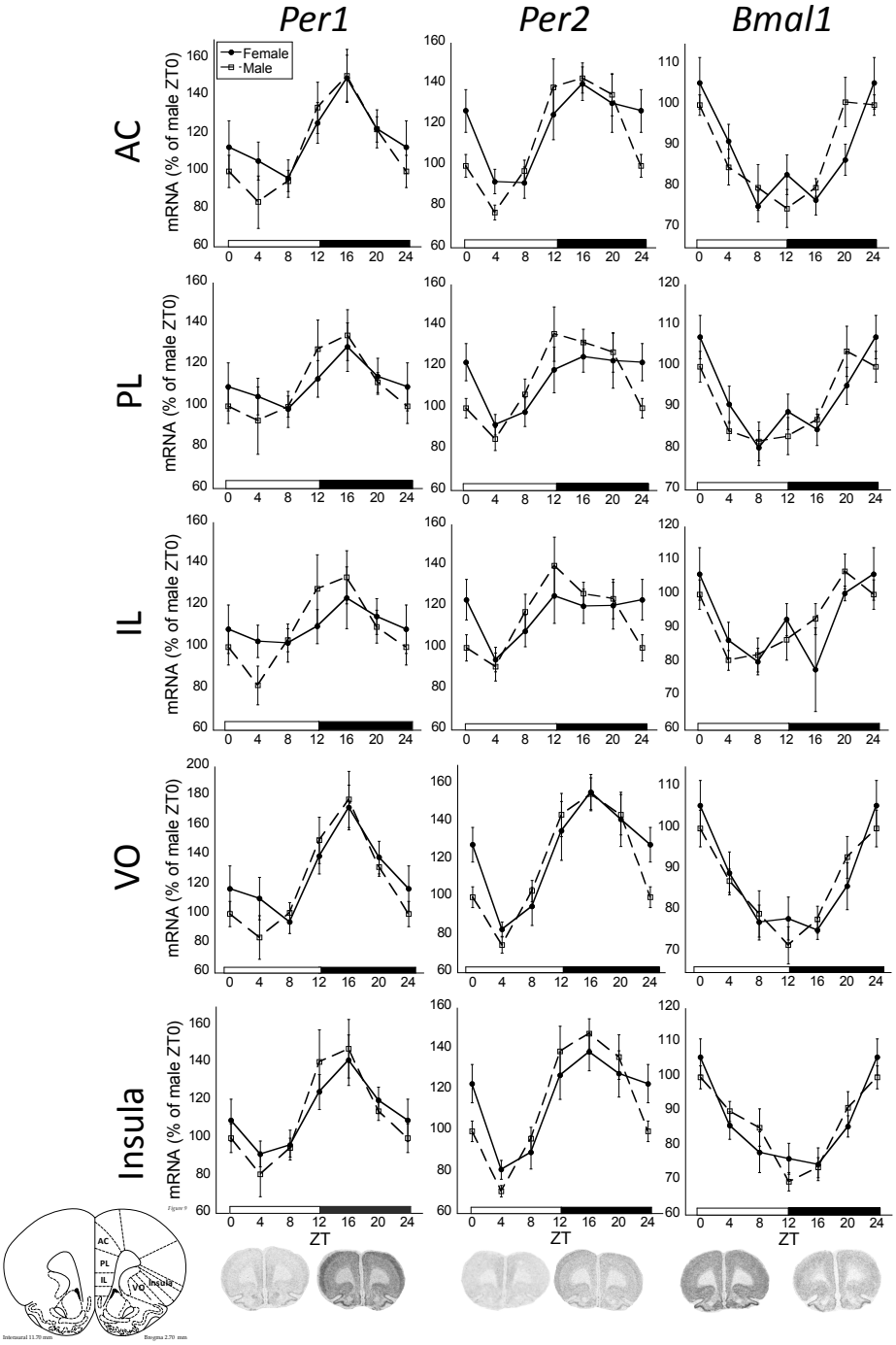


Figure 2.4. 24-h rhythmic expression of *Per1*, *Per2*, and *Bmal1* mRNA in the CA1 and CA3 subregions of the hippocampus in male and female rats. There was an overall significant ZT effect for all clock genes in the CA1 and CA3 (two-way ANOVA, $p < 0.05$; n=8 for each sex and ZT time). Whereas females had significant rhythmic expression of each clock gene in both CA1 and CA3 (cosinor analysis, $p < 0.05$), males failed to have significant rhythmic *Per1* and *Bmal1* mRNA expression in the CA1 and CA3. All clock genes failed to exhibit rhythmic expression in the inferior (not pictured) and superior blades of the dentate gyrus (ZT effect, 2-way ANOVA, $p > 0.05$), however, *Per2* mRNA expression was rhythmic in the supra DG for females (cosinor analysis, $p < 0.05$). Female profiles are denoted by filled circles connected by a solid line; male profiles are denoted by open squares connected by a dashed line.

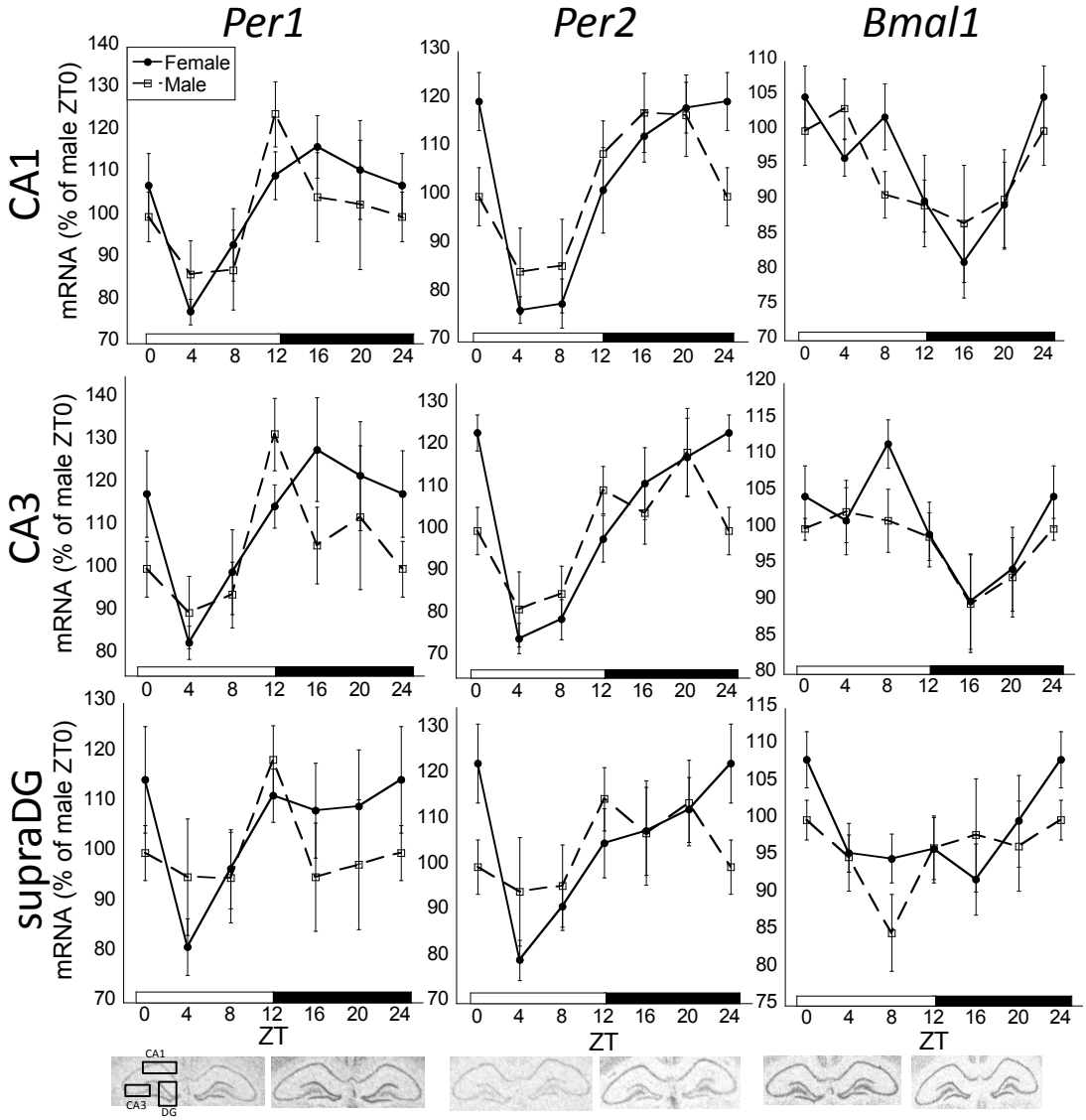
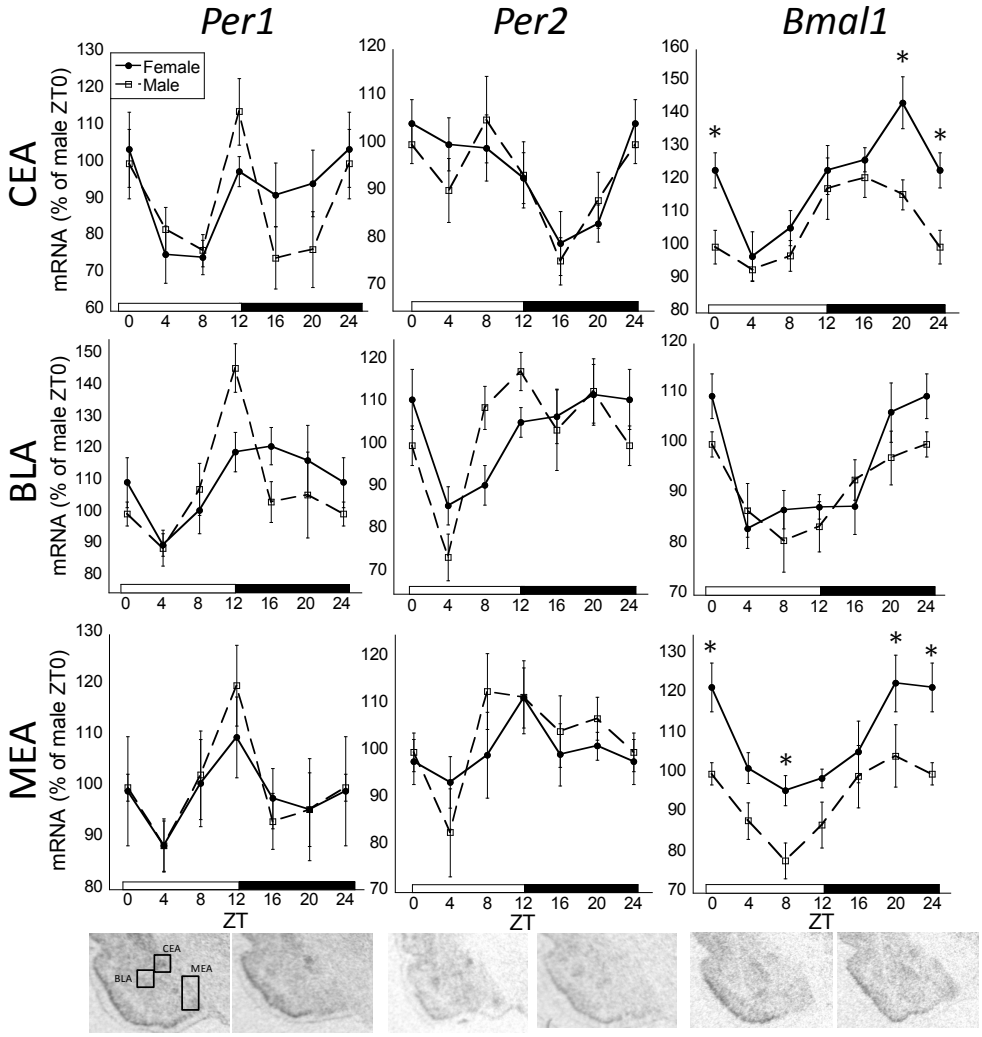


Figure 2.5. 24-h rhythmic expression of *Per1*, *Per2*, and *Bmal1* mRNA in the amygdala. There was an overall significant ZT effect for all clock genes in the BLA, MEA, and CEA (two-way ANOVA, $p < 0.05$; n=8 for each sex and ZT time). There was also a significant sex effect for *Bmal1* mRNA in the CEA and MEA (two-way ANOVA, $p < 0.05$; *$p < 0.05$, FLSD). Cosinor analysis found all clock genes to be rhythmic in the BLA for both males and females ($p < 0.05$). In the CEA, cosinor analysis found *Bmal1* mRNA rhythmic for both males and females ($p < 0.05$), and *Per2* mRNA rhythmic in females ($p < 0.05$) with males trending toward rhythmic expression ($p = 0.06$). In the MEA, only *Bmal1* mRNA was rhythmic for both males and females ($p < 0.05$). Female profiles are denoted by filled circles connected by a solid line; male profiles are denoted by open squares connected by a dashed line.



Although there was not a significant sex by ZT interaction for clock gene expression in any of the brain regions examined, cosinor analysis indicated that males but not females had a robust 24-h rhythm of *Per1* (prelimbic and infralimbic cortex) and *Per2* (infralimbic cortex) mRNA in subregions of the medial prefrontal cortex [Table 2.1]. On the other hand, females but not males

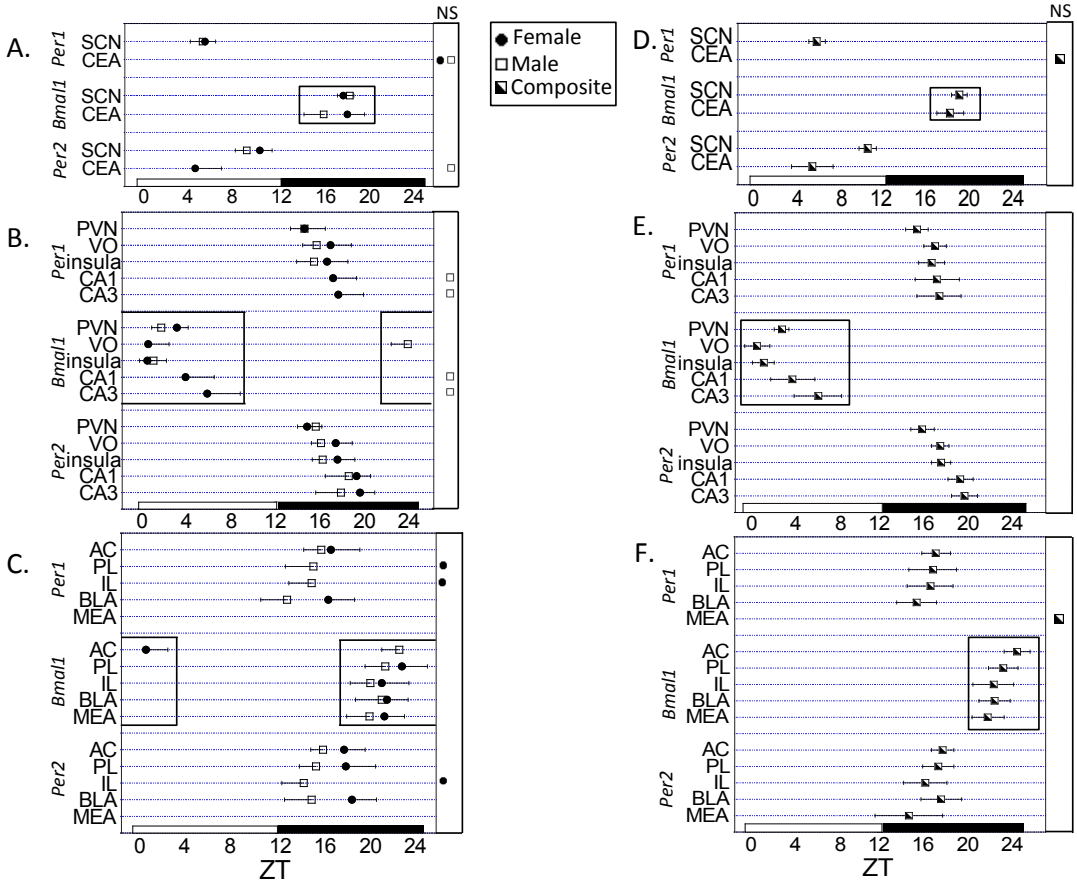
had a robust 24-h rhythm of *Per1* (CA1 and CA3), *Bmal1* (CA1 and CA3), and *Per2* (supra blade of the dentate gyrus) mRNA in subregions of the hippocampus, and a more robust 24-h rhythm of *Per2* mRNA in the central nucleus of the amygdala (CEA) [Table 2.1]. The only main effect of sex observed was for *Bmal1* mRNA expression in the CEA and medial amygdala (MEA) [Table 2.1]. Post-hoc analysis revealed that this sex effect is due to greater *Bmal1* mRNA expression in females compared to males at ZT0 and ZT20 in the CEA (greater peak levels in females), and ZT0, ZT8, and ZT20 in the MEA (greater peak and trough levels in females).

There was an absence of rhythmic *Per1* and *Bmal1* mRNA in the dentate gyrus (DG) [Table 2.1]. Although there was a significant ZT effect for *Per1* mRNA in all three amygdala subdivisions, only in the BLA did that expression have a significant 24-h rhythm. Similarly, there was a significant ZT effect for *Per1* and *Per2* mRNA in the MEA, but that expression did not have a significant 24-h rhythm.

Clock Gene Analysis – Acrophase Comparison Within and Across Brain Regions

The 24-h rhythmic expression of *Bmal1, Per1,* and *Per2* mRNA in the brain regions examined revealed three distinct composite profiles, suggesting that the molecular clock varies in its overall phase relationship between different brain regions. The three main clock profiles are: 1) *Bmal1* mRNA acrophase in the dark phase with *Per1/Per2* mRNA antiphasic to *Bmal1* mRNA; 2) *Bmal1* mRNA acrophase in the light phase with *Per1/Per2* mRNA antiphasic to *Bmal1* mRNA; 3) *Bmal1* mRNA acrophase around the transition from dark to light phase with *Per1/Per2* mRNA acrophase in the early/mid dark phase [Fig. 2.6].

Figure 2.6. Plot of acrophases (± 95% CI) for *Per1*, *Bmal1*, and *Per2* mRNA in all brain regions examined in males and females separately (A-C) and males and females combined (D-F). There were 3 distinct molecular clock profiles: 1) *Bmal1* mRNA acrophase in the dark phase with *Per1*/*Per2* mRNA antiphasic to *Bmal1* mRNA (A,D), 2) *Bmal1* mRNA acrophase in the light phase with *Per1/Per2* mRNA antiphasic to *Bmal1* mRNA (B, E), and 3) *Bmal1* mRNA acrophase around the transition from dark to light phase with *Per1/Per2* mRNA acrophase in the early/mid dark phase (C,F). The *Bmal1* mRNA acrophase clusters for each of the 3 distinct profiles are highlighted within black rectangles. Acrophase estimates are for rhythms with a significant 24-h period, $p < 0.05$; cosinor analysis (NS = not significant).



Bmal1 mRNA acrophase in the dark phase with Per1/Per2 mRNA antiphasic to Bmal1 mRNA

The SCN and the CEA both had *Bmal1* mRNA acrophase at ~ZT18. In the SCN, the acrophase of *Per1* mRNA was antiphasic to *Bmal1* mRNA and occurred at ~ZT5 [Fig 2.6]. The acrophase of *Per2* mRNA expression occurred at ~ZT9, between that of *Per1* and *Bmal1* mRNA. This is the only brain region examined in which the acrophase of *Per2* mRNA was significantly different than that of *Per1* mRNA (no overlap of 95% CIs). In the CEA, *Per1* mRNA failed to be rhythmic,

but *Per2* mRNA was rhythmic and antiphasic (acrophase ~ZT4) to *Bmal1* mRNA expression. Considered together, both the SCN and CEA had a *Bmal1* mRNA acrophase in the dark phase and an antiphasic acrophase of a negative component of the molecular clock (either *Per1* or *Per2* mRNA).

Bmal1 mRNA acrophase in the light phase with Per1/Per2 mRNA antiphasic to Bmal1 mRNA

The PVN, insula, VO, and the CA1 and CA3 subregions of the hippocampus had a *Bmal1* mRNA acrophase in the beginning of the light phase [Fig 2.6]. In these brain areas, both *Per1* and *Per2* mRNA expression had an acrophase (~ZT13-18) that was approximately antiphasic to the *Bmal1* mRNA acrophase (~ZT0-5). It is noteworthy that the acrophases for *Bmal1* and *Per1* mRNA in these brain areas were also anti-phasic to their respective acrophases in the SCN, demonstrating distinct patterns of rhythmicity between the two hypothalamic nuclei. In these brain regions, the acrophases for *Per1* and *Per2* mRNA were similar to each other.

Bmal1 mRNA acrophase at the transition from dark to light with Per1/Per2 mRNA acrophase during the early-mid dark phase

Subregions of the medial PFC (AC, PL, IL), the BLA, and the MEA had an acrophase of *Bmal1* mRNA around the late dark phase (ZT20-24), and an acrophase of *Per1* and *Per2* mRNA during the early to mid dark phase (ZT11-16) [Fig 2.6]. The acrophases of *Per1* and *Per2* mRNA of these brain regions were similar to the *Per1* and *Per2* mRNA acrophases seen in the PVN, insula, VO, and the CA1 and CA3 subregions of the hippocampus. The exception is the MEA, which lacked 24-h rhythmic expression of both *Per1* and *Per2*.

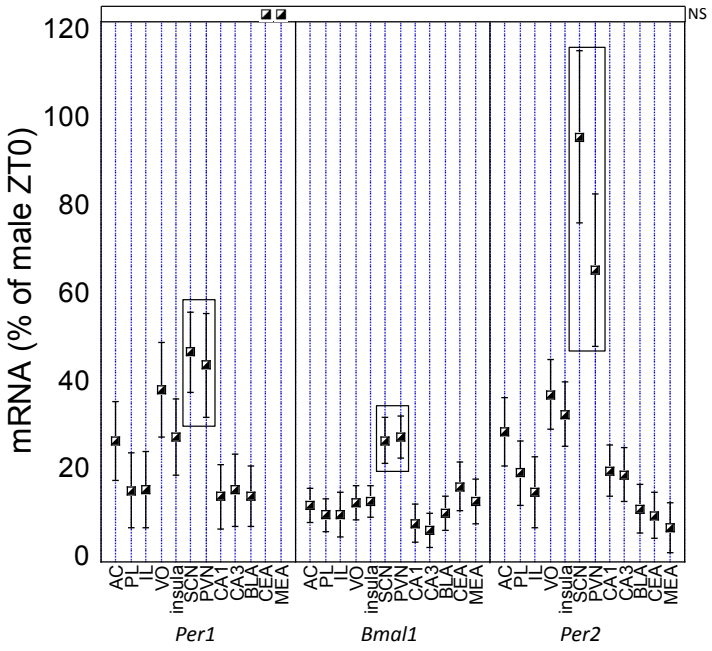
Throughout nearly all brain regions examined, there was a distinct trend for the acrophase of each of the three clock genes to be slightly phase-delayed in females compared to

males, although within any one brain region this sex difference was not statistically significant [Fig 2.6].

Rhythmic Clock Gene Amplitude Comparison Across Brain Regions

There was robust *Per1*, *Per2*, and *Bmal1* mRNA expression in the SCN and PVN, with relatively large amplitudes for each 24-h rhythm [Fig 2.7]. Consistent with other reports (Girotti et al., 2009; Harbour et al., 2014; Harbour et al., 2013), in these two brain regions there was a larger amplitude of *Per1* and *Per2* mRNA 24-h rhythms than there was for *Bmal1* mRNA. Notably, the amplitudes of the 24-h rhythms of *Bmal1* and *Per2* mRNA were lower in all other brain regions examined. The amplitude of *Per1* mRNA 24-h rhythm was somewhat lower in the PFC subregions and insula compared to the SCN and PVN, and was distinctly lower in the hippocampus and amygdala.

Figure 2.7. Plot of amplitudes (± 95% CI) for the rhythmic expression of *Per1, Bmal1,* and *Per2* mRNA in all brain regions examined in males and females combined. The SCN and PVN had the greatest amplitudes for *Per1*, *Bmal1* and *Per2* mRNA rhythmic expression compared to all other brain regions examined. In all cases where there was a significant 24 h rhythm for both sexes (Table 1, Fig. 6), there was no significant sex difference in amplitude. Brain regions of interest are arranged in rostral to caudal order on the x-axis. Instances where there was not a significant rhythm have been marked as not significant (NS).



Experiment 2: Basal clock gene expression throughout the brain of normally cycling versus non-cycling female rats

Estrous Cyclicity and CORT levels

A large percentage of female rats (43%) in this experiment were considered non-cyclers. This lack of cycling could be due to some cases of vaginal lavage-induced pseudopregnancy (Becker et al., 2004), but perhaps largely due to the Lee-Boot effect, as females were pair-housed and in rooms with only females (Van der Lee & Boot, 1955; 1956). Similar to Experiment 1, females had a large diurnal rhythm in basal plasma CORT levels (two-way ANOVA and cosinor analysis, $p < 0.05$) with an acrophase of 11.2±1.7 95% CI. There was no effect of cyclicity or ZT by cyclicity interaction.

Clock gene expression

Two-way ANOVA and cosinor analysis found similar acrophases and amplitudes for 24-h rhythms of *Bmal1* and *Per2* mRNA expression as in Experiment 1 [Table 2.2].

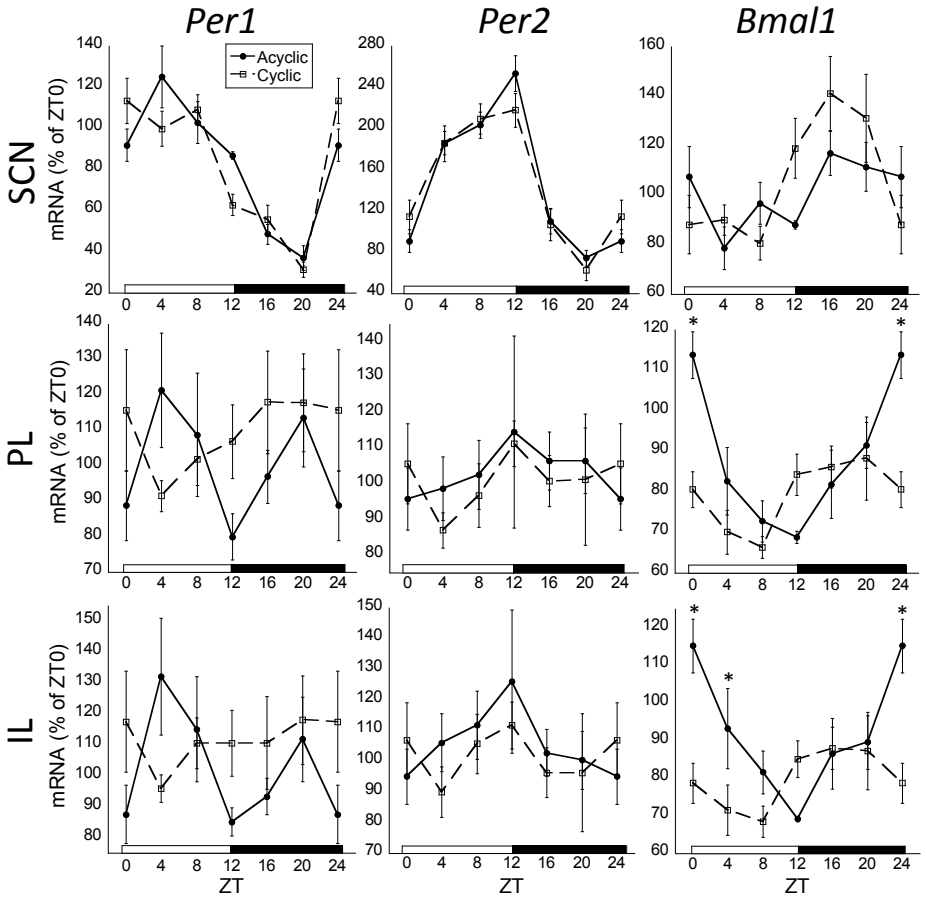
Table 2.2. Experiment 2: Statistical analysis of basal *Per1*, *Per2*, and *Bmal1* mRNA throughout the brain. Two-way ANOVA results for effect of time of day (ZT), estrous cyclicity, and their interaction, *p < 0.05, **p < 0.001. Cosinor analysis test for 24 h rhythmicity was analyzed separately for female rats that displayed a regular estrous cycle (Cyclic; n=5-10 per ZT time; N=42) and those that did not (Acyclic; n=2-8, per ZT time; N=32).

					Cosinor Analysis (x = p < 0.05)	
		ZT	Cyclicity	Cyclicity × ZT	Acyclic	Cyclic
Per1	SCN	$F_{(5,63)} = 30.5^{**}$	$F_{(1,63)} = 0.4$	$F_{(5,63)} = 2.3$	x	x
	PVN	$F_{(5,63)} = 5.1^{*}$	$F_{(1,63)} = 2.8$	$F_{(5,63)} = 0.6$	x	x
	AC	$F_{(5,62)} = 0.6$	$F_{(1,62)} = 0.3$	$F_{(5,62)} = 1.4$		
	PL	$F_{(5,62)} = 0.5$	$F_{(1,62)} = 0.9$	$F_{(5,62)} = 1.6$		
	IL	$F_{(5,62)} = 0.6$	$F_{(1,62)} = 0.7$	$F_{(5,62)} = 1.8$		
	VO	$F_{(5,62)} = 0.9$	$F_{(1,62)} = 1.0$	$F_{(5,62)} = 1.5$		x
	Insula	$F_{(5,62)} = 1.2$	$F_{(1,62)} = 0.1$	$F_{(5,62)} = 1.4$		x
	CA1	$F_{(5,63)} = 0.8$	$F_{(1,63)} = 0.4$	$F_{(5,63)} = 1.8$		
	CA3	$F_{(5,63)} = 0.6$	$F_{(1,63)} = 0.3$	$F_{(5,63)} = 1.2$		
	supraDG	$F_{(5,63)} = 1.0$	$F_{(1,63)} = 0.9$	$F_{(5,63)} = 1.4$		
	infraDG	$F_{(5,63)} = 1.1$	$F_{(1,63)} = 1.7$	$F_{(5,63)} = 1.5$		
	CEA	$F_{(5,62)} = 0.8$	$F_{(1,62)} = 0.0$	$F_{(5,62)} = 1.4$		
	BLA	$F_{(5,62)} = 2.2$	$F_{(1,62)} = 0.0$	$F_{(5,62)} = 1.9$	x	
	MEA	$F_{(5,62)} = 1.5$	$F_{(1,62)} = 0.0$	$F_{(5,62)} = 1.5$		
Bmal	SCN	$F_{(5,63)} = 4.6^{*}$	$F_{(1,63)} = 1.3$	$F_{(5,63)} = 1.4$	0.07	x
	PVN	$F_{(5,63)} = 3.6^{*}$	$F_{(1,63)} = 0.1$	$F_{(5,63)} = 1.2$	x	
	AC	$F_{(5,62)} = 7.0^{**}$	$F_{(1,62)} = 4.6^{*}$	$F_{(5,62)} = 2.8^{*}$	x	x
	PL	$F_{(5,62)} = 4.7^{*}$	$F_{(1,62)} = 2.3$	$F_{(5,62)} = 2.8^{*}$	x	x
	IL	$F_{(5,62)} = 2.1$	$F_{(1,62)} = 4.3^{*}$	$F_{(5,62)} = 2.6^{*}$	x	x
	VO	$F_{(5,62)} = 6.3^{**}$	$F_{(1,62)} = 2.4$	$F_{(5,62)} = 2.6^{*}$	x	x
	Insula	$F_{(5,62)} = 10.97^{**}$	$F_{(1,62)} = 4.1^{*}$	$F_{(5,62)} = 2.1$	x	x
	CA1	$F_{(5,63)} = 1.9$	$F_{(1,63)} = 0.7$	$F_{(5,63)} = 0.5$		
	CA3	$F_{(5,63)} = 2.2$	$F_{(1,63)} = 0.1$	$F_{(5,63)} = 0.5$		
	supraDG	$F_{(5,63)} = 1.2$	$F_{(1,63)} = 0.3$	$F_{(5,63)} = 0.2$		
	infraDG	$F_{(5,63)} = 0.8$	$F_{(1,63)} = 0.0$	$F_{(5,63)} = 0.3$		
	CEA	$F_{(5,62)} = 2.6^{*}$	$F_{(1,62)} = 0.5$	$F_{(5,62)} = 0.4$		x
	BLA	$F_{(5,62)} = 4.4^{*}$	$F_{(1,62)} = 0.8$	$F_{(5,62)} = 0.4$		x
	MEA	$F_{(5,62)} = 6.0^{**}$	$F_{(1,62)} = 0.1$	$F_{(5,62)} = 1.5$		x
Per2	SCN	$F_{(5,63)} = 34.6^{**}$	$F_{(1,63)} = 0.1$	$F_{(5,63)} = 0.7$	x	x
	PVN	$F_{(5,63)} = 10.1^{**}$	$F_{(1,63)} = 0.1$	$F_{(5,63)} = 1.1$	x	x
	AC	$F_{(5,62)} = 4.0^{*}$	$F_{(1,62)} = 1.0$	$F_{(5,62)} = 0.3$	x	x
	PL	$F_{(5,62)} = 0.7$	$F_{(1,62)} = 0.4$	$F_{(5,62)} = 0.3$		
	IL	$F_{(5,62)} = 0.8$	$F_{(1,62)} = 0.8$	$F_{(5,62)} = 0.4$		
	VO	$F_{(5,62)} = 5.7^{**}$	$F_{(1,62)} = 0.5$	$F_{(5,62)} = 0.4$	x	x
	Insula	$F_{(5,62)} = 7.2^{**}$	$F_{(1,62)} = 0.5$	$F_{(5,62)} = 0.3$	x	x
	CA1	$F_{(5,62)} = 1.8$	$F_{(1,62)} = 0.0$	$F_{(5,62)} = 1.5$		
	CA3	$F_{(5,62)} = 1.6$	$F_{(1,62)} = 0.3$	$F_{(5,62)} = 1.4$		x
	supraDG	$F_{(5,62)} = 0.7$	$F_{(1,62)} = 0.0$	$F_{(5,62)} = 1.9$		
	infraDG	$F_{(5,62)} = 1.3$	$F_{(1,62)} = 0.0$	$F_{(5,62)} = 2.0$		
	CEA	$F_{(5,62)} = 3.9^{*}$	$F_{(1,62)} = 0.3$	$F_{(5,62)} = 1.5$	x	
	BLA	$F_{(5,62)} = 0.7$	$F_{(1,62)} = 0.2$	$F_{(5,62)} = 0.5$		
	MEA	$F_{(5,62)} = 0.5$	$F_{(1,62)} = 0.0$	$F_{(5,62)} = 1.0$		
CORT		$F_{(5,63)} = 5.5^{**}$	$F_{(1,63)} = 0.0$	$F_{(5,63)} = 0.4$	x	x

Per1 mRNA failed to have a significant main effect of ZT, with the SCN and PVN as the exceptions. Similar to the profile for females in Experiment 1, *Per1* and *Per2* expression in the

prelimibc cortex (PL) and infralimbic cortex (IL) failed to be rhythmic, suggestive of an attenuated amplitude in females compared to males. There was a significant cyclicity × ZT interaction for *Bmal1* mRNA expression in the AC ($F_{(5,62)}$ = 2.8, p = 0.02), PL ($F_{(5,62)}$ = 2.8, p = 0.02), IL ($F_{(5,62)}$ = 2.6, p = 0.03), and VO (F (5,62) = 2.6, p = 0.03), where non-cyclers had more robust 24-h *Bmal1* expression compared to cyclers [Fig 2.8].

Figure 2.8. Comparison of *Per1, Per2* and *Bmal1* mRNA in the SCN, PL, and IL of female rats separated according to estrous cyclicity status. Note that there was a blunted rhythm and significant phase-advance for *Bmal1* mRNA in the PL and IL, but not SCN, of female rats that had regular estrous cycles (cyclic; n=5-10 per ZT time; N=42) compared to female rats with less regular estrous cycles (acyclic; n=2-8 per ZT time; N=32) (Significant cyclicity status by ZT interaction, two-way ANOVA, p < 0.05). There were also significant differences of cyclicity status on *Bmal1* mRNA at ZT0 (PL, IL) and ZT4 (IL) (two-way ANOVA, FLSD, *p < 0.05). Cyclicity status had no significant effect on clock gene expression in the SCN. *Per1* and *Per2* mRNA were not rhythmic in the PL and IL (p > 0.05). Acyclic profiles are denoted by filled circles connected by a solid line; cyclic profiles are denoted by open squares connected by a dashed line.



Bmal1 mRNA expression in the subregions of the PFC were the only instances where there was a significant cyclicity by ZT interaction [Table 2.2]. Examination of cosinor analysis revealed that while both non-cyclers and cyclers had rhythmic *Bmal1* mRNA expression in the PFC, cyclers had a blunted rhythm (smaller amplitude) compared to non-cyclers. In addition to a blunted amplitude, cyclers also had a significant difference (cosinor analysis) in acrophase of *Bmal1* mRNA rhythm in the PL and IL subregions. When including all of these brain regions in an ANOVA analysis that treated brain region as a repeated measure factor, there was a significant cyclicity by ZT interaction ($F_{5,62}$ = 2.9; p = 0.02) [Fig 2.8].

DISCUSSION

We found a 24-h rhythmic expression profile for both positive (*Bmal1*) and negative (*Per1*, *Per2*) regulatory clock genes across a range of brain structures that are important for emotion-related learning and control. The phase relationship between the expression of these clock genes varied within and between brain regions with three distinct profiles evident. Overall, these rhythms were similar between male and female rats. However, in female rats, the acrophase of all clock genes in nearly all brain regions examined was slightly phase-delayed compared to male rats, though not significant. There were also some brain region specific sex differences in the robustness of rhythmic clock gene expression. In addition, the PFC had less robust rhythmic clock gene expression in the composite data of female rats at various phases of the estrous cycle compared to females that were not cycling.

Three Distinct Molecular Clock Profiles

The molecular clock in the SCN represents one of the distinct molecular clocks found in this study – *Bmal1* mRNA acrophase in the dark phase with antiphasic expression of *Per1/Per2* mRNA. These acrophases matched that of previous studies seen in the SCN (Dunlap, 1999; Girotti et al., 2009, Harbour et al., 2014). This was the only tissue examined where there was a

significant difference in the acrophase of *Per1* and *Per2* mRNA with a ~4-h phase-delay of *Per2* mRNA relative to *Per1* mRNA, as previously reported in several different rodent species (Albrecht et al., 1997). The CEA was the only extra-SCN brain region examined in this study that had a phase relationship for the positive and negative clock gene components similar to the SCN. *Per2* mRNA was rhythmic in the CEA and its acrophase was antiphasic to the acrophase of *Bmal1* mRNA. Our results are consistent with the previous finding that PER2 protein diurnal expression in the CEA has a similar, although somewhat advanced phase relationship with PER2 protein expression in the SCN, which differs from other brain regions (BLA, DG) (Lamont et al., 2005). Our results are also in close agreement with the recent finding that *Bmal1* and *Per2* mRNA in the CEA and SCN have similar phase-relationships, with acrophase estimates very close to ours (Harbour et al., 2014). The CEA integrates neural input to produce expression or inhibition of fear (LeDoux et al., 1988; Wilensky et al., 2006). Circadian variations have been observed in conditioned fear expression and conditioned fear extinction memory (Chaudhury & Colwell, 2002; Eckel-Mahan et al., 2008; Valentinuzzi et al., 2001; Woodruff et al., 2015; Pace-Schott et al., 2013). Whether the similar phase-relationship of clock gene expression between the SCN and CEA has a functional significance for circadian modulation of conditioned fear remains to be determined. The SCN and CEA are comprised predominantly of GABAergic neurons (Sun & Cassell, 1993; Sun et al., 1994; Wagner et al., 1997), which may contribute to the somewhat unique circadian clock relationship of those two brain regions.

The second distinct molecular clock observed was characterized by a *Bmal1* mRNA acrophase during the light phase with anti-phasic expression of *Per1/Per2* mRNA. This was evident in the PVN, the CA1 and CA3, the VO, and the rostral insula. In the PVN there were no sex differences in acrophase or amplitude of any of the clock genes despite a robust sex difference seen in the amplitude of diurnal CORT levels. In the hippocampal DG, the only significant clock gene expression rhythm that we observed was in female rats (*Per2* mRNA), consistent with the more robust CA1 and CA3 rhythms evident in female rats compared to

males [Fig 2.4 & 2.6]. Despite the overall lack of statistical significance, the general diurnal expression profile for each clock gene in the DG was similar to that seen in the CA1 and CA3 [Fig 2.4]. Previous studies have shown rhythmic clock gene expression in rodent DG (Feillet et al., 2008a; Gilhooley et al., 2011; Harbour et al., 2014; Lamont et al., 2005), whereas others failed to see rhythmic *Per1* or *Per2* mRNA in DG or whole hippocampus of rodents (Shieh et al., 2003). Wang et al. (2009) observed rhythmic but blunted amplitude of *Per2* mRNA and PER2 protein in the DG compared to CA1, CA2, and CA3 of mouse hippocampus. Consequently, there may be some rhythmic clock gene expression in the DG with an expression profile similar, but less robust, to the profile in the CA1 and CA3. Performance on hippocampal-dependent learning and memory tasks has been shown to have diurnal differences (Eckel-Mahan et al., 2008; Smarr et al., 2014), possibly reflecting rhythmic clock gene expression in the hippocampus.

The third distinct molecular clock observed had an acrophase of *Bmal1* mRNA that occurred at the transition from the dark to the light phase and acrophases of *Per1* and *Per2* mRNA that occurred during the early to mid dark phase. Subregions of the medial PFC (AC, PL, IL) and BLA shared this molecular clock. This is the first study to show rhythmic expression of these clock genes throughout the mPFC. A key feature of this third molecular clock profile is the lack of fully anti-phasic rhythms between *Bmal1* and *Per1/Per2* mRNA due to the distinct acrophase of *Bmal1* compared to other brain regions. This lack of anti-phasic expression may be due to phenotypic variations in clock gene mRNA or protein half-lives, resulting in an anti-phasic molecular clock evident primarily at the protein level. Additionally, CRY in these tissues may play a bigger role in the negative regulation of *Bmal1* mRNA expression than PER1 or PER2 (Shearman et al., 2000). It should also be noted that in the SCN, only the rhythmic expression of *Per1* mRNA, not *Per2* mRNA, is fully anti-phasic to *Bmal1* mRNA.

Possible Factors Contributing to Tissue Differences in the Molecular Clock

The 24-h rhythmic expression of *Per1* and *Per2* mRNA in the brain regions examined had two distinct acrophases: in the mid-light phase or in the early to mid-dark phase. The timing/entrainment of these rhythms may be related to the presence of a glucocorticoid response element (GRE) and cAMP response element (CRE) in the promoter region of both genes (Colwell, 2011; So et al., 2009; Yamamoto et al., 2005). In the SCN, light increases activation of cAMP response element binding protein (CREB), which results in rapid increase of *Per1,* and somewhat delayed *Per2* transcription (Miyake et al., 2000; Shearman, 2000; Tischkau et al., 2002; Zylka et al., 1998). Thus, it may be expected that the acrophase of *Per1* and *Per2* expression occurs during the light phase in the SCN. In all other brain regions examined except the CEA, the acrophase of *Per1* and *Per2* mRNA occurred during the first half of the dark phase. The increased nocturnal physical activity and general arousal during the dark phase is associated with increased neural activation, which could increase *Per1* and *Per2* expression via CREB activation. Interestingly, in humans and a diurnal rodent (degu) *Per1/2* gene expression in extra-SCN brain regions has been found to have an acrophase during the light phase (Li et al., 2013; Vosko et al., 2009).

Glucocorticoids can rapidly induce *Per1* mRNA in fibroblasts, liver, and hippocampus, and *Per2* mRNA in mesenchymal stem cells, likely via the GRE associated with these genes' promoter region (Balsalobre et al., 2000; Conway-Campbell et al., 2010; So et al., 2009; Yamamoto et al., 2005). Peak *Per1* and *Per2* mRNA levels seen during the early dark phase in extra-SCN brain regions could be influenced by the daily peak in circulating CORT levels present at the onset of the dark phase. The diurnal CORT peak may contribute to daily GRE-mediated induction of *Per1* and *Per2* mRNA expression and act as an entrainment factor for extra-SCN clocks (Balsalobre et al., 2000; Pezük et al., 2012). There is very little GR expression in the rodent SCN (Balsalobre et al., 2000; Rosenfeld et al., 1988; 1993). Thus, *Per1* and *Per2* mRNA expression in the SCN would not be subject to this daily entraining influence.

While there were two distinct acrophases for *Per1* and *Per2* mRNA evident in the brain regions examined, there were three distinct acrophases for *Bmal1* mRNA. This suggests that the phase relationship of rhythmic *Bmal1* mRNA is not solely determined by *Per1/Per2* mRNA profiles. Retinoid-related orphan receptor (ROR) is a protein that positively induces *Bmal1* transcription (Sato et al., 2004). There are tissue variations in the expressed isoforms of ROR that may differentially modulate the oscillatory relationship between *Bmal1* gene expression and the other molecular clock elements (Emery & Reppert, 2004).

Tissue Comparison of the Molecular Clock Amplitude

Overall, there was a robust amplitude of rhythmic *Per1, Per2,* and *Bmal1* mRNA in the SCN and PVN of male and female rats, with no differences between the sexes. Clock gene expression in all other brain regions, while still rhythmic, had attenuated amplitudes compared to the SCN and PVN. These other brain regions may have less intercellular synchronization compared to the SCN and PVN. Increased synchronization could be due to a greater shared phenotypic response to the entraining influence of local neural, paracrine or hormonal factors. It is also possible that clock gene rhythmic expression patterns (phase, amplitude) may differ between cell types within some brain regions, resulting in a blunted composite amplitude at the tissue level.

Gonadal Steroid Modulation of the Molecular Clock

There were small sex differences in the robustness of rhythmic clock gene expression in certain extra-SCN brain regions. In females, there were less robust *Per1* and *Per2* rhythms in the PL and IL, brain areas important in stress adaptation, fear extinction, and emotional control (Dalley et al., 2004; Jones et al., 2011; Quirk et al., 2006). This may reflect individual rat variations in rhythm parameters due to fluctuating gonadal hormone levels as data for female rats in Experiment 1 were pooled regardless of estrous cycle status. Perrin et al. (2006)

demonstrated that PER2 protein amplitude changes depending on estrous cycle stage in the CEA and oval nucleus of the bed nucleus of the stria terminalis. There are sex differences in mPFC-modulated behaviors that rely on gonadal hormone status (Farrell et al., 2013; Fenton et al., 2014; Sutcliffe et al., 2007), and diurnal variations in performance of these tasks has also been shown in male rodents (Chaudhury & Colwell, 2002; Eckel-Mahan et al., 2008; Smarr et al., 2014; Woodruff et al., 2015). In contrast to the PFC, female rats exhibited more robust rhythmic *Per1* and *Bmal1* mRNA in the CA1 and CA3 hippocampal subregions compared to males, which in this brain region may be related to the greater amplitude of diurnal CORT levels in females due to the high density of GRs in the hippocampus [Fig 2.1] (Atkinson & Waddell, 1997; Goel et al., 2014; Viau & Meaney, 1991).

There was a distinct trend for the acrophase of female rats to be slightly phase-delayed compared to males for all clock genes in nearly all brain regions [Fig 2.6], which may be due to the influence of gonadal hormones. Previous studies have found that administration of estradiol or progesterone to ovariectomized rats, whether acute or chronic, alters the acrophase of clock gene expression in some peripheral tissues and the SCN (He et al., 2007; Nakamura et al., 2001; 2005; 2010; Smith et al., 2010). In addition, ovariectomy has been found to increase *Per1* transcriptional synchrony between cells in the SCN and decrease synchrony in some peripheral tissues (Murphy et al., 2013). We also observed significant sex differences in *Bmal1* mRNA expression within the CEA and MEA. Females had greater peak *Bmal1* mRNA expression in the CEA, and overall greater *Bmal1* mRNA levels (mesor) in the MEA compared to males. The MEA has high concentrations of gonadal hormone receptors (Gray & Bingaman, 1996; Li et al., 1993; Merchenthaler et al., 2004; Simerly et al., 1990), and gonadal hormones may regulate overall *Bmal1* mRNA expression levels in the amygdala subregions.

There was a trend for blunted aggregate clock gene rhythmic expression in females that have robust cycling of the estrous cycle compared to non-cyclers. This difference was predominantly evident for *Bmal1* mRNA expression in the PFC where, in addition to a blunted

amplitude, cycling females also had a slightly phase-advanced acrophase of *Bmal1* mRNA compared to noncycling females [Fig 2.8]. These results suggest that fluctuating gonadal hormone levels in female rats contributes to varying clock gene expression, resulting in blunted amplitude at the composite level. Our results are consistent with a study that found robust diurnal rhythm of PER2 protein in the CEA and the oval nucleus of the bed nucleus of the stria terminalis of ovariectomized female rats, whereas in non-ovariectomized rats, the amplitude of PER2 protein rhythm varied with estrous cycle phase (Perrin et al., 2006).

Concluding Summary

We found 24-h rhythmic expression of both positive and negative regulatory clock genes within a network of brain regions important for emotional regulation in male and female rats. However, there was diversity in acrophase of these molecular clocks that varied depending on brain region. In addition, females had a consistent and distinct trend to have acrophases slightly phase-delayed compared to males. There were also small differences in the robustness of rhythmic clock gene expression that depended on sex and estrous cycle status, which may be due to activational effects of gonadal hormones. Further research is necessary to determine the basis and functional significance of the brain region variations in molecular clock phase-relationships and how gonadal hormones may influence these molecular clocks. This knowledge may lead to a better understanding of the neurobiological basis of sex differences in risk for certain psychological disorders.

CHAPTER III: ADRENAL DEPENDENT AND INDEPENDENT STRESS-INDUCED *PER1* MRNA IN THE HYPOTHALAMIC PARAVENTRICULAR NUCLEUS AND PREFRONTAL CORTEX OF MALE AND FEMALE RATS

ABSTRACT

A molecular clock is expressed not only in the body's master circadian clock, the suprachiasmatic nucleus of the hypothalamus (SCN), but also in many extra-SCN brain regions. Although oscillatory clock gene expression in these extra-SCN brain regions depends on the SCN for entrainment to a light:dark cycle, the SCN has a limited extent of direct neural projections. The SCN may entrain extra-SCN molecular clocks through the circadian release of glucocorticoid hormones (CORT). CORT can induce the expression of the core clock gene, *Per1*, which may be how CORT regulates extra-SCN molecular clocks. Untimely increases in CORT due to acute stress may compromise extra-SCN molecular clock function. This study examined whether acute stress administered at ZT4 or ZT16 can rapidly increase clock gene expression (*Per1, Per2, Bmal1* mRNA; in situ hybridization) in the paraventricular nucleus of the hypothalamus (PVN) and prefrontal cortex (PFC) of male and female rats within 30 minutes. The immediate early gene *c-Fos* was also examined. Restraint stress selectively increased *Per1* and *c-Fos* mRNA, but not *Per2* or *Bmal1* mRNA in the PVN and PFC, but not SCN, of male and female rats. This increase was comparable between sexes and time of day. A second experiment using only male rats examined whether endogenous CORT is necessary for stress-induced *Per1* mRNA. In the PVN, adrenalectomy significantly attenuated stress-induced *Per1* in the PVN at ZT4, but not ZT16. In the PFC, adrenalectomy had no significant effect on stress-induced *Per1* mRNA. *c-Fos* mRNA was increased by stress regardless of adrenal status in both brain regions. These data suggest that altered Per1 expression may be the gateway by which extra-SCN molecular clocks adapt to changing environmental stimuli, such as a stressor. However, rapid increases of *Per1* mRNA levels after acute stress may be largely independent of the presence of endogenous CORT.

INTRODUCTION

Properly entrained circadian rhythms optimize an organism's survival and are essential for healthy behavior and physiology(Reppert & Weaver, 2002). Underlying these circadian rhythms are cellular molecular circadian clocks(Hastings et al., 2011; Takahashi et al., 2008). The molecular clock induces a positive regulatory component that depends on certain clock genes (*Bmal1*, *Clock/Npas2*) whose protein products dimerize and increase the expression of negative regulatory component clock genes (*Period1,2,3,* and *Cryptochrome1,2*). PER and CRY proteins heterodimers then feedback to repress the actions of the positive regulatory component. This self-regulating transcription-translation cycle approximates a 24-hour period(Albrecht, 2002; Reppert & Weaver, 2002). An oscillating molecular clock has been well-established in the body's master clock, the suprachiasmatic nucleus of the hypothalamus (SCN) (Hastings, 2004; Oishi et al., 1998; Welsh et al., 2010), and appears to be operational in many peripheral tissues (e.g., kidney, liver, heart, adrenal gland)(Yamamoto et al., 2004) and extra-SCN brain regions (e.g., prefrontal cortex, amygdala, hippocampus, hypothalamus) (Amir et al., 2004; Ángeles-Castellanos et al., 2007; Chun et al., 2015; Harbour et al., 2014; Rath et al., 2012). Previous rodent studies demonstrate that knockdown of *Bmal1* or *Per1/Per2* expression can lead to behavioral arrhythmicity (Bae et al., 2001; Bunger et al., 2000). Furthermore, *ClockΔ19* mutant mice exhibit manic-like behavior (Dzirasa et al., 2011), and selective knockdown of *Per1/Per2* in the nucleus accumbens of rats leads to anxiety-like behavior in rats(Spencer et al., 2012). In humans, mutations of a variety of clock genes are associated with mental health disorders (Bunney et al., 2014; Etain et al., 2011; Johansson et al., 2002; Lamont et al., 2009; Li et al., 2013; McCarthy & Welsh, 2012; McClung, 2007; Partonen et al., 2007).

The SCN has few direct neural projections (Sylvester et al., 2002), raising the question as to how the master clock is able to entrain all of the extra-SCN cellular clocks. Glucocorticoid hormones (CORT; cortisol in humans, corticosterone in rats) are an ideal candidate as a means

by which the SCN communicates with extra-SCN clocks. CORT has a prominent diurnal rhythm that is generated by the SCN(Herman et al., 2003), and glucocorticoid receptors are ubiquitously expressed, with the SCN as a notable exception (Balsalobre et al., 2000). Furthermore, the *Per1* gene has a functional glucocorticoid response element (GRE) associated with its promoter region(Yamamoto et al., 2005). Thus, *Per1* expression may be the gateway by which CORT can modulate entrainment of molecular clocks in extra-SCN tissues. Such a process would be analogous to the pivotal role that *Per1* expression plays in light entrainment of the SCN molecular clock. We have recently provided support for the importance of an appropriately timed diurnal peak in CORT to maintain normal clock gene expression profiles in the PFC (Woodruff et al., 2016). Per2 also has GRE sequences associated within its promoter region, although the functionality of these GREs is less well established (Cheon et al., 2013; Reddy et al., 2009; So et al., 2009). If a circadian peak in CORT modulates entrainment of extra-SCN molecular clocks, then untimely stress-induced surges in CORT may compromise the integrity of the molecular clock.

Studies using *Per2:luc* reporter mice found that chronic stress produced alterations in luciferase rhythm amplitude in the nucleus accumbens (Logan et al., 2015). Restraint stress for 2 hours per day for three consecutive days resulted in phase shifts of *Per1* and *Per2* mRNA, as well as other clock genes in peripheral tissues, hippocampus, and cortex (Tahara et al., 2015). These data collectively suggest that stress can compromise the integrity of molecular clocks. However, few studies have examined whether a single stressor has an acute effect on clock gene expression, which may be the mechanism by which stress produces subsequent changes in molecular clock rhythms (e.g., altered amplitude or phase). Takahashi et al. (2001) found that several different stressors increased *Per1* mRNA, but not *Per2* mRNA, in the PVN of male mice when examined 60 min after stressor onset. Al-Safadi et al. (2014) demonstrated that several different stressors also increased PER1 protein immunoreactivity in the PVN, dorsomedial hypothalamus, and piriform cortex 60 min after stressor onset in male rats. Stress-induced

alterations in clock gene expression have yet to be examined in female subjects, or in the prefrontal cortex (PFC), a brain region important in emotional control and stress modulation (Arnsten & Rubia, 2012; Goldman-Rakic, 1996; Robbins, 1996; Vertes, 2003). PFC dysfunction is linked to disorders associated with both stress and disruptions in circadian rhythms (Mayberg et al., 1999).

Certain mood disorders (e.g., depression, anxiety, post-traumatic stress disorder) that are associated with stress, as well as disruptions in circadian rhythms have a greater prevalence rate in women compared to men (Kessler, 2003). Stress may lead to mood disorders in part by disrupting the coordination of extra- SCN molecular clocks via untimely CORT surges. Examining the interplay between stress and disrupted circadian rhythms is a novel approach to understanding possible mechanisms underlying these mood disorders. There are also sex differences in stress reactivity, with female rats exhibiting greater CORT response to stress compared to male rats (Babb et al., 2014; Critchlow et al., 1963; Viau & Meaney, 1991). Thus, greater CORT response to stress may cause females to be more susceptible to disruptions in the molecular clock, contributing to the greater prevalence of stress-related mood disorders in females.

The objective of this study was to examine whether 30 min of acute restraint stress can induce clock gene expression in the SCN, PFC and PVN of male and female rats, as the immediate and rapid (within 30 min) induction of clock gene expression may underlie the mechanism of stress-mediated changes of the molecular clock. Each of these brain regions of interest have been shown to have rhythmic clock gene expression in both male and female rats (Chun et al., 2015). The PVN was chosen as a region of interest due to its importance as the neural component of the stress-reactive HPA axis, while the PFC was chosen due to its role in stress modulation and emotional control. The SCN was chosen for comparison purposes as the body's master clock. Because the SCN lacks glucocorticoid receptors, we predicted that there should be no changes in clock gene expression in the SCN due to stress. In order to examine

whether stress-induced clock gene expression is dependent on the presence of endogenous CORT, a second experiment examined in male rats whether adrenalectomy would prevent stress-induced clock gene expression. For all experiments, rats were stressed at two time points, one at zeitgeber time (ZT; hours after light onset) 4 and ZT16 in order to examine if there would be diurnal differences in stress-induced clock gene expression. In addition to examination of *Per1, Per2,* and *Bmal1* mRNA, we also examined *c-Fos* mRNA*,* an immediate early gene that is a marker of neuronal activation and has been shown to be rapidly induced by restraint stress in the PVN and PFC. We hypothesized that restraint stress would increase *Per1* mRNA, and possibly *Per2* and *Bmal1* mRNA, in the PFC and PVN of both male and female rats, and that this stress induction would be dependent on the presence of endogenous CORT.

MATERIALS AND METHODS

Subjects

Sprague Dawley rats (Harlan, Indianapolis, IN) were used, and all rats were ~3 months old at the time of sacrifice. Upon arrival to the facility, rats were pair-housed and evenly distributed between two adjacent individual rooms that were humidity and temperature controlled. Half of the rats were kept on a standard 12:12 hour light:dark cycle (time of lights on ranged from 0500-0800). The other half were kept on a reverse 12:12 h light:dark cycle (time of lights on ranged from 1700-2000). Rats were allowed 2 weeks to acclimate to their new environment and light:dark cycle prior to experimental manipulations. Food and water were available ad libitum. All procedures were approved by the University of Colorado Boulder's Institutional Animal Care and Use Committee, and were in accordance with the guidelines found within the *Guide for the Care and Use of Laboratory Animals* (DHHS Publication No. [NIH] 80-23, revised 2010 eighth edition).

Experiment 1: Does acute stress rapidly induce clock gene expression in male and female rats?

25 male and 25 female Sprague Dawley rats were used. Males and females were housed in separate rooms. Female rats' estrous cycles were not tracked. After two weeks of acclimation, rats received 30 minutes of restraint stress, or remained in their home cage until time of sacrifice. Time of sacrifice centered around ZT4 and ZT16.

Experiment 2: Does stress-altered clock gene expression depend on the presence of endogenous CORT in male rats?

50 male Sprague Dawley rats (Harlan, Indianapolis, IN) were used. Half of the rats received bilateral adrenalectomy surgery (ADX). This consisted of bilateral incisions within the peritoneum, then carefully locating the adrenal glands with intestinal tissue forceps, and removing them for the surrounding adipose tissue. The other half of the rats received SHAM surgeries, which followed the same procedure as for adrenalectomy, but the adrenals were left undisturbed. Surgeries occurred 2 weeks after arrival to the facility. Rats were allowed 1 week to recover before test day, where animals were acutely exposed to 30 min of restraint stress, or remained in their home cage until time of sacrifice (ZT4 or ZT16).

Experiment 3: Test of the effect of stress in total darkness on *Per1* and *c-Fos* mRNA in the SCN and PVN of male and female rats at ZT16.

Experiments 1 and 2 exhibited an increase in *Per1* mRNA expression in the SCN of stressed male rats at ZT16, and an increase in *c-Fos* mRNA expression in the SCN of both male and female stressed rats. These results contradict previous findings that stress does not affect *Per1* or *c-Fos* expression in the SCN (Al-Safadi et al., 2014; Takahashi et al., 2001). In order to determine if the results of experiments 1 and 2 were due to inadvertent light stimulation of SCN activity with the red light conditions used, 12 male and 12 female Sprague Dawley rats (Harlan,

Indianapolis, IN) were used for this experiment. Males and females were housed in separate rooms. Female rats' estrous cycles were not tracked. Testing only occurred at ZT16, during the rats' active phase. Rats were taken from their home cage, or were exposed to 30 minutes of restraint stress under complete darkness.

Test Day and Tissue Processing

For each experiment, half of the rats were stressed for 30 min in restraint tubes (6.3 cm in diameter, 16 cm length for males; 5 cm in diameter, 15.5 cm in length for females) and then immediately killed by guillotine decapitation. Non-stressed control rats remained in their home cage before time of day matched decapitation. Restraint stress was administered in a separate room than the rats' home room. Trunk blood was collected in EDTA tubes and immediately put on wet ice before being centrifuged at 4000 rpms at 4°C for 10 min. Plasma was then aliquoted, snap frozen on dry ice, and stored at -70°C until further use. Brains were extracted and then immediately flash frozen in isopentane cooled to -20°C to -30°C with dry ice. Brains were then stored at -70°C until further use.

Brains were sliced using a cryostat (Leica CM1850). 12μm coronal sections were taken at the level of the prefrontal cortex (2.2-3.2 mm anterior to bregma), the suprachiasmatic nucleus of the hypothalamus (1.3-1.4 mm posterior to bregma), and the paraventricular nucleus of the hypothalamus (1.80-1.88 mm posterior to bregma). Brain slices were thaw-mounted onto Colorfrost plus microscope slides, then stored at -70°C until use in in situ hybridization.

In Situ Hybridization

In situ hybridization for *Per1, Per2, Bmal1,* and *Cfos* followed procedures previously reported (Chun et al., 2015; Ginsberg et al., 2003; Girotti et al., 2009; 2006), with one modification concerning the concentration of the RNase incubation (0.02 g/L). Slides were set on Kodak BioFilm Maximum Resolution Autoradiography Film (Carestream Health, Windsor,

CO) for 2-4 weeks, then developed in a medical film processor SRX-101A (Konica Minolta, Tokyo, Japan).

Optical Density

Brain images from the X-ray film were captured using a lightbox Northern Light model B95 (Imaging Res Inc., St. Catharines, Ontario, Candada) and a Sony CCD video camera model XC-ST70 fitted with a Navitar 7000 zoom lens (Rochester, NY) and connected to LG3-01 frame grabber (Scion Corp., Frederick, MD) inside a Dell Dimension 500, and captured with Scion Image beta rel. 4.0.2. Once digitized, images were analyzed using ImageJ Software (NIH). Mean gray values were converted to uncalibrated optical density, and densitometry was performed on images whose gray levels fell within a linear range of the gray level to optical density ratio.

An experimenter blind to the treatment group of the rats took each measurement. Regions of interest were selected by using anatomical landmarks from *Paxinos & Watson* (4th Edition). Regions of interest at the level of the PFC include: anterior cingulate cortex (AC), prelimbic medial prefrontal cortex (PL), infralimbic medial prefrontal cortex (IL), ventral orbital cortex (VO), and insula. A virtual tool was used to draw a circle centered within each ROI to measure representative portions of each ROI. Six slices/brain were used for analysis of the PFC and insula subregions. In more caudal sections, SCN and PVN were measured by the experimenter using free-hand outline of each ROI. 4 slices/brain were used for analysis for the SCN and PVN. Measurements were taken from both hemispheres for all brain slices analyzed. The average optical density for each ROI for each brain was used for treatment group averages and statistical analysis.

Corticosterone (CORT) Assay

An enzyme-linked immunoabsorbent assay kit (cat No. K014-H1, Arbor Assays, Ann Arbor, MI) was used according to manufacturer's instructions. Plasma samples were run in duplicate and diluted 1:50 in assay buffer. Samples were then heated for 1 hour at 65°C to inactivate corticosteroid-binding globulin. Intra- and inter-assay coefficients of variation for 5 µg/100 mL (low CORT) and 20 µg/100mL (high CORT) were ≤ 10%.

Statistical Analysis

Statistical Package for Social Science (SPSS; IBM Mac version 22, 2012) was used for statistical analysis. For Experiment 1, multifactorial analysis of variance (ANOVA) was used to determine whether there were main effects of time, sex, and stress, and if there were any significant interactions. For Experiment 2, multifactorial ANOVA was used to determine whether there were main effects of time, stress, and adrenal status, and if there were any significant interactions. For Experiment 3, multifactorial ANOVA was used to assess whether there were main effects of stress and sex. Main effects and interactions were considered to be significant if $p < 0.05$, and were followed up by Fischer's Least Significant Difference (FLSD) post-hoc analysis for pair-wise group comparisons of interest. Outliers were determined using the Grubbs' Test criteria with GraphPad Software QuickCalcs (alpha = 0.05).

RESULTS

EXPERIMENT 1: Does acute stress rapidly induce clock gene expression in male and female rats?

Corticosterone (CORT)

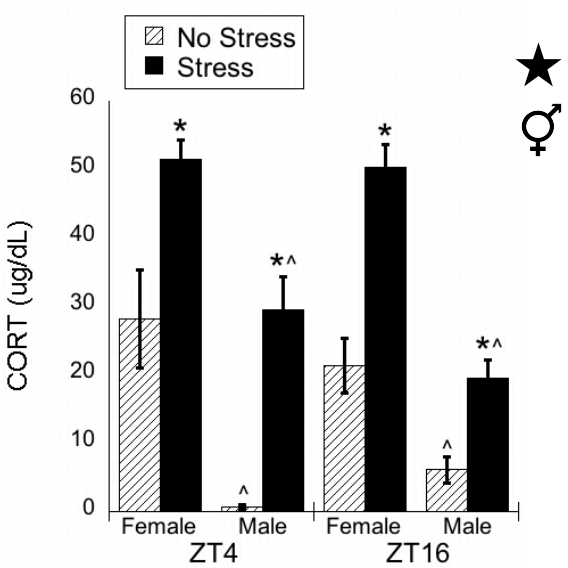
There were significant main effects for stress and sex [Table 3.1].

Table 3.1. Experiment 1: Statistical analysis of *Per1*, *Per2*, *Bmal1,* and *c-Fos* mRNA in the suprachiasmatic nucleus of the hypothalamus (SCN), paraventricular nucleus of the hypothalamus (PVN), and prefrontal cortex, as well as plasma CORT. Three-way ANOVA results for effect of stress, sex, and time, and their interaction, *p < 0.05, **p < 0.01, ***p < 0.001. [AC = anterior cingulate cortex; PL = prelimbic medial prefrontal cortex; IL = infralimbic medial prefrontal cortex; VO = ventral orbital cortex].

		Stress	Sex	Time	Stress x Sex	Stress x Time	Sex x Time	Stress x Sex x Time
Per1	**SCN**	$F_{(1,38)} = 8.38^{**}$	$F_{(1,38)} = 1.00$	$F_{(1,38)} = 36.37^{***}$	$F_{(1,38)} = 0.08$	$F_{(1,38)} = 1.32$	$F_{(1,38)} = 1.06$	$F_{(1,38)} = 1.85$
	PVN	$F_{(1,38)} = 16.24^{**}$	$F_{(1,38)} = 0.22$	$F_{(1,38)} = 5.31^{*}$	$F_{(1,38)} = 0.10$	$F_{(1,38)} = 1.52$	$F_{(1,38)} = 0.44$	$F_{(1,38)} = 1.06$
	AC	$F_{(1,40)} = 57.36^{***}$	$F_{(1,40)} = 2.04$	$F_{(1,40)} = 9.26^{**}$	$F_{(1,40)} = 0.00$	$F_{(1,40)} = 0.31$	$F_{(1,40)} = 0.85$	$F_{(1,40)} = 0.00$
	PL	$F_{(1,40)} = 71.06^{***}$	$F_{(1,40)} = 5.69^{*}$	$F_{(1,40)} = 0.04$	$F_{(1,40)} = 0.34$	$F_{(1,40)} = 0.31$	$F_{(1,40)} = 0.15$	$F_{(1,40)} = 0.50$
	IL	$F_{(1,40)} = 55.09^{***}$	$F_{(1,40)} = 2.42$	$F_{(1,40)} = 0.49$	$F_{(1,40)} = 0.04$	$F_{(1,40)} = 0.20$	$F_{(1,40)} = 0.07$	$F_{(1,40)} = 0.59$
	VO	$F_{(1,40)} = 75.19^{***}$	$F_{(1,40)} = 0.12$	$F_{(1,40)} = 14.30^{***}$	$F_{(1,40)} = 1.22$	$F_{(1,40)} = 0.73$	$F_{(1,40)} = 0.15$	$F_{(1,40)} = 0.34$
	Insula	$F_{(1,41)} = 4.38^{*}$	$F_{(1,40)} = 4.02$	$F_{(1,40)} = 27.67^{***}$	$F_{(1,40)} = 0.08$	$F_{(1,40)} = 2.89$	$F_{(1,40)} = 0.49$	$F_{(1,40)} = 0.01$
Per2	**SCN**	$F_{(1,40)} = 5.08^{*}$	$F_{(1,40)} = 0.17$	$F_{(1,40)} = 6.56^{*}$	$F_{(1,40)} = 1.39$	$F_{(1,40)} = 0.06$	$F_{(1,40)} = 4.22^{*}$	$F_{(1,40)} = 0.10$
	PVN	$F_{(1,40)} = 3.17$	$F_{(1,40)} = 0.14$	$F_{(1,40)} = 247.6^{***}$	$F_{(1,40)} = 1.09$	$F_{(1,40)} = 0.36$	$F_{(1,40)} = 0.47$	$F_{(1,40)} = 0.10$
	AC	$F_{(1,41)} = 0.32$	$F_{(1,41)} = 0.21$	$F_{(1,41)} = 43.58^{***}$	$F_{(1,41)} = 1.36$	$F_{(1,41)} = 0.11$	$F_{(1,41)} = 0.75$	$F_{(1,41)} = 0.00$
	PL	$F_{(1,41)} = 1.06$	$F_{(1,41)} = 0.41$	$F_{(1,41)} = 7.51^{**}$	$F_{(1,41)} = 0.59$	$F_{(1,41)} = 0.06$	$F_{(1,41)} = 1.03$	$F_{(1,41)} = 0.00$
	IL	$F_{(1,41)} = 0.85$	$F_{(1,41)} = 1.06$	$F_{(1,41)} = 0.24$	$F_{(1,41)} = 1.25$	$F_{(1,41)} = 0.04$	$F_{(1,41)} = 1.13$	$F_{(1,41)} = 0.01$
	VO	$F_{(1,41)} = 3.53$	$F_{(1,41)} = 1.14$	$F_{(1,41)} = 66.20^{***}$	$F_{(1,41)} = 1.27$	$F_{(1,41)} = 0.20$	$F_{(1,41)} = 0.26$	$F_{(1,41)} = 0.00$
	Insula	$F_{(1,40)} = 0.13$	$F_{(1,40)} = 0.14$	$F_{(1,40)} = 89.87^{***}$	$F_{(1,40)} = 0.48$	$F_{(1,40)} = 0.26$	$F_{(1,40)} = 0.46$	$F_{(1,40)} = 0.01$
Bmal 1	**SCN**	$F_{(1,40)} = 0.75$	$F_{(1,40)} = 1.61$	$F_{(1,40)} = 113.8^{***}$	$F_{(1,40)} = 0.14$	$F_{(1,40)} = 0.95$	$F_{(1,40)} = 5.02^{*}$	$F_{(1,40)} = 0.28$
	PVN	$F_{(1,41)} = 0.02$	$F_{(1,41)} = 0.10$	$F_{(1,41)} = 139.6^{***}$	$F_{(1,41)} = 0.64$	$F_{(1,41)} = 0$	$F_{(1,41)} = 0.19$	$F_{(1,41)} = 3.13$
	AC	$F_{(1,40)} = 0.57$	$F_{(1,40)} = 0.06$	$F_{(1,40)} = 9.73^{**}$	$F_{(1,40)} = 0.00$	$F_{(1,40)} = 0.12$	$F_{(1,40)} = 0.34$	$F_{(1,40)} = 0.26$
	PL	$F_{(1,40)} = 0.52$	$F_{(1,40)} = 0.07$	$F_{(1,40)} = 3.00$	$F_{(1,40)} = 0.53$	$F_{(1,40)} = 0.15$	$F_{(1,40)} = 0.08$	$F_{(1,40)} = 0.02$
	IL	$F_{(1,40)} = 0.21$	$F_{(1,40)} = 0.00$	$F_{(1,40)} = 0.22$	$F_{(1,40)} = 0.20$	$F_{(1,40)} = 0.07$	$F_{(1,40)} = 0.03$	$F_{(1,40)} = 0.05$
	VO	$F_{(1,40)} = 0.18$	$F_{(1,40)} = 0.02$	$F_{(1,40)} = 11.77^{***}$	$F_{(1,40)} = 0.60$	$F_{(1,40)} = 0.29$	$F_{(1,40)} = 1.18$	$F_{(1,40)} = 0.02$
	Insula	$F_{(1,40)} = 0.17$	$F_{(1,40)} = 0.03$	$F_{(1,40)} = 19.86^{***}$	$F_{(1,40)} = 0.36$	$F_{(1,40)} = 0.06$	$F_{(1,40)} = 0.48$	$F_{(1,40)} = 0$
C-fos	**SCN**	$F_{(1,40)} = 61.05^{***}$	$F_{(1,40)} = 1.31$	$F_{(1,40)} = 27.29^{***}$	$F_{(1,40)} = 1.40$	$F_{(1,40)} = 32.4^{**}$	$F_{(1,40)} = 7.99^{**}$	$F_{(1,40)} = 0.17$
	PVN	$F_{(1,40)} = 285.7^{***}$	$F_{(1,40)} = 0.78$	$F_{(1,40)} = 6.67^{*}$	$F_{(1,40)} = 0.04$	$F_{(1,40)} = 5.71^{*}$	$F_{(1,40)} = 0.01$	$F_{(1,40)} = 4.29^{*}$
	AC	$F_{(1,39)} = 201.5^{***}$	$F_{(1,39)} = 1.89$	$F_{(1,39)} = 5.02^{*}$	$F_{(1,39)} = 0.59$	$F_{(1,39)} = 0.04$	$F_{(1,39)} = 1.86$	$F_{(1,39)} = 2.47$
	PL	$F_{(1,41)} = 546.8^{***}$	$F_{(1,41)} = 7.5^{**}$	$F_{(1,41)} = 0.67$	$F_{(1,41)} = 3.27$	$F_{(1,41)} = 2.69$	$F_{(1,41)} = 0.52$	$F_{(1,41)} = 0.00$
	IL	$F_{(1,40)} = 624.3^{***}$	$F_{(1,40)} = 2.80$	$F_{(1,40)} = 1.50$	$F_{(1,40)} = 4.67^{*}$	$F_{(1,40)} = 0.05$	$F_{(1,40)} = 0.14$	$F_{(1,40)} = 0.39$
	VO	$F_{(1,41)} = 250.5^{***}$	$F_{(1,41)} = 0.51$	$F_{(1,41)} = 25.60^{***}$	$F_{(1,41)} = 0.80$	$F_{(1,41)} = 6.02^{*}$	$F_{(1,41)} = 3.46$	$F_{(1,41)} = 0.55$
	Insula	$F_{(1,41)} = 3.97$	$F_{(1,41)} = 9.0^{**}$	$F_{(1,41)} = 17.29^{***}$	$F_{(1,41)} = 0.07$	$F_{(1,41)} = 1.94$	$F_{(1,41)} = 0.06$	$F_{(1,41)} = 0.47$
CORT	**CORT**	$F_{(1,42)} = 75.95^{***}$	$F_{(1,42)} = 77.7^{***}$	$F_{(1,42)} = 1.35$	$F_{(1,42)} = 0.92$	$F_{(1,42)} = 0.80$	$F_{(1,42)} = 0.11$	$F_{(1,42)} = 3.77$

As expected, acute restraint stress significantly increased CORT for both sexes at both times of day ($p < 0.05$). In line with what is well-established (Atkinson & Waddell, 1997; Babb et al., 2014; Viau & Meaney, 1991), females had significantly higher basal and stress-induced CORT than males for both ZT4 and ZT16 ($p < 0.01$) [Figure 3.1]. Females also had much greater variability in basal CORT levels at ZT4 than males, consistent with previous findings that female but not male Sprague-Dawley rats have pronounced ultradian variations in basal CORT at this time of day (Spiga, Walker, Terry, & Lightman, 2014).

Figure 3.1. Experiment 1: effect of stress, sex and time of day on plasma CORT. Plasma CORT was increased by 30 min of acute restraint stress at both ZT4 and ZT16. Females had greater CORT compared to males during both basal and stressed conditions. [Star symbol indicates main effect of stress, and sex symbol indicates main effect of sex, $p < 0.05$, 3-way ANOVA. * = stress effect; # = time of day effect; ^ = sex effect; $p < 0.05$, FLSD post hoc pair-wise tests, n = 6-7].

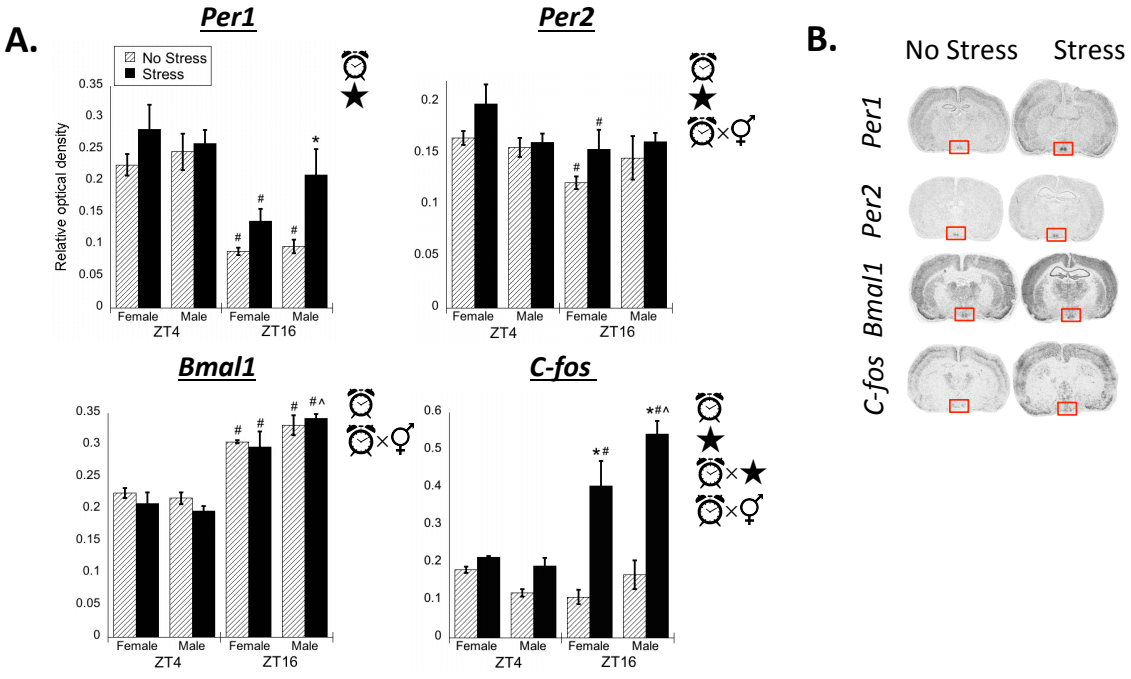


Suprachiasmatic Nucleus of the Hypothalamus (SCN)

Unexpectedly, there was a significant main effect of stress on *Per1, Per2,* and *c-Fos* mRNA in the SCN and a significant stress x time of day interaction for *c-Fos* mRNA [Table 3.1]. Post-hoc analysis revealed that stressed males only at ZT16 had significantly higher *Per1* mRNA compared to no stress individuals (FLSD, $p < 0.05$). In addition, both males and females at ZT16 had increased *c-Fos* mRNA compared to no stress controls (FLSD, $p < 0.05$). These apparent effects of stress on SCN *Per1* and *c-Fos* mRNA are inconsistent with prior studies (Al-Safadi et al., 2014; Takahashi et al., 2001). Because we observed a significant stress effect only at ZT16, we questioned whether our red light conditions present during stress may have confounded the results in the SCN. Thus, Experiment 3 (see below) examined whether stress at ZT16 would still induce *Per1* and *c-Fos* mRNA under complete dark conditions. Although there was a significant overall effect of acute stress on *Per2* mRNA, post-hoc analysis did not reveal any significant differences between stress and no stress groups for a particular stress and time of day condition. There was a significant time of day effect for *Per1, Per2*, *Bmal1,* and *c-Fos*

[Table 3.1], where *Per1, Per2,* and *c-Fos* mRNA were greater at ZT4, and *Bmal1* mRNA was greater at ZT16 (FLSD, $p < 0.05$). These results confirm previous studies that have determined the acrophases of the clock genes under basal conditions in the SCN of male and female rats (Chun et al., 2015; Girotti et al., 2009; Harbour et al., 2014). There was no significant sex effect for the expression of any genes examined [Figure 3.2], although *Per2, Bmal1,* and *c-Fos* all had significant sex x time of day interactions, suggesting that gonadal hormones may differentially influence SCN clock gene expression under these conditions.

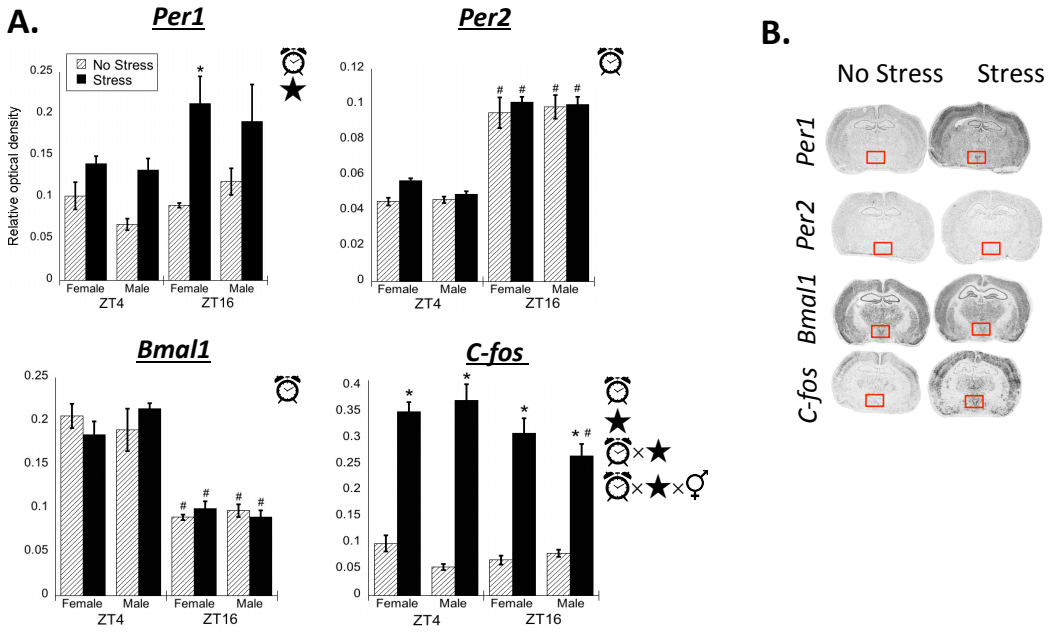
Figure 3.2. Experiment 1: effect of stress, sex and time of day on gene expression in the SCN. There was a significant main effect of time of day for *Per1, Per2, Bmal1* and *c-Fos* mRNA in the SCN of male and female rats. There was also an apparent effect of stress on *Per1* and *c-Fos* mRNA when administered at ZT16. This effect was not present in Experiment 3 when stress was administered in total darkness (see Figure 10). [Clock symbol indicates main effect of time of day, star symbol indicates main effect of stress, and sex symbol indicates main effect of sex. Interactions between these factors are also indicated, $p < 0.05$, 3-way ANOVA. * = stress effect; # = time of day effect; ^ = sex effect; $p < 0.05$, FLSD post hoc pair-wise tests, n=6-7] B) Representative autoradiographs of clock gene expression under no stress and stress conditions. All images were taken from females at ZT4.



Paraventricular Nucleus of the Hypothalamus (PVN)

Stress produced a significant overall increase in Per1 and *c-Fos* mRNA in the PVN [Table 3.1, Figure 3.3].

Figure 3.3. Experiment 1: effect of stress, sex and time of day on gene expression in the PVN. A) 30 min of acute restraint stress increased *Per1* mRNA in the PVN of male and female rats, but not *Per2* or *Bmal1. c-Fos* mRNA was also increased in the PVN. There was also a time of day effect for all genes. [Clock symbol indicates main effect of time of day, star symbol indicates main effect of stress, and sex symbol indicates main effect of sex. Interactions between these factors are also indicated, $p < 0.05$, 3-way ANOVA. * = stress effect; # = time of day effect; ^ = sex effect; $p < 0.05$, FLSD post hoc pair-wise tests, n=6-7] B) Representative autoradiographs of females at ZT4 that were home cage controls or stressed.



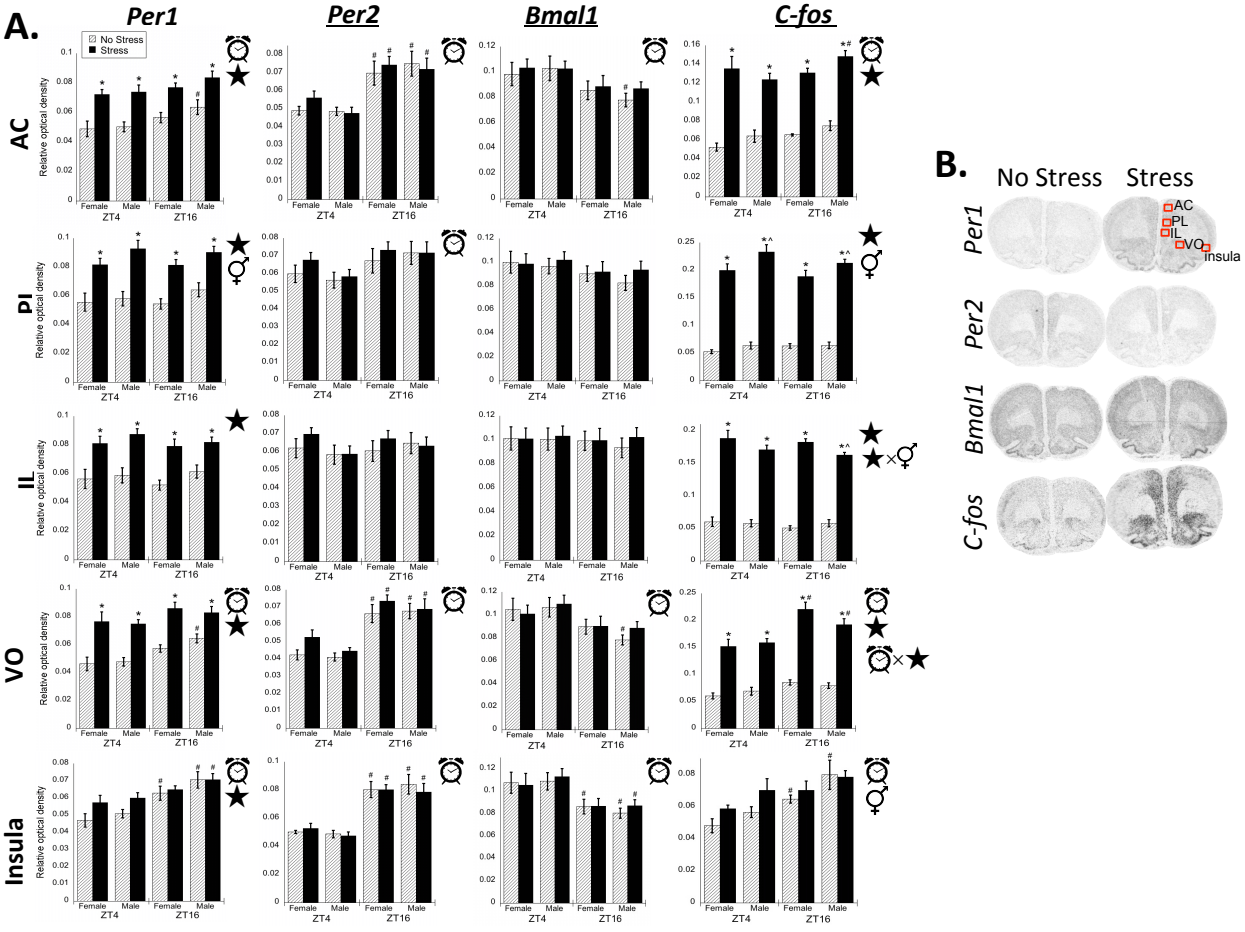
In the case of *c-Fos* mRNA this effect was significantly moderated by time of day with somewhat higher stress-induced *c-Fos* mRNA at Z4 than ZT16 (time of day X stress interaction, $p < 0.05$). There was also an overall significant time of day effect for *Per1, Per2*, *Bmal1*, and *c-Fos* mRNA [Table 3.1]. Post-hoc comparisons show that basal (no stress) *Per2* mRNA levels were greater at ZT16 while *Bmal1* mRNA levels were greater at ZT4, which aligns with previous studies examining the acrophases of these clock genes under basal conditions across the 24 hour day (Chun et al., 2015; Girotti et al., 2009). There was also an overall time of day difference for *Per1* mRNA (higher at ZT16) and *c-Fos* mRNA (higher at ZT4), but the time of day difference pattern for the expression of these two genes was somewhat variable across sex and stress status. There were no significant main effects of sex for the expression of these genes, however, there

were significant time of day × stress and time of day × stress × sex interactions for *c-Fos* mRNA, where *c-Fos* mRNA was greater at ZT4 versus ZT16 for stressed males only [FLSD, $p < 0.05$; Table 3.1; Figure 3.3].

Prefrontal Cortex (PFC)

Stress increased *Per1* and *c-Fos* mRNA in all subregions of the prefrontal cortex (AC, PL, IL, VO) [Table 3.1; Figure 3.4].

Figure 3.4. Experiment 1: effect of stress, sex and time of day on gene expression in the PFC and insula. A) Acute stress increased *Per1* and *c-Fos* mRNA within the AC, PL, IL, and VO – but not the agranular insula – of male and female rats at both ZT4 and ZT16. *Per2* and *Bmal1* mRNA were unaffected by stress. The insula did not have stress-induced *Per1* or *c-Fos* mRNA. [Clock symbol indicates main effect of time of day, star symbol indicates main effect of stress, and sex symbol indicates main effect of sex. Interactions between these factors are also indicated, $p < 0.05$, 3-way ANOVA. * = stress effect; # = time of day effect; ^ = sex effect; $p < 0.05$, FLSD post hoc pair-wise tests, n=6-7] B) Representative autoradiographs of females at ZT4 under no stress or stress conditions.



This stress effect was significant for both males and females at both ZT4 and ZT16 (FLSD, $p < 0.05$). There was also a significant main effect of time of day for all clock genes and *c-Fos* mRNA within the AC and VO, but only a significant effect of time of day in the PL for *Per2* [Table 3.1]. *Per1, Per2,* and *c-Fos* mRNA levels were higher at ZT16 for both males and females, whereas *Bmal1* mRNA levels were higher at ZT4 for both males and females. These findings correspond with the diurnal profile of rhythmic clock gene expression in the PFC subregions under basal conditions seen in previous studies in male and female rats (Chun et al., 2015; Woodruff et al., 2016). Additionally, the lack of a robust time of day effect seen in the PL and IL of this study reflects the lower amplitude of rhythmic clock gene expression seen under basal conditions for these PFC subregions (Chun et al., 2015). There was a significant main effect of sex for *Per1* and *c-Fos* mRNA in the PL, but FLSD post-hoc analysis revealed only significantly higher *c-Fos* mRNA in stressed males compared to females at both times of day. There was also a significant time of day × stress interaction for *c-Fos* in the VO, where for both sexes there was a greater effect of stress at ZT16 compared to ZT4 [Table 3.1; Figure 3.4]. There was also a significant stress x sex interaction for *c-Fos* mRNA in the IL, where stressed females had greater *c-Fos* mRNA than males at ZT16 [Figure 3.4].

Rostral Agranular Insula

Although there was a significant main effect of stress in the insula for *Per1* mRNA [Table 3.1], this effect was small and FLSD post-hoc analysis did not show any significant pair-wise comparisons for a stress effect within either sex at either time of day. Moreover, there was no significant effect of stress for *c-Fos* mRNA, suggesting that in contrast to the PVN and PFC, acute stress had limited effect on the expression of these genes in the insula. There was a significant main effect of time of day for all clock genes and *c-Fos* [Table 3.1], with greater mRNA expression for *Per1, Per2,* and *c-Fos* mRNA at ZT16, and greater *Bmal1* mRNA at ZT4. These data are consistent with previous studies that observed peak diurnal expression of *Per1*

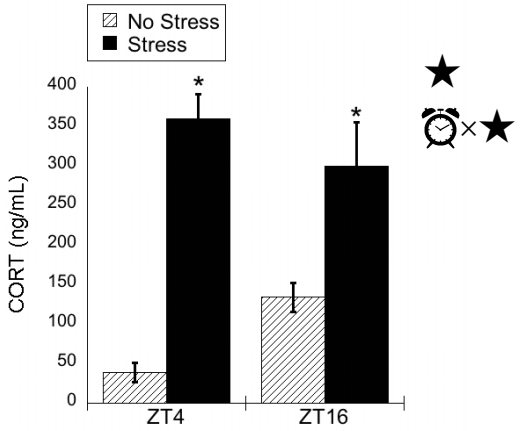
and *Per2* mRNA in the insula around ZT16, and peak *Bmal1* mRNA expression in the insula around ZT4 (Chun et al., 2015). There was also a main effect of sex on *c-Fos* mRNA in the insula with overall higher levels in males than females [Table 3.1; Figure 3.4].

EXPERIMENT 2: Does stress-altered clock gene expression depend on the presence of endogenous CORT in male rats?

Corticosterone (CORT)

CORT levels for ADX rats were below detectable levels, thus only CORT plasma values for SHAM rats were analyzed and are displayed [Table 3.2; Figure 3.5].

Figure 3.5. Experiment 2: effect of stress and time of day on plasma CORT levels of male SHAM rats. Stress increased plasma CORT levels to similar levels at both times of day. There were no detectable CORT levels in ADX rats. [Clock symbol indicates main effect of time of day, star symbol indicates main effect of stress. Interactions between these factors are also indicated, $p < 0.05$, 2-way ANOVA; * = stress effect;; $p < 0.05$; FLSD post hoc pair-wise tests, n=6-7].



There was a significant main effect of stress and a significant time of day x stress interaction [Table 3.2].

Table 3.2. Experiment 2: Statistical analysis of *Per1*, *Per2*, *Bmal1,* and *c-Fos* mRNA in the suprachiasmatic nucleus of the hypothalamus (SCN), paraventricular nucleus of the hypothalamus (PVN), and prefrontal cortex, as well as plasma CORT. Three-way ANOVA results for effect of stress, adrenal status, time, and their interaction, *p < 0.05, **p < 0.01, ***p < 0.001. [AC = anterior cingulate cortex; PL = prelimbic medial prefrontal cortex; IL = infralimbic medial prefrontal cortex; VO = ventral orbital cortex].

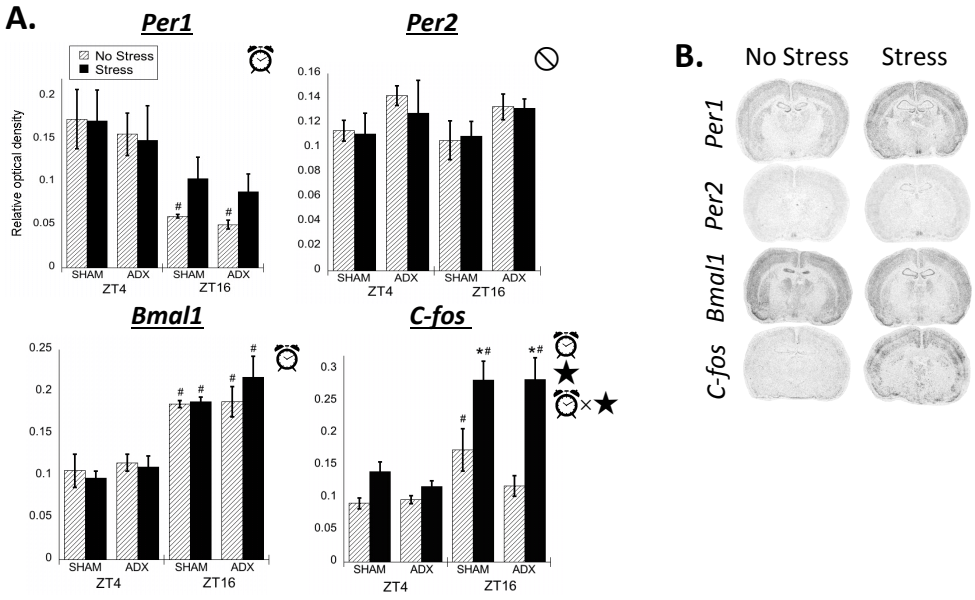
		Stress	Adrenal Status	Time	Stress x Adrenal Status	Stress x Time	Adrenal Status x Time	Stress x Adrenal Status x Time
Per1	SCN	$F_{(1,40)}$ =0.88	$F_{(1,40)}$ =0.70	$F_{(1,40)}$ = 19.75***	$F_{(1,40)}$ = 0.02	$F_{(1,40)}$ = 1.36	$F_{(1,40)}$ =0.04	$F_{(1,40)}$ = 0.00
	PVN	$F_{(1,36)}$ = 30.50***	$F_{(1,36)}$ = 7.28*	$F_{(1,36)}$ = 8.36**	$F_{(1,36)}$ = 0.95	$F_{(1,36)}$ = 0.22	$F_{(1,36)}$ = 0.02	$F_{(1,36)}$ = 2.96
	AC	$F_{(1,40)}$ = 6.32*	$F_{(1,40)}$ = 0.08	$F_{(1,40)}$ = 19.30***	$F_{(1,40)}$ = 0.22	$F_{(1,40)}$ = 0.14	$F_{(1,40)}$ = 0.14	$F_{(1,40)}$ = 0.07
	PL	$F_{(1,39)}$ = 20.22***	$F_{(1,39)}$ = 0.32	$F_{(1,39)}$ = 13.26**	$F_{(1,39)}$ = 0.05	$F_{(1,39)}$ = 0.24	$F_{(1,39)}$ = 1.70	$F_{(1,39)}$ = 0.80
	IL	$F_{(1,40)}$ = 7.79**	$F_{(1,40)}$ = 0.66	$F_{(1,40)}$ = 10.58**	$F_{(1,40)}$ = 0.01	$F_{(1,40)}$ = 0.09	$F_{(1,40)}$ = 1.12	$F_{(1,40)}$ = 0.57
	VO	$F_{(1,38)}$ = 13.17**	$F_{(1,38)}$ = 1.00	$F_{(1,38)}$ = 35.48***	$F_{(1,38)}$ = 1.09	$F_{(1,38)}$ = 0.31	$F_{(1,38)}$ = 0.20	$F_{(1,38)}$ = 0.55
	Insula	$F_{(1,38)}$ = 0.54	$F_{(1,38)}$ = 1.89	$F_{(1,38)}$ = 40.67***	$F_{(1,38)}$ = 0.16	$F_{(1,38)}$ = 0.01	$F_{(1,38)}$ = 0.84	$F_{(1,38)}$ = 0.02
Per2	SCN	$F_{(1,42)}$ = 0.14	$F_{(1,42)}$ = 5.62*	$F_{(1,42)}$ = 0.13	$F_{(1,42)}$ = 0.16	$F_{(1,42)}$ = 0.22	$F_{(1,42)}$ = 0.01	$F_{(1,42)}$ = 0.03
	PVN	$F_{(1,40)}$ = 0.03	$F_{(1,40)}$ = 2.09	$F_{(1,40)}$ = 82.82***	$F_{(1,40)}$ = 1.47	$F_{(1,40)}$ = 0.37	$F_{(1,40)}$ = 7.44**	$F_{(1,40)}$ = 0.51
	AC	$F_{(1,41)}$ = 0.02	$F_{(1,41)}$ = 1.60	$F_{(1,41)}$ = 17.24***	$F_{(1,41)}$ = 0.19	$F_{(1,41)}$ = 0.31	$F_{(1,41)}$ = 0.48	$F_{(1,41)}$ = 0.09
	PL	$F_{(1,41)}$ = 0.27	$F_{(1,41)}$ = 1.14	$F_{(1,41)}$ = 4.63*	$F_{(1,41)}$ = 010	$F_{(1,41)}$ = 0.34	$F_{(1,41)}$ = 0.07	$F_{(1,41)}$ = 0.04
	IL	$F_{(1,41)}$ = 0.24	$F_{(1,41)}$ = 1.54	$F_{(1,41)}$ = 2.128	$F_{(1,41)}$ = 0.06	$F_{(1,41)}$ = 0.37	$F_{(1,41)}$ = 0.02	$F_{(1,41)}$ = 0.01
	VO	$F_{(1,41)}$ = 0.72	$F_{(1,41)}$ = 1.75	$F_{(1,41)}$ = 23.70***	$F_{(1,41)}$ = 0.57	$F_{(1,41)}$ = 0.32	$F_{(1,41)}$ = 0.65	$F_{(1,41)}$ = 0.06
	Insula	$F_{(1,41)}$ = 0.00	$F_{(1,41)}$ = 1.58	$F_{(1,41)}$ = 40.37***	$F_{(1,41)}$ = 0.94	$F_{(1,41)}$ = 0.13	$F_{(1,41)}$ = 1.67	$F_{(1,41)}$ = 0.02
Bmal1	SCN	$F_{(1,41)}$ = 0.18	$F_{(1,41)}$ = 1.61	$F_{(1,41)}$ = 66.09***	$F_{(1,41)}$ = 0.49	$F_{(1,41)}$ = 1.09	$F_{(1,41)}$ = 0.05	$F_{(1,41)}$ = 0.29
	PVN	$F_{(1,39)}$ = 0.35	$F_{(1,39)}$ = 9.58**	$F_{(1,39)}$ = 36.53***	$F_{(1,39)}$ = 0.44	$F_{(1,39)}$ = 0.18	$F_{(1,39)}$ = 6.17*	$F_{(1,39)}$ = 0.71
	AC	$F_{(1,41)}$ = 0.10	$F_{(1,41)}$ = 4.14*	$F_{(1,41)}$ = 2.72	$F_{(1,41)}$ = 0.09	$F_{(1,41)}$ = 4.00	$F_{(1,41)}$ = 1.36	$F_{(1,41)}$ = 1.33
	PL	$F_{(1,42)}$ = 0.54	$F_{(1,42)}$ = 6.43*	$F_{(1,42)}$ = 1.16	$F_{(1,42)}$ = 0.00	$F_{(1,42)}$ = 1.64	$F_{(1,42)}$ = 0.39	$F_{(1,42)}$ = 0.23
	IL	$F_{(1,41)}$ = 1.66	$F_{(1,41)}$ = 3.49	$F_{(1,41)}$ = 5.16*	$F_{(1,41)}$ = 0.11	$F_{(1,41)}$ = 3.53	$F_{(1,41)}$ = 0.46	$F_{(1,41)}$ = 0.68
	VO	$F_{(1,40)}$ = 2.30	$F_{(1,40)}$ = 10.85**	$F_{(1,40)}$ = 2.50	$F_{(1,40)}$ = 0.15	$F_{(1,40)}$ = 9.59**	$F_{(1,40)}$ = 0.41	$F_{(1,40)}$ = 1.00
	Insula	$F_{(1,39)}$ = 2.14	$F_{(1,39)}$ = 2.49	$F_{(1,39)}$ = 12.13**	$F_{(1,39)}$ = 0.22	$F_{(1,39)}$ = 15.43***	$F_{(1,39)}$ = 1.61	$F_{(1,39)}$ = 1.12
C-fos	SCN	$F_{(1,39)}$ = 29.84***	$F_{(1,39)}$ = 1.34	$F_{(1,39)}$ = 43.41***	$F_{(1,39)}$ = 0.20	$F_{(1,39)}$ = 10.7**	$F_{(1,39)}$ = 0.37	$F_{(1,39)}$ = 1.83
	PVN	$F_{(1,39)}$ = 124.7***	$F_{(1,39)}$ = 0.13	$F_{(1,39)}$ = 1.77	$F_{(1,39)}$ = 0.40	$F_{(1,39)}$ = 0.20	$F_{(1,39)}$ = 0.00	$F_{(1,39)}$ = 0.53
	AC	$F_{(1,41)}$ = 73.12***	$F_{(1,41)}$ = 5.37*	$F_{(1,41)}$ = 37.08***	$F_{(1,41)}$ = 0.60	$F_{(1,41)}$ = 0.07	$F_{(1,41)}$ = 0.00	$F_{(1,41)}$ = 1.28
	PL	$F_{(1,41)}$ = 120.3***	$F_{(1,41)}$ = 4.24*	$F_{(1,41)}$ = 11.63**	$F_{(1,41)}$ = 1.01	$F_{(1,41)}$ = 0.70	$F_{(1,41)}$ = 1.23	$F_{(1,41)}$ = 2.40
	IL	$F_{(1,41)}$ = 105.5***	$F_{(1,41)}$ = 5.91*	$F_{(1,41)}$ = 8.67**	$F_{(1,41)}$ = 1.61	$F_{(1,41)}$ = 0.46	$F_{(1,41)}$ = 0.27	$F_{(1,41)}$ = 1.04
	VO	$F_{(1,39)}$ = 60.41***	$F_{(1,39)}$ = 1.56	$F_{(1,39)}$ = 46.34***	$F_{(1,39)}$ = 0.28	$F_{(1,39)}$ = 0.80	$F_{(1,39)}$ = 0.31	$F_{(1,39)}$ = 0.35
	Insula	$F_{(1,41)}$ = 1.59	$F_{(1,41)}$ = 2.37	$F_{(1,41)}$ = 105.7***	$F_{(1,41)}$ = 1.12	$F_{(1,41)}$ = 2.06	$F_{(1,41)}$ = 0.90	$F_{(1,41)}$ = 0.06
CORT	CORT	$F_{(1,20)}$ = 45.24***	N/A	$F_{(1,20)}$ = 0.24	N/A	$F_{(1,20)}$ = 4.61*	N/A	N/A

As expected (Spencer & Deak, 2016), despite higher basal CORT levels at night (ZT16) than day (ZT4), acute stress increased CORT to similar levels at both times of day (time of day X stress interaction, $p < 0.05$).

Suprachiasmatic Nucleus of the Hypothalamus (SCN)

There was a significant effect of stress on *c-Fos* mRNA in the SCN at ZT16 [Table 3.2; Fig. 3.6].

Figure 3.6. Experiment 2: effect of stress, adrenalectomy and time of day on gene expression in the SCN. A) Overall, acute stress had no effect on clock gene expression. Stress increased *c-Fos* mRNA in the SCN of male rats only at ZT16. This effect was not present in experiment 3 when stress was administered in total darkness (see Figure 10). There was a significant adrenal effect for *Per2* mRNA, but post-hoc tests did not reveal a significant difference. [Clock symbol indicates main effect of time of day, star symbol indicates main effect of stress, and "not" symbol indicates main effect of adrenal status. Interactions between these factors are also indicated, $p < 0.05$, 3-way ANOVA. * = stress effect; # = time of day effect $p < 0.05$; FLSD post hoc pair-wise tests, n=6-7] B) Representative autoradiographs of SHAM rats at ZT4 under no stress or stress conditions.

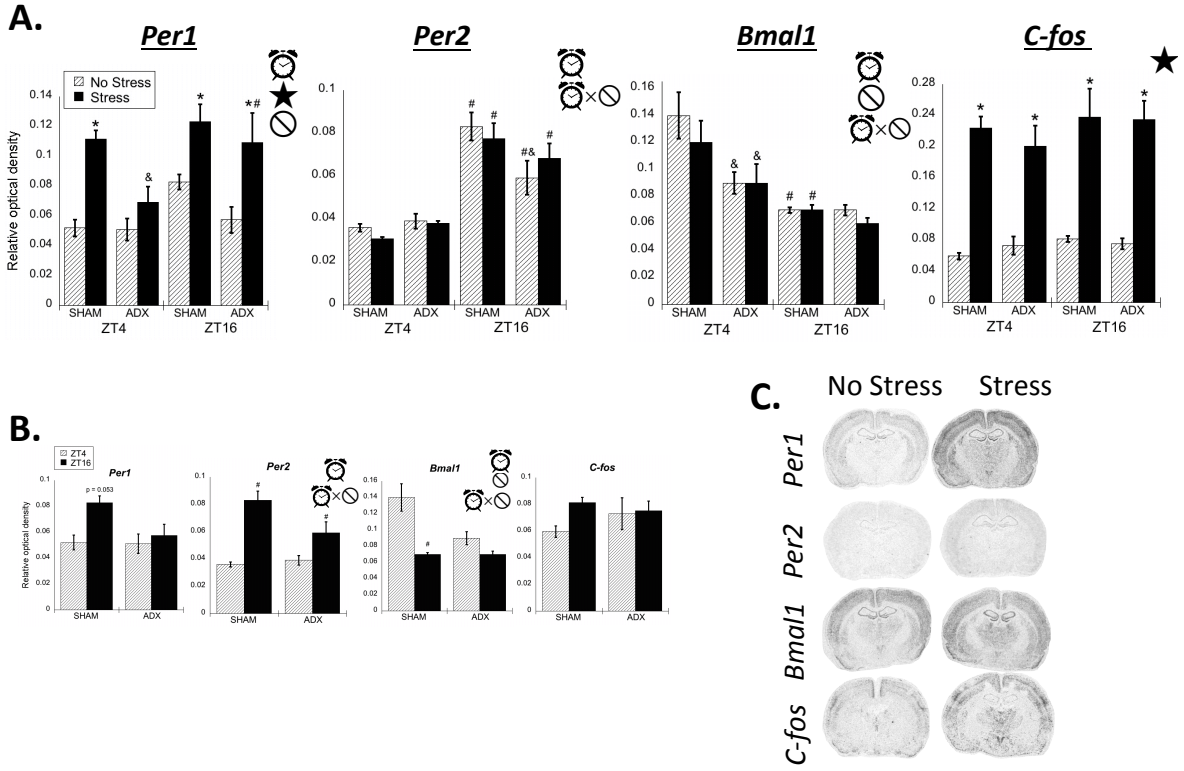


As in the first experiment, this effect was likely due to the inadequate red light condition present during stress exposure (see Experiment 3), rather than stress per se. There was a significant effect of adrenal status on *Per2* mRNA in the SCN, reflected by higher average levels at both times of day in ADX rats compared to sham rats. There was a significant time of day effect for the expression of all genes except for *Per2* [Table 3.2, Figure 3.6]. As in the first experiment, *Per1* mRNA levels were greater at ZT4 compared to ZT16, and *Bmal1* mRNA levels were greater at ZT16 compared to ZT4 (FLSD, $p < 0.05$). The lack of a time of day effect in *Per2* mRNA may be explained by the fact that its acrophase occurs around ZT10 (Chun et al., 2015), and thus, its diurnal variation may not be well-represented by the two time points chosen for this study.

Paraventricular Nucleus of the Hypothalamus (PVN)

Consistent with Experiment 1, stress significantly increased *Per1* and *c-Fos* mRNA levels at both times of day in the PVN [Table 3.2; Figure 3.7].

Figure 3.7. Experiment 2: effect of stress, adrenalectomy and time of day on gene expression in the PVN. A) Acute stress increased *Per1* mRNA, but not *Per2* or *Bmal1* mRNA in the PVN of male rats. This effect was attenuated by ADX at ZT4, but not ZT16. *c-Fos* mRNA was induced by stress, and unaffected by ADX. B) Replot of the no stress only conditions from Panel A. Under no stress conditions, *Per2* and *Bmal1* mRNA had a significant ZT x adrenal status interaction, where the time of day effect was more pronounced in the SHAM animals. [Clock symbol indicates main effect of time of day, star symbol indicates main effect of stress, and "not" symbol indicates main effect of adrenal status. Interactions between these factors are also indicated, $p < 0.05$, 3-way ANOVA. * = stress effect; # = time of day effect; & = adrenal status effect; $p < 0.05$; FLSD post hoc pair-wise tests, n=6-7] C) Representative autoradiographs of SHAM rats at ZT4 under no stress or stress conditions.



Interestingly, at ZT4 there was an absence of a significant stress effect on *Per1* mRNA in ADX rats (FLSD, $p < 0.05$), suggesting that at ZT4, stress-induced *Per1* mRNA is dependent on endogenous CORT. This CORT-dependency did not extend to *c-Fos* mRNA, as stress increased *c-Fos* mRNA for both SHAM and ADX rats at both times of day [Figure 3.7A; FLSD, p

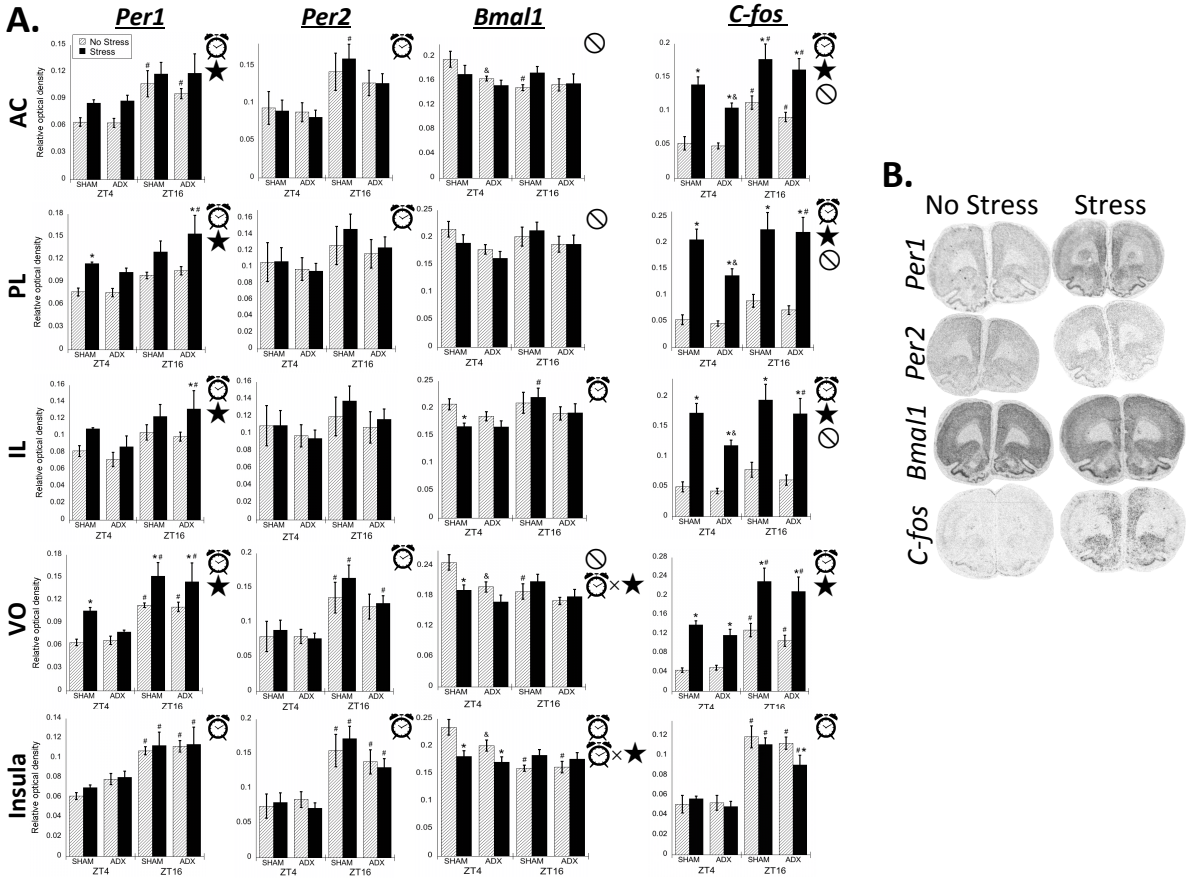
< 0.05]. There was also a significant main effect of adrenal status on *Per1* mRNA, largely due to the absence of a stress effect at ZT4 in ADX rats, as well as a lack of time of day increase in basal *Per1* mRNA at ZT16 (FLSD, $p < 0.05$).

There was a significant effect of time of day on the expression of each of the 3 clock genes, and the pattern was similar to Experiment 1, with higher *Per1* and *Per2* mRNA at ZT16, and higher *Bmal1* mRNA at ZT4 [Figure 3.7, Table 3.2]. Interestingly, this time of day difference was attenuated for all 3 clock genes in ADX rats (significant time of day by adrenal status interaction for *Per2* and *Bmal1* mRNA, and for *Per1* mRNA if stress condition was excluded from the analysis) [Figure 3.7B]. This suggests that diurnal CORT was necessary for normal diurnal fluctuations of clock gene expression within the PVN, which has been demonstrated previously in some other brain regions (Segall et al., 2010; Woodruff et al., 2016).

Prefrontal Cortex (PFC)

Similar to the PVN, in all subregions examined (AC, PL, IL, VO), stress increased *Per1* and *c-Fos* mRNA, but not *Per2* or *Bmal1* mRNA [Table 3.2; Fig 3.8].

Figure 3.8. Experiment 2: effect of stress, adrenalectomy and time of day on gene expression in the PFC and insula. A) Overall, acute stress increased *Per1* and *c-Fos* mRNA, but not *Per2* or *Bmal1* mRNA, in the AC, PL, IL, and VO of SHAM and ADX male rats. [Clock symbol indicates main effect of time of day, star symbol indicates main effect of stress, and "not" symbol indicates main effect of adrenal status. Interactions between these factors are also indicated, $p < 0.05$, 3-way ANOVA. * = stress effect; # = time of day effect; & = adrenal effect; $p < 0.05$; FLSD post hoc pair-wise tests, n=6-7] B) Representative autoradiographs of SHAM rats at ZT4 under no stress or stress conditions.



Importantly, this stress-induced increase in *Per1* and *c-Fos* mRNA was evident in both sham and ADX rats. There was also a significant main effect of time of day for all subregions for *Per1* and *c-Fos* mRNA [Table 3.2], and for all subregions except for IL for *Per2* mRNA [Table 3.2]. There was a significant main effect of time of day for *Bmal1* mRNA only in the IL [Figure 3.8]. There were significantly greater *Per1* and *Per2* mRNA levels at ZT16 (FLSD, $p < 0.05$), which matches with Experiment 1 and previous determination of a dark phase acrophase for the expression of both genes in the PFC (Chun et al., 2015). The lack of a significant time of day

effect in *Bmal1* mRNA may be reflective of its acrophase occurring closer to ZT0 (Chun et al., 2015). There was also significantly higher *c-Fos* mRNA present at ZT16 compared to ZT4 in all PFC subregions, and the same pattern was seen in Experiment 1. There was a significant time of day x stress interaction for *Bmal1* mRNA in the VO, where unstressed rats had greater *Bmal1* mRNA than stressed rats at ZT4, suggesting suppression of *Bmal1* mRNA expression with stress at ZT4. There was also a significant main effect of adrenal status for *Bmal1* mRNA expression in the AC, PL, and VO and for *c-Fos* mRNA in the AC, PL, and IL. There was a general pattern for lower *Bmal1* mRNA in ADX rats supporting the importance of endogenous CORT in basal clock gene expression.

Rostral Agranular Insula

There were no significant main effects for stress or adrenal status for all clock genes and *c-Fos* mRNA in the rostral agranular insula [Table 3.2; Figure 3.8] which is consistent with Experiment 1, and suggests that *Per1* and *c-Fos* mRNA levels in the insula are not affected by acute stress. Although there was no significant effect of stress, there was a significant time of day × stress interaction for *Bmal1* mRNA [Table 3.2], where stress decreased *Bmal1* mRNA expression for both SHAM and ADX rats at ZT4, but not ZT16. This same time of day X stress interaction pattern was also seen in the VO.

EXPERIMENT 3: Test of the effect of stress in total darkness on *Per1* and *c-Fos* mRNA in the SCN and PVN of male and female rats at ZT16.

Corticosterone (CORT)

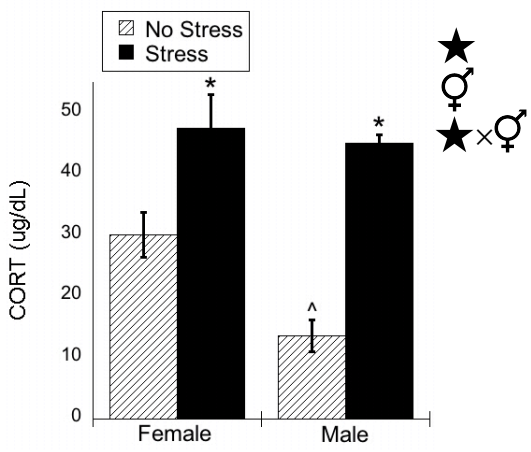
There were significant main effects of stress and sex on plasma CORT levels [Table 3.3].

Table 3.3. Experiment 3: Statistical analysis of *Per1* and *c-Fos* mRNA in the suprachiasmatic nucleus of the hypothalamus (SCN) and paraventricular nucleus of the hypothalamus (PVN), as well as plasma CORT. Two-way ANOVA results for effect of stress, sex, and their interaction, *p < 0.05, **p < 0.01, ***p < 0.001.

		Stress	Sex	Stress x Sex
Per1	SCN	$F_{(1,19)} = 4.31$	$F_{(1,19)} = 0.12$	$F_{(1,19)} = 0.08$
	PVN	$F_{(1,20)} = 16.58$**	$F_{(1,20)} = 1.07$	$F_{(1,20)} = 0.03$
C-fos	SCN	$F_{(1,20)} = 0.25$	$F_{(1,20)} = 7.41$*	$F_{(1,20)} = 0.04$
	PVN	$F_{(1,19)} = 37.38$***	$F_{(1,19)} = 0.94$	$F_{(1,19)} = 0.31$
CORT	CORT	$F_{(1,17)} = 68.47$***	$F_{(1,17)} = 6.32$*	$F_{(1,17)} = 14.63$**

There was also a significant stress x sex interaction. Stress significantly increased plasma CORT in both males and females, and females had greater plasma CORT than males under no stress conditions (FLSD, $p < 0.05$) [Figure 3.9].

Figure 3.9. Experiment 3: effect of stress at ZT16 in total darkness on plasma CORT in male and female rats. 30 minutes of acute restraint stress increased plasma CORT levels in both male and female rats [Star symbol indicates main effect of stress, and sex symbol indicates main effect of sex. Interactions between these factors are also indicated, p < 0.05, 2-way ANOVA. * = stress effect; ^ = sex effect; p < 0.05; FLSD post hoc pair-wise tests, n=6].

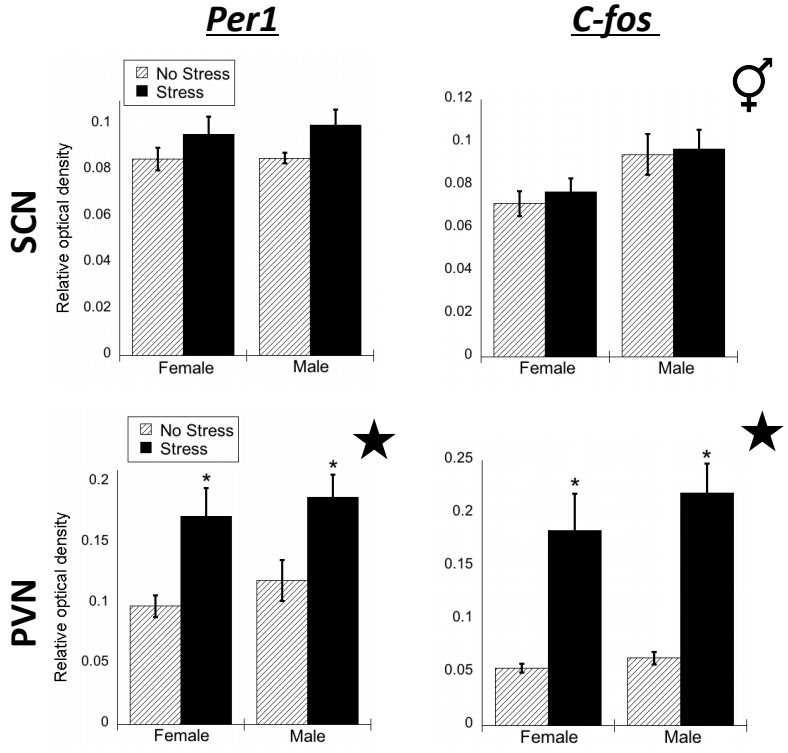


This sex difference in plasma CORT levels is typically seen under both non-stressed and stressed conditions (Babb et al., 2014; Viau, 2002), and replicates the basal CORT results seen in Experiment 1 at ZT16.

Suprachiasmatic Nucleus of the Hypothalamus (SCN)

There was no overall main effect of stress on *Per1* or *c-Fos* mRNA in the SCN of both male and female rats [Table 3.3; Figure 3.10].

Figure 3.10. Experiment 3: effect of stress at ZT16 in total darkness on SCN and PVN *Per1* and *c-Fos* mRNA in male and female rats. When rats were exposed to 30 min of acute restraint stress under complete darkness, there was no increase of *Per1* and *c-Fos* mRNA in the SCN of both male and female rats. However, in the PVN, both males and females showed a significant increase in both *Per1* and *c-Fos* mRNA. [Star symbol indicates main effect of stress, and sex symbol indicates main effect of sex, $p < 0.05$, 2-way ANOVA * = stress effect $p < 0.05$; FLSD post hoc pair-wise tests, n=6].



These results differ from the stress-induced *Per1* mRNA in the SCN seen in Experiment 1 at ZT16, and the stress-induced *c-Fos* mRNA at ZT16 in the SCN seen in both Experiments 1 and 2. This indicates that the red light condition used in the first two experiments was likely responsible for the increased *Per1* and *c-Fos* mRNA seen at ZT16 in those experiments. There were significantly greater *c-Fos* mRNA levels in males than females. This pattern was also observed at this time of day in Experiment 1.

Paraventricular Nucleus of the Hypothalamus (PVN)

As expected there was a significant main effect of stress, where restraint stress significantly increased *Per1* and *c-Fos* mRNA in the PVN for both males and females [Table 3.3; Figure 3.10; FLSD, $p < 0.05$], which is consistent with the results seen in Experiment 1 and 2. There was no main effect of sex.

DISCUSSION

This study has demonstrated that 30 min of acute restraint stress can selectively and rapidly increase *Per1 mRNA*, but not *Per2* or *Bmal1* mRNA, in male and female rat medial prefrontal cortex (AC, PL, IL, and VO subregions) and PVN. These data extend the range of tissues that exhibit stress-induced *Per1* mRNA or PER1 protein expression as previously reported in several other brain regions (hippocampus, dorsal medial hypothalamus, and piriform cortex) (Al-Safadi et al., 2014; Al-Safadi et al., 2015; Bohacek et al., 2015; Mifsud et al., 2016) and peripheral tissues (liver, heart, lung and stomach) (Yamamoto et al., 2005). *Per1* expression in the SCN was unaffected by acute stress, in keeping with the general observation that SCN clock function is largely resistant to environmental conditions other than light:dark cycle (Al-Safadi et al., 2014; Takahashi et al., 2001). There was also no effect of acute stress on *Per1* mRNA in the rostral agranular insula, indicating that there are differences between extra-SCN brain regions in the ability of acute stress to rapidly increase *Per1 mRNA*. Surprisingly, stress-induced *Per1* mRNA in both the PVN and PFC did not necessarily depend on the presence of endogenous CORT. These data indicate that there are non-CORT mediated mechanisms by which acute stress can rapidly increase *Per1* mRNA in extra-SCN brain regions.

Selectivity of clock gene response to acute stress

The selectivity of *Per1* mRNA to be increased by acute restraint stress suggests that direct modulation of *Per1* expression may be the gateway by which stress, or other environmental cues, can alter the molecular clock in extra-SCN brain tissue. *Per1*, but not *Per2* mRNA has also been shown to be increased by acute stress in the mouse PVN (Takahashi et al., 2001). In mouse peripheral tissues the *Per1* gene was the only clock gene of several examined that responded to acute stress (Yamamoto et al., 2005). Although *Per2* and *Bmal1* are not rapidly induced by acute stress, they have been shown, in addition to *Per1*, to have altered rhythmic expression in response to repeated or chronic stress. These repeated stress effects have been observed in both brain and peripheral tissues (Christiansen et al., 2016; Logan et al., 2015; Razzoli et al., 2014; Tahara et al., 2015; K. Takahashi et al., 2013). For example, restraint applied at the same time for three consecutive days was sufficient to induce a phase-shift in diurnal *Per2* and *Bmal1* mRNA expression, as well as *Per1, Dbp,* and *Rev-erbα* mRNA in mouse adrenal, liver, kidneys, and submandibular glands (Tahara et al., 2015). This phase shift also occurred for *Per1* and *Per2* mRNA in the hippocampus and cortex. A separate study found that chronic variable stress altered *Per1* and *Bmal1* diurnal expression in the nucleus accumbens of male mice (Logan et al., 2015). Thus, it appears that although *Per2* and *Bmal1* are not rapidly induced by an acute stressor, their subsequent rhythms are altered, particularly after repeated or chronic stress.

The altered phase and amplitude of rhythmic *Per2* and *Bmal1* expression after repeated stress may be due to the rapid induction of *Per1* mRNA after each stressor exposure. Modulation of the molecular clock by rapid *Per1* induction in extra-SCN brain tissue may be related to the process by which light entrains the phase of the molecular clock in the SCN. A light pulse during the dark phase rapidly induces SCN *Per1* mRNA, which results in a subsequent phase shift in SCN clock gene expression and the animal's circadian rhythm (usually measured by circadian locomotor activity) (Oster et al., 2003; Shigeyoshi et al., 1997). Light also increases *Per2* expression in the SCN, however with some delay (~180 min)

compared to *Per1* (Shearman et al., 1997). It is possible that there may also be a delay in stress-induced *Per2* gene expression outside the SCN. Glucocorticoid treatment of various cell lines had produced a delayed increase in *Per2* mRNA relative to the rapid increase in *Per1* mRNA (Cheon et al., 2013; Nagy et al., 2016; So et al., 2009). Thus, our examination of gene expression 30 min after stressor onset may have been too short of a period of time to see induction of clock genes other than *Per1* that may require more than 30 min to reflect a significant increase in mRNA levels.

Intercellular signals mediating stress-induced *Per1* expression

Stress-increased *Per1* expression in peripheral and brain tissue has been believed to be due primarily to an increase in CORT (Balsalobre et al., 2000; Takahashi et al., 2001; Yamamoto et al., 2005). There is a functional glucocorticoid response element (GRE) within the promoter region of *Per1*, and glucocorticoids rapidly induce *Per1* expression in some cell lines, liver and hippocampus (Balsalobre et al., 2000; Conway-Campbell et al., 2010; Reddy et al., 2012; Yamamoto et al., 2005). In addition, acute stress increases in *Per1* RNA expression in the hippocampus are accompanied by increased glucocorticoid receptor binding to a GRE associated with the *Per1* gene (Mifsud & Reul, 2016).

GRE consensus sequences are also associated with promoter regions of *Per2* and *Bmal1*, although the functionality of those GREs in vivo has been less well established (Cheon et al., 2013; Reddy et al., 2006; Reddy et al., 2012; So et al., 2009). It has been proposed that while *Per2* does have a GRE within its promoter region, the GRE is constituitively active (So et al., 2009), thereby limiting its ability to respond to acute CORT elevations.

Despite the compelling evidence for a functional GRE associated with the mammalian *Per1* gene, we found in the second experiment that removal of endogenous CORT via adrenalectomy had no significant effect on stress-induced *Per1* mRNA in all subregions of the PFC (AC, PL, IL, and VO). In the PVN, ADX attenuated stress-induced *Per1* mRNA, but only at

ZT4. This may reflect a time of day gating mechanism for acute CORT-dependent modulation of PVN *Per1* expression, which could possibly occur through CRY protein interference of GR-mediated function (Lamia et al., 2011). Two other studies recently reported that adrenalectomy or GR antagonist treatment failed to prevent acute stress increased *Per1* mRNA or PER1 protein immunoreactivity in select rodent brain regions (Al-Safadi et al., 2015; Bohacek et al., 2015). In addition to a GRE, the *Per1* gene also contains a functional cAMP response element (CRE) within its promoter region. In the SCN, *Per1* expression is rapidly induced by retinal light exposure during the subjective night. This induction depends on cAMP response element binding protein (CREB) activation and its binding to the *Per1* associated CRE (Tischkau et al., 2002). It is possible that outside of the SCN, this same CRE-dependent process is responsible for increased *Per1* expression in response to stress-induced alteration of neuronal activation.

Consistent with this possibility is the largely parallel changes in *Per1* and *c-Fos* expression that we observed in this study. *c-Fos* is a well-characterized immediate early gene (IEG) that is rapidly induced throughout most neuronal populations under conditions associated with increased neuronal excitation (Hughes et al., 1995; Kovács et al., 1998). This activity-dependent *c-Fos* expression is largely a result of changes in the activity of intracellular signal transduction pathways that lead to increased CREB activation (Nestler et al., 2015). In our study acute stress increased both *Per1* mRNA and *c-Fos* mRNA in all of the PFC subregions and the PVN, but not in the agranular insula. We also observed parallel increases in *Per1* and *c-Fos* mRNA in the SCN when in the first two experiments rats were inadvertently exposed to pseudo red-light conditions during restraint applied during the dark phase. In the third experiment when rats were exposed to restraint in total darkness, neither *Per1* or *c-Fos* mRNA were altered in the SCN, which aligns with previous studies (Al-Safadi et al., 2014; Takahashi et al., 2001) and indicates that SCN neuroactivity is not increased by acute stress. In contrast stress-induced *Per1* mRNA in the PVN was present in all 3 experiments, indicating that the effect depended

solely on the stress associated with restraint. In a previous study we found that night-time light exposure alone does not induce FOS protein in PVN (Highland et al., 2014).

Thus the *Per1* gene seems to operate much like an IEG. In the SCN it is rapidly induced by photic related signals transmitted to the SCN during the subjective night. But outside the SCN, *Per1* expression may be rapidly increased by a wide range of experiences, including acute stress, that effectively induce other IEGs. The PFC and PVN are well-established stress-reactive brain regions, in terms of IEG induction (Pace et al., 2005; Weinberg et al., 2009). This study suggests that neural activity in the rostral agranular insula is not acutely stress-reactive.

One dissociation that we observed between *Per1* and *c-Fos* expression changes with treatment conditions was the attenuation of stress-induced *Per1* mRNA levels in the PVN of ADX rats at ZT4. The lack of a parallel effect of ADX on *c-Fos* mRNA is consistent with the fact that the *c-Fos* gene is not associated with a GRE and is not directly regulated by acute increases in CORT (Ginsberg et al., 2003).

Although in most cases stress-induced *Per1* mRNA increases did not depend on CORT, we did see some evidence of CORT sensitivity of clock gene expression in this study. In the PVN of unstressed adrenal-intact rats, the relative expression of all three clock genes exhibited the expected time of day variation with higher levels of *Bmal1* mRNA at ZT4 compared to ZT16 and the opposite time of day relationship for *Per1* and *Per2* mRNA (Chun et al., 2015; Girotti et al., 2009). However, in ADX rats the time of day variation in PVN *Per1*, *Per2* and *Bmal1* mRNA expression was significantly blunted. This result may reflect an impairment in the proper entrainment of PVN rhythmic clock gene expression of ADX rats, similar to what we previously reported in the PFC (Woodruff et al., 2016). In this study the two times of day sampled may not have been optimal to see a phase shift in Per1 and Per2 diurnal expression in the PFC that we previously observed in ADX rats, but we did see an effect of ADX on *Bmal1* mRNA in the PFC, consistent with our previous results (Woodruff et al., 2016). In our previous study we found that normal PFC clock gene diurnal expression patterns was restored in ADX rats that were treated

with an appropriately timed daily pulse of CORT. The results of this study suggest that CORT's regulation of daily clock gene expression entrainment in extra-SCN brain regions may operate over a longer time-scale than 30 min.

Sex comparison

Despite the fact that female rats had substantially higher basal and stress-induced plasma CORT levels than male rats, female rats did not have higher basal or stress-induced *Per1* mRNA levels compared to male rats. This result further supports the case that stress-induced *Per1* mRNA levels were not primarily dependent on acute elevations in CORT. It should be noted, however, that Droste et al. (2009) found that restraint stress increased free CORT within the hippocampus equally between male and female rats. Thus, while there may be a sex difference in plasma CORT, this may not be the case in restraint-induced free CORT within certain brain regions. In contrast, another study observed greater increases in hippocampal *Per1* mRNA in female mice compared to male mice after swim stress, whereas restraint produced no change in *Per1* mRNA for either sex (Bohacek et al., 2015). In that study pretreatment with a GR antagonist did not block increases in *Per1* mRNA after swim stress, again pointing to a CORT independent mechanism.

We did observe in this study a few instances of sex differences in gene expression. In the PL, male rats had overall higher *Per1* and *c-Fos* mRNA levels compared to female rats, and this difference was primarily a result of higher levels after acute stress. Another study also observed higher stress-induced *c-Fos* mRNA levels in the PL of male rats compared to female rats, and that sex difference was most pronounced when female rats were stressed during proestrus (Bland et al., 2005). Thus this is an example of a sex difference in stress-induced gene expression that appears dissociated from relative CORT levels, but may be related to gonadal steroid levels. In this study there was also overall significantly higher *c-Fos* mRNA levels in the rostral agranular insula of male rats compared to female rats. This sex difference

was mirrored in relative *Per1* mRNA levels, although it did not quite reach statistical significance ($p = 0.052$). Thus, overall there were minimal sex differences in basal or acute stress-induced gene expression in the PVN and cortical regions examined despite the greater prevalence of mood disorders associated with both stress and circadian disruption in females (Bailey & Silver, 2014; Kessler, 2003).

It is possible, though unexplored, that sex differences in stress-induced *Per1* expression may appear with a more chronic stressor, as repeated insults may cause greater disruption to the molecular clock within various brain regions (Christiansen et al., 2016; Logan et al., 2015). Additionally, it is currently unknown whether there are sex differences in subsequent phase-shifting of the molecular clock after an acute stressor. Further research examining sex differences in stress-induced alterations of the molecular clock in extra-SCN brain regions are necessary.

Concluding Comments

In closing, 30 minutes of acute restraint stress rapidly and selectively induced *Per1* mRNA expression in the PVN and PFC of male and female rats. These results suggest that altered *Per1* expression may be the gateway by which stress can subsequently alter the phase and amplitude of extra-SCN molecular clocks. However, in addition to GRE/CORT-mediated mechanisms of altered clock gene expression, stress-induced *Per1* mRNA expression was largely CORT independent and may depend on CRE-mediated mechanisms. The potential for daily acute induction of *Per1* expression and the subsequent alterations to the molecular clock in extra-SCN tissue may be important for phase adjustment of extra-SCN molecular clocks to periodic non photic environmental changes. This is further supported by previous studies that show that temporal shifts in daily food access, or repeated stress at the same time each day can alter the phase of clock gene expression in extra-SCN brain regions (Girotti et al., 2009; Tahara et al., 2015). On the other hand, temporally unpredictable recurring alterations of *Per1*

expression by environmental factors such as chronic variable stress may result in disrupted profiles of extra-SCN molecular clocks. This disruption may contribute to some of the adverse neurobiological effects of chronic stress.

CHAPTER IV: THE ROLE OF GLUCOCORTICOIDS AND ESTRADIOL IN STRESS-INDUCED *PER1* MRNA IN THE PREFRONTAL CORTEX, HYPOTHALAMIC PARAVENTRICULAR NUCLEUS, AND HIPPOCAMPUS IN FEMALE RATS

ABSTRACT

Properly entrained circadian rhythms are critical to the normal health and behavior of an organism. Underlying these circadian rhythms is the presence of oscillating molecular clocks. The proper entrainment of extra-SCN clocks is dependent on the diurnal presence of glucocorticoids (CORT), as adrenalectomy has been shown to disrupt the molecular clock in the prefrontal cortex (PFC). If diurnal peaks in CORT are important for normal clock gene expression, then untimely stress-induced CORT may also interfere with clock gene expression. Furthermore, female rats have greater stress reactivity, especially in stress-induced CORT. This effect is believed to be due to the activational effects of estradiol. Furthermore, women have greater prevalence rates of mood disorders associated with stress an disruptions in circadian rhythms. Thus, the objective of this study was to determine whether 30 min of acute restraint stress could rapidly alter clock gene expression in the PFC and hippocampus, limbic brain regions important in stress reactivity, and in the hypothalamic paraventricular nucleus (PVN), which controls the release of CORT. It was also examined if stress-induced changes in clock gene expression are dependent on CORT and if estradiol can modulate this effect either acutely or permissively. Female rats were ovariectomized and had estradiol replaced or not. Half of the rats also received adrenalectomy to assess if endogenous CORT is necessary for stress-induced clock gene expression. It was found that stress increases *Per1* mRNA (in situ hybridization), and that this effect was largely independent of CORT, as stress-induced *Per1* mRNA was only partially attenuated in only the PFC. Furthermore, estradiol had no effect on stress-induced *Per1* mRNA.

INTRODUCTION

There are circadian rhythms in many behaviors and physiology, such as the sleep/wake cycle, fluctuations in hormone levels, cognition, body temperature, and alertness. These rhythms allow for the organism to be synchronized with their environment in order to optimize their survival. Properly entrained rhythms contribute to the overall health of the organism, as disruptions in circadian rhythms are associated with diseased states, such as depression, anxiety, and post-traumatic stress disorder (PTSD), which often have disrupted sleep/wake patterns and disrupted rhythms in glucocorticoid release (Drevets et al., 1997; Johansson et al., 2002; Lamont et al., 2009; Mayberg et al., 1999). Additionally, shift work is associated with an increased rate of depression (Caruso, 2014). Thus, properly entrained circadian rhythms are essential for healthy behaviors and physiology.

Circadian rhythms are maintained by the existence of molecular clocks that exist in virtually all cells of the organism. The molecular clock is comprised of the oscillating expression of core clock genes from both the positive and negative component of the molecular clock. The positive component of the molecular clock consists of the core clock genes *Bmal1* and *Clock/Npas2*, whose protein products dimerize to initiate the expression of the negative component of the molecular clock, *Period 1, 2, 3* (*Per1, Per2, Per3*) and *Cryptochrome 1,2* (*Cry1, Cry2*). Per and Cry then feedback to inhibit their own transcription. This self-regulating transcriptional/translational loop takes approximately 24 hours (Reppert & Weaver, 2002). The molecular clock has been well-characterized in the body's master clock, the suprachiasmatic nucleus of the hypothalamus (SCN). More recently, this molecular clock has also been found in extra-SCN brain regions (e.g., cortex, amygdala, hippocampus, prefrontal cortex, other hypothalamic nuclei) (Amir et al., 2004; Ángeles-Castellanos et al., 2007; Chun et al., 2015; Feillet et al., 2008a; Harbour et al., 2014; Rath et al., 2012) and peripheral tissue (e.g., liver, kidney, heart, lungs, spleen, bone) (Yamamoto et al., 2004). The ubiquitous nature of these clock genes suggests their importance in the normal functioning of the organism. Knockdown or

knockout of clock genes lead to behavioral arrhythmicity, as well as depressive-like or manic-like behaviors (Bae et al., 2001; Bunger et al., 2000; Spencer et al., 2012). Thus, normal expression of clock genes is critical to healthy physiology and behavior.

The body's master clock, the SCN, communicates with extra-SCN molecular clocks in order to synchronize the organism to its environment. Light input from the environment enters the retina and travels via the retinohypothalamic tract (RHT) to the SCN (Sollars & Pickard, 2015), thereby entraining the molecular clock within the SCN to the organism's environment. However, the SCN has very limited efferent projections (Balsalobre et al., 2000; Sollars & Pickard, 2015), which raises the issue of how the SCN communicates with extra-SCN clocks.

Glucocorticoids (CORT) are an ideal candidate for how the SCN communicates with extra-SCN clocks. CORT has a diurnal rhythm, with peak plasma levels occurring immediately upon the animals' active phase [Herman et al., 2003] and is controlled by the SCN (Moore & Eichler, 1972). Glucocorticoid receptors (GR) are ubiquitously expressed throughout the brain and body, with the SCN as a notable exception (Balsalobre et al., 2000; Rosenfeld et al., 1988; 1993). Thus, nearly all cells can be affected by the actions of CORT, while the master clock remains unaffected. CORT can induce rhythmic expression of clock genes (*Per1, Per2, Bmal1*) in mesenchymal stem cells (So et al., 2009). Furthermore, Woodruff et al. (2016) has demonstrated in adrenalectomized rats that the timing of a daily CORT injection is critical to restore the proper circadian rhythm of clock gene expression in male prefrontal cortex.

Acutely, CORT can rapidly induce the expression of *Per1* in hippocampal cells (Conway-Campbell et al., 2010), liver, rat fibroblast cells (Balsalobre et al., 2000), and human peripheral blood mononuclear cells (Burioka et al., 2005). The rapid induction of *Per1* gene expression is due to a functional glucocorticoid response element (GRE) within the promoter region of Per1 (Yamamoto et al., 2005), and may be how CORT can entrain the rhythm of extra-SCN molecular clocks.

If an appropriately timed peak in diurnal CORT is responsible for the entrainment of extra-SCN molecular clocks, then untimely stress-induced peaks in CORT may adversely alter rhythmic clock gene expression. Thus, untimely stress-induced CORT could lead to disruptions in the circadian rhythm of these clock genes by initially altering *Per1* expression. Many mood disorders that are characterized by disruptions in circadian rhythms are often precipitated by stress (Johnson & Sarason, 1978; Pittenger & Duman, 2008). Thus, if stress can alter clock gene expression, then that may lead to disruptions in extra-SCN molecular clocks, resulting in disruptions in circadian rhythms and mental illness.

There is growing evidence for the ability of chronic stress to alter both positive and negative components of the molecular clock (Jiang et al., 2013; Logan et al., 2015; Takahashi et al., 2013). Additionally, it has been demonstrated that acute stress (cold swim stress, restraint stress) can rapidly induce Per1 gene expression in peripheral tissue and different brain regions, which depends on the stressor (Chapter 2; Al-Safadi et al., 2015; Bohacek et al., 2015; Gray et al., 2014; Takahashi et al., 2001; Yamamoto et al., 2005). Thus, Per1 may be the gateway by which an acute stressor can subsequently (especially after repeated stress) alter the molecular clock.

While it is largely hypothesized that stress induces Per1 expression via GRE-mediated mechanisms, limited studies have begun to test this hypothesis. It was found that ADX had minimal or variable effects on stress-induced *Per1* expression depending on the brain region (Chapter 2; (Al-Safadi et al., 2015). Other studies have administered a GR antagonist prior to an acute stressor and found no effect on stress-induced *Per1* expression (Bohacek et al., 2015; Gray et al., 2012; Roszkowski et al., 2016). These studies challenge the existing dogma of CORT-mediated Per1, and thus more studies need to replicate these findings. Additionally, many of these studies have only examined male subjects. In many mental health disorders that are associated with both disruptions in circadian rhythms and with stress (e.g., depression, anxiety, PTSD) there is a greater prevalence rate in women compared to men (Kessler, 2003).

There are sex differences in both circadian rhythms (Bailey & Silver, 2014) and in stress reactivity (Viau & Meaney, 1991). These sex differences are believed to be due to the activational effects of estradiol in the female rat brain. Thus, examining stress-induced changes in clock gene expression and how this may be modulated by estradiol in female subjects is critical. Additionally, there is evidence to suggest that there are sex differences in stress-induced HPA axis activation and in stress-induced neuronal activation. Chapter 2 and Al-Safadi et al. (2015) studies suggest CORT-independent mediation of stress-induced *Per1* expression. It is possible that there may be sex differences in the mechanism of stress-induced clock gene expression (e.g., stress-induced Per1 by hormonal versus neuronal mechanisms). Thus, there may be sex differences in the dependency of CORT on stress-induced *Per1* expression.

The objective of this study was to determine whether stress-induced clock gene expression is dependent on CORT in female rats, and if any of these effects are modulated by the acute or permissive actions of estradiol. Furthermore, previous studies suggest that there may be brain region specificity in stress-induced Per1 expression. Thus, this study also examined stress-induced Per1 mRNA in the PFC and PVN, as well as the hippocampus (HPC). The HPC is a brain region that is part of the limbic system, and integral to stress reactivity, emotional regulation, and learning and memory (Herman et al., 2003), all functions that are decreased in mental health disorders (Videbech & Ravnkilde, 2004). This brain region is also important to study in order to obtain a more complete picture in understanding stress and disruptions in clock gene expression.

MATERIALS AND METHODS

Subjects

Three cohorts of 32-34 female Sprague Dawley rats (Harlan, Indianapolis, IN), for a total of 98 rats, were used. Rats were approximately 3 months of age at the time of sacrifice. Upon arrival to the facility, rats were pair-housed and evenly distributed between two rooms. Each room was

temperature and humidity controlled and had its separately controlled light:dark cycle. Rats were maintained on a standard 12:12 hour light:dark cycle, with lights on at 07:00 or 08:00. Food and water were available ad libitum. Rats were allowed to acclimate for two weeks to their new housing conditions before surgery was performed. All procedures used were approved by University of Colorado Boulder's Institutional Animal Care and Use Committee, and were in accordance with the guidelines found within the *Guide for the Care and Use of Laboratory Animals* (DHHS Publication No. [NIH] 80-23, revised 2010 eighth edition).

Surgery

Surgery occurred during the animals' light phase. All rats received bilateral ovariectomy. Additionally, half of the rats received bilateral adrenalectomy (ADX), while the other half received SHAM adrenalectomy surgery (SHAM). Rats were anesthetized with halothane gas. Bilateral incisions in the dorsal portion of the peritoneal cavity immediately posterior to the rib cage were made in order to expose the ovaries. Ovaries were carefully isolated from surrounding organs and adipose tissue. A suture directly under the base of the ovaries and around the uterus and was made before ovaries were removed with a sharp scalpel in order to avoid blood loss. For the rats that received ADX, adrenals were located after ovaries had been removed. They were carefully separated from surrounding tissue, and removed with intestinal forceps. For the SHAM surgeries, the adrenal glands were located, but remained intact. Rats' peritoneal cavity was sutured, and skin stapled shut.

Hormone Replacement Regimen Post-Surgery

Corticosterone

Immediately after surgery, ADX rats received CORT in their drinking water in order to restore the rats' diurnal rhythm of CORT. Rats drink almost exclusively in the dark phase (their active phase), with the majority at the onset of the dark cycle. Thus, CORT in the drinking water is an

ideal, non-invasive way to restore the rats' normal diurnal plasma CORT rhythm (Jacobson et al., 1988). Replacement of diurnal CORT prevents any changes in clock gene expression and other circadian physiological measures that may be affected with ADX (Woodruff et al., 2016). ADX rats were given 25 µg/mL of CORT in 0.2% ethanol and 0.9% saline with tap water, which has been shown to restore plasma levels of diurnal CORT (Jacobson et al., 1988). SHAM rats received 0.2% ethanol in their drinking water as a control.

Estradiol

Rats received either estradiol benzoate (Sigma, St. Louis, MO) or vehicle injections subcutaneously once every four days in order to mimic plasma estradiol levels across the rats' estrous cycle (Asarian & Geary, 2002). Injections began 4 days after surgery, allowing enough time for any remaining endogenous estrogens in the rat to dissipate (Becker et al., 2004). A total of 3 injections were given, each at zeitgeber time 2 (ZT2, 2 hours after lights are turned on). This time was chosen so that testing could occur at ZT4. Estradiol-treated rats received 2 µg of estradiol benzoate (EB) crystal dissolved in 0.1 mL sesame oil (Foreway, New Taipei City, Taiwan), for a dose of 10 µg/kg per rat. Rats who received vehicle received 0.1 mL sesame oil. Testing occurred either 2 hours after the last EB injection (high plasma estradiol levels) in order to test for the acute effects of estradiol on stress-induced clock gene expression, or 48 hours after the last EB injection (low plasma estradiol levels) in order to test the permissive effects of estradiol on stress-induced clock gene expression.

Test Day and Tissue Processing

Test Day occurred either 2 h or 48 h after the last EB or vehicle injection. Procedures were the same between each test day. Half of the rats received 30 minutes of acute restraint stress at ZT3.5 in Plexiglas restraint tubes (5 cm in diameter, 15.5 cm in length). Upon termination of the stressor, rats were immediately decapitated at ZT4. No stress controls remained in their home

cage until rapid decapitation, which centered around ZT4. ZT4 was chosen as the time of sacrifice because previous studies have determined that there are no time of day effects in stress-induced clock gene expression [Chapter 2], and ZT4 is when *Per1* mRNA levels are at its trough in extra-SCN brain regions (Chun et al., 2015).

Upon decapitation, brains were carefully extracted and flash frozen in ~ -25°C isopentane chilled with dry ice. Brains were stored at -70°C until further use. Brains were cut at -20°C on a cryostat (Leica CM1850). 12µm coronal sections were taken at the level of the prefrontal cortex (2.2-3.2 mm anterior to bregma), the suprachiasmatic nucleus of the hypothalamus (1.3-1.4 mm posterior to bregma), the paraventricular nucleus of the hypothalamus (1.80-1.88 mm posterior to bregma), and the hippocampus (2.56 to 3.60 mm posterior to bregma) [Paxinos & Watson, Rat Brain Atlas, 4th edition]. Brain slices were thaw-mounted onto Colorfrost plus microscope slides, then stored at -70°C until use in in situ hybridization. Trunk blood was collected in EDTA-coated tubes and centrifuged at 4000 rpms for 10 minutes at 4°C. Plasma was then aliquoted out on dry ice and stored at -70°C until further use in a CORT assay.

Uteri were extracted and connecting adipose tissue was carefully cut away with a razor blade. Uteri were briefly stored in 0.9% saline in microfuge tubes before being blotted with a Kimwipe and weighed. This was done in order to assess the validity of the ovariectomies and subsequent EB replacement or vehicle injections. Thymi were also carefully removed and its wet weight determined in order to assess the validity of the adrenalectomies and subsequent CORT replacement in the drinking water.

Corticosterone (CORT) Assay

Plasma CORT levels were measured using a commercially available enzyme-linked immunoabsorbent assay kit (cat No. K014-H1, Arbor Assays, Ann Arbor, MI) according to manufacturer's instructions. Plasma samples were run in duplicate and diluted 1:50 in assay

buffer. Samples were then heated for 1 hour at 65°C to inactive corticosteroid-binding globulin. Sensitivity for CORT was 27 pg/mL, according to the manufacturer. The intra-assay coefficient of variation was 8.0%.

In situ hybridization

In situ hybridization for *Per1, Per2, Bmal1,* and *Cfos* followed procedures previously reported (Chun et al., 2015; Ginsberg et al., 2003; Girotti et al., 2009). Briefly, riboprobes for each gene of interest were made using radioactively labeled ^{35}S-UTP. The riboprobe was mixed into hybridization buffer. 97.5 µL (for PFC slide) or 65µL (for SCN, PVN, and HPC slides) of this mixture were applied to each slide and allowed to incubate at 54°C for 16-20 hours. Coverslips were gently floated off in 2X saline citrate solution (SSC), slides were then subjected to an incubation of 0.02 g/L of RNase (Fischer, Waltham, MA) at 37°C for an hour, put in decreasing concentrations of SSC, then subjected to a stringency wash of 0.1X SSC at 65°C for an hour, then dehydrated in increasing concentrations of ethanol. Slides were then air dried before putting down X-ray film. Slides were set on film for 2-4 weeks, then developed in a medical film processor SRX-101A (Konica Minolta, Tokyo, Japan).

Densitometry

Brain images from the X-ray film were captured using a Northern Light lightbox model B95 (Imaging Res Inc., St. Catharines, Ontario, Candada) and a Sony CCD video camera model XC-ST70 fitted with a Navitar 7000 zoom lens (Rochester, NY) and connected to LG3-01 frame grabber (Scion Corp., Frederick, MD) inside a Dell Dimension 500, and captured with Scion Image beta rel. 4.0.2. Digitized images were analyzed with ImageJ Software (NIH). Mean gray values were converted to uncalibrated optical density, and densitometry was performed on images whose gray levels fell within a linear range of the gray level:optical density ratio.

Measurements were taken within each region of interest by an experimenter blind to the rats' treatment groups. A circle or line was manually drawn and selected using anatomical landmarks from *Paxinos & Watson* (4th Edition). Regions of interest at the level of the PFC include: anterior cingulate cortex (AC), prelimbic medial prefrontal cortex (PL), infralimbic medial prefrontal cortex (IL), ventral orbital cortex (VO), and insula. 6 slices were used for analysis for the PFC. In more caudal sections, SCN and PVN were measured by the experimenter using free-hand selection of each ROI. Hippocampus (HPC) sections were also measured free hand, but with a line function. Subregions of the HPC that were measured include the CA1, CA3, the superior dentate gyrus (supraDG), and inferior dentate gyrus (infraDG). 4 slices were used for analysis for the SCN, PVN, and HPC. Measurements were taken from both hemispheres for all brain slices analyzed. The average optical density for each animal was used for treatment group averages and statistical analysis.

Statistical Analysis

Statistical Package for Social Science (SPSS; IBM version 24, 2016) was used to perform multivariate analysis of variance (ANOVA). Four-way ANOVA was used to determine whether there were main effects of stress, adrenal status, estradiol status, and time after last injection, and if there were any significant interactions. Main effects and interactions were considered to be significant if $p < 0.05$, and were followed up by Fischer's Least Significant Difference (FLSD) post-hoc analysis.

RESULTS

Corticosterone (CORT)

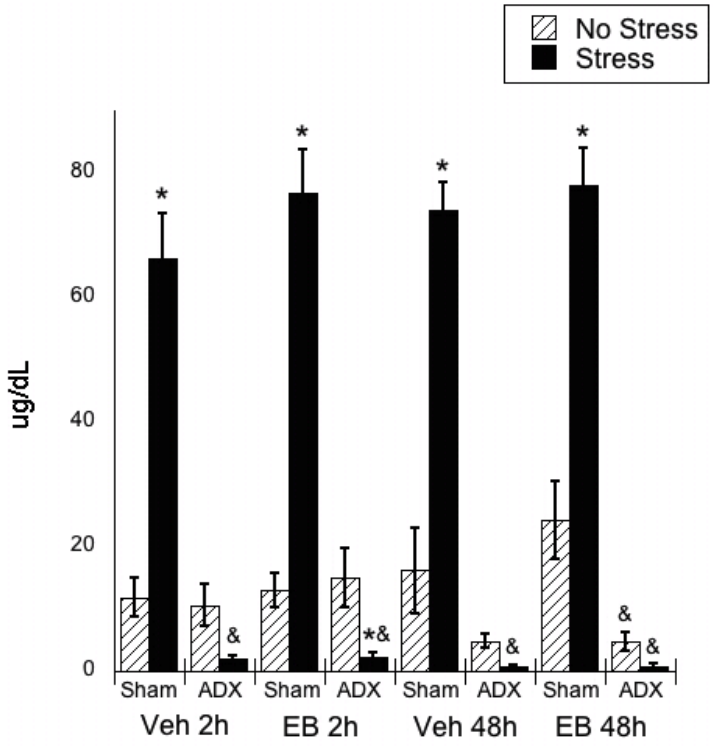
There were significant main effects of stress and of adrenal status, a significant stress x adrenal status interaction, and a significant adrenal status x time interaction [Table 4.1].

Table 4.1. Statistical analysis of a four-way ANOVA (stress x adrenal status x estradiol treatment x time of last injection) of CORT and *Per1* mRNA in the HPC, *Per1* and *c-Fos* mRNA in the SCN, PVN, and of *Per1, c-Fos, Bmal1* and *Per2* mRNA in the PFC of female rats. Significant main effects (S = stress; A = adrenal status; E = estradiol status; T = time after last injection) and significant interactions are denoted: * = p < 0.05; ** p < 0.01; *** = p < 0.001

		Stress	Adrenal	Estradiol	Time	S*A	S*E	S*T	A*E	A*T	E*T	S*A*E	S*A*T	S*E*T	A*E*T	S*A*E*T
Per1	SCN	$F_{(1,78)} = 0$	$F_{(1,78)} = 1.7$	$F_{(1,78)} = 0.7$	$F_{(1,78)} = 3.1$	$F_{(1,78)} = 1.4$	$F_{(1,78)} = 0.1$	$F_{(1,78)} = 0.4$	$F_{(1,78)} = 1.2$	$F_{(1,78)} = 0$	$F_{(1,78)} = 0.3$	$F_{(1,78)} = 0.5$	$F_{(1,78)} = 0.1$	$F_{(1,78)} = 1.7$	$F_{(1,78)} = 0.4$	$F_{(1,78)} = 0.1$
	PVN	$F_{(1,78)} = 80.3$***	$F_{(1,78)} = 1.6$	$F_{(1,78)} = 0$	$F_{(1,78)} = 1.7$	$F_{(1,78)} = 0.2$	$F_{(1,78)} = 0.1$	$F_{(1,78)} = 2.4$	$F_{(1,78)} = 1.9$	$F_{(1,78)} = 3.5$	$F_{(1,78)} = 0.1$	$F_{(1,78)} = 0.8$	$F_{(1,78)} = 1.1$	$F_{(1,78)} = 0.1$	$F_{(1,78)} = 2.5$	$F_{(1,78)} = 4.5$*
	AC	$F_{(1,81)} = 52.6$***	$F_{(1,81)} = 0.2$	$F_{(1,81)} = 1.2$	$F_{(1,81)} = 0.8$	$F_{(1,81)} = 11.04$**	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 3.1$	$F_{(1,81)} = 2.2$	$F_{(1,81)} = 1.4$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.3$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0$
	PL	$F_{(1,81)} = 60.5$***	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0.3$	$F_{(1,81)} = 0.6$	$F_{(1,81)} = 7.42$**	$F_{(1,81)} = 0$	$F_{(1,81)} = 1.1$	$F_{(1,81)} = 2.9$	$F_{(1,81)} = 0.8$	$F_{(1,81)} = 0.9$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.6$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.1$
	IL	$F_{(1,81)} = 53.2$***	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0.3$	$F_{(1,81)} = 1.1$	$F_{(1,81)} = 6.53$*	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.7$	$F_{(1,81)} = 2.0$	$F_{(1,81)} = 1.3$	$F_{(1,81)} = 0.4$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.4$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.4$
	VO	$F_{(1,81)} = 92.4$***	$F_{(1,81)} = 0.3$	$F_{(1,81)} = 0.9$	$F_{(1,81)} = 1.0$	$F_{(1,81)} = 9.5$**	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0.8$	$F_{(1,81)} = 1.5$	$F_{(1,81)} = 1.0$	$F_{(1,81)} = 0.7$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0.1$
	insula	$F_{(1,81)} = 0.7$	$F_{(1,81)} = 0.5$	$F_{(1,81)} = 1.8$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 9.4$**	$F_{(1,81)} = 0.3$	$F_{(1,81)} = 0.6$	$F_{(1,81)} = 2.3$	$F_{(1,81)} = 2.4$	$F_{(1,81)} = 1.8$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.2$	$F_{(1,81)} = 0.3$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0.5$
	CA1	$F_{(1,81)} = 0.8$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0.2$	$F_{(1,81)} = 0.7$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 1.0$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.2$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0.2$	$F_{(1,81)} = 0.2$	$F_{(1,81)} = 1.0$	$F_{(1,81)} = 0.5$
	CA3	$F_{(1,81)} = 0.3$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0.2$	$F_{(1,81)} = 1.3$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 1.4$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0.8$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.4$	$F_{(1,81)} = 0.3$
	supDG	$F_{(1,80)} = 0$	$F_{(1,80)} = 1.0$	$F_{(1,80)} = 0.1$	$F_{(1,80)} = 2.2$	$F_{(1,80)} = 0.2$	$F_{(1,80)} = 0$	$F_{(1,80)} = 0.2$	$F_{(1,80)} = 0.6$	$F_{(1,80)} = 0$	$F_{(1,80)} = 1.0$	$F_{(1,80)} = 0$	$F_{(1,80)} = 0$	$F_{(1,80)} = 0.3$	$F_{(1,80)} = 0.5$	$F_{(1,80)} = 0.1$
	infDG	$F_{(1,81)} = 0.3$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0.3$	$F_{(1,81)} = 2.8$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.2$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.4$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0.5$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 1.1$	$F_{(1,81)} = 0.4$
c-Fos	SCN	$F_{(1,78)} = 8.5$**	$F_{(1,78)} = 1.0$	$F_{(1,78)} = 0.5$	$F_{(1,78)} = 4.9$*	$F_{(1,78)} = 0$	$F_{(1,78)} = 0.2$	$F_{(1,78)} = 0.1$	$F_{(1,78)} = 0.7$	$F_{(1,78)} = 0.2$	$F_{(1,78)} = 0$	$F_{(1,78)} = 0.1$	$F_{(1,78)} = 1.1$	$F_{(1,78)} = 0$	$F_{(1,78)} = 0.7$	$F_{(1,78)} = 0.6$
	PVN	$F_{(1,81)} = 915$***	$F_{(1,81)} = 5.3$*	$F_{(1,81)} = 1.7$	$F_{(1,81)} = 0.2$	$F_{(1,81)} = 4.0$*	$F_{(1,81)} = 1.2$	$F_{(1,81)} = 1.0$	$F_{(1,81)} = 1.0$	$F_{(1,81)} = 0.6$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.2$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.8$	$F_{(1,81)} = 1.0$
	AC	$F_{(1,81)} = 36.9$***	$F_{(1,81)} = 2.8$	$F_{(1,81)} = 3.9$*	$F_{(1,81)} = 1.6$	$F_{(1,81)} = 2.6$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.9$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.6$	$F_{(1,81)} = 2.0$	$F_{(1,81)} = 0.3$	$F_{(1,81)} = 0.3$	$F_{(1,81)} = 1.0$	$F_{(1,81)} = 0$
	PL	$F_{(1,81)} = 64.8$***	$F_{(1,81)} = 4.0$*	$F_{(1,81)} = 3.6$	$F_{(1,81)} = 2.6$	$F_{(1,81)} = 0.4$	$F_{(1,81)} = 0$	$F_{(1,81)} = 2.2$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0$	$F_{(1,81)} = 1.5$	$F_{(1,81)} = 0.6$	$F_{(1,81)} = 0.4$	$F_{(1,81)} = 1.3$	$F_{(1,81)} = 1.8$	$F_{(1,81)} = 0.1$
	IL	$F_{(1,81)} = 69.0$***	$F_{(1,81)} = 5.5$*	$F_{(1,81)} = 5.8$*	$F_{(1,81)} = 2.5$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.3$	$F_{(1,81)} = 2.4$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0.9$	$F_{(1,81)} = 1.4$	$F_{(1,81)} = 0.3$	$F_{(1,81)} = 1.2$	$F_{(1,81)} = 0.4$	$F_{(1,81)} = 0.1$
	VO	$F_{(1,81)} = 61.9$***	$F_{(1,81)} = 6.2$*	$F_{(1,81)} = 4.6$*	$F_{(1,81)} = 2.6$	$F_{(1,81)} = 0.3$	$F_{(1,81)} = 0$	$F_{(1,81)} = 1.8$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0.6$	$F_{(1,81)} = 0.8$	$F_{(1,81)} = 0.9$	$F_{(1,81)} = 0.3$	$F_{(1,81)} = 1.1$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0.1$
	insula	$F_{(1,81)} = 1.0$	$F_{(1,81)} = 30.5$***	$F_{(1,81)} = 12.9$**	$F_{(1,81)} = 0.2$	$F_{(1,81)} = 38$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.5$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.5$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0.5$	$F_{(1,81)} = 1.0$	$F_{(1,81)} = 0.6$
Per2	AC	$F_{(1,81)} = 0$	$F_{(1,81)} = 1.5$	$F_{(1,81)} = 3.0$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 2.0$	$F_{(1,81)} = 1.0$	$F_{(1,81)} = 0$	$F_{(1,81)} = 1.3$	$F_{(1,81)} = 0.2$	$F_{(1,81)} = 0.6$	$F_{(1,81)} = 0.2$	$F_{(1,81)} = 0.4$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.2$	$F_{(1,81)} = 0.1$
	PL	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 2.2$	$F_{(1,81)} = 0.3$	$F_{(1,81)} = 0.6$	$F_{(1,81)} = 0.5$	$F_{(1,81)} = 0$	$F_{(1,81)} = 1.3$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0.5$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0.3$	$F_{(1,81)} = 0.3$	$F_{(1,81)} = 0$
	IL	$F_{(1,81)} = 0.5$	$F_{(1,81)} = 0$	$F_{(1,81)} = 2.0$	$F_{(1,81)} = 0.2$	$F_{(1,81)} = 0.9$	$F_{(1,81)} = 0.6$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0.5$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.4$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.5$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0$
	VO	$F_{(1,81)} = 0.8$	$F_{(1,81)} = 2.6$	$F_{(1,81)} = 3.7$	$F_{(1,81)} = 0$	$F_{(1,81)} = 1.9$	$F_{(1,81)} = 1.6$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.5$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0.5$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0$
	insula	$F_{(1,81)} = 0.9$	$F_{(1,81)} = 5.7$*	$F_{(1,81)} = 3.7$	$F_{(1,81)} = 0$	$F_{(1,81)} = 1.2$	$F_{(1,81)} = 1.7$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 1.8$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.5$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0.2$
Bmal1	AC	$F_{(1,81)} = 4.6$*	$F_{(1,81)} = 0.9$	$F_{(1,81)} = 0.5$	$F_{(1,81)} = 2.2$	$F_{(1,81)} = 0.7$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 1.4$	$F_{(1,81)} = 0.2$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.6$	$F_{(1,81)} = 1.1$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0.7$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0.6$
	PL	$F_{(1,81)} = 9.7$**	$F_{(1,81)} = 3.6$	$F_{(1,81)} = 0.3$	$F_{(1,81)} = 2.1$	$F_{(1,81)} = 1.2$	$F_{(1,81)} = 0.3$	$F_{(1,81)} = 0.3$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0.9$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0$
	IL	$F_{(1,81)} = 6.0$*	$F_{(1,81)} = 4.5$*	$F_{(1,81)} = 0.4$	$F_{(1,81)} = 2.5$	$F_{(1,81)} = 2.7$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.9$	$F_{(1,81)} = 0.2$	$F_{(1,81)} = 0.3$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.2$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0.3$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.2$
	VO	$F_{(1,81)} = 7.8$**	$F_{(1,81)} = 2.15$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0.2$	$F_{(1,81)} = 2.2$	$F_{(1,81)} = 0.5$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.3$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.8$	$F_{(1,81)} = 0.3$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.06$ [
	insula	$F_{(1,81)} = 6.8$**	$F_{(1,81)} = 1.9$	$F_{(1,81)} = 0$	$F_{(1,81)} = 1.2$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0$	$F_{(1,81)} = 2.6$	$F_{(1,81)} = 1.5$	$F_{(1,81)} = 0.9$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.6$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.5$	$F_{(1,81)} = 0.1$
	CORT	$F_{(1,81)} = 137$***	$F_{(1,81)} = 348$***	$F_{(1,81)} = 2.7$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 229$***	$F_{(1,81)} = 0$	$F_{(1,81)} = 0.2$	$F_{(1,81)} = 1.3$	$F_{(1,81)} = 6.32$*	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 0.3$	$F_{(1,81)} = 1.4$	$F_{(1,81)} = 0.3$	$F_{(1,81)} = 0.1$	$F_{(1,81)} = 1.1$

Post-hoc analysis revealed significant pair-wise comparisons for the following: stress significantly increased CORT plasma levels for all SHAM groups, but not for any ADX groups (FLSD, $p < 0.05$) [Figure 4.1].

Figure 4.1. Effect of stress, adrenal status, estradiol status, and time after last injection on plasma corticosterone (CORT) levels at ZT4. Acute stress increased CORT levels in SHAM animals only, as expected. ADX rats still had basal CORT levels similar to that of SHAM rats, as CORT replacement had been put in their drinking water. Estradiol had no significant effects [* = $p < 0.05$ for stress; & = $p < 0.05$ for adrenal status; FLSD post-hoc pair-wise test].



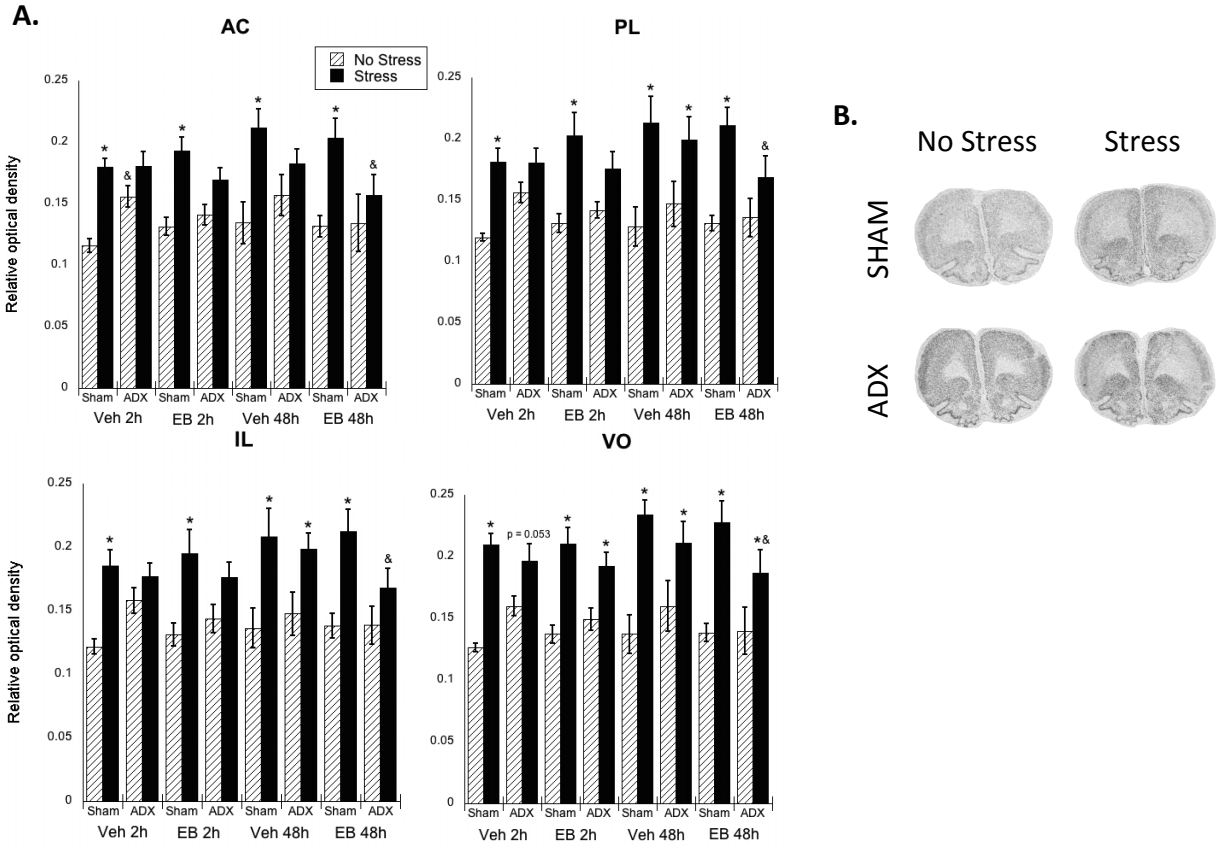
This was to be expected since ADX rats cannot mount a CORT response. SHAM rats also had greater basal CORT compared to ADX rats when treated with EB 48 hours prior to sacrifice (FLSD, $p < 0.05$). This is the only treatment group under no stress conditions to have a significant adrenal effect. All rats were sacrificed at ZT4, during the rats' trough of diurnal plasma CORT levels, thus little to no difference are expected. For ADX rats that received EB 2 hours prior to challenge, stress decreased plasma CORT (FLSD, $p < 0.05$). ADX rats should

have equal amounts of plasma CORT regardless if exposed to stress or not. This surprising result may be due to the fact that the preceding EB injection at ZT2 was stimulating enough to wake the rats and increase their drinking, thus increasing plasma CORT. Stress rats did not have access to their CORT water during restraint, while the no stress rats in their home cage did.

Prefrontal Cortex (PFC)

Per1, Per2, Bmal1, and *c-Fos* mRNA were examined in the anterior cingulate (AC), prelimbic (PL), infralimbic (IL), and ventral orbital cortex (VO) subregions of the prefrontal cortex. There was a significant main effect of stress, as well as a significant stress x adrenal interaction for *Per1* mRNA expression in all subregions of the PFC [Table 4.1]. Stress significantly increased *Per1* mRNA in all SHAM animals regardless of estradiol treatment and time after last injection. However, in ADX rats, there were few instances for stress to increase *Per1* mRNA in the PL and IL for ADX rats treated with vehicle 48 hours prior to sacrifice, and in the VO of ADX vehicle rats (FLSD, $p < 0.05$) [Figure 4.2].

Figure 4.2: Effect of stress, adrenal status, estradiol treatment, and time of last injection on *Per1* mRNA throughout the PFC. A) There was a significant main effect of stress and a significant stress x adrenal status interaction throughout all subregions (AC = anterior cingulate; PL = prelimbic; IL = infralimbic; VO = ventral orbital). Acute stress increases *Per1* mRNA, and ADX partially attenuates this effect. There were no effects for estradiol treatment [* = $p < 0.05$ for stress; & = $p < 0.05$ for adrenal status, for FLSD post-hoc pair-wise test]. B) Representative autoradiograms of stress-induced *Per1* mRNA in SHAM and ADX rats.



These results suggest that throughout the PFC, stress-induced *Per1* mRNA expression is largely independent from the presence of endogenous CORT, as ADX attenuated stress-induced *Per1* mRNA in only a few treatment groups.

There was a significant main effect of stress for *Bmal1* mRNA in all subregions of the PFC [Table 4.1]. Post-hoc analysis failed to find any significant pair-wise comparisons, though there was an overall trend for stress to decrease *Bmal1* mRNA expression [Fig 4.3]. *Per2* mRNA in all subregions of the PFC is not induced by stress and had no other significant main effects or interactions [Table 4.1; Fig 4.4].

Figure 4.3. Effect of stress, adrenal status, estradiol treatment, and time of last injection on *Bmal1* mRNA throughout the PFC. A) There was a significant main effect of stress in decreasing *Bmal1* mRNA throughout all subregions of the PFC, however there was no significant pair-wise comparisons. B) Because there was no difference in regards to estradiol treatment and time of last injection, data was condensed in order to better visually compare the main effects of stress and adrenal status. C) Representative autoradiograms of stress-induced *Bmal1* mRNA in SHAM and ADX rats.

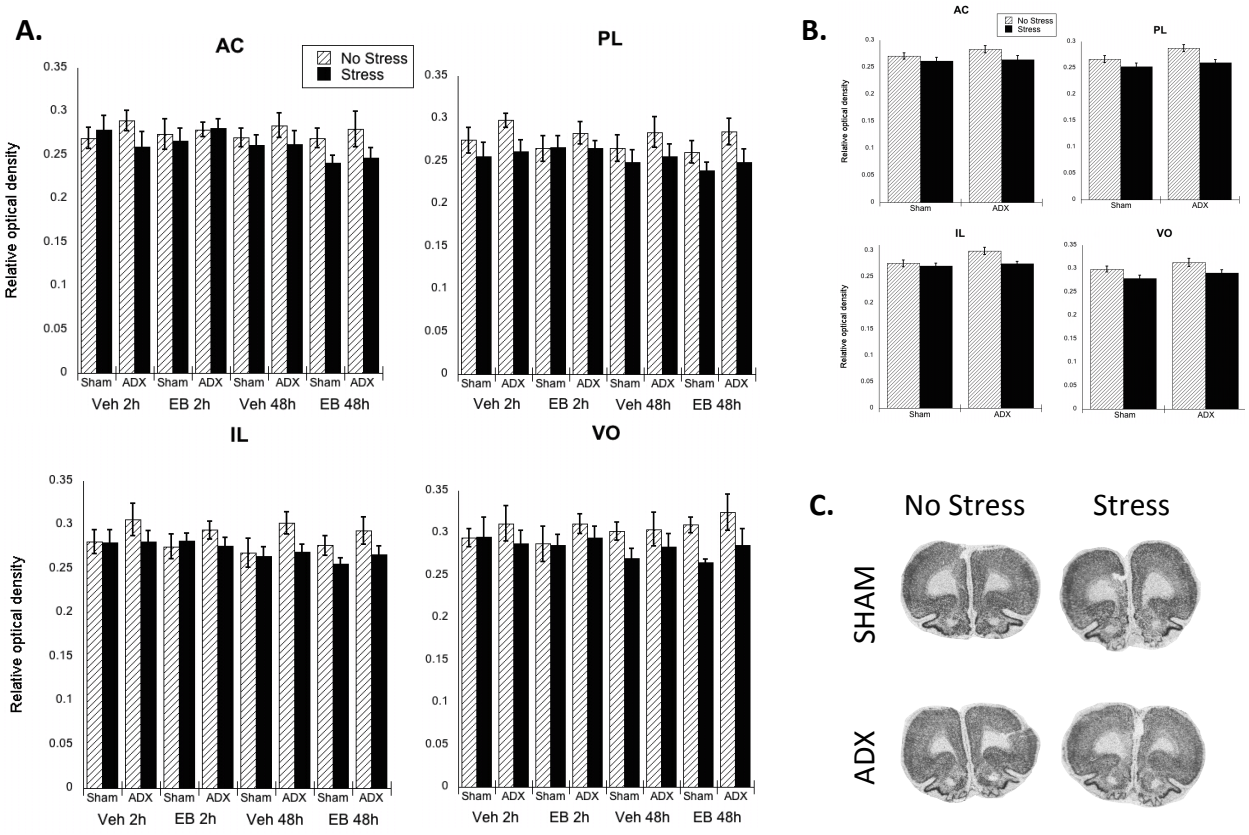
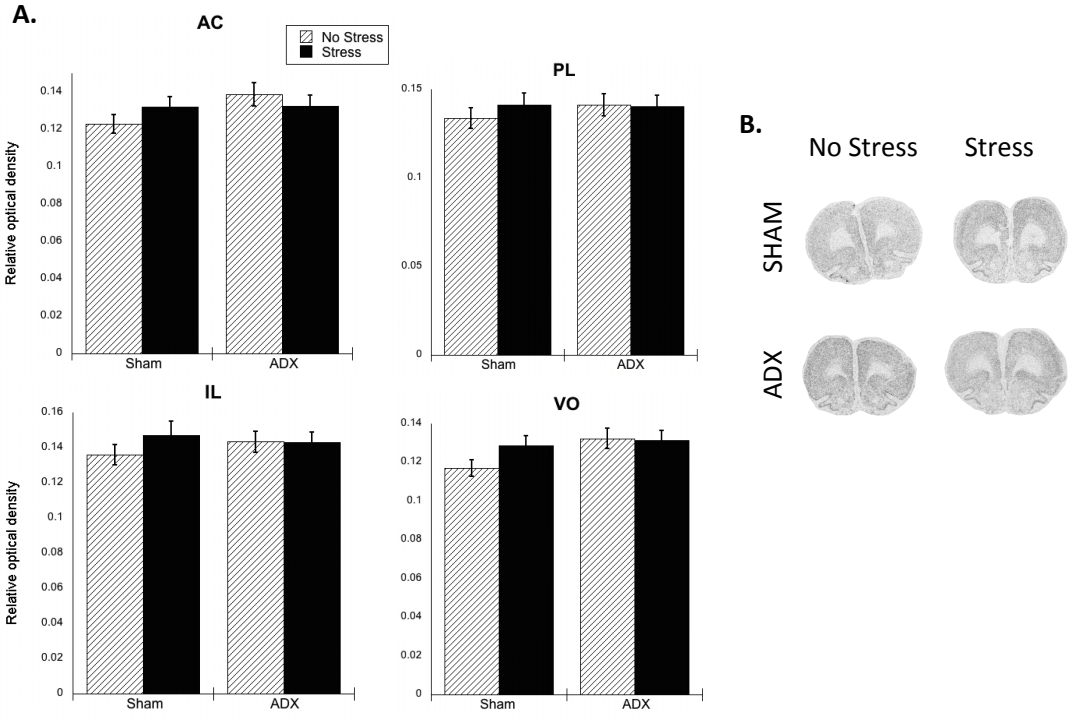
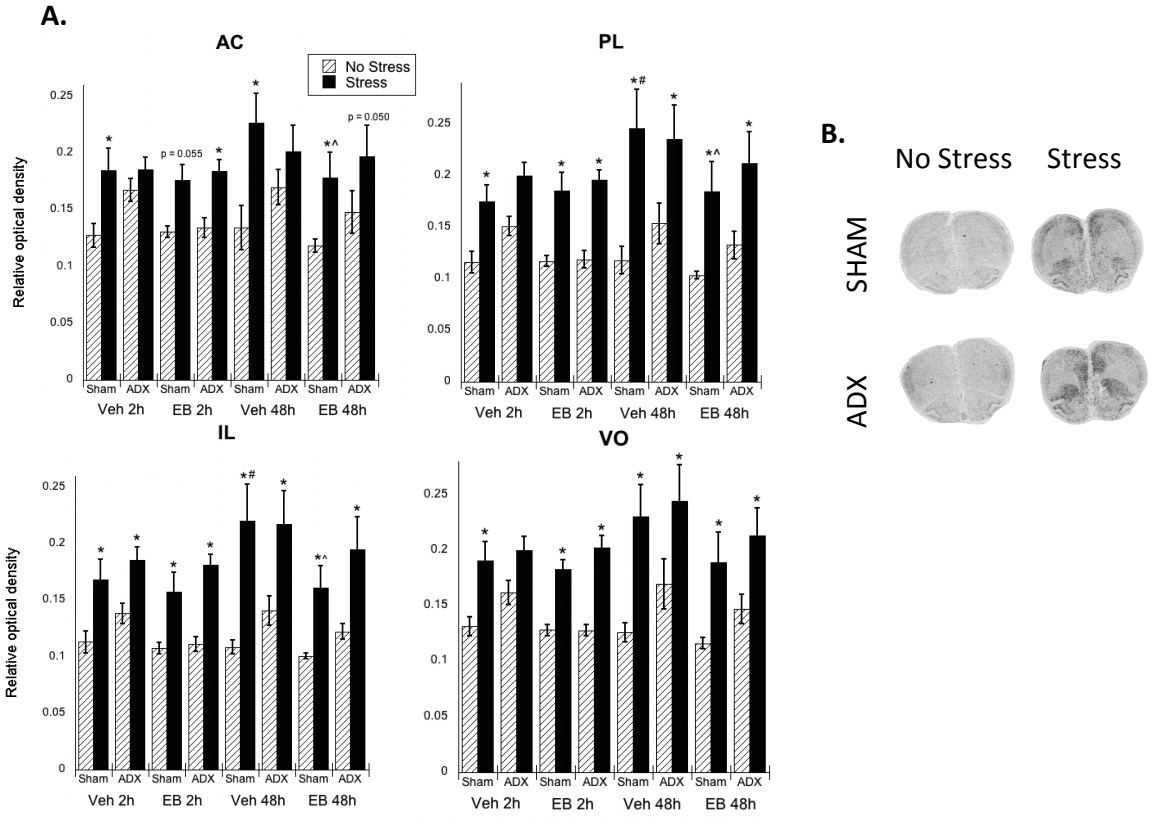


Figure 4.4. Effect of stress, adrenal status, estradiol treatment, and time of last injection on *Per2* mRNA throughout the PFC. A) Because there were no significant main effects or interactions, estradiol and time of last injection were condensed in order to better display no stress versus stress effects. B) Representative autoradiograms of stress-induced *Per2* mRNA in SHAM and ADX rats.



There was a significant main effect of stress for *c-Fos* mRNA expression for all subregions of the PFC [Table 4.1]. Post-hoc analysis (FLSD, $p < 0.05$) revealed that stress increased *c-Fos* mRNA for nearly all treatment groups in all subregions, with a few exceptions (in VO: ADX vehicle 2h; in AC: ADX EB 2h, SHAM vehicle 2h, ADX EB 48h, and SHAM EB 48h) [Fig 4.5].

Figure 4.5. Effect of stress, adrenal status, estradiol treatment, and time of last injection on *c-Fos* mRNA throughout the PFC. A) There was a main effect of stress, where acute restraint stress rapidly (within 30 min) increases *c-Fos* mRNA expression throughout all subregions of the PFC (AC, PL, IL, VO). ADX had no effect on this stress-induction [* = p < 0.05 for stress, & = p < 0.05 for adrenal status; ^ = p < 0.05 for estradiol status; # = p < 0.05 for time after last injection; FLSD post-hoc pair-wise test). B) Representative autoradiograms of stress-induced *c-Fos* mRNA in SHAM and ADX rats.

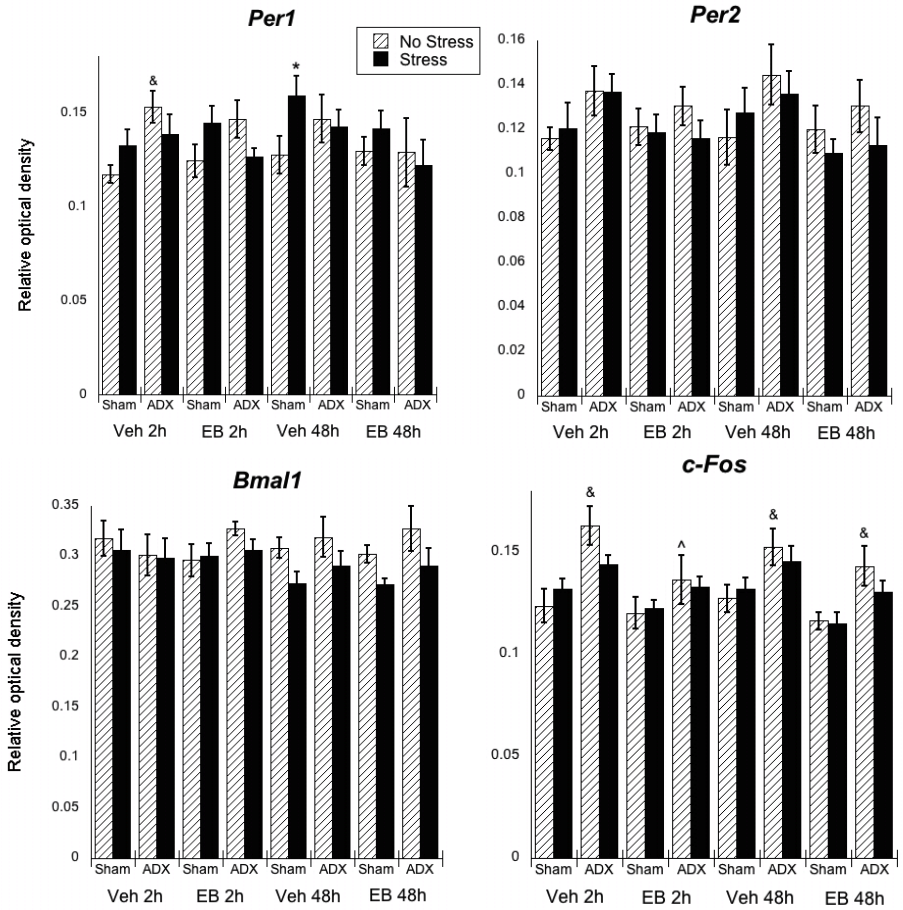


There was also a significant main effect of adrenal status in the PL, IL, and VO subregions [Table 4.1], though post-hoc tests failed to indicate any significant pair-wise comparisons. Additionally, the AC, IL, and VO subregions had a significant main effect of estradiol status [Table 4.1], where EB treated rats had less stress-induced *c-Fos* mRNA expression in the AC, PL, and IL subregions compared to vehicle treated rats in the SHAM 48 hour treatment group (FLSD, $p < 0.05$). This suggests that estradiol decreases stress-induced *c-Fos* mRNA expression, and that permissive actions of estradiol are enough to cause this effect.

Rostral Agranular Insula

Per1, Per2, Bmal1, and *c-Fos* mRNA were examined in the insula. There were no significant main effects for *Per1* mRNA, but there was a significant adrenal status x stress interaction [Table 4.1]. Stress increased *Per1* mRNA levels only for SHAM vehicle treated rats at 48 hours after their last injection (FLSD, $p < 0.05$). Additionally, ADX rats had greater *Per1* mRNA expression compared to SHAM rats under no stress vehicle 2 h conditions (FLSD, $p < 0.05$) [Fig 4.6]. Thus, compared to the PFC, there were minimal effects of stress on *Per1* mRNA in the insula.

Figure 4.6. Effect of stress, adrenal status, estradiol treatment, and time of last injection on *Per1, Per2, Bmal1,* and *c-Fos* mRNA in the rostral agranular insula. Overall, effects in the insula were limited. *Per1* had a significant adrenal status x stress interaction, *Per2* had a significant effect of adrenal status, and *c-Fos* had a main effect of adrenal status and estradiol treatment. [* = $p < 0.05$ for stress; & = $p < 0.05$ for adrenal status; ^ = $p < 0.05$ for estradiol treatment; FLSD post-hoc pair-wise test].



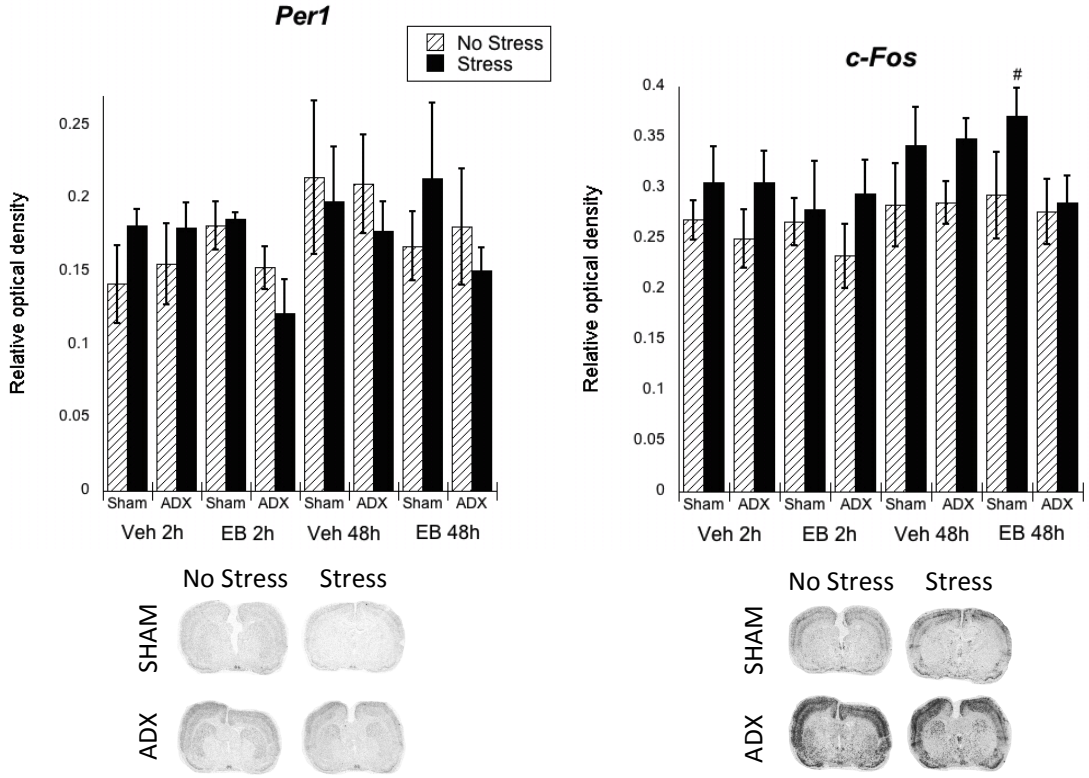
Per2 mRNA in the insula had a significant main effect of adrenal status [Table 4.1], but FLSD post-hoc analysis did not find any significant pair-wise comparisons. *Bmal1* mRNA had a significant main effect of stress, but FLSD post-hoc analysis also failed to find any significant pair-wise comparisons. Similar to the PFC, stress had a tendency to decrease *Bmal1* mRNA expression in the insula.

C-Fos mRNA in the insula had significant main effects of adrenal status and estradiol status [Table 4.1]. While EB decreased stress-induced *c-Fos* mRNA in the PFC, EB treatment decreased *c-Fos* mRNA in the insula under no stress conditions in ADX 2 hour rats (FLSD, $p < 0.05$) [Fig 4.6]. ADX increased *c-Fos* mRNA in the insula under no stress conditions in rats treated with vehicle at 2 h and in rats treated with EB or vehicle at 48 h. This suggests that under basal conditions, EB decreases and ADX increases *c-Fos* mRNA expression in the insula. Overall, the insula was generally not reactive to stress, which differs from that seen in the prefrontal cortex.

Suprachiasmatic Nucleus of the Hypothalamus (SCN)

Per1 and *c-Fos* mRNA expression were examined in the SCN. There were no main effects or interactions for *Per1* mRNA [Table 4.1], which supports the idea that the SCN is impervious to non-light environmental stimuli that could disrupt the molecular clock within the body's master clock. *C-Fos* mRNA had a significant main effect of stress and time after last injection [Table 4.1], but no significant pair-wise comparisons between no stress and stress groups. Under conditions of stress, SHAM rats treated with EB 48 h prior to stress had greater *c-Fos* mRNA expression compared to EB treatment 2 h prior to sacrifice (FLSD, $p < 0.05$) [Figure 4.7].

Figure 4.7. Effect of stress, adrenal status, estradiol treatment, and time of last injection on *Per1* and *c-Fos* mRNA in the SCN. Overall, there was a lack of effect on *Per1* and *c-Fos* mRNA expression in the SCN. Representative autoradiograms (in situ hybridication) are below the corresponding gene of interest. (# = $p < 0.05$ for time after last injection; FLSD post-hoc pair-wise test).

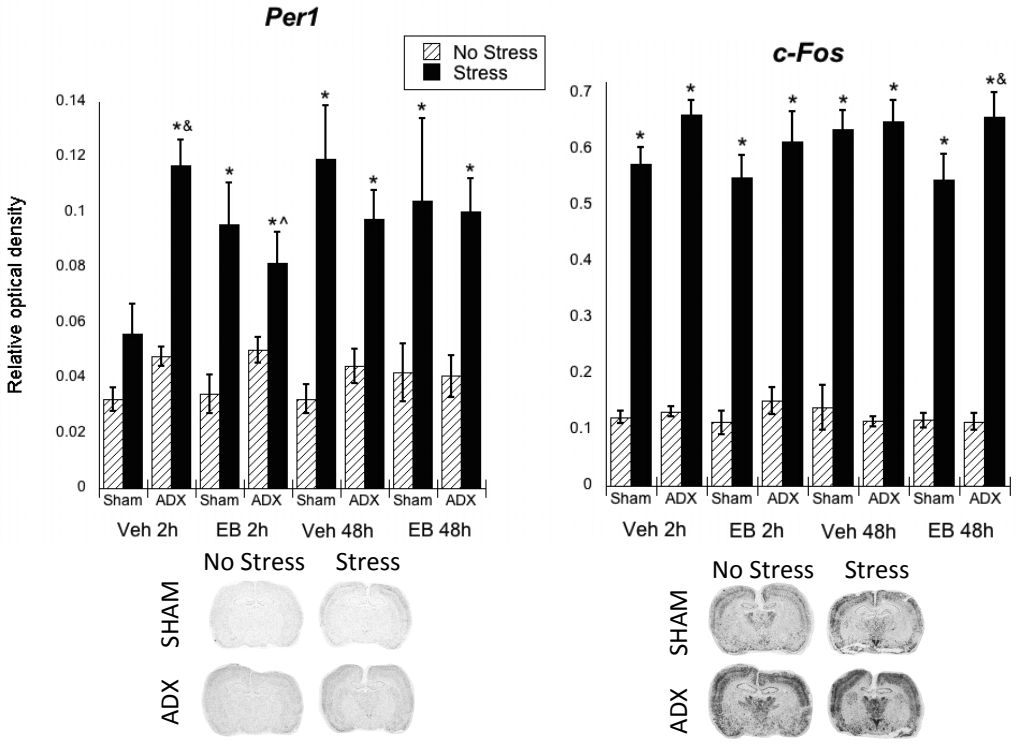


This suggests that EB may acutely decrease *c-Fos* mRNA expression in the SCN under conditions of stress. This matches results in the PFC where EB treatment decreases stress-induced *c-Fos* mRNA. However, in the PFC permissive actions of EB were enough to decrease stress-induced *c-Fos* mRNA.

Paraventricular Nucleus of the Hypothalamus (PVN)

Per1 and *c-Fos* mRNA were examined in the PVN. For *Per1* mRNA, there was a significant main effect of stress and a significant stress x adrenal status x hormone status x time interaction [Table 4.1]. Stress significantly increased *Per1* mRNA for all treatment groups except ADX EB 2 h and SHAM vehicle 2 h (FLSD, $p < 0.05$) [Figure 4.8].

Figure 4.8. Effect of stress, adrenal status, estradiol treatment, and time of last injection on *Per1* and *c-Fos* mRNA in the PVN. There was a significant main effect of stress for both *Per1* and *c-Fos* mRNA in the PVN. There was no effect of adrenal status nor estradiol status on stress-induced gene expression. [* = p < 0.05 for stress; & = p < 0.05 for adrenal status; ^ = p < 0.05 for estradiol treatment; # = p < 0.05 for time after last injection; FLSD post-hoc pair-wise test]. Representative autoradiograms (in situ hybridization) are below the graphs.



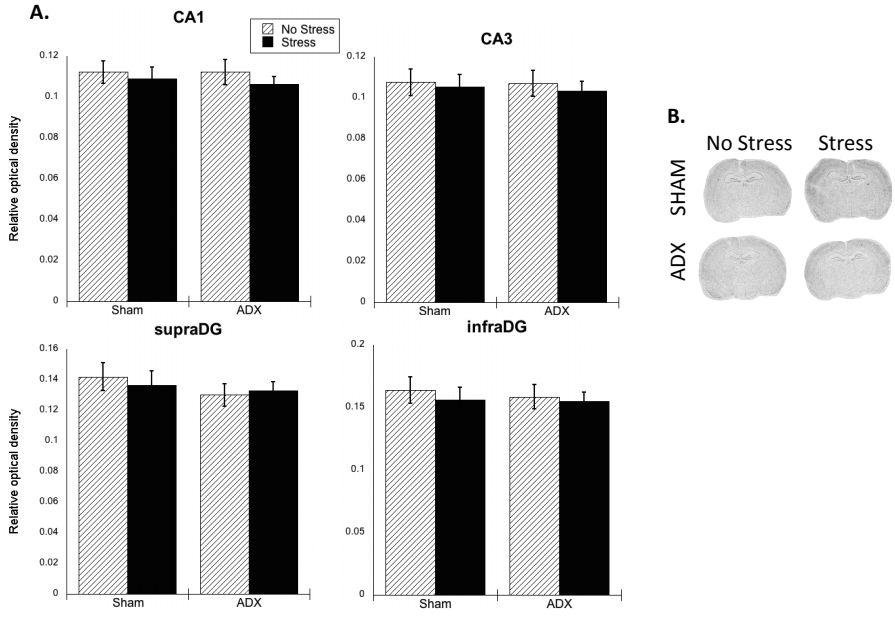
Thus, stress did not induce *Per1* mRNA as robustly (e.g., within all treatment groups) compared to the PFC. There was no significant main effect of adrenal status on this stress induction, indicating that endogenous CORT is not necessary for stress-induced *Per1* mRNA in the PVN. This differs from the PFC, which demonstrated partial attenuation of stress-induced *Per1* mRNA with ADX. Post-hoc tests revealed that ADX rats treated with EB 2 h prior to stress had lower *Per1* expression compared to vehicle treated rats (FLSD, $p < 0.05$). Thus, EB decreases stress-induced *Per1* mRNA acutely for this treatment group. However, for stress SHAM 2h rats, EB actually increased *Per1* expression compared to vehicle treated rats. These contradictory results suggest no strong conclusions can be made in regards to the effect of EB on stress-induced *Per1* mRNA in the PVN.

C-Fos mRNA expression had significant main effects of stress and adrenal status, and a stress x adrenal status interaction with a p-value of 0.050 [Table 4.1]. Stress increased *c-Fos* mRNA for all treatment groups (FLSD, $p < 0.05$) [Figure 4.8]. ADX had significantly greater *c-Fos* expression compared to SHAM rats that were stressed and treated with EB 48 h prior to sacrifice (FLSD, $p < 0.05$). Stressed SHAM rats that were treated with EB 48 h prior to stress had decreased *c-Fos* mRNA compared to rats treated with vehicle (FLSD, $p = 0.050$), which coincides with the effect of EB overall decreasing stress-induced *c-Fos* mRNA expression in the PFC [Figure 4.2] and insula [Figure 4.6].

Hippocampus (HPC)

Per1 mRNA expression was examined in the CA1, CA3, superior dentate gyrus (supraDG), and inferior dentate gyrus (infraDG) subregions of the hippocampus (HPC). There were no significant main effects or interactions for *Per1* mRNA expression in any of the subregions examined [Table 4.1; Figure 4.9].

Figure 4.9. Effect of stress and adrenal status on *Per1* mRNA throughout the HPC. A) There were no significant main effects, thus estradiol treatment and time after last injection were condensed in order to better visually compare the no stress an stress groups. B) Representative autoradiograms (in situ hybridization) of no stress versus stress *Per1* mRNA in SHAM and ADX rats.



Thus, unlike the PFC and PVN, restraint stress has no effect on *Per1* mRNA expression, suggesting regional specificity in stress-induced *Per1* mRNA expression. Additionally, any adrenal effect on *Per1* mRNA was undetectable at ZT4. Estradiol manipulation under basal conditions also had no effect on *Per1* mRNA expression in the HPC.

DISCUSSION

The present study examined whether stress-induced clock gene expression, particularly *Per1* mRNA, in the prefrontal cortex (PFC), paraventricular nucleus of the hypothalamus (PVN), suprachiasmatic nucleus (SCN), rostral agraunular insula, and hippocampus (HPC), is dependent on the presence of endogenous CORT in female rats. Additionally, it was examined whether estradiol could modulate stress-induced clock gene expression acutely and/or permissively. Female rats had their ovaries removed and received estradiol replacement or vehicle. Additionally, half of the rats were adrenalectomized (ADX) while the other half had their adrenals intact (SHAM). Rats received 30 minutes of acute restraint stress or were left undisturbed in their home cage before sacrifice. Stress selectively increased *Per1* mRNA expression, but not *Per2* or *Bmal1,* in the PFC, as measured by in situ hybridization. In the PVN, only *Per1* and *c-Fos* mRNA were examined, and both were induced by stress. Restraint stress had no effect on *Per1* mRNA induction in the insula, SCN, and throughout the HPC. In the PFC, ADX partially attenuated stress-induced *Per1* mRNA, while there was no significant effect of ADX on stress-induced *Per1* mRNA in the PVN. *C-Fos* mRNA in these brain areas was also induced by stress, but unaffected by ADX. While estradiol had no effect on stress-induced *Per1* mRNA, estradiol attenuated stress-induced *c-Fos* mRNA in the PFC. The results from this study suggest that in female rat PFC and PVN, stress-induced *Per1* mRNA is largely independent on the presence of endogenous CORT, and unaffected by estradiol. These data also suggest regional specificity in stress-induced *Per1* mRNA, as stress does not induce *Per1* mRNA in the insula, SCN, and HPC.

Stress-induced Per1 mRNA is only partially dependent on the presence of endogenous CORT

Similar to the results seen in Chapter 2 with male rats, stress-induction of *Per1* mRNA in the PFC and PVN is largely independent of the presence of endogenous CORT in female rats as well. This study also found a significant main effect of stress for *Bmal1* mRNA in the PFC, where stress decreased *Bmal1* mRNA expression. This is the first study to show rapid changes in *Bmal1* mRNA expression in response to an acute stressor. However, there were no significant pair-wise comparisons, suggesting that this stress effect is not robust. In contrast, *Per1* mRNA is robustly induced by stress, which suggests that Per1 may be the gateway by which stress or other environmental stimuli can entrain extra-SCN molecular clocks. There was a partial attenuation of stress-induced *Per1* mRNA in all subregions of the PFC with ADX, where all SHAM treatment groups exhibited a stress effect, but only a handful of ADX treatment groups did [Figure 4.2]. However, in the PVN, acute stress induced *Per1* mRNA regardless of ADX status [Figure 4.8]. These results suggest that there is regional specificity in how much the presence of endogenous CORT can influence stress-induced clock gene expression. These limited effects of ADX on stress-induced *Per1* mRNA seen in this study are somewhat similar to that seen in Chapter 2 with male rats. However, in Chapter 2, the PFC was completely unaffected by ADX, while the PVN had attenuation of stress-induced *Per1* mRNA expression at ZT4, but not ZT16. These differences in brain region may be due to differences between the sexes, but may also be due to the fact that in the current study, female rats received replacement CORT in their drinking water, while male rats in Chapter 2 did not. Woodruff et al. (2016) has demonstrated that appropriately timed diurnal CORT is necessary for normal clock gene expression in the PFC of male rats. Thus, CORT replacement in the drinking water may alter how stress induces *Per1* mRNA in extra-SCN brain regions. Al-Safadi et al. (2015) demonstrated that acute stress increases PER1 protein expression in the PVN, but decreases PER1 protein in the BNSTov and CEA, and that ADX had opposing effects in these different

brain regions. This study used CORT replacement in the rats' drinking water, and still demonstrated variable ADX effects on stress-induced PER1. Thus, the effect of ADX on stress-induced clock gene expression may vary based on brain region. However, all these results collectively suggest that there is partial dependence of stress-induced *Per1* mRNA on CORT.

These results are somewhat surprising, as it is generally believed that the body's master clock, the SCN, is able to entrain extra-SCN molecular clocks via glucocorticoids (Balsalobre et al., 2000; Dickmeis, 2009). CORT has a diurnal rhythm with peak levels immediately upon the animal's active phase, which is controlled by the SCN (Herman et al., 2003; Moore & Eichler, 1972) Additionally, CORT has been shown to rapidly induce Per1 gene expression in many peripheral tissues (e.g., liver, blood mononuclear cells, kidney, mesenchymal stem cells, fibroblast cells) (Balsalobre et al., 2000; Burioka et al., 2005; Cuesta et al., 2015; So et al., 2009; Sujino et al., 2012; Yamamoto et al., 2004) as well as hippocampus (Bohacek et al., 2015; Conway-Campbell et al., 2010; Gray et al., 2014) presumably via the glucocorticoid response element (GRE) located within the promoter region of the Per1 gene (Yamamoto et al., 2005). Thus, it has been suggested that untimely peaks in CORT may alter the expression of *Per1* mRNA (Balsalobre et al., 2000). There has been evidence in the literature to suggest that even under no stress conditions, normal clock gene expression is dependent on the presence of endogenous CORT, as ADX disrupts rhythmic *Per1, Per2,* and *Bmal1* mRNA, in male rat PFC. Furthermore, the timing of CORT peak (via CORT injection) is critical in restoring normal clock gene expression (Woodruff et al., 2016). Thus, while CORT is necessary for basal clock gene expression and CORT alone is sufficient to induce *Per1* gene expression in peripheral tissues and hippocampus, few studies have actually begun to examine if CORT is necessary for stress-induced clock gene expression.

Previous studies that have examined stress-induced clock gene expression at an acute level has also shown that *Per1* gene expression is induced in the PVN (Takahashi et al., 2001), DMH, piriform cortex, and hippocampus (Bohacek et al., 2015; Roszkowski et al., 2016).

However, other than the current study and those from Chapter 2, there has only been one other study examining the affect of ADX on stress-induced clock gene expression. Al-Safadi et al. (2015) have found that ADX of male rats exposed to 30 minutes of restraint stress attenuated the stress-induced decrease of PER1 protein in the BNST and CeA, but had no effect on the stress-induced increase in PER1 protein in the PVN. Thus, similar to the results of this study, stress-induced *Per1* expression in the PVN is not dependent on the presence of endogenous CORT. This study found a robust ADX effect on stress-induced PER1 in the BNST and CEA, which differend in robustness from the only partial attenuation int eh PFC of this current study. Other studies have used GR antagonists in lieu of ADX in order to examine whether stress-induced Per1 is mediated by GR. Bohacek et al. (2015) has demonstrated that while swim stress induces both *c-Fos* and *Per1* expression in both male and female rat HPC, CORT injection fails to replicate this effect. Additionally, a GR antagonist given prior to stress does not alter swim stress-induced *Per1.* This study suggests that stress-induced Per1 is via a non-GRE mediated mechanism. Roszkowski et al. (2016) also confirms that GR antagonsists have no effect on blocking swim stress-induced *Per1* mRNA in the HPC.

Collectively, these studies suggest CORT independence in stress-induced *Per1* gene expression. There is a well-established cAMP response element (CRE) located within the promoter region of the Per1 gene. In the SCN, light increases the phosphorylation of CREB, which then binds to the CRE within the promoter region of Per1, rapidly (within 30 minutes) inducing *Per1* expression (Tischkau et al., 2002). It is possible that stress, which rapidly increases CREB in the PFC (Shansky & Morrison, 2009) rapidly increases the expression of Per1 in extra-SCN brain regions via the CRE within Per1's promoter region. In this study, throughout all brain regions examined, stress-induced *Per1* mRNA was largely mirrored by *c-Fos* mRNA, where stress rapidly induces *c-Fos* mRNA in the PFC and PVN. Stress-induced *c-Fos* mRNA was completely independent of CORT, as expected. Additionally, both *c-Fos* and *Per1* mRNA are rapidly induced (within 30 minutes). CREB is known to increase *c-Fos* mRNA

via the CRE located within the promoter region of c-Fos (Sheng & Greenberg, 1990). Thus, *c-Fos* mRNA induction is a good indicator of CREB activity. Further studies are needed in order to assess with certainty stress-induced *Per1* gene expression via CRE-mediated mechanisms (e.g., measuring phosphorylated/activated CREB) and to determine the neural mediators that increase CREB activity and induce *Per1* mRNA expression in the PFC and PVN.

Lack of a stress effect in the insula and hippocampus suggests regional differences in stress-induced Per1 mRNA

There was a robust induction of *Per1* mRNA by stress throughout the subregions of the PFC, as well as the PVN. However, restraint stress did not induce *Per1* mRNA in the rostral agranular insula [Fig 6] and all subregions of the HPC (CA1, CA3, superior DG, inferior DG) [Fig 9]. Even though glucocorticoid receptors (GRs) are ubiquitously expressed throughout the brain, with the SCN as a notable exception (Balsalobre et al., 2000; Rosenfeld et al., 1988; 1993), there are differences in stress-induced *Per1* mRNA depending on the brain region. The PVN is head of the HPA-axis, and thus it is unsurprising that a brain region with such an integral role in the stress response would have stress-induced *Per1* mRNA. The PFC is a brain region extremely important in stress reactivity, emotional control, and assessment of stressful/aversive stimuli, and also plays an important role in modulating HPA axis/stress reactivity. The HPC is also a brain region important in the limbic system, fear learning, and stress reactivity. Furthermore, previous studies have found stress-induced *Per1* mRNA in the HPC using acute swim stress (Bohacek et al., 2015; Roszkowski et al., 2016). Thus, it was surprising that stress had no effect on the induction of *Per1* mRNA in any subregion of the hippocampus in the present study.

While only *Per1* mRNA was examined in the HPC in this study, it is known from previous studies that restraint stress does not induce *c-Fos* mRNA in the hippocampus (Bohacek et al., 2015; Pace et al., 2005; Roszkowski et al., 2016). Thus, if stress-induced *Per1* mRNA is

independent of CORT, then the lack of *c-Fos* mRNA induction in the HPC after an acute stressor may suggest that CREB phosphorylation is not increased, and *Per1* mRNA is not induced. The lack of *c-Fos* and *Per1* mRNA induction may be stressor-specific. Pace et al. (2005) have found that stress-mediated *c-Fos* mRNA induction in the hippocampus is particularly sensitive to type of stressor used. Stressors that utilize the hippocampus (e.g., engaging spatial memory, but not restraint) can induce *c-Fos* mRNA, and therefore these stressor may induce *Per1* mRNA. This is further supported by Bohacek et al. (2015), where it was found that cold swim stress acutely induced both *Per1* and *c-Fos* mRNA in the HPC, but restraint stress does not. Roszkowski et al. (2016) also found cold swim stress to induce *Per1* in the HPC. Both studies found stress-induced *Per1* mRNA to be CORT-independent, suggesting that restraint stress may not induce *Per1* mRNA in the HPC if *c-Fos* mRNA is also not being induced.

The lack of a stress effect in both the insula and HPC also provide further evidence that stress-induced *Per1* gene expression is largely independent of CORT. This is because both the insula and the HPC are dense with glucocorticoid receptors (McEwen et al., 1986; Meaney & Aitken, 1985), and thus have the ability for stress-induced CORT to bind the GRs, which could then potentiate *Per1* gene expression via the GRE within the promoter region of Per1. Another possibility for brain region specificity in stress-induced *Per1* mRNA is that there may be differences in Per1 gene promoter region dynamics depending on the brain region. A possibility would be that in the insula and HPC, stress-induced CORT can only induce *Per1* gene expression if other response elements, coactivators, or enhancers are simultaneously activated. Another possibility is that it may take longer (e.g., more than 30 minutes) for *Per1* mRNA to be induced by stress in the insula or HPC.

Effect of estradiol on stress-induced Per1 and c-Fos mRNA expression

Estradiol had no effect on stress-induced *Per1* mRNA expression in all brain regions examined. This is somewhat surprising, as female rats have been shown to have greater stress reactivity compared to male rats, and this effect is largely due to the activational effects of estradiol. Female rats with high estradiol levels have greater CORT release under basal and stress conditions, compared to females with low estradiol levels and males (Atkinson & Waddell, 1997; Babb et al., 2014; Critchlow et al., 1963; Viau & Meaney, 1991). However, if stress-induced *Per1* mRNA is largely independent of CORT, then this may explain the lack of an effect of EB on *Per1* mRNA expression.

While EB treatment had no effect on stress-induced *Per1,* it was found that EB significantly decreased stress-induced *c-Fos* mRNA in the PFC, but not the PVN. Most of the existing literature on the effect of gonadal hormones on stress-induced neuronal activation (e.g., as measured by CREB or c-Fos expression) have conflicting results that often depend on the brain region examined and type of stressor used. In the oval nucleus of the BNST, females have greater stress-induced c-Fos (Sterrenburg et al., 2011), while other studies show that females have lower stress-induced c-Fos compared to males in the HPC and cortex (Figueiredo et al., 2002; Horst et al., 2009). There are conflicting results as to whether there are sex differences in stress-induced *c-Fos* expression in the PVN (Figueiredo et al., 2002; Horst et al., 2009; Sterrenburg et al., 2011). Additionally, Bland et al. (2005) demonstrated that sex differences in c-Fos expression depends on the duration after stressor onset in which brains are examined, where peak *c-Fos* mRNA occurs 30 minutes after stressor onset in males, and 60 minutes after stressor onset in females. In our hands with female rats that have been ovariectomized, EB replacement treatment attenuated the stress-induced increase in *c-Fos* mRNA expression in the PFC. This effect was both for acute and permissive treatment conditions, suggesting that cycling, but low levels of estradiol is enough to limit the stress-induced *c-Fos* mRNA expression.

Activation of the mPFC in response to stress is thought to contribute to glucocorticoid negative feedback by sending inhibitory signals to the HPA axis (Herman et al., 2003). The

decrease in *c-Fos* mRNA expression by estradiol may be related to the subsequent increase and prolonged release of CORT in female rats (e.g., females have less negative feedback). Estradiol is thought to decrease c-Fos expression in the PFC in order to decrease the inhibitory input the PFC has to the HPA axis, resulting in decreased glucocorticoid negative feedback and overall sustained HPA axis activation (Figueiredo et al., 2002). However, the mechanism by which estradiol is able to alter stress-induced c-Fos (and possibly CREB) is still unknown. Despite largely parallel inductions of *c-Fos* and *Per1* mRNA by stress, the estradiol effect seen in this study only occurred with *c-Fos*. These results also point to the possibility that *Per1* mRNA in the PFC is in fact dependent on both GRE- and CRE-mediated effects. If EB decreases CREB but increases CORT, then that may explain the overall lack of an effect of EB on stress-induced *Per1* mRNA expression. It would be important for future studies to examine if estradiol may be impacting other aspects of stress-induced clock gene expression (e.g., how estradiol may impact stress-induced clock gene expression in durations longer than 30 minutes, or how estradiol may impact any subsequent phase changes that may be seen following an acute or chronic stressor).

Closing Statement

30 minutes of an acute restraint stress rapidly induces *Per1* mRNA in female rat PFC and PVN, but not HPC, SCN, or insula. In the PFC, stress-induced *Per1* mRNA was partially dependent on the presence of endogenous CORT. In the PVN, stress-induced *Per1* mRNA was completely independent of the presence of endogenous CORT, as ADX had no effect on stress-induced *Per1* mRNA. These results suggest that there is non-GRE mediated induction of *Per1* mRNA by stress in extra-SCN brain regions in female rats. CORT independent mechanisms that may contribute to stress-induced *Per1* expression include neuronal activation via the CRE within the promoter region of the Per1 gene. Estradiol had no effect on stress-induced *Per1* mRNA expression in the PFC and PVN of female rats. However, EB treatment significantly

decreased stress-induced *c-Fos* mRNA expression in the PFC. Thus, while stress-induced *Per1* mRNA expression is thought to be mediated via CORT- and/or CREB-mediated mechanisms, both of which are modulated by the activational effects of estradiol, this study has found a lack of an activational effect, both acute and permissive, on stress-induced *Per1* mRNA expression. Future studies will tease apart the different mechanisms involved in the induction of stress-induced *Per1* mRNA (e.g., CORT-mediated versus CREB-mediated).

CHAPTER V: DISCUSSION

Summary of Findings

These studies have demonstrated for the first time that there is rhythmic expression of clock genes from both the positive (*Bmal1* mRNA) and negative (*Per1, Per2* mRNA) components of the molecular clock throughout the prefrontal cortex (PFC), the amygdala (AMYG), the hippocampus (HPC), and the paraventricular nucleus of the hypothalamus (PVN) in both male and female rats. This provides further evidence for the ubiquitous nature of extra-SCN molecular clocks, suggesting their importance in maintaining normal health and behaviors. There was diversity in phase and amplitude of these extra-SCN molecular clocks depending on brain region, with limited sex differences. A subsequent study found that non-cycling females had greater amplitude in *Bmal1* mRNA in the PFC compared to cycling females, suggesting a modulatory role of gonadal hormones on basal clock gene expression. In Chapter 3, acute restraint stress rapidly and selectively induced *Per1*, but not *Per2* or *Bmal1* mRNA expression in both male and female rat PFC and PVN, despite the greater release of stress-induced CORT in females. Male rats that received adrenalectomy (ADX) had decreased stress-induced *Per1* mRNA in the PVN at ZT4, but not ZT16, and no effect in the PFC, suggesting that stress-induced *Per1* mRNA is largely independent from CORT. In Chapter 4, stress-induced *Per1* mRNA in female rats was also largely independent on CORT, as ADX only partially attenuated stress-induced *Per1* mRNA in the PFC, and had no effect in the PVN. Estradiol treatment had no effect on stress-induced *Per1* mRNA, but did decrease stress-induced *c-Fos* mRNA. Chapter 4 also found further evidence for brain regional specificity, where stress did not alter *Per1* mRNA in the HPC or insula. Collectively, the data suggest that there is regional specificity in both basal and stress-induced clock gene expression, selectivity of *Per1* mRNA to be rapidly induced by stress, large independence of stress-induced *Per1* mRNA from CORT, and no sex

differences or effect of estradiol in stress-induced *Per1* mRNA despite sex differences in other measures of stress reactivity.

The ubiquitous existence of extra-SCN molecular clocks

The studies within Chapter 2 discovered that there is rhythmic clock gene expression of both the positive (*Bmal1* mRNA) and negative (*Per1, Per2* mRNA) components of the molecular clock throughout the PFC (AC, PL, IL, and VO subregions), insula, PVN, SCN, HPC (CA1, CA3, superior DG, and inferior DG subregions), and AMYG (CEA, BLA, and MEA subregions) of both male and female rats. This was the first study to examine rhythmic clock gene expression throughout the PFC. This was critical due to the role the PFC plays in emotional control, executive function, stress reactivity, and working memory (Arnsten & Rubia, 2012; Goldman-Rakic, 1996; Herman et al., 2003; Robbins, 1996), all functions that are disrupted in mood disorders associated with both stress and disruptions in circadian rhythms (Baxter et al., 1989; Drevets et al., 1997; Elliott et al., 1997; Mayberg et al., 1999; Radkovsky et al., 2014). Furthermore, these results significantly contribute to the existing literature because it has determined that a molecular clock exists in many extra-SCN brain regions importantly involved in emotional control and stress reactivity. These brain regions (PFC, AMYG, HPC) are dysfunctional in human patients with mood disorders that are associated with both stress and circadian rhythms (Lebow & Chen, 2016; Videbech & Ravnkilde, 2004). These data corroborate previous findings that have also found rhythmic clock gene expression in a variety of extra-SCN brain regions, including the bed nucleus of the stria terminalis, nucleus accumbens, lateral septum, hippocampus, amygdala, hypothalamic nuclei, thalamic nuclei, striatum, caudate putamen, and cortex (Ángeles-Castellanos et al., 2007; Feillet et al., 2008a; Harbour et al., 2013; 2014; Jilg et al., 2009; Lamont et al., 2005; Masubuchi et al., 2000; Rath et al., 2012).

The expanse of brain regions that have an oscillating molecular clock suggests its importance in normal health and behavior (Takahashi et al., 2008). In rodent studies, complete

knockdown of the *Bmal1* gene (Bunger et al., 2000), or complete knockdown of both *Per1* and *Per2* genes (Bae et al., 2001), lead to behavioral arrhythmicity. These data suggest that rhythmic expression of both the positive and the negative components of the molecular clock are necessary for normal circadian behavior. In addition to disruptions in circadian behavior, disruptions in clock gene expression results in abnormal affective behaviors as well. ClockΔ19 mutations in mice cause "manic-like" behaviors and has been proposed to as a rodent model for bipolar disorder (McClung et al., 2005; Mukherjee et al., 2010; Roybal et al., 2007). Additionally, disruptions in *Per1* and *Per2* expression in the nucleus accumbens, a brain region involved in reward and motivation, is associated with anxiety-like behaviors. Spencer et al. (2013) have found that mice deficient in both *Per1* and *Per2* exhibit increased anxiety-like behaviors. Furthermore, wild type mice exposed to chronic social stress exhibit greater anxiety-like behavior and decreased *Per1* and *Per2* gene expression in the nucleus accumbens. More recently, Woodruff et al. (in preparation) found that selective knockdown of *Per1* mRNA in the medial PFC (mPFC) leads to disruptions in the learning of extinction of conditioned fear, a memory task that is dependent on the mPFC. Taken together, these rodent studies suggest the importance of normal clock gene expression for maintenance mental health and proper function. These results are corroborated by human studies that have found an association between mutations in clock genes and a variety of mental health disorders, including major depressive disorder, Alzheimer's disease, seasonal affective disorder, bipolar disorder, and schizophrenia (Bunney et al., 2014; Bunney & Bunney, 2000; Etain et al., 2011; Johansson et al., 2002; Lamont et al., 2009; Landgraf et al., 2014; Li et al., 2013; McCarthy & Welsh, 2012; McClung, 2007; Partonen et al., 2007).

There is diversity in phase and amplitude of the molecular clocks

Although rhythmic clock gene expression is expansive in regards to expression in brain areas and peripheral tissue, studies from Chapter 2 have determined that there is diversity

amongst these molecular clocks that vary in acrophase and amplitude. This diversity depends on the brain region. There are three main patterns that were found: acrophase of *Bmal1* mRNA expression in the light phase, in the dark phase, or in the transition between the light to dark phase. *Per1* and *Per2* mRNA expression peaked either in the light or dark phase. The brain region specificity in the phase of the molecular clock suggests tight regulation of rhythmic clock gene expression. The precision of the phase suggests that the circadian nature of the molecular clocks is critical to their function. This is exemplified by the characterization of basal clock gene expression in extra-SCN brain areas of diurnal rodents. Their active phase is anti-phasic to nocturnal lab rats, and this is reflected in anti-phasic clock gene expression in extra-SCN brain regions (Li et al., 2013; Otalora et al., 2013; Ramanathan et al., 2006; Ramanathan et al., 2008; Vosko et al., 2009).

Furthermore, it is estimated that at least ten percent of the transcriptome has a circadian rhythm in its expression (Marcheva et al., 2013; Miller et al., 2007; Oishi et al., 1998; Panda et al., 2002; Rey et al., 2011; Storch et al., 2002). The circadian expression of the overall transcriptome is believed to be due to the clock genes acting as transcription factors for clock controlled genes (CCG). Thes first-order CCGs have E-boxes in their promoter region where protein products of the core clock genes can act directly as transcription factors to modulate the rhythmic expression of CCGs. These first-order CCGs can ultimately lead to the tightly controlled circadian expression of ~10% of the transcriptome. Many of these genes that are expressed in a circadian manner are involved in metabolism and other critical cell function. This suggests two important things: 1) clock genes can act as transcription factors, implying their potential for non-circadian function; and 2) the specificity in brain region in regards to acrophase suggests that appropriate circadian timing is critically important to the health and survival of the organism.

In addition to diversity in acrophase of clock gene expression, there were also differences in the amplitude of rhythmic clock gene expression. Rhythmic *Per1, Per2,* and

Bmal1 mRNA in the SCN and PVN had much greater amplitude compared to *Per1, Per2,* and *Bmal1* mRNA in all other brain regions examined. Amplitude often indicates how robust the rhythm is. The greater amplitude of clock gene expression in the SCN may confer greater robustness in oscillations of the molecular clock, which may help protect the SCN from perturbations in the environment that may be irrelevant to circadian function. The PVN may have robust rhythm as well because as head of the HPA axis, it controls the circadian rhythm of CORT, and if CORT is a critical entrainment factor, then it is important that the PVN remain robust in its rhythmic expression of the molecular clock. Additionally, amplitude may be directly related to how autonomous (e.g., how able the molecular clock can maintain rhythmicity in cell culture without input from the environment) the molecular clocks are. The master clock is autonomous because it can sustain rhythmic expression of the molecular clock for ~32 days ex vivo, without any input (Yamazaki et al., 2000). Extra-SCN molecular clocks are considered semi-autonomous or slave oscillators, because their rhythms are dependent on input from the SCN. Rhythmic clock gene expression ex vivo becomes arrhythmic rapidly (e.g., within 2-7 cycles) (Yamazaki et al., 2000). Thus, the amplitudes of these slave oscillators are smaller.

Mechanistically, the increased amplitude of clock gene expression in the SCN may be due to the presence of the accessory circadian loop that modulates the expression of Rev-erbα, which adds to the robustness of the rhythmic expression of *Bmal1* mRNA (Preitner et al., 2002; Sato et al., 2004). While Rev-erbα is expressed in the PFC, AMYG, and HPC, the majority of its expression is in the SCN (Onishi et al., 2002), which could explain the amplitude differences. Additionally, it is still unknown which cellular phenotype has rhythmic clock gene expression that dictates the circadian rhythm of that particular brain region. This information cannot be determined in the radioactive in situ hybridization and has yet to be examined in other studies. If the SCN, whose neurons almost all contain GABA, is more homogenous, then this may exhibit more synchronized and robust rhythm. Furthermore, it is possible that the SCN has greater amplitude in clock gene rhythm due to better intercellular synchronization. It has been shown

that VIP is an essential neuropeptide that promotes synchronization between cells within the SCN (Colwell et al., 2003; Cutler et al., 2003; Harmar et al., 2002). These amplitude differences between brain regions may suggest that ability of some extra-SCN clocks to be more easily affected by environmental stimuli compared to the more robust clocks in the SCN and PVN. Further studies are needed to assess what mechanisms contribute to amplitude differences.

Gonadal hormones have a limited effect on basal clock gene rhythm

Overall, *Per1, Per2,* and *Bmal1* mRNA had rhythmic expression that was similar between male and female rats. However, there were few instances where rhythmic clock gene expression was more robust for one sex but not the other. This was demonstrated by the fact that even if there was a significant ZT effect for both sexes, as assessed by ANOVA, subsequent cosinor analysis was only significant for one sex. This may be due to differences in amplitude size. Male rats, but not female rats, had rhythmic *Per1* mRNA in the PL and IL, and *Per2* mRNA in the IL. Females had rhythmic expression of *Bmal1* and *Per1* mRNA in CA1 and CA3, and of *Per2* mRNA in the supraDG and CEA, compared to males. Only females also had overall greater *Bmal1* mRNA expression in the CEA and MEA. These results indicate that male rats have more robust rhythmic clock gene expression in the mPFC, while female rats have more robust rhythmic clock gene expression in the HPC and AMYG.

In a subsequent study using only female rats, non-cycling females had much greater amplitude and robustness of *Bmal1* mRNA expression in the mPFC compared to normally cycling females; the SCN remained unaffected. This provides more evidence that gonadal hormones may play a modulatory role in rhythmic clock gene expression in extra-SCN tissue. Perrin et al. (2006) has previously shown that rhythmic PER2 protein levels in the BNST and CEA are blunted during metestrus and diestrus, compared to protestrus and estrus. The blunting in amplitude during metestrus and diestrus is inhibited by ovariectomy, suggesting that ovarian steroids are necessary for this estrous cycle stage difference in the robustness of PER2

protein expression in these brain regions. Other studies in peripheral tissue have found that both estradiol and progesterone alter the acrophase and amplitude of clock gene expression (He et al., 2007; Nakamura et al., 2001; 2005; 2010; Smith et al., 2010). Thus, the normal fluctuation in gonadal hormones may contribute to the blunted *Bmal1* mRNA rhythmic expression in the mPFC in non-cycling females, as well as the sex differences seen in rhythmic clock gene expression in the PFC and HPC. Further studies are needed in order to determine the exact mechanism by which ovarian steroids modulate basal clock gene expression.

Acute restraint stress robustly and rapidly induces Per1 mRNA in the PFC and PVN

Glucocorticoids (CORT) are believed to be the signal by which the SCN is able to entrain extra-SCN molecular clocks, as the SCN has limited efferent neural projections (Sollars & Pickard, 2015). Additionally, CORT has a diurnal rhythm and glucocorticoid receptors (GRs) are ubiquitously expressed, with the SCN as an exception. The diurnal rhythm of CORT is necessary for normal clock gene expression (Woodruff et al., 2016). Studies have also determined that CORT can induce *Per1* expression rapidly due to the glucocorticoid response element (GRE) within its promoter region (Yamamoto et al., 2005). In order to assess if untimely, stress-induced CORT could induce the expression of clock genes, the following studies examined the effect of acute stress on clock gene expression. 30 minutes of acute restraint stress selectively induced *Per1,* but not *Per2* or *Bmal1* mRNA, throughout the PFC (AC, PL, IL, and VO subregions) and PVN in both male and female rats. This rapid induction of *Per1* mRNA was largely mirrored by the induction of *c-Fos* mRNA. The rapid *Per1* mRNA induction by acute stress was replicated in Chapters 3 and 4. Additionally, other studies have found selective induction of the *Per1* gene expression in response to an acute stressor (Al-Safadi et al., 2015; Bohacek et al., 2015; Roszkowski et al., 2016; Takahashi et al., 2001; Yamamoto et al., 2005). Thus, the robust and rapid induction of *Per1* mRNA is a reproducible effect. In the female only study, there was also a significant main effect of stress in *Bmal1*

mRNA in the PFC, but no significant pair-wise comparisons were found. There was a trend for stress to decrease *Bmal1* mRNA throughout the PFC and this effect seemed independent of CORT. This is the first reported case that demonstrates a stress-induced decrease in *Bmal1* mRNA expression. This result could be a sex-specific effect, where stress decreases *Bmal1* mRNA in females only. However, female rats in Chapter 3 Experiment 1 did not exhibit stress induced decreases in *Bmal1* mRNA. Further studies are needed to reproduce this effect. Additionally, in the female study, ADX rats received replacement CORT in the drinking water, restoring their diurnal CORT rhythm. In the male study, ADX rats had no replacement CORT, and thus they lacked a diurnal rhythm in CORT. Woodruff et al. (2016) found that ADX without replacement CORT disrupted the basal rhythm of clock genes (*Per1, Per2, Bmal1* mRNA), which could be restored with an appropriately timed injection of CORT. Further work by Woodruff et al. (unpublished) found that in fear extinction learning, both diurnal and stress-induced CORT are necessary for time of day dependent learning. Thus, presence of diurnal CORT may impact the outcome of stress-induced clock gene expression.

Stress-induced *Per1* mRNA expression is believed to be due to the functional GRE located within the promoter region of the Per1 gene (Yamamoto et al., 2005). However, there is evidence for a GRE within the promoter region of the Per2 and Bmal1 gene as well (Cheon et al., 2013; Reddy et al., 2007; So et al., 2009), although evidence for the functionality of the GRE is less well-established. So et al. (2009) has proposed that the GRE within the Per2 promoter region is constitutively active, which may explain why increased CORT by stress would not affect *Per2* gene induction. It is also important to consider that throughout these experiments, clock gene expression was only examined 30 minutes after stressor onset. In the SCN, light induces *Per1* mRNA within 30 minutes, but it takes ~120 minutes for *Per2* mRNA levels to peak (Tischkau et al., 2002). It is possible that *Per2* or *Bmal1* mRNA may be altered by stress, but may take longer than 30 minutes for this effect to be seen. Though Chapter 4 did see a slight decrease in *Bmal1* mRNA in the PFC 30 minutes after stressor onset. Further studies looking at

different time points after stressor onset are needed in order to conclusively assess stress-induced alterations in clock gene expression.

It is striking that *Per1* mRNA is rapidly (within 30 minutes) and robustly induced by an acute stressor, which is characteristic of an immediate early gene. Consequently, the immediate early gene, *c-Fos,* largely mirrors *Per1* mRNA induction by stress throughout all studies. This observation emphasizes an important, but non-circadian role of Per1 in cellular function, where its rapid induction makes it an ideal candidate for altering transcription, which would allow an organism to respond to changes in their environment.

Stress-induced Per1 mRNA was largely independent of CORT in both male and female rat PFC and PVN

In order to determine if CORT is necessary for stress-induced *Per1* mRNA in male and female rats, endogenous CORT was removed by adrenalectomy (ADX), and rats were challenged with 30 minutes of acute restraint stress. In Chapter 3, stress-induced *Per1* mRNA in the PFC was completely independent from CORT, whereas stress-induced *Per1* in the PVN at ZT4, but not ZT16, was ablated by ADX in male rats. In Chapter 4, ADX had no effect on stress-induced *Per1* mRNA in the PVN, but had partial inhibition of stress-induced *Per1* mRNA in the PFC of female rats. Overall, these two studies indicate that stress-induced *Per1* mRNA in the PFC (AC, PL, IL, and VO subregions) and PVN are largely independent from the presence of endogenous CORT, as ADX had limited effects. The slight difference in brain region specificity in ADX effect on stress-induced *Per1* mRNA between these two experiments may be due to sex differences. However, a direct comparison would be needed. It is also important to consider that the male rats did not receive replacement CORT in their drinking water, thus lacking diurnal CORT rhythm. This differs from the females who did receive CORT replacement in their drinking water, thus restoring the circadian rhythm of CORT. Thus, the presence or absence of the diurnal rhythm of CORT may contribute to these regional differences. Future studies need to be

done in order to access the importance of basal diurnal CORT in the impact of stress on circadian outcomes (e.g., clock genes, behavior).

Due to the lack of an ADX effect, a non-CORT-mediated mechanism, such as neuronal activation, must then underlie stress-induced *Per1* mRNA in the PFC and PVN of male and female rats. In addition to a GRE, there is also a well-established and functional cAMP response element (CRE) within the promoter region of the Per1 gene. In the SCN, a light pulse can rapidly (within 30 minutes) induce *Per1* mRNA expression. Light ultimately causes an increase in CREB in the SCN, which can then bind to the CRE within the Per1 promoter region to promote its transcription. Thus, it is reasonable to assume that other stimuli that can increase CREB, such as an acute stressor, can also increase *Per1* mRNA expression via binding to the CRE in extra-SCN tissue. Notably, the induction of *Per1* mRNA was largely mirrored by the induction of *c-Fos* mRNA, suggesting that CREB may act as a transcription factor for both genes.

There are no sex differences and no effect of estradiol in stress-induced Per1 mRNA in the PFC or PVN

When directly comparing male and female rats, there were no sex differences in stress-induced clock gene expression in the PFC and PVN. This is despite the fact that females had significantly greater stress-induced plasma CORT levels. Greater HPA axis activation in females in response to stress is well-established in rodents (Babb et al., 2014; Viau & Meaney, 1991), and is believed to be due to the activational effects of estradiol (Burgess & Handa, 1992; Carey et al., 1995; Ogle & Kitay, 1977). In addition to the lack of a sex difference, in Chapter 4, there was no effect of estradiol treatment on stress-induced *Per1* mRNA in OVX female rats, suggesting that gonadal hormones have seemingly limited influence on stress-induced *Per1* mRNA.

It is important to consider that even if there are no sex differences at the level of *Per1* mRNA expression, there may be sex differences in the induction of *Per1* by stress. In Chapter 4, estradiol treatment significantly decreased stress-induced *c-Fos* mRNA levels in the PFC of female rats. This corroborates previous studies that have shown males have increased stress-induced *c-Fos* and/or CREB gene expression compared to females in certain brain regions (Figueiredo et al., 2002; Ter Horst et al., 2009; Sterrenburg et al., 2011). Thus, stress may predominantly increase *Per1* mRNA via the CRE in male rats, whereas females, who have greater CORT in response to stress, may have stress-induced *Per1* mRNA that is mediated by the GRE. Even though there was only a partial attenuation of stress-induced *Per1* mRNA with ADX, that small dependence on CORT may be enough to negate any sex differences seen at the *Per1* mRNA level. Further studies that examine exactly how stress (e.g., CRE-mediated versus GRE-mediated) can induce *Per1* expression, and how estradiol modulates both CRE- and GRE-mediated processes, are necessary.

Stress-induced Per1 mRNA is brain region specific

Across all stress studies performed here, acute restraint stress selectively affected clock gene expression in all subregions of the PFC (AC, PL, IL, VO) and the PVN. Clock gene expression in the SCN, insula, and HPC remained unaltered by stress. Thus, despite the ubiquitous nature of rhythmic clock gene expression under basal conditions as seen in Chapter 2, and the nearly ubiquitous nature of GRs, the induction of *Per1* mRNA by stress is tightly regulated in a brain region specific manner.

It is vital that the master clock, the SCN, is not easily susceptible to phase changes in response to potentially unimportant and non-circadian stimuli in the environment. Light is the main entrainment factor of the SCN. A stressor in the environment that may or may not be a salient cue that indicates changes in the circadian environment, should not be able to alter the master clock. Additionally, the studies in Chapters 3 and 4 have found a lack of an effect of

stress on clock gene expression in the SCN. This was believed to be due to the lack of GRs in the SCN.

However, while the SCN lacks GRs, they are densely expressed in the HPC and insula. The rostral agranular insula has a role in pain (Burkey et al., 1996) and nictone addiction (Pushparaj et al., 2015), but has a limited role in stress (Imbe et al., 2014), and thus may seem unsurprising that stress does not affect clock gene expression. However, the HPC plays a vital role in stress regulation, emotional control, and memory. Due to its dense concentration of GRs, it is a brain structure that is stress reactive. The HPC provides major inhibitory input to the HPA axis and plays a critical role in glucocorticoid negative feedback. Thus, it is surprising that the HPC lacks stress-induced *Per1* mRNA as seen in the current studies. However, other studies have shown that acute restraint stress has no effect on *Per1* mRNA in the HPC, while cold swim stress rapidly increases *Per1* mRNA in the HPC (Bohacek et al., 2015; Roszkowski et al., 2016). Thus, the HPC may exhibit stress-induced *Per1* gene expression in a stressor-specific manner.

Notably, throughout Chapters 2, 3, and 4, in all brain regions where there was no stress-induced *Per1* mRNA, there was also a lack of stress-induced *c-Fos* mRNA. Acute restraint stress is unable to induce *c-Fos* gene expression in the HPC of rats (Bohacek et al., 2015; Pace et al., 2005). The HPC is an important structure in spatial memory. Pace et al. (2005) has proposed that a stressor, such as restraint, which does not engage the HPC, would not lead to *c-Fos* mRNA induction. The parallel induction of *c-Fos* and *Per1* mRNA further supports Chapter 2 and 3's findings that stress-induced *Per1* mRNA is largely independent of CORT and may be mediated via the CRE within the promoter region of Per1. Thus, consideration of more neuronal activation and CREB-mediated mechanisms of *Per1* mRNA induction by acute stress needs to be explored.

Closing Statement

Incident Detectors

The ubiquitous nature of rhythmic clock gene expression underscores the importance of these genes in circadian function, and how this circadian function is critical to normal health and behavior. Unlike the ubiquity of basal clock gene expression, stress-induced clock gene expression has specificity in two ways: 1) Overall, Per1 is selectively increased by acute stress; and 2) there is specificity in which brain regions exhibit stress-induced *Per1* mRNA expression. The selective induction of *Per1* suggests that Per1 acts as an incident detector at the cellular level for the molecular clock. It is sensitive to salient cues (e.g., aversive stressors, appetitive rewards) in the environment in order to signal to the molecular clock if it should adapt via a phase shift to changes in the environment. This adaptability allows for an organism to optimize its survival to its environment by avoiding aversive stimuli or to optimize time spent with an appetitive reward (e.g., food). Further studies need to examine how other components of the molecular clock also change after an acute stressor and how long these changes remain. A time course study, where clock gene expression is examined every 4 hours up to 48 hours after stressor onset, would be critical in testing the hypothesis of *Per1* as an incident detector for the molecular clock.

Furthermore, the fact that Per1 acts like an immediate early gene suggests its importance in acting as the gateway between stimuli from the environment (e.g., light in the SCN; stressors in certain extra-SCN brain regions), and that it also has important non-circadian roles. Non-circadian roles of Per1 may include initiation of synaptic plasticity, including increases in spine density and formation of new synapses, which would allow *Per1* to be a mechanism by which the organism adapts to its environment. If Per1 is truly an incident detector, assisting the brain to increases in synaptic plasticity would increase learned behaviors to reflect adaptation to changes in one's environment. There is some evidence for a link between clock gene expression and synaptic plasticity, where Per2 mutant mice have decreased p-CREB expression in the HPC after a HPC-dependent task (Wang et al., 2009) and

melatonin injections increase *Per1* and *Bmal1* mRNA in the HPC along with increased dendritic spines in the CA1 subregion (Ikeno & Nelson, 2015). Furthermore, there is circadian expression of brain-derived neurotrophic factor (BDNF), a protein essential in learning and memory, and its receptor TrkB, in the HPC (Martin-Fairey & Nunez, 2014), rhythmic changes in spine and synapse density in rodent cortex across the 24 hour day (Jasinska et al., 2015; Perez-Cruz et al., 2009), and diurnal CORT is necessary for the stabilization of new spines (Liston et al., 2013). These circadian fluctuations point to the potential role of clock genes in synaptic plasticity.

The brain region specificity of stress-induced Per1 suggests that extra-SCN molecular clocks may be the incident detectors for the body's master clock, the SCN. It is critical that the SCN remain impervious to environmental stimuli so that its entrainment can remain stable. However, if the organism needs to adapt to new factors in the environment that will optimize its survival (e.g., avoidance of stressors, acquisition of food or reward), it is able to do so by input from these extra-SCN incident detectors. Once an incident repeats and becomes a predictable environmental stimuli, these extra-SCN molecular clocks phase shift in order to synchronize an organism to its new environment. Anticipation of either aversive or appetitive stimuli has a strong influence on the molecular clock.

Role of acute stress, circadian rhythms, and mood disorders

Disruptions in circadian rhythms are often associated with mood disorders, such as major depressive disorder (MDD), anxiety disorders, bipolar disorder, and post-traumatic stress disorder (PTSD). These disorders are not only associated with mutations in a variety of clock genes, such as *Per1, Per2, Npas2, Clock*, but also a disruption in the diurnal rhythm of CORT. Thus, understanding how these mood disorders manifest at a circadian level is critical. Furthermore, many of these mood disorders are often precipitated by an acute traumatic stressor, or by chronic stress. The studies herein examined the role of an acute stressor on

clock gene expression in brain regions (PFC, PVN) that are often disrupted in these mood disorders, as a starting point in understanding how stress-induced clock gene expression may contribute to disruptions in circadian rhythms and subsequent diseased states.

Acute restraint stress can impact *Per1,* and possibly *Bmal1,* mRNA expression in the PFC and PVN of male and female rats. The robust and specific induction of *Per1* mRNA by an acute stressor indicates a role for *Per1* as an incident detector between salient environmental stimuli (e.g., stress) and the rest of the molecular clock. If stress becomes chronic, resulting in the constant untimely peaks in *Per1* mRNA expression, the entire molecular clock (e.g., not just Per1) may become disrupted. This may begin in extra-SCN molecular tissue, but as the stressor persists, then the molecular clocks in the SCN may also become disrupted. Chronic stress studies are inconsistent in whether clock gene expression is disrupted in the SCN (Jiang et al., 2011; Kinoshita et al., 2012; Logan et al., 2015; Takahashi et al., 2013). Regardless of whether or not the SCN is affected, disrupted rhythms in these molecular clocks can result in the desynchronization of the organism from its environment, leading to diseased states, such as depression and anxiety disorders.

This research stresses the importance of maintaining proper circadian rhythms as a means to help an organism be resilient to disruptive entrainment factors in their environment. Chronotherapy, which is the tight regulation of one's social environment (e.g., maintaining a consistent sleep/wake cycle, bright light in the morning, avoidance of blue light at night, exercising at the appropriate time of day, meals at the same time each day), may be a critical component in the treatment of mood disorders. Having a robustly oscillating molecular clock will help the organism become more resilient to stimuli in the organism's environment that may disrupt circadian rhythms.

BIBLIOGRAPHY

Abe, M., Herzog, E. D., Yamazaki, S., Straume, M., Tei, H., Sakaki, Y., et al. (2002). Circadian rhythms in isolated brain regions. *Journal of Neuroscience*, *22*(1), 350–356.

Adhikari, A. (2014). Distributed circuits underlying anxiety. *Frontiers in Behavioral Neuroscience*, *8*.

Al-Safadi, S., Al-Safadi, A., Branchaud, M., Rutherford, S., Dayanandan, A., Robinson, B., & Amir, S. (2014). Stress-Induced Changes in the Expression of the Clock Protein PERIOD1 in the Rat Limbic Forebrain and Hypothalamus: Role of Stress Type, Time of Day, and Predictability. *PLoS ONE*, *9*(10), e111166.

Al-Safadi, S., Branchaud, M., Rutherford, S., & Amir, S. (2015). Glucocorticoids and Stress-Induced Changes in the Expression of PERIOD1 in the Rat Forebrain. *PLoS ONE*, *10*(6), e0130085.

Albrecht, U. (2002). Invited review: regulation of mammalian circadian clock genes. *Journal of Applied Physiology*, *92*(3), 1348–1355.

Albrecht, U., Sun, Z. S., Eichele, G., & Lee, C. C. (1997). A differential response of two putative mammalian circadian regulators, mper1 and mper2, to light. *Cell*, *91*(7), 1055–1064.

Amir, S., Waddington Lamont, E., Robinson, B., & Stewart, J. (2004). A Circadian Rhythm in the Expression of PERIOD2 Protein Reveals a Novel SCN-Controlled Oscillator in the Oval Nucleus of the Bed Nucleus of the Stria Terminalis. *Journal of Neuroscience*, *24*(4), 781–790.

Arnsten, A. F. T., & Rubia, K. (2012). Neurobiological circuits regulating attention, cognitive control, motivation, and emotion: disruptions in neurodevelopmental psychiatric disorders. *Journal of the American Academy of Child and Adolescent Psychiatry*, *51*(4), 356–367.

Asarian, L., & Geary, N. (2002). Cyclic Estradiol Treatment Normalizes Body Weight and Restores Physiological Patterns of Spontaneous Feeding and Sexual Receptivity in Ovariectomized Rats. *Hormones and Behavior*, *42*(4), 461–471.

Atkinson, H. C., & Waddell, B. J. (1997). Circadian variation in basal plasma corticosterone and adrenocorticotropin in the rat: sexual dimorphism and changes across the estrous cycle. *Endocrinology*, *138*(9), 3842–3848.

Ángeles-Castellanos, M., Mendoza, J., & Escobar, C. (2007). Restricted feeding schedules phase shift daily rhythms of c-Fos and protein Per1 immunoreactivity in corticolimbic regions in rats. *Neuroscience*, *144*(1), 344–355.

Babb, J. A., Masini, C. V., Day, H. E. W., & Campeau, S. (2014). Habituation of hypothalamic-pituitary-adrenocortical axis hormones to repeated homotypic stress and subsequent heterotypic stressor exposure in male and female rats. *Stress*, 17(3), 224-234.

Bae, K., Jin, X., Maywood, E. S., Hastings, M. H., Reppert, S. M., & Weaver, D. R. (2001). Differential Functions of mPer1, mPer2, and mPer3 in the SCN Circadian Clock. *Neuron*,

30(2), 525–536.

Bailey, M., & Silver, R. (2014). Sex differences in circadian timing systems: implications for disease. *Frontiers in Neuroendocrinology*, *35*(1), 111–139.

Balsalobre, A. (2000). Resetting of Circadian Time in Peripheral Tissues by Glucocorticoid Signaling. *Science*, *289*(5488), 2344–2347.

Baxter, L. R., Schwartz, J. M., Phelps, M. E., Mazziotta, J. C., Guze, B. H., Selin, C. E., et al. (1989). Reduction of prefrontal cortex glucose metabolism common to three types of depression. *Archives of General Psychiatry*, *46*(3), 243–250.

Becker, J. B., Arnold, A. P., Berkley, K. J., Blaustein, J. D., Eckel, L. A., Hampson, E., et al. (2004). Strategies and Methods for Research on Sex Differences in Brain and Behavior. *Endocrinology*, *146*(4), 1650–1673.

Bland, S. T., Schmid, M. J., Der-Avakian, A., Watkins, L. R., Spencer, R. L., & Maier, S. F. (2005). Expression of c-fos and BDNF mRNA in subregions of the prefrontal cortex of male and female rats after acute uncontrollable stress. *Brain Research*, *1051*(1-2), 90–99.

Bohacek, J., Manuella, F., Roszkowski, M., & Mansuy, I. M. (2015). Hippocampal gene expression induced by cold swim stress depends on sex and handling. *Psychoneuroendocrinology*, *52*, 1–12.

Bremner, J. D., Narayan, M., & Anderson, E. R. (2000). Hippocampal volume reduction in major depression. *American Journal of Psychiatry,* 157(1), 115-118.

Bunger, M. K., Wilsbacher, L. D., Moran, S. M., Clendenin, C., Radcliffe, L. A., Hogenesch, J. B., et al. (2000). Mop3 Is an Essential Component of the Master Circadian Pacemaker in Mammals. *Cell*, *103*(7), 1009–1017.

Bunney, B. G., Li, J. Z., Walsh, D. M., Stein, R., Vawter, M. P., Cartagena, P., et al. (2014). Circadian dysregulation of clock genes: clues to rapid treatments in major depressive disorder. *Molecular Psychiatry, 20*(1): 48-55.

Bunney, W. E., & Bunney, B. G. (2000). Molecular clock genes in man and lower animals: possible implications for circadian abnormalities in depression. *Neuropsychopharmacology*, *22*(4), 335–345.

Burgess, L. H., & Handa, R. J. (1992). Chronic estrogen-induced alterations in adrenocorticotropin and corticosterone secretion, and glucocorticoid receptor-mediated functions in female rats. *Endocrinology*, *131*(3), 1261–1269.

Burioka, N., Takata, M., Okano, Y., Ohdo, S., Fukuoka, Y., Miyata, M., et al. (2005). Dexamethasone influences human clock gene expression in bronchial epithelium and peripheral blood mononuclear cells in vitro. *Chronobiology International*, *22*(3), 585–590.

Burkey, A. R., Carstens, E., & Wenniger, J. J. (1996). An opioidergic cortical antinociception triggering site in the agranular insular cortex of the rat that contributes to morphine antinociception. *Journal of Neuroscience,* 16(20), 6612-6623.

Calabrese, F., Savino, E., Papp, M., Molteni, R., & Riva, M. A. (2016). Chronic mild stress-induced alterations of clock gene expression in rat prefrontal cortex: modulatory effects of prolonged lurasidone treatment. *Pharmacological Research*, *104*, 140–150.

Carey, M. P., Deterd, C. H., de Koning, J., Helmerhorst, F., & de Kloet, E. R. (1995). The influence of ovarian steroids on hypothalamic-pituitary-adrenal regulation in the female rat. *The Journal of Endocrinology*, *144*(2), 311–321.

Carlezon, W. A., Duman, R. S., & Nestler, E. J. (2005). The many faces of CREB. *Trends in Neurosciences*, 28(8), 436-445.

Caruso, C. C. (2014). Negative impacts of shiftwork and long work hours. *Rehabilitation Nursing : the Official Journal of the Association of Rehabilitation Nurses*, *39*(1), 16–25.

Chaudhury, D., & Colwell, C. S. (2002). Circadian modulation of learning and memory in fear-conditioned mice. *Behavioural Brain Research*, *133*(1), 95–108.

Cheon, S., Park, N., Cho, S., & Kim, K. (2013). Glucocorticoid-mediated Period2 induction delays the phase of circadian rhythm. *Nucleic Acids Research*, *41*(12), 6161–6174.

Christiansen, S. L., Bouzinova, E. V., Fahrenkrug, J., & Wiborg, O. (2016). Altered Expression Pattern of Clock Genes in a Rat Model of Depression. *The International Journal of Neuropsychopharmacology*, *19*(11): pyw061.

Chun, L. E., Woodruff, E. R., Morton, S., Hinds, L. R., & Spencer, R. L. (2015). Variations in Phase and Amplitude of Rhythmic Clock Gene Expression across Prefrontal Cortex, Hippocampus, Amygdala, and Hypothalamic Paraventricular and Suprachiasmatic Nuclei of Male and Female Rats. *Journal of Biological Rhythms*, *30*(5), 417–436.

Colwell, C. S. (2011). Linking neural activity and molecular oscillations in the SCN. *Nature Reviews Neuroscience*, *12*(10), 553–569.

Colwell, C. S., Michel, S., Itri, J., Rodriguez, W., Tam, J., Lelievre, V., et al. (2003). Disrupted circadian rhythms in VIP- and PHI-deficient mice. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, *285*(5), R939–49.

Committee for the Update of the Guide for the Care and Use of Laboratory Animals. (2011). Guide for the Care and Use of Laboratory Animals. *National Academies Press, Washington DC,* 8th Edititon.

Conrad, C. D. (2008). Chronic stress-induced hippocampal vulnerability: the glucocorticoid vulnerability hypothesis. *Reviews in the Neurosciences*, *19*(6), 395–411.

Conway-Campbell, B. L., Sarabdjitsingh, R. A., McKenna, M. A., Pooley, J. R., Kershaw, Y. M., Meijer, O. C., et al. (2010). Glucocorticoid Ultradian Rhythmicity Directs Cyclical Gene Pulsing of the Clock Gene Period 1 in Rat Hippocampus. *Journal of Neuroendocrinology*, *22*(10), 1093–1100.

Critchlow, V., Liebelt, R. A., Bar-Sela, M., Mountcastle, W., & Lipscomb, H. S. (1963). Sex differences in resting pituitary-adrenal function in the rat, *205*(5), 807–815.

Cuesta, M., Cermakian, N., & Boivin, D. B. (2015). Glucocorticoids entrain molecular clock components in human peripheral cells. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *29*(4), 1360–1370.

Cutler, D. J., Haraura, M., Reed, H. E., Shen, S., Sheward, W. J., Morrison, C. F., et al. (2003). The mouse VPAC2 receptor confers suprachiasmatic nuclei cellular rhythmicity and responsiveness to vasoactive intestinal polypeptide in vitro. *European Journal of Neuroscience*, *17*(2), 197–204.

Dalley, J. W., Cardinal, R. N., & Robbins, T. W. (2004). Prefrontal executive and cognitive functions in rodents: neural and neurochemical substrates. *Neuroscience & Biobehavioral Reviews*, *28*(7), 771–784.

Darlington, T. K., Wager-Smith, K., Ceriani, M. F., Staknis, D., Gekakis, N., Steeves, T. D., et al. (1998). Closing the circadian loop: CLOCK-induced transcription of its own inhibitors per and tim. *Science*, *280*(5369), 1599–1603.

DeBruyne, J. P., Weaver, D. R., & Reppert, S. M. (2007). CLOCK and NPAS2 have overlapping roles in the suprachiasmatic circadian clock. *Nature Neuroscience*, *10*(5), 543–545.

Del Casale, A., Serata, D., Rapinesi, C., Kotzalidis, G. D., Angeletti, G., Tatarelli, R., et al. (2013). Structural neuroimaging in patients with panic disorder: findings and limitations of recent studies. *Psychiatria Danubina*, *25*(2), 108–114.

Dickmeis, T. (2009). Glucocorticoids and the circadian clock. *Journal of Endocrinology*, *200*, 3–22.

Drevets, W. C., Price, J. L., & Furey, M. L. (2008a). Brain structural and functional abnormalities in mood disorders: implications for neurocircuitry models of depression. *Brain Structure and Function*, *213*(1-2), 93–118.

Drevets, W. C., Price, J. L., Simpson, J. R., Todd, R. D., Reich, T., Vannier, M., & Raichle, M. E. (1997). Subgenual prefrontal cortex abnormalities in mood disorders. *Nature*, *386*(6627), 824–827.

Drevets, W. C., Savitz, J., & Trimble, M. (2008b). The subgenual anterior cingulate cortex in mood disorders. *CNS Spectrums*, *13*(8), 663–681.

Droste, S. K., de Groote, L., Lightman S.L., Reul, J.M.H.M., & Linthorst, A.C.E. (2009). The ultratdian and circadian rhythms of free corticosterone in the brain are not affected by gender: an in vivo microdialysis study in Wistar rats. *Journal of Neuroendocrinology,* 21(2), 132-140.

Duez, H., & Staels, B. (2008). The nuclear receptors Rev-erbs and RORs integrate circadian rhythms and metabolism. *Diabetes & Vascular Disease Research*, *5*(2), 82–88.

Dunlap, J. C. (1999). Molecular bases for circadian clocks. *Cell*, *96*(2), 271–290.

Dzirasa, K., McGarity, D. L., Bhattacharya, A., Kumar, S., Takahashi, J. S., Dunson, D., et al. (2011). Impaired Limbic Gamma Oscillatory Synchrony during Anxiety-Related Behavior in a Genetic Mouse Model of Bipolar Mania. *Journal of Neuroscience*, *31*(17), 6449–6456.

Eckel-Mahan, K. L., Phan, T., Han, S., Wang, H., Chan, G. C. K., Scheiner, Z. S., & Storm, D. R. (2008). Circadian oscillation of hippocampal MAPK activity and cAmp: implications for memory persistence. *Nature Neuroscience*, *11*(9), 1074–1082.

Elliott, R., Baker, S. C., Rogers, R. D., O'Leary, D. A., Paykel, E. S., Frith, C. D., et al. (1997). Prefrontal dysfunction in depressed patients performing a complex planning task: a study using positron emission tomography. *Psychological Medicine*, *27*(4), 931–942.

Emery, P., & Reppert, S. M. (2004). A Rhythmic Ror. *Neuron*, *43*(4), 443–446.

Etain, B., Milhiet, V., Bellivier, F., & Leboyer, M. (2011). Genetics of circadian rhythms and mood spectrum disorders. *European Neuropsychopharmacology*, *21*, S676–S682.

Farrell, M. R., Sengelaub, D. R., & Wellman, C. L. (2013). Sex differences and chronic stress effects on the neural circuitry underlying fear conditioning and extinction. *Physiology & Behavior*, *122*, 208–215.

Feillet, C. A., Mendoza, J., Albrecht, U., Pévet, P., & Challet, E. (2008a). Forebrain oscillators ticking with different clock hands. *Molecular and Cellular Neuroscience*, *37*(2), 209–221.

Feillet, C. A., Mendoza, J., Pévet, P., & Challet, E. (2008b). Restricted feeding restores rhythmicity in the pineal gland of arrhythmic suprachiasmatic-lesioned rats. *European Journal of Neuroscience*, *28*(12), 2451–2458.

Fenton, G. E., Pollard, A. K., Halliday, D. M., Mason, R., Bredy, T. W., & Stevenson, C. W. (2014). Persistent prelimbic cortex activity contributes to enhanced learned fear expression in females. *Learning & Memory*, *21*(2), 55–60.

Figueiredo, H. F., Dolgas, C. M., & Herman, J. P. (2002). Stress activation of cortex and hippocampus is modulated by sex and stage of estrus. *Endocrinology*, *143*(7), 2534–2540.

Gallego, M., & Virshup, D. M. (2007). Post-translational modifications regulate the ticking of the circadian clock. *Nature Reviews. Molecular Cell Biology*, *8*(2), 139–148.

Gekakis, N. (1998). Role of the CLOCK Protein in the Mammalian Circadian Mechanism. *Science*, *280*(5369), 1564–1569.

Gilhooley, M. J., Pinnock, S. B., & Herbert, J. (2011). Rhythmic expression of per1 in the dentate gyrus is suppressed by corticosterone: Implications for neurogenesis. *Neuroscience Letters*, *489*(3), 177–181.

Ginsberg, A. B., Campeau, S., Day, H. E., & Spencer, R. L. (2003). Acute glucocorticoid pretreatment suppresses stress-induced hypothalamic-pituitary-adrenal axis hormone secretion and expression of corticotropin-releasing hormone hnRNA but does not affect c-fos mRNA or fos protein expression in the paraventricular nucleus of the hypothalamus. *Journal of Neuroendocrinology*, *15*(11), 1075–1083.

Girotti, M., Pace, T. W. W., Gaylord, R. I., Rubin, B. A., Herman, J. P., & Spencer, R. L. (2006). Habituation to repeated restraint stress is associated with lack of stress-induced c-fos expression in primary sensory processing areas of the rat brain. *Neuroscience*, *138*(4),

1067–1081.

Girotti, M., Weinberg, M. S., & Spencer, R. L. (2009). Diurnal expression of functional and clock-related genes throughout the rat HPA axis: system-wide shifts in response to a restricted feeding schedule. *AJP: Endocrinology and Metabolism*, *296*(4), E888–E897.

Goel, N., Workman, J. L., Lee, T. T., Innala, L., & Viau, V. (2011). Sex Differences in the HPA Axis (pp. 1121–1155). Hoboken, NJ, USA: John Wiley & Sons, Inc.

Goldman, J. M., Murr, A. S., & Cooper, R. L. (2007). The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies. *Birth Defects Research Part B: Developmental and Reproductive Toxicology*, *80*(2), 84–97.

Goldman-Rakic, P. S. (1996). The prefrontal landscape: implications of functional architecture for understanding human mentation and the central executive. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *351*(1346), 1445–1453.

Gray, J. D., Rubin, T. G., Hunter, R. G., & McEwen, B. S. (2014). Hippocampal gene expression changes underlying stress sensitization and recovery. *Molecular Psychiatry*, *19*(11), 1171–1178.

Gray, M., Bingham, B., & Viau, V. (2012). A Comparison of Two Repeated Restraint Stress Paradigms on Hypothalamic-Pituitary-Adrenal Axis Habituation, Gonadal Status and Central Neuropeptide Expression in Adult Male Rats. *Journal of Neuroendocrinology*, *22*(2), 92–101.

Gray, T. S., & Bingaman, E. W. (1996). The amygdala: corticotropin-releasing factor, steroids, and stress. *Critical Reviews in Neurobiology*, *10*(2), 155–168.

Guilding, C., & Piggins, H. D. (2007). Challenging the omnipotence of the suprachiasmatic timekeeper: are circadian oscillators present throughout the mammalian brain? *European Journal of Neuroscience*, *25*(11), 3195–3216.

Handa, R. J., Burgess, L. H., Kerr, J. E., & O'Keefe, J. A. (1994). Gonadal steroid hormone receptors and sex differences in the hypothalamo-pituitary-adrenal axis. *Hormones and Behavior*, *28*(4), 464–476.

Harbour, V. L., Weigl, Y., Robinson, B., & Amir, S. (2013). Comprehensive mapping of regional expression of the clock protein PERIOD2 in rat forebrain across the 24-h day. *PLoS ONE*, *8*(10), e76391.

Harbour, V. L., Weigl, Y., Robinson, B., & Amir, S. (2014). Phase Differences in Expression of Circadian Clock Genes in the Central Nucleus of the Amygdala, Dentate Gyrus, and Suprachiasmatic Nucleus in the Rat. *PLoS ONE*, *9*(7), e103309.

Harmar, A. J., Marston, H. M., Shen, S., Spratt, C., West, K. M., Sheward, W. J., et al. (2002). The VPAC(2) receptor is essential for circadian function in the mouse suprachiasmatic nuclei. *Cell*, *109*(4), 497–508.

Hastings, M. H. (2004). Clock Genes, Oscillators, and Cellular Networks in the Suprachiasmatic Nuclei. *Journal of Biological Rhythms*, *19*(5), 400–413.

Hastings, M., O'Neill, J. S., & Maywood, E. S. (2011). Circadian clocks: regulators of endocrine and metabolic rhythms. *Journal of Endocrinology*, *195*(2), 187–198.

He, P. J., Hirata, M., Yamauchi, N., & Hattori, M. A. (2007). Up-regulation of Per1 expression by estradiol and progesterone in the rat uterus. *Journal of Endocrinology*, *194*(3), 511–519.

Heinsbroek, R. P., Van Haaren, F., Feenstra, M. G., Endert, E., & Van de Poll, N. E. (1991). Sex- and time-dependent changes in neurochemical and hormonal variables induced by predictable and unpredictable footshock. *Physiology & Behavior*, *49*(6), 1251–1256.

Herman, J. P., Figueiredo, H., Mueller, N. K., Ulrich-Lai, Y., Ostrander, M. M., Choi, D. C., & Cullinan, W. E. (2003). Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo–pituitary–adrenocortical responsiveness. *Frontiers in Neuroendocrinology*, *24*(3), 151–180.

Highland, J. A., Weiser, M. J., Hinds, L. R., & Spencer, R. L. (2014). CRTC2 activation in the suprachiasmatic nucleus, but not paraventricular nucleus, varies in a diurnal fashion and increases with nighttime light exposure. *American Journal of Physiology. Cell Physiology*, *307*(7), C611–21.

Horst, Ter, G. J., Wichmann, R., Gerrits, M., Westenbroek, C., & Lin, Y. (2009). Sex differences in stress responses: focus on ovarian hormones. *Physiology & Behavior*, *97*(2), 239–249.

Hughes, P., & Dragunow, M. (1995). Induction of immediate-early genes and the control of neurotransmitter-regulated gene expression within the nervous system. *Pharmacological Reviews*, *47*(1), 133–178.

Ikeno, T., & Nelson, R. J. (2015). Acute melatonin treatment alters dendritic morphology and circadian clock gene expression in the hippocampus of Siberian hamsters. *Hippocampus*, *25*(2), 142–148.

Imbe, H., Kimura, A., Donishi, T., & Kaneoke, Y. (2014). Repeated forced swim stress enhances CFA-evoked thermal hyperalgesia and affects the expressions of pCREB and c-Fos in the insular cortex. *Neuroscience*, 259, 1-11.

Iwasaki-Sekino, A., Mano-Otagiri, A., Ohata, H., Yamauchi, N., & Shibasaki, T. (2009). Gender differences in corticotropin and corticosterone secretion and corticotropin-releasing factor mRNA expression in the paraventricular nucleus of the hypothalamus and the central nucleus of the amygdala in response to footshock stress or psychological stress in rats. *Psychoneuroendocrinology*, *34*(2), 226–237.

Jacobson, L., Akana, S. F., Cascio, C. S., Shinsako, J., & Dallman, M. F. (1988). Circadian variations in plasma corticosterone permit normal termination of adrenocorticotropin responses to stress. *Endocrinology*, *122*(4), 1343–1348.

Jasinska, M., Grzegorczyk, A., & Woznicka, O. (2015). Circadian rhythmicity of synapses in mouse somatosensory cortex. *European Journal of Neuroscience,* 42(8), 2585-2594.

Jiang, W. G., Li, S. X., Liu, J. F., Sun, Y., Zhou, S. J., & Zhu, W. L. (2013). Hippocampal CLOCK protein participates in the persistence of depressive-like behavior induced by

chronic unpredictable stress. *Psychopharmacology,* 227(1), 79-92.

Jiang, W.-G., Li, S.-X., Zhou, S.-J., Sun, Y., Shi, J., & Lu, L. (2011). Chronic unpredictable stress induces a reversible change of PER2 rhythm in the suprachiasmatic nucleus. *Brain Research*, *1399*, 25–32.

Jilg, A., Lesny, S., Peruzki, N., Schwegler, H., Selbach, O., Dehghani, F., & Stehle, J. H. (2009). Temporal dynamics of mouse hippocampal clock gene expression support memory processing. *Hippocampus*, 20, 377-388.

Joëls, M., Pasricha, N., & Karst, H. (2013). The interplay between rapid and slow corticosteroid actions in brain. *European Journal of Pharmacology*, *719*(1-3), 44–52.

Johansson, C., Willeit, M., Smedh, C., Ekholm, J., Paunio, T., Kieseppä, T., et al. (2002). Circadian Clock-Related Polymorphisms in Seasonal Affective Disorder and their Relevance to Diurnal Preference. *Neuropsychopharmacology*, *28*(4), 734–739.

Johnson, J. H., & Sarason, I. G. (1978). Life stress, depression and anxiety: internal-external control as a moderator variable. *Journal of Psychosomatic Research*, *22*(3), 205–208.

Jones, K. R., Myers, B., & Herman, J. P. (2011). Stimulation of the prelimbic cortex differentially modulates neuroendocrine responses to psychogenic and systemic stressors. *Physiology & Behavior*, *104*(2), 266–271.

Kessler, R. (2003). Epidemiology of women and depression. *Journal of Affective Disorders*, *74*(1), 5–13.

Kinoshita, C., Miyazaki, K., & Ishida, N. (2012). Chronic stress affects PERIOD2 expression through glycogen synthase kinase-3β phosphorylation in the central clock. *Neuroreport*, *23*(2), 98–102.

Kitay, J. I. (1961). Sex differences in adrenal cortical secretion in the rat. *Endocrinology*, *68*, 818–824.

Koenigs, M., & Grafman, J. (2009). Posttraumatic Stress Disorder: The Role of Medial Prefrontal Cortex and Amygdala. *The Neuroscientist*, *15*(5), 540–548.

Kovács, K. J. (1998). Invited review c-Fos as a transcription factor: a stressful (re)view from a functional map. *Neurochemistry International*, *33*(4), 287–297.

Lamia, K. A., Papp, S. J., Yu, R. T., Barish, G. D., Uhlenhaut, N. H., Jonker, J. W., et al. (2011). Cryptochromes mediate rhythmic repression of the glucocorticoid receptor. *Nature*, *480*(7378), 552–556.

Lamont, E. W., Coutu, D. L., Cermakian, N., & Boivin, D. B. (2009). Circadian rhythms and clock genes in psychotic disorders. *The Israel Journal of Psychiatry and Related Sciences*, *47*(1), 27–35.

Lamont, E. W., Robinson, B., Stewart, J., & Amir, S. (2005). The central and basolateral nuclei of the amygdala exhibit opposite diurnal rhythms of expression of the clock protein Period2. *Proceedings of the National Academy of Sciences*, *102*(11), 4180–4184.

Landgraf, D., McCarthy, M. J., & Welsh, D. K. (2014). Circadian clock and stress interactions in the molecular biology of psychiatric disorders. *Current Psychiatry Reports*, *16*(10), 483.

Landgraf, D., Wang, L. L., Diemer, T., & Welsh, D. K. (2016). NPAS2 Compensates for Loss of CLOCK in Peripheral Circadian Oscillators. *PLoS Genetics*, *12*(2), e1005882.

Lebow, M. A., & Chen, A. (2016). Overshadowed by the amygdala: the bed nucleus of the stria terminalis emerges as key to psychiatric disorders. *Molecular Psychiatry*, *21*(4), 450–463.

LeDoux, J. E., Iwata, J., Cicchetti, P., & Reis, D. J. (1988). Different projections of the central amygdaloid nucleus mediate autonomic and behavioral correlates of conditioned fear. *The Journal of Neuroscience*, *8*(7), 2517–2529.

Lee, C., Etchegaray, J. P., Cagampang, F. R., Loudon, A. S., & Reppert, S. M. (2001). Posttranslational mechanisms regulate the mammalian circadian clock. *Cell*, *107*(7), 855–867.

Lee, H. Y., Kim, M. S., Kim, O. S., & Lee, I. H. (2015). Association between shift work and severity of depressive symptoms among female nurses: the Korea Nurses' Health Study. *Journal of Nursing Management,* 24, 192-200.

Lehman, M. N., Silver, R., Gladstone, W. R., Kahn, R. M., Gibson, M., & Bittman, E. L. (1987). Circadian rhythmicity restored by neural transplant. Immunocytochemical characterization of the graft and its integration with the host brain. *The Journal of Neuroscience*, *7*(6), 1626–1638.

Li, H. Y., Blaustein, J. D., De Vries, G. J., & Wade, G. N. (1993). Estrogen-receptor immunoreactivity in hamster brain: preoptic area, hypothalamus and amygdala. *Brain Research*, *631*(2), 304–312.

Li, J. Z., Bunney, B. G., Meng, F., Hagenauer, M. H., Walsh, D. M., Vawter, M. P., et al. (2013). Circadian patterns of gene expression in the human brain and disruption in major depressive disorder. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(24), 9950–9955.

Liston, C., Cichon, J. M., Jeanneteau, F., Jia, Z., Chao, M. V., & Gan, W.-B. (2013). Circadian glucocorticoid oscillations promote learning-dependent synapse formation and maintenance. *Nature Neuroscience*, *16*(6), 698–705.

Liu, C., & Reppert, S. M. (2000). GABA synchronizes clock cells within the suprachiasmatic circadian clock. *Neuron*, *25*(1), 123–128.

Logan, R. W., Edgar, N., Gillman, A. G., Hoffman, D., Zhu, X., & McClung, C. A. (2015). Chronic Stress Induces Brain Region-Specific Alterations of Molecular Rhythms that Correlate with Depression-like Behavior in Mice. *Biological Psychiatry*, 78(4), 249-58.

Lowrey, P. L., & Takahashi, J. S. (2004). MAMMALIAN CIRCADIAN BIOLOGY: Elucidating Genome-Wide Levels of Temporal Organization. *Annual Review of Genomics and Human Genetics*, *5*(1), 407–441.

Ma, C. C., Andrew, M. E., Fekedulegn, D., Gu, J. K., Hartley, T. A., Charles, L. E., et al. (2015). Shift work and occupational stress in police officers. *Safety and Health at Work*, *6*(1), 25–29.

Marcheva, B., Ramsey, K. M., Peek, C. B., Affinati, A., Maury, E., & Bass, J. (2013). Circadian Clocks and Metabolism. In *Handbook of Experimental Pharmacology*, 217, 127–155.

Marcondes, F. K., Bianchi, F. J., & Tanno, A. P. (2002). Determination of the estrous cycle phases of rats: some helpful considerations. *Brazilian Journal of Biology*, *62*(4A), 609–614.

Martin-Fairey, C. A., & Nunez, A. A. (2014). Circadian modulation of memory and plasticity gene products in a diurnal species. *Brain Research*, *1581*, 30–39.

Martinek, S., Inonog, S., Manoukian, A. S., & Young, M. W. (2001). A role for the segment polarity gene shaggy/GSK-3 in the Drosophila circadian clock. *Cell*, 105(6), 769-779.

Masubuchi, S., Honma, S., Abe, H., Ishizaki, K., Namihira, M., Ikeda, M., & Honma, K.-I. (2000). Clock genes outside the suprachiasmatic nucleus involved in manifestation of locomotor activity rhythm in rats. *European Journal of Neuroscience*, *12*(12), 4206–4214.

Mayberg, H. S., Liotti, M., Brannan, S. K., McGinnis, S., Mahurin, R. K., Jerabek, P. A., et al. (1999). Reciprocal Limbic-Cortical Function and Negative Mood: Converging PET Findings in Depression and Normal Sadness. *American Journal of Psychiatry*, *156*(5), 675–682.

Mayberg, H. S., Lozano, A. M., Voon, V., McNeely, H. E., Seminowicz, D., Hamani, C., et al. (2005). Deep Brain Stimulation for Treatment-Resistant Depression. *Neuron*, *45*(5), 651–660.

McCarthy, M. J., & Welsh, D. K. (2012). Cellular Circadian Clocks in Mood Disorders. *Journal of Biological Rhythms*, *27*(5), 339–352.

McClung, C. A. (2007). Circadian genes, rhythms and the biology of mood disorders. *Pharmacology & Therapeutics*, *114*(2), 222–232.

McClung, C. A., Sidiropoulou, K., Vitaterna, M., Takahashi, J. S., White, F. J., Cooper, D. C., & Nestler, E. J. (2005). Regulation of dopaminergic transmission and cocaine reward by the Clock gene. *Proceedings of the National Academy of Sciences*, *102*(26), 9377–9381.

McEwen, B. S., de Kloet, E. R., & Rostene, W. (1986). Adrenal steroid receptors and actions in the nervous system. *Physiological Reviews*, *66*(4), 1121–1188.

Meaney, M. J., & Aitken, D. H. (1985). [3H]Dexamethasone binding in rat frontal cortex. *Brain Research*, *328*(1), 176–180.

Merchenthaler, I., Lane, M. V., Numan, S., & Dellovade, T. L. (2004). Distribution of estrogen receptor alpha and beta in the mouse central nervous system: In vivo autoradiographic and immunocytochemical analyses. *Journal of Comparative Neurology*, *473*(2), 270–291.

Mifsud, K. R., & Reul, J. M. H. M. (2016). Acute stress enhances heterodimerization and binding of corticosteroid receptors at glucocorticoid target genes in the hippocampus. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(40), 11336–

11341.

Miller, B. H., McDearmon, E. L., Panda, S., Hayes, K. R., Zhang, J., Andrews, J. L., et al. (2007). Circadian and CLOCK-controlled regulation of the mouse transcriptome and cell proliferation. *Proceedings of the National Academy of Sciences*, *104*(9), 3342–3347.

Miyake, S., Sumi, Y., Yan, L., Takekida, S., Fukuyama, T., Ishida, Y., et al. (2000). Phase-dependent responses of Per1 and Per2 genes to a light-stimulus in the suprachiasmatic nucleus of the rat. *Neuroscience Letters*, *294*(1), 41–44.

Mojón, A., Fernández, J. R., & Hermida, R. C. (1992). Chronolab: An Interactive Software Package for Chronobiologic Time Series Analysis Written for the Macintosh Computer. *Chronobiology International,* 9(6), 403-412.

Moore, R. Y., & Eichler, V. B. (1972). Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. *Brain Research*, *42*(1), 201–206.

Morin, L. P. (2013). Neuroanatomy of the extended circadian rhythm system. *Experimental Neurology*, 243, 4-20.

Mukherjee, S., Coque, L., Cao, J.-L., Kumar, J., Chakravarty, S., Asaithamby, A., et al. (2010). Knockdown of Clock in the Ventral Tegmental Area Through RNA Interference Results in a Mixed State of Mania and Depression-Like Behavior. *Biological Psychiatry*, *68*(6), 503–511.

Murphy, Z. C., Pezuk, P., Menaker, M., & Sellix, M. T. (2013). Effects of Ovarian Hormones on Internal Circadian Organization in Rats. *Biology of Reproduction*, *89*(2), 35–35.

Nagy, Z., Marta, A., Butz, H., Liko, I., Racz, K., & Patocs, A. (2016). Modulation of the circadian clock by glucocorticoid receptor isoforms in the H295R cell line. *Steroids*, *116*, 20–27.

Nakamura, T. J., Moriya, T., Inoue, S., Shimazoe, T., Watanabe, S., Ebihara, S., & Shinohara, K. (2005). Estrogen differentially regulates expression of Per1 andPer2 genes between central and peripheral clocks and between reproductive and nonreproductive tissues in female rats. *Journal of Neuroscience Research*, *82*(5), 622–630.

Nakamura, T. J., Sellix, M. T., Kudo, T., Nakao, N., Yoshimura, T., Ebihara, S., et al. (2010). Influence of the estrous cycle on clock gene expression in reproductive tissues: Effects of fluctuating ovarian steroid hormone levels. *Steroids*, *75*(3), 203–212.

Nakamura, T. J., Shinohara, K., Funabashi, T., & Kimura, F. (2001). Effect of estrogen on the expression of Cry1 and Cry2 mRNAs in the suprachiasmatic nucleus of female rats. *Neuroscience*, *41*(3), 251–255.

Nestler, E., Hyman, S., & Malenka, R. (2015). Molecular Neuropharmacology: A Foundation for Clinical Neuroscience, Third Edition. McGraw Hill Professional.

Ogle, T. F., & Kitay, J. I. (1977). OVARIAN AND ADRENAL STEROIDS DURING PREGNANCY AND THE OESTROUS CYCLE IN THE RAT. *Journal of Endocrinology*, *74*(1), 89–98.

Oishi, K., Sakamoto, K., Okada, T., Nagase, T., & Ishida, N. (1998). Antiphase Circadian Expression between BMAL1 and period Homologue mRNA in the Suprachiasmatic Nucleus

and Peripheral Tissues of Rats. *Biochemical and Biophysical Research Communications*, *253*(2), 199–203.

Onishi, H., Yamaguchi, S., & Yagita, K. (2002). Rev‐erbα gene expression in the mouse brain with special emphasis on its circadian profiles in the suprachiasmatic nucleus. *Journal of Neuroscience Research,* 68(5), 551-557.

Oster, H., Werner, C., Magnone, M. C., Mayser, H., Feil, R., Seeliger, M. W., et al. (2003). cGMP-dependent protein kinase II modulates mPer1 and mPer2 gene induction and influences phase shifts of the circadian clock. *Current Biology : CB*, *13*(9), 725–733.

Otalora, B. B., Hagenauer, M. H., Rol, M. A., Madrid, J. A., & Lee, T. M. (2013). Period gene expression in the brain of a dual-phasing rodent, the Octodon degus. *Journal of Biological Rhythms*, *28*(4), 249–261.

Pace, T. W. W., Gaylord, R., Topczewski, F., Girotti, M., Rubin, B., & Spencer, R. L. (2005). Immediate-early gene induction in hippocampus and cortex as a result of novel experience is not directly related to the stressfulness of that experience. *European Journal of Neuroscience*, *22*(7), 1679–1690.

Pace-Schott, E. F., Spencer, R. M. C., Vijayakumar, S., Ahmed, N. A. K., Verga, P. W., Orr, S. P., et al. (2013). Extinction of conditioned fear is better learned and recalled in the morning than in the evening. *Journal of Psychiatric Research*, *47*(11), 1776–1784.

Panda, S., Hogenesch, J. B., & Kay, S. A. (2002). Circadian rhythms from flies to human. *Nature*, *417*(6886), 329–335.

Pando, M. P., Morse, D., Cermakian, N., & Sassone-Corsi, P. (2002). Phenotypic rescue of a peripheral clock genetic defect via SCN hierarchical dominance. *Cell*, *110*(1), 107–117.

Partonen, T., Treutlein, J., Alpman, A., Frank, J., Johansson, C., Depner, M., et al. (2007). Three circadian clock genes Per2, Arntl, and Npas2 contribute to winter depression. *Annals of Medicine*, *39*(3), 229–238.

Paxinos, G. & Watson, C. (1998). The Rat Brain in Stereotaxic Coordinates. Academic Press, Elsevier, 4th edition.

Perez-Cruz, C., Simon, M., Flügge, G., Fuchs, E., & Czéh, B. (2009). Diurnal rhythm and stress regulate dendritic architecture and spine density of pyramidal neurons in the rat infralimbic cortex. *Behavioural Brain Research*, *205*(2), 406–413.

Perrin, J. S., Segall, L. A., Harbour, V. L., Woodside, B., & Amir, S. (2006). The expression of the clock protein PER2 in the limbic forebrain is modulated by the estrous cycle. *Proceedings of the National Academy of Sciences*, *103*(14), 5591–5596.

Pezük, P., Mohawk, J. A., Wang, L. A., & Menaker, M. (2012). Glucocorticoids as entraining signals for peripheral circadian oscillators. *Endocrinology*, *153*(10), 4775–4783.

Pittenger, C., & Duman, R. S. (2008). Stress, depression, and neuroplasticity: a convergence of mechanisms. *Neuropsychopharmacology*, *33*(1), 88–109.

Preitner, N., Damiola, F., Lopez-Molina, L., Zakany, J., Duboule, D., Albrecht, U., & Schibler, U. (2002). The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell*, *110*(2), 251–260.

Pushparaj, A., Kim, A. S., Musiol, M., Trigo, J. M., & Le Foll, B. (2015). Involvement of the rostral agranular insular cortex in nicotine self-administration in rats. *Behavioural Brain Research*, *290*, 77–83.

Quirk, G. J., Garcia, R., & González-Lima, F. (2006). Prefrontal Mechanisms in Extinction of Conditioned Fear. *Biological Psychiatry*, *60*(4), 337–343.

Radkovsky, A., McArdle, J. J., Bockting, C. L. H., & Berking, M. (2014). Successful emotion regulation skills application predicts subsequent reduction of symptom severity during treatment of major depressive disorder. *Journal of Consulting and Clinical Psychology*, *82*(2), 248–262.

Ralph, M. R., Foster, R. G., Davis, F. C., & Menaker, M. (1990). Transplanted suprachiasmatic nucleus determines circadian period. *Science*, *247*(4945), 975–978.

Ramanathan, C., Nunez, A. A., Martinez, G. S., Schwartz, M. D., & Smale, L. (2006). Temporal and spatial distribution of immunoreactive PER1 and PER2 proteins in the suprachiasmatic nucleus and peri-suprachiasmatic region of the diurnal grass rat (Arvicanthis niloticus). *Brain Research*, *1073-1074*, 348–358.

Ramanathan, C., Smale, L., & Nunez, A. A. (2008). Rhythms in expression of PER1 protein in the amygdala and bed nucleus of the stria terminalis of the diurnal grass rat (Arvicanthis niloticus). *Neuroscience Letters*, *441*(1), 86–89.

Rath, M. F., Rohde, K., Fahrenkrug, J., & Møller, M. (2012). Circadian clock components in the rat neocortex: daily dynamics, localization and regulation. *Brain Structure and Function*, *218*(2), 551–562.

Rath, M. F., Rovsing, L., & Møller, M. (2014). Circadian oscillators in the mouse brain: molecular clock components in the neocortex and cerebellar cortex. *Cell and Tissue Research*, *357*(3), 743–755.

Razzoli, M., Karsten, C., Yoder, J. M., Bartolomucci, A., & Engeland, W. C. (2014). Chronic subordination stress phase advances adrenal and anterior pituitary clock gene rhythms. *AJP: Regulatory, Integrative and Comparative Physiology*, *307*(2), R198–205.

Reddy, A. B., Karp, N. A., Maywood, E. S., Sage, E. A., Deery, M., O'Neill, J. S., et al. (2006). Circadian orchestration of the hepatic proteome. *Current Biology*, *16*(11), 1107–1115.

Reddy, A. B., Maywood, E. S., Karp, N. A., King, V. M., Inoue, Y., Gonzalez, F. J., et al. (2007). Glucocorticoid signaling synchronizes the liver circadian transcriptome. *Hepatology*, *45*(6), 1478–1488.

Reddy, T. E., Gertz, J., & Crawford, G. E. (2012). The hypersensitive glucocorticoid response specifically regulates period 1 and expression of circadian genes. *Molecular and Cellular Biology*, 32(18), 3756-3767.

Reddy, T. E., Pauli, F., Sprouse, R. O., Neff, N. F., Newberry, K. M., Garabedian, M. J., & Myers, R. M. (2009). Genomic determination of the glucocorticoid response reveals unexpected mechanisms of gene regulation. *Genome Research*, *19*(12), 2163–2171.

Reick, M. (2001). NPAS2: An Analog of Clock Operative in the Mammalian Forebrain. *Science*, *293*(5529), 506–509.

Reppert, S. M., & Weaver, D. R. (2002). Coordination of circadian timing in mammals. *Nature*, *418*(6901), 935–941.

Rey, G., Cesbron, F., Rougemont, J., Reinke, H., Brunner, M., & Naef, F. (2011). Genome-wide and phase-specific DNA-binding rhythms of BMAL1 control circadian output functions in mouse liver. *PLoS Biology*, *9*(2), e1000595.

Rivier, C. (1999). Gender, sex steroids, corticotropin-releasing factor, nitric oxide, and the HPA response to stress. *Pharmacology Biochemistry and Behavior*, *64*(4), 739–751.

Robbins, T. W. (1996). Dissociating executive functions of the prefrontal cortex. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *351*(1346), 1463–71.

Rosenfeld, P., Eekelen, J., & Levine, S. (1993). Ontogeny of corticosteroid receptors in the brain. *Cellular and Molecular Neurobiology,* 13(4), 295-319.

Rosenfeld, P., Van Eekelen, J. A., Levine, S., & de Kloet, E. R. (1988). Ontogeny of the type 2 glucocorticoid receptor in discrete rat brain regions: an immunocytochemical study. *Brain Research*, *470*(1), 119–127.

Roszkowski, M., Manuella, F., Ziegler, von, L., Durán-Pacheco, G., Moreau, J.-L., Mansuy, I. M., & Bohacek, J. (2016). Rapid stress-induced transcriptomic changes in the brain depend on beta-adrenergic signaling. *Neuropharmacology*, *107*, 329–338.

Roybal, K., Theobold, D., Graham, A., DiNieri, J. A., Russo, S. J., Krishnan, V., et al. (2007). Mania-like behavior induced by disruption of CLOCK. *Proceedings of the National Academy of Sciences*, *104*(15), 6406–6411.

Sakamoto, K., Nagase, T., Fukui, H., Horikawa, K., Okada, T., Tanaka, H., et al. (1998). Multitissue circadian expression of rat period homolog (rPer2) mRNA is governed by the mammalian circadian clock, the suprachiasmatic nucleus in the brain. *The Journal of Biological Chemistry*, *273*(42), 27039–27042.

Sanada, K., Okano, T., & Fukada, Y. (2002). Mitogen-activated protein kinase phosphorylates and negatively regulates basic helix-loop-helix-PAS transcription factor BMAL1. *Journal of Biological Chemistry*, 277, 267-271.

Sato, T. K., Panda, S., Miraglia, L. J., Reyes, T. M., Rudic, R. D., McNamara, P., et al. (2004). A Functional Genomics Strategy Reveals Rora as a Component of the Mammalian Circadian Clock. *Neuron*, *43*(4), 527–537.

Seale, J. V., Wood, S. A., Atkinson, H. C., Harbuz, M. S., & Lightman, S. L. (2004). Gonadal Steroid Replacement Reverses Gonadectomy-Induced Changes in the Corticosterone

Pulse Profile and Stress-Induced Hypothalamic-Pituitary-Adrenal Axis Activity of Male and Female Rats. *Journal of Neuroendocrinology*, *16*(12), 989–998.

Segall, L. A., & Amir, S. (2010). Exogenous Corticosterone Induces the Expression of the Clock Protein, PERIOD2, in the Oval Nucleus of the Bed Nucleus of the Stria Terminalis and the Central Nucleus of the Amygdala of Adrenalectomized and Intact Rats. *Journal of Molecular Neuroscience*, *42*(2), 176–182.

Shansky, R. M., & Morrison, J. H. (2009). Stress-induced dendritic remodeling in the medial prefrontal cortex: Effects of circuit, hormones and rest. *Brain Research*, *1293*, 108–113.

Shearman, L. P. (2000). Interacting Molecular Loops in the Mammalian Circadian Clock. *Science*, *288*(5468), 1013–1019.

Shearman, L. P., Zylka, M. J., Weaver, D. R., Kolakowski, L. F., & Reppert, S. M. (1997). Two period homologs: circadian expression and photic regulation in the suprachiasmatic nuclei. *Neuron*, *19*(6), 1261–1269.

Sheng, M., & Greenberg, M. E. (1990). The regulation and function of c-fos and other immediate early genes in the nervous system. *Neuron*, *4*(4), 477–485.

Shieh, K. R. (2003). Distribution of the rhythm-related genes rPERIOD1, rPERIOD2, and rCLOCK, in the rat brain. *Neuroscience*, *118*(3), 831–843.

Shigeyoshi, Y., Taguchi, K., Yamamoto, S., Takekida, S., Yan, L., Tei, H., et al. (1997). Light-induced resetting of a mammalian circadian clock is associated with rapid induction of the mPer1 transcript. *Cell*, *91*(7), 1043–1053.

Simerly, R. B., Chang, C., Muramatsu, M., & Swanson, L. W. (1990). Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an in situ hybridization study. *The Journal of Comparative Neurology*, *294*(1), 76–95.

Smarr, B. L., Jennings, K. J., Driscoll, J. R., & Kriegsfeld, L. J. (2014). A time to remember: The role of circadian clocks in learning and memory. *Behavioral Neuroscience*, *128*(3), 283–303.

Smith, B. J., Sutton, G. M., Wu, X., Yu, G., Goh, B. C., Hebert, T., et al. (2010). Ovariectomy and genes encoding core circadian regulatory proteins in murine bone. *Osteoporosis International*, *22*(5), 1633–1639.

So, A. Y. L., Bernal, T. U., Pillsbury, M. L., Yamamoto, K. R., & Feldman, B. J. (2009). Glucocorticoid regulation of the circadian clock modulates glucose homeostasis. *Proceedings of the National Academy of Sciences*, *106*(41), 17582–17587.

Sollars, P. J., & Pickard, G. E. (2015). The Neurobiology of Circadian Rhythms. *Psychiatric Clinics of North America*, *38*(4), 645–665.

Spencer, R. L., & Deak, T. (2016). A users guide to HPA axis research. *Physiology & Behavior*. http://doi.org/10.1016/j.physbeh.2016.11.014

Spencer, S., Falcon, E., Kumar, J., Krishnan, V., Mukherjee, S., Birnbaum, S. G., & McClung, C. A. (2012). Circadian genes Period 1and Period 2in the nucleus accumbens regulate

anxiety-related behavior. *European Journal of Neuroscience*, *37*(2), 242–250.

Spiga, F., Walker, J. J., Terry, J. R., & Lightman, S. L. (2014). HPA Axis-Rhythms (Vol. 4, pp. 1273–1298). Hoboken, NJ, USA: John Wiley & Sons, Inc.

Stephan, F. K., & Zucker, I. (1972). Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions. *Proceedings of the National Academy of Sciences*, *69*(6), 1583–1586.

Sterrenburg, L., Gaszner, B., Boerrigter, J., Santbergen, L., Bramini, M., Roubos, E. W., et al. (2011). Sex-dependent and differential responses to acute restraint stress of corticotropin-releasing factor-producing neurons in the rat paraventricular nucleus, central amygdala, and bed nucleus of the stria terminalis. *Journal of Neuroscience Research*, *90*(1), 179–192.

Storch, K.-F., Lipan, O., Leykin, I., Viswanathan, N., Davis, F. C., Wong, W. H., & Weitz, C. J. (2002). Extensive and divergent circadian gene expression in liver and heart. *Nature*, *417*(6884), 78–83.

Sujino, M., Furukawa, K., Koinuma, S., Fujioka, A., Nagano, M., Iigo, M., & Shigeyoshi, Y. (2012). Differential entrainment of peripheral clocks in the rat by glucocorticoid and feeding. *Endocrinology*, *153*(5), 2277–2286.

Sun, N., & Cassell, M. D. (1993). Intrinsic GABAergic neurons in the rat central extended amygdala. *Journal of Comparative Neurology*, *330*(3), 381–404.

Sun, N., Yi, H., & Cassell, M. D. (1994). Evidence for a GABAergic interface between cortical afferents and brainstem projection neurons in the rat central extended amygdala. *The Journal of Comparative Neurology*, *340*(1), 43–64.

Sutcliffe, J. S., Marshall, K. M., & Neill, J. C. (2007). Influence of gender on working and spatial memory in the novel object recognition task in the rat. *Behavioural Brain Research*, *177*(1), 117–125.

Sylvester, C. M., Krout, K. E., & Loewy, A. D. (2002). Suprachiasmatic nucleus projection to the medial prefrontal cortex: a viral transneuronal tracing study. *Neuroscience*, *114*(4), 1071–1080.

Szafarczyk, A., Ixart, G., Alonso, G., & Malaval, F. (1983). CNS control of the circadian adrenocortical rhythm. *Journal of Steroid Biochemistry,* 19(1), 1009-1015.

Tahara, Y., Shiraishi, T., Kikuchi, Y., & Haraguchi, A. (2015). Entrainment of the mouse circadian clock by sub-acute physical and psychological stress. *Scientific Reports,* 5:14207.

Takahashi, J. S., Hong, H.-K., Ko, C. H., & McDearmon, E. L. (2008). The genetics of mammalian circadian order and disorder: implications for physiology and disease. *Nature Reviews Genetics*, *9*(10), 764–775.

Takahashi, K., Yamada, T., Tsukita, S., Kaneko, K., Shirai, Y., Munakata, Y., et al. (2013). Chronic mild stress alters circadian expressions of molecular clock genes in the liver. *AJP: Endocrinology and Metabolism*, *304*(3), E301–9.

Takahashi, S., Yokota, S.-I., Hara, R., Kobayashi, T., Akiyama, M., Moriya, T., & Shibata, S. (2001). Physical and inflammatory stressors elevate circadian clock gene mPer1 mRNA levels in the paraventricular nucleus of the mouse. *Endocrinology*, *142*(11), 4910–4917.

Thomas, E. M., & Armstrong, S. M. (1989). Effect of ovariectomy and estradiol on unity of female rat circadian rhythms. *The American Journal of Physiology*, *257*(5 Pt 2), R1241–50.

Tischkau, S. A. (2002). Ca2+/cAMP Response Element-binding Protein (CREB)-dependent Activation of Per1 Is Required for Light-induced Signaling in the Suprachiasmatic Nucleus Circadian Clock. *Journal of Biological Chemistry*, *278*(2), 718–723.

Togo, F., Yoshizaki, T., & Komatsu, T. (2017). Association between depressive symptoms and morningness-eveningness, sleep duration and rotating shift work in Japanese nurses. *Journal of Biological and Medical Rhythm Research,* 34(3), 349-359.

Toh, K. L., Jones, C. R., He, Y., Eide, E. J., Hinz, W. A., Virshup, D. M., et al. (2001). An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome. *Science*, *291*(5506), 1040–1043.

Ueda, H. R., Chen, W., Adachi, A., Wakamatsu, H., Hayashi, S., Takasugi, T., et al. (2002). A transcription factor response element for gene expression during circadian night. *Nature*, *418*(6897), 534–539.

Valentinuzzi, V. S., Kolker, D. E., Vitaterna, M. H., Ferrari, E. A., Takahashi, J. S., & Turek, F. W. (2001). Effect of circadian phase on context and cued fear conditioning in C57BL/6J mice. *Animal Learning & Behavior*, *29*(2), 133–142.

Van der Lee, S., & Boot, L. M. (1955). Spontaneous pseudopregnancy in mice. *Acta Physiologica Et Pharmacologica Neerlandica*, *4*(3), 442.

Vertes, R. P. (2003). Differential projections of the infralimbic and prelimbic cortex in the rat. *Synapse*, *51*(1), 32–58.

Viau, V. (2002). Functional cross-talk between the hypothalamic-pituitary-gonadal and-adrenal axes. *Journal of Neuroendocrinology*, *14*(6), 506–513.

Viau, V., & Meaney, M. J. (1991). Variations in the hypothalamic-pituitary-adrenal response to stress during the estrous cycle in the rat. *Endocrinology*, *129*(5), 2503–2511.

Videbech, P., & Ravnkilde, B. (2004). Hippocampal volume and depression: a meta-analysis of MRI studies. *American Journal of Psychiatry*, *161*(11), 1957–1966.

Vosko, A. M., Hagenauer, M. H., Hummer, D. L., & Lee, T. M. (2009). Period gene expression in the diurnal degu (Octodon degus) differs from the nocturnal laboratory rat (Rattus norvegicus). *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, *296*(2), R353–61.

Wagner, S., Castel, M., Gainer, H., & Yarom, Y. (1997). GABA in the mammalian suprachiasmatic nucleus and its role in diurnal rhythmicity. *Nature*, *387*(6633), 598–603.

Wakamatsu, H., Yoshinobu, Y., Aida, R., Moriya, T., Akiyama, M., & Shibata, S. (2001).

Restricted-feeding-induced anticipatory activity rhythm is associated with a phase-shift of the expression of mPer1 and mPer2 mRNA in the cerebral cortex and hippocampus but not in the suprachiasmatic nucleus of mice. *European Journal of Neuroscience*, *13*(6), 1190–1196.

Wang, L., Dragich, J. M., Kudo, T., & Odom, I. H. (2009). Expression of the circadian clock gene Period2 in the hippocampus: possible implications for synaptic plasticity and learned behaviour. *ASN Neuro,* 10;1(3). pii. e00012.

Weinberg, M. S., Bhatt, A. P., Girotti, M., Masini, C. V., Day, H. E. W., Campeau, S., & Spencer, R. L. (2009). Repeated ferret odor exposure induces different temporal patterns of same-stressor habituation and novel-stressor sensitization in both hypothalamic-pituitary-adrenal axis activity and forebrain c-fos expression in the rat. *Endocrinology*, *150*(2), 749–761.

Weiser, M. J., & Handa, R. J. (2011). Estrogen impairs glucocorticoid dependent negative feedback on the hypothalamic–pituitary–adrenal axis via estrogen receptor alpha within the hypothalamus. *Neuroscience*, *159*(2), 883–895.

Welsh, D. K., Takahashi, J. S., & Kay, S. A. (2010). Suprachiasmatic Nucleus: Cell Autonomy and Network Properties. *Annual Review of Physiology*, *72*(1), 551–577.

Wilensky, A. E., Schafe, G. E., Kristensen, M. P., & LeDoux, J. E. (2006). Rethinking the Fear Circuit: The Central Nucleus of the Amygdala Is Required for the Acquisition, Consolidation, and Expression of Pavlovian Fear Conditioning. *Journal of Neuroscience*, *26*(48), 12387–12396.

Woodruff, E. R., Chun, L. E., Hinds, L. R., & Spencer, R. L. (2016). Diurnal Corticosterone Presence and Phase Modulate Clock Gene Expression in the Male Rat Prefrontal Cortex. *Endocrinology*, *157*(4), 1522–1534.

Woodruff, E. R., Greenwood, B. N., Chun, L. E., Fardi, S., Hinds, L. R., & Spencer, R. L. (2015). Adrenal-dependent diurnal modulation of conditioned fear extinction learning. *Behavioural Brain Research*, *286*, 249–255.

Yamamoto, T., Nakahata, Y., Soma, H., Akashi, M., Mamine, T., & Takumi, T. (2004). Transcriptional oscillation of canonical clock genes in mouse peripheral tissues. *BMC Molecular Biology*, *5*(1), 18.

Yamamoto, T., Nakahata, Y., Tanaka, M., Yoshida, M., Soma, H., Shinohara, K., et al. (2005). Acute physical stress elevates mouse period1 mRNA expression in mouse peripheral tissues via a glucocorticoid-responsive element. *Journal of Biological Chemistry*, *280*(51), 42036–42043.

Yamazaki, S., Numano, R., Abe, M., Hida, A., Takahashi, R., Ueda, M., et al. (2000). Resetting central and peripheral circadian oscillators in transgenic rats. *Science*, *288*(5466), 682–685.

Zylka, M. J., Shearman, L. P., Weaver, D. R., & Reppert, S. M. (1998). Three period homologs in mammals: differential light responses in the suprachiasmatic circadian clock and oscillating transcripts outside of brain. *Neuron*, *20*(6), 1103–1110.