Characterization of diurnal core clock gene expression across brain regions of mice with differential degree of glucocorticoid receptor knockout

Sarah Morton

University of Colorado Boulder

Department of Integrative Physiology (IPHY)

Defense Date: October 28, 2015

Committee Members: Kenneth P. Wright Jr., Ph.D. (thesis advisor; IPHY), Robert L. Spencer, Ph.D. (Psychology), David Sherwood, Ph.D. (IPHY)

Abstract

Circadian rhythms are maintained through the self-regulatory, oscillatory molecular clock, which includes *Per1*, *Per2*, and *Bmal1* clock genes, among others. Disruptions to clock gene expression have been associated with numerous disorders including major depressive disorder, anxiety disorders, and bipolar disorders. The molecular clock has been well characterized in the hypothalamic suprachiasmatic nucleus (SCN), the master clock of the body. Many peripheral tissues and extra-SCN brain regions have also been shown to express these core clock genes rhythmically, but as the SCN has few direct projections to extra-hypothalamic regions, the question remains as to how the SCN communicates to extra-SCN molecular clocks. Glucocorticoids (CORT) are a promising candidate by which the SCN signals to other brain and body regions, as glucocorticoid receptors (GR) are found ubiquitously throughout the body, with the notable exception of the SCN. Furthermore, CORT is released in a circadian manner, with peak plasma levels occurring at the beginning of the animal's active phase. Interestingly, there is a hypersensitive glucocorticoid response element (GRE) in the promoter region of the *Per1* gene, which may be a mechanism by which CORT can modulate the molecular clock. We compared clock gene expression in mice that had a conditional forebrain glucocorticoid receptor knockout (FBGRKO) to expression in GR floxed mice (control genotype comparison) to determine the necessity of GRs in diurnal core clock gene expression. FBGRKO (C57BL/6 pure strain of the T29-1 founder line containing Cre+ recombinase transgene) mice have been previously well characterized to have disruptions in GR expression in the forebrain including the hippocampus (HPC), amygdala (AMY), cortex, and nucleus accumbens, while the central nucleus of the amygdala (CEA) had a 50% deletion and the paraventricular nucleus (PVN) was not affected. Mice were sacrificed under basal conditions in the light phase (zeitgeber time, ZT, 1.5) or dark phase (ZT13). In situ hybridization was used to measure mRNA expression. Our results show there is a time of day difference for *Per1*, *Per2*, and *Bmal1* clock genes mRNA expression in the SCN and for *Per1* and *Bmal1* mRNA in the PVN. Only *Bmal1* mRNA showed a time of day difference in subregions of the prefrontal cortex (PFC; anterior cingulate, prelimbic, infralimbic, ventral orbital), insula, subregions of the HPC (CA1, CA3, supra dentate gyrus, infra dentate gyrus), and subregions of the AMY (central, basolateral, medial). *Per1* mRNA expression had a significant time of day effect only in the medial amygdala, and *Per2* mRNA only in CA3. CA1 of the hippocampus did not show time of day differences for any clock gene investigated. The lack of a time of day effect in some brain regions may be due to the limited temporal resolution of the brain samples (only 2 time-points). There were no genotype differences for all brain regions examined. These results are expected in the SCN and PVN, as hypothalamic GRs would not be affected by the FBGRKO. The lack of a genotype effect in the HPC, AMY, PFC, and insula may be due to some phenotypic sparing of GR expression in those brain regions. It is also possible that CORT is not necessary for diurnal clock gene expression in these tissues or other possible mechanisms have been upregulated to compensate for the lack of GRs. CORT may also modulate the diurnal rhythm of *Per1, Per2,* and *Bmal1* mRNA in the forebrain regions through a series of neuronal projections that had spared GR expression.

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Introduction

Core clock gene expression underlies circadian rhythms in the master clock of the body, the suprachiasmatic nucleus (SCN), and extra-SCN tissues (Amir et al., 2004; Angeles-Castellanos et al., 2007; Masubuchi et al., 2000; Girotti et al., 2009; Chun et al., 2015). Glucocorticoid receptors (GRs) are found ubiquitously throughout the brain and body with the notable exception of the SCN. One of the core clock genes, *Per1*, has a glucocorticoid response element (GRE) in its promoter region where GRs bind after being activated by glucocorticoids (Yamamoto et al., 2005; So et al., 2009). The GRE in *Per1*'s promoter region may be a way in which glucocorticoids can entrain the molecular clock in extra-SCN tissue without disrupting the master clock functioning. In this study, mice had a forebrain-specific glucocorticoid receptor knockout. We hypothesized that *Per1* mRNA expression would be altered, which will influence the expression of other core clock genes in the forebrain, leading to a disrupted molecular clock in these brain regions. Core clock gene mRNA expression was then analyzed in SCN and extra-SCN brain tissues to investigate the necessity of GRs in regulating the molecular clock.

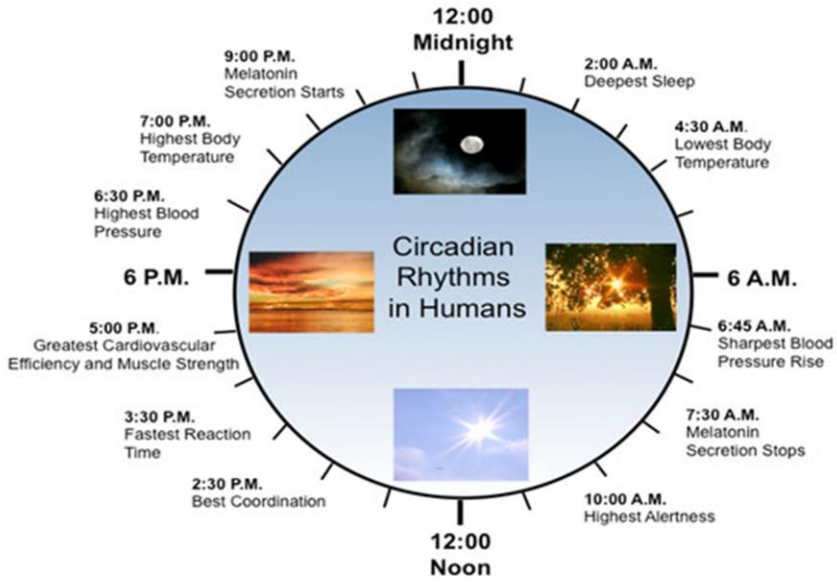


Figure 1. A few examples of mechanisms that oscillate in a circadian manner in humans. https://lookfordiagnosis.com/mesh_info.php?term=circadian+rhythm&lang=1

Regulation of circadian rhythms and clock gene expression

Circadian rhythms are essential in coordinating the proper timing of physiology and behavior (Hastings et al., 2003). Many aspects of physiology and behavior, including sleeping, eating, temperature regulation, and hormone secretion, have circadian rhythms (Figure 1). These rhythms oscillate in a circadian fashion, meaning they follow a 24-hour cycle. In humans, the circadian clock promotes sleeping during the night and being active during the day. Disruptions to these circadian rhythms, such as shifted or blunted cycles of body temperature, sleep/wake, blood pressure, and pulse, have been associated with many mood and anxiety disorders, including major depressive disorder, bipolar disorder, panic disorder, general anxiety disorder, post-traumatic stress disorder (PTSD), and seasonal affective disorder (Bunney et al., 2000; Atkinson et al., 1975; Kripke et al., 1978; Souetre et al., 1989).

Circadian rhythms are regulated by a few core clock genes that comprise the internal molecular clock. The molecular clock operates through a series of transcription and translation mechanisms of these core clock genes. This internal clock has a positive regulatory arm composed of Brain and Muscle Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT)-like protein-1 (*Bmal1*) and Circadian Locomotor Output Cycles Kaput (*Clock*) or Neuronal Per-Arnt-Sim (PAS) Domain Protein 2 (Npas2), a homolog to *Clock*. The negative regulatory arm consists of *Period (Per)* 1, 2, 3, and *Cryptochrome (Cry)* 1 and 2. Gene products from the positive arm enhance the transcription of the negative arm genes and the protein products of the negative arm inhibit their own transcription. As seen in Figure 2, BMAL1/CLOCK proteins dimerize in the nucleus of a cell, which serves as the transcription factor that binds to an Enhancer Box (E-Box), a DNA sequence within the promoter region of the clock genes of the negative arm, *Per1, 2, 3*,

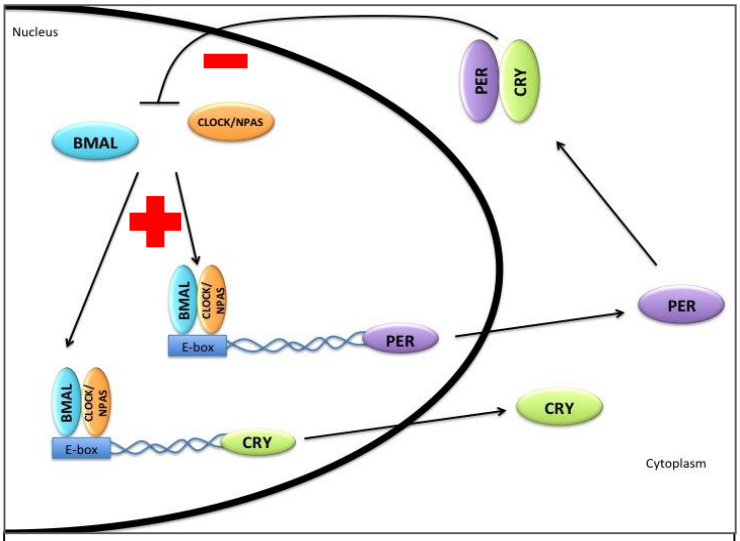


Figure 2. Self-regulatory negative and positive feedback loops comprised of the core clock genes: *Bmal1*, *Clock*/Npas1, *Per*, and *Cry*.

and *Cry1* and 2. *Per* and *Cry* mRNA are then brought into the cytosol where they are translated into proteins. PER/CRY proteins dimerize and enter the nucleus of the cell. Inside the nucleus, PER/CRY dimers inhibit the dimerization of BMAL1 and CLOCK/NPAS2 proteins, resulting in decreased transcription of the *Per* and *Cry* mRNA (Griffin et al., 1999; Kume et al., 1999; Shearman et al., 2000a). This creates a self-regulatory transcription and translation feedback loop system that approximates 24 hours to complete one cycle of activity.

24 hour basal clock gene expression has been well studied in the SCN and research is growing for extra-SCN central and peripheral tissues (Amir et al., 2004; Angeles-Castellanos et al., 2007; Masubuchi et al., 2000; Girotti et al., 2009; Chun et al., 2015). *Per1/2* and *Bmal1* mRNA expression tends to be antiphasic to each other in most brain and body regions, meaning that when one gene is at its peak levels, the other is at its trough levels (Girotti et al., 2009). Furthermore, mRNA expression in the SCN tends to be antiphasic to most other brain and body regions in nocturnal rodents. For example, the acrophase of *Per1* mRNA in the SCN occurs at zeitgeber time (ZT) 4, while *Per1* mRNA in the paraventricular nucleus (PVN) is at its trough. In circadian research time is referred to in zeitgeber time (ZT). For a 12:12 hour light:dark cycle, ZT0 is the time when lights are turned on, and ZT12 is the time

when lights are turned off. Similarly, when *Bmal1* mRNA peaks in the SCN at ZT16, it is at its trough in the PVN (Figure 3). Interestingly, basal clock gene expression of *Per2* mRNA in the central nucleus of the amygdala (CEA) seems to be in phase, rather than antiphasic like the PVN, with the SCN in rats (Chun et al., 2015; Harbour et al. 2013). The CEA is the only extra-SCN tissue that is known to have the same phase relationship as the SCN which may be due to the fact that they are both comprised predominantly of GABAergic neurons (Sun and Cassell 1993; Sun et al., 1994; Wagner et al., 1997). Whether this distinction has a functional significance and whether it is comparable in mice is yet to be determined.

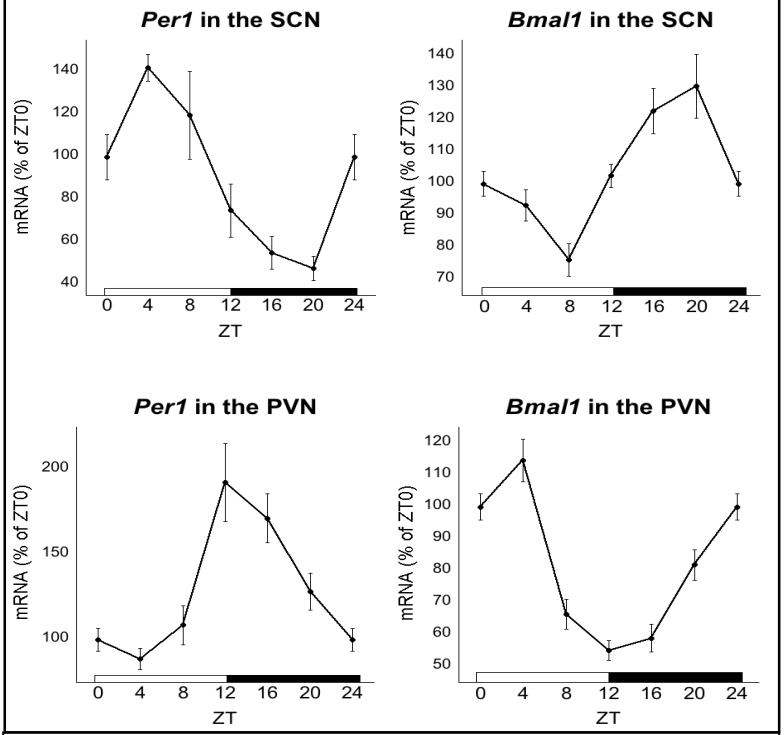


Figure 3. *Per1* and *Bmal1* basal clock gene expression in the SCN and PVN in male rats. Antiphasic relationships exist between *Per1* and *Bmal1* mRNA, as well as between the SCN and extra-SCN tissue (adapted from Chun et al., 2015).

The SCN of the hypothalamus is the master clock of the body and is believed to control the body's main circadian rhythm and communicate with peripheral brain and body regions about how and when to oscillate. The SCN is the primary region essential for receiving light input from the external environment and relaying this information to the rest of the body. Light is sensed by rods and cones in the retinas of the eyes and proceeds through the retinohypothalamic tract towards the SCN, where it serves as a strong entrainment factor (Figure 4; Ueyama et al., 1999). Melanopsin, a photopigment expressed in retinal ganglion cells in the retina of the eye directly project photic input to the SCN

synchronizing the period and phase of the circadian clock to the environment. Melanopsin photoreception has the ability to entrain circadian rhythms in mice lacking rods and cones, the primary visual photoreceptors in the eyes (Gooley et al., 2001; Warren et al., 2006). Retinal ganglion cells are primarily glutamatergic and act on AMPA/kainite receptors creating a response to light (Zhang et al., 2008; Wong et al., 2007). Thus, glutamatergic signaling from melanopsin in the retinal ganglion cells is believed to be one of the primary photopigment candidates that entrain the circadian clock to the light:dark cycle, especially in the absence of rods and cones. In the SCN, light has a significant impact on the body's natural circadian rhythms making it one of the most influential zeitgebers (time givers) to the molecular clock. Clock gene expression in the SCN has been well characterized, and light has been shown to be capable of resetting the circadian clock in the SCN by inducing *Per1* and *Per2* mRNA expression most potently during the dark phase (Oishi et al., 1998; Amir et al., 2004; Angeles-Castellanos et al., 2007; Masubuchi et al., 2000; Girotti et al., 2009; Chun et al., 2015; Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997).

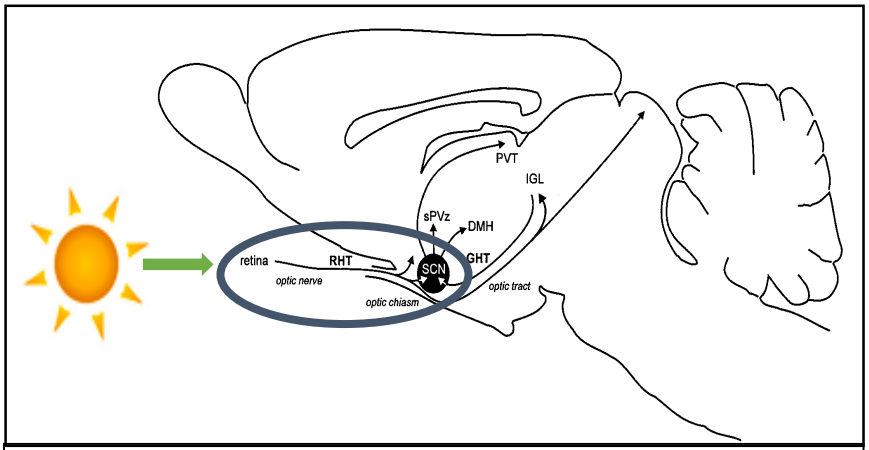


Figure 4. Light from the external environment enters into the eye through the retina and passes through the retinohypothalamic tract (RHT) to the SCN (source for sagittal brain slice).

Disruptions to the molecular clock have been associated with mood and anxiety disorders, and may underlie the alterations in circadian rhythms associated with these disorders (Bunney et al., 2008; Li et al., 2013). For example, Johansson et al. (2003) found that Npas2 may be associated with the development of seasonal affective disorder. Additionally, Benedetti et al. (2003) found that a single

nucleotide polymorphism (SNP) in the flanking region of *Clock* has a higher association with bipolar episodes and greater insomnia. Schizophrenia, alcoholism, and bipolar disorder may all be associated with SNPs in *Bmal1* and *Per3* genes (Nievergelt et al., 2006; Mansour et al., 2006). Further, in rodent studies, disruptions to normal clock gene expression have been associated with impaired cognition and emotional function (Takahashi et al., 2008; Garcia et al., 2000; McClung et al., 2007; Rawashdeh et al., 2014). These negative implications from disrupted clock gene expression show that normal, rhythmic clock gene expression is essential for maintaining mental health.

Role of glucocorticoids and their receptors

Glucocorticoids (CORT) are steroid hormones that fluctuate in a circadian manner with peak blood plasma levels of CORT occurring upon the beginning of the animal's active phase. In addition to circadian drive, CORT, cortisol in humans and corticosterone in rodents, also responds to stress and is the end product of the hypothalamic-pituitary-adrenal (HPA) axis, which is the body's hormonal stress response system. When the body receives a stressor, brain regions that are part of the limbic system (hippocampus, amygdala) and the prefrontal cortex receive input of the stress stimuli and then signal to the PVN of the hypothalamus (Herman et al., 2005). The PVN reacts to the stimulus by releasing corticotropin releasing hormone (CRH) which acts on the anterior pituitary gland. The anterior pituitary gland then releases adrenocorticotropic hormone (ACTH) which acts on the adrenal cortex of the adrenal gland to synthesize and release CORT. CORT enters the bloodstream, then negatively feeds back onto the HPA axis, thereby decreasing the release of CRH from the PVN and ACTH from the anterior pituitary, ultimately limiting its own future immediate release (Figure 5).

CORT is an important hormone in the body with various effects including glucose regulation, lipid metabolism, immune activity, stress response, learning, and memory (Kassel and Herrlich 2007; Popoli et al., 2011; Chung et al., 2011). It has been reported that CORT may have a wide range of effects because glucocorticoid receptors (GRs) are found ubiquitously throughout the brain and body, with the notable exception of the SCN (Reul and De Kloet 1985; Morimoto et al., 1996; Rosenfeld et al., 1988; Rosenfeld et al., 1993; Balsalobre et al., 2000). Under basal conditions, circulating CORT levels are low during the inactive phase and peak upon the beginning of the animal's active phase. Humans, for example, have low CORT at night and peak levels in the morning, around the time they wake. Many rodents, including rats and mice, are nocturnal animals and therefore have peak CORT levels at the onset of the dark phase and trough CORT levels during their light phase. Disruptions in the CORT circadian rhythm and hypersecretion of CORT have been associated with major depressive disorder, bipolar disorder, Cushing syndrome, diabetes, obesity, Alzheimer's disease, and metabolic syndrome (Daban et al., 2005; Chung et al., 2011; Dickmeis T, 2009; Herichova et al., 2005; Cermakian et al, 2011; Tahira et al., 2011).

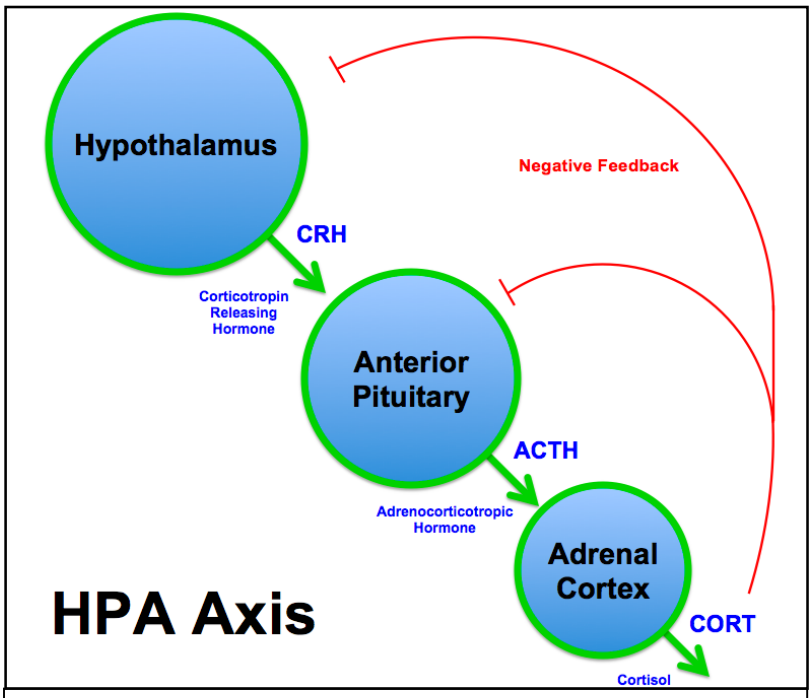


Figure 5. Representative image of negative feedback loops controlled by hypothalamic-pituitary-adrenal axis. CRH is released from the PVN of the hypothalamus which acts on the anterior pituitary to release ACTH. ACTH then acts on the adrenal cortex of the adrenal gland to release CORT. CORT can feedback and inhibit CRH release from the hypothalamus and ACTH release from the anterior pituitary.

In most extra-SCN brain regions and peripheral tissues, *Per1* mRNA peaks in the middle of the animal's active phase. Rats, for example, have peak *Per1* mRNA levels around ZT16, depending on the region of interest. Interestingly, there is a molecular connection between CORT and the *Per1* gene. The SCN has few direct projections to other brain and body regions, whereas GR is expressed throughout the brain and periphery. Due to this molecular connection between CORT and the *Per1* gene, and the fact that diurnal CORT circulation is under the control of the SCN, CORT should be considered a candidate secondary entrainer (Buijs et al., 1993). As seen in Figure 6, CORT binds to a glucocorticoid receptor (GR) which dimerizes and binds to a glucocorticoid response element (GRE). There is a GRE in the promoter region of the *Per1* gene which may be the path by which CORT acts to induce *Per1* mRNA expression, thereby entraining the molecular clock (Yamamoto et al., 2005; So et al., 2009). Considering that GRs are found throughout the brain and body, CORT may be a regulator of clock gene expression without affecting the SCN.

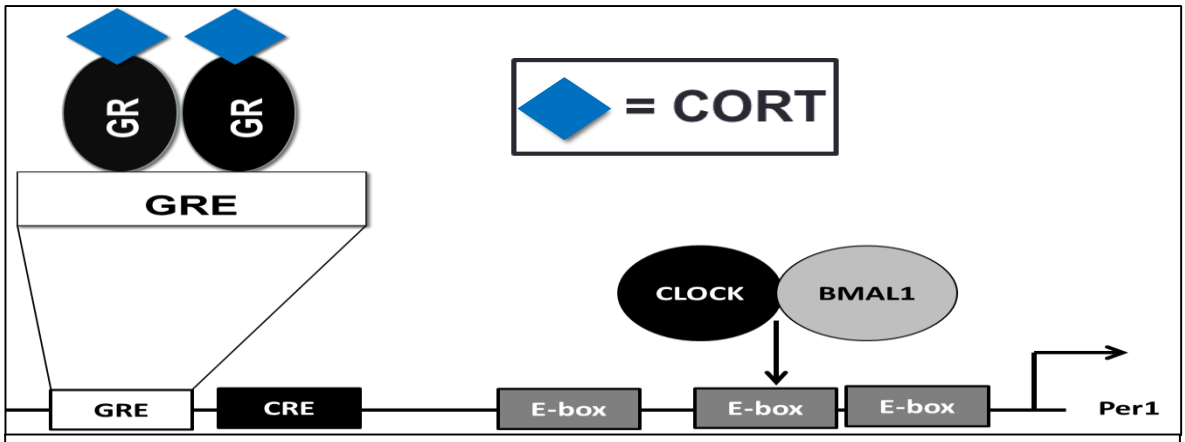


Figure 6. Representative image showing the binding of CORT to GRs which dimerize and bind to a GRE. The GRE is in the promoter region of *Per1* which is a clock gene that is also induced by the positive components of *Bmal1* and Clock/Npas2. Image adapted from Yamamoto et al., 2005.

Experimental design and study goals

Transgenic mice were bred to test the functionality of CORT, through GRs, as a regulatory entrainment factor of clock gene expression from the SCN to other brain and body regions. The breeder mice were created using a Cre recombinase and loxP system specifically using the CaMKIIα promoter. The loxP system is a molecular technique used to flank a specific region of DNA by inserting the loxP DNA sequence into the genome of embryonic stem cells. As the embryonic stem cells develop, the gene that is flanked by the loxP sites is then referred to as being "floxed". Once a sequence of the genome is floxed, the Cre recombinase recognizes these sequences and cuts at the loxP sites, thereby deleting or inverting the portion of the DNA enclosed by the loxP sites (Figure 7; Tsein et al., 1996; Lamont, K.).

The CaMKIIα promoter used in these mice is important because previous studies have demonstrated that the actions of CaMKIIα are restricted to the forebrain thereby creating a gene manipulation that is forebrain-specific (Mayford et al., 1995, 1996a, 1996b). In the mice used for this

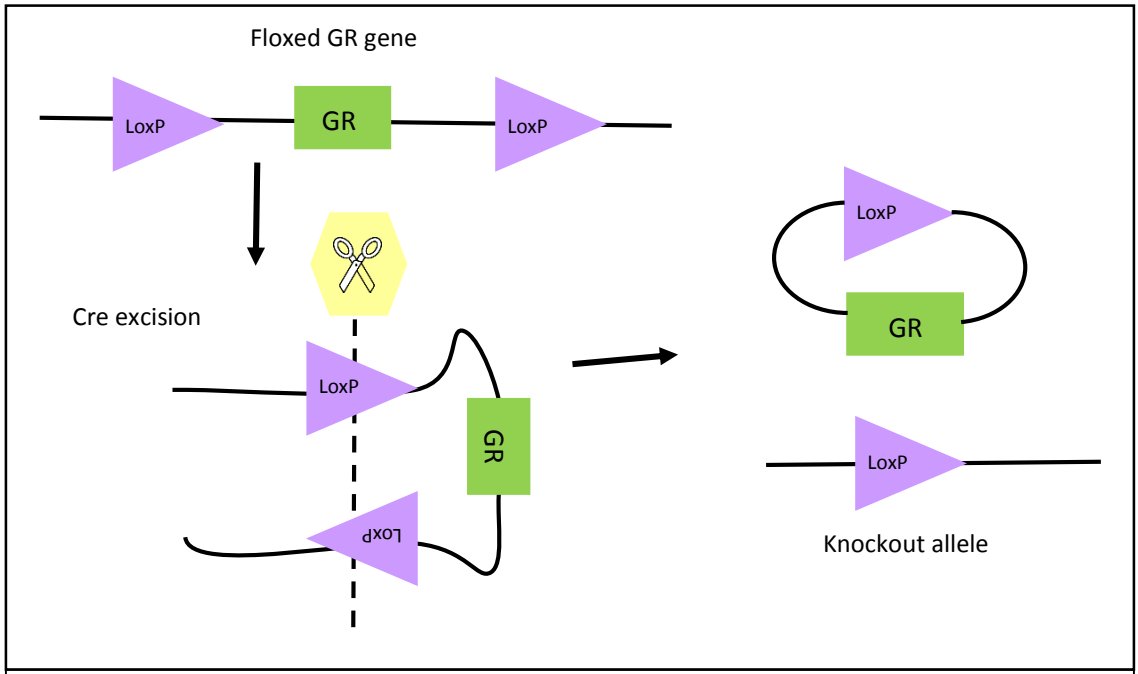


Figure 7. Mechanism for Cre recombinase and loxP system. The gene of interest (GR) is floxed by two loxP sites. Cre will then cut at the loxP sites, excising the gene of interest. Image adapted from Lamont, K.

study, DNA for the GR exon 2 is floxed and the Cre recombinase and CaMKIIα promoter (CaMKIIα-Cre) transgene ultimately deletes the GR exon 2 from the forebrain (Figure 8). Thus, mice that have a forebrain-specific knockout of glucocorticoid receptors were created and used for the current investigation to determine whether GRs are necessary for core clock gene expression. The mice that were GR floxed but lack the CaMKIIα-Cre transgene will be referred to as the "floxed" mice in this study

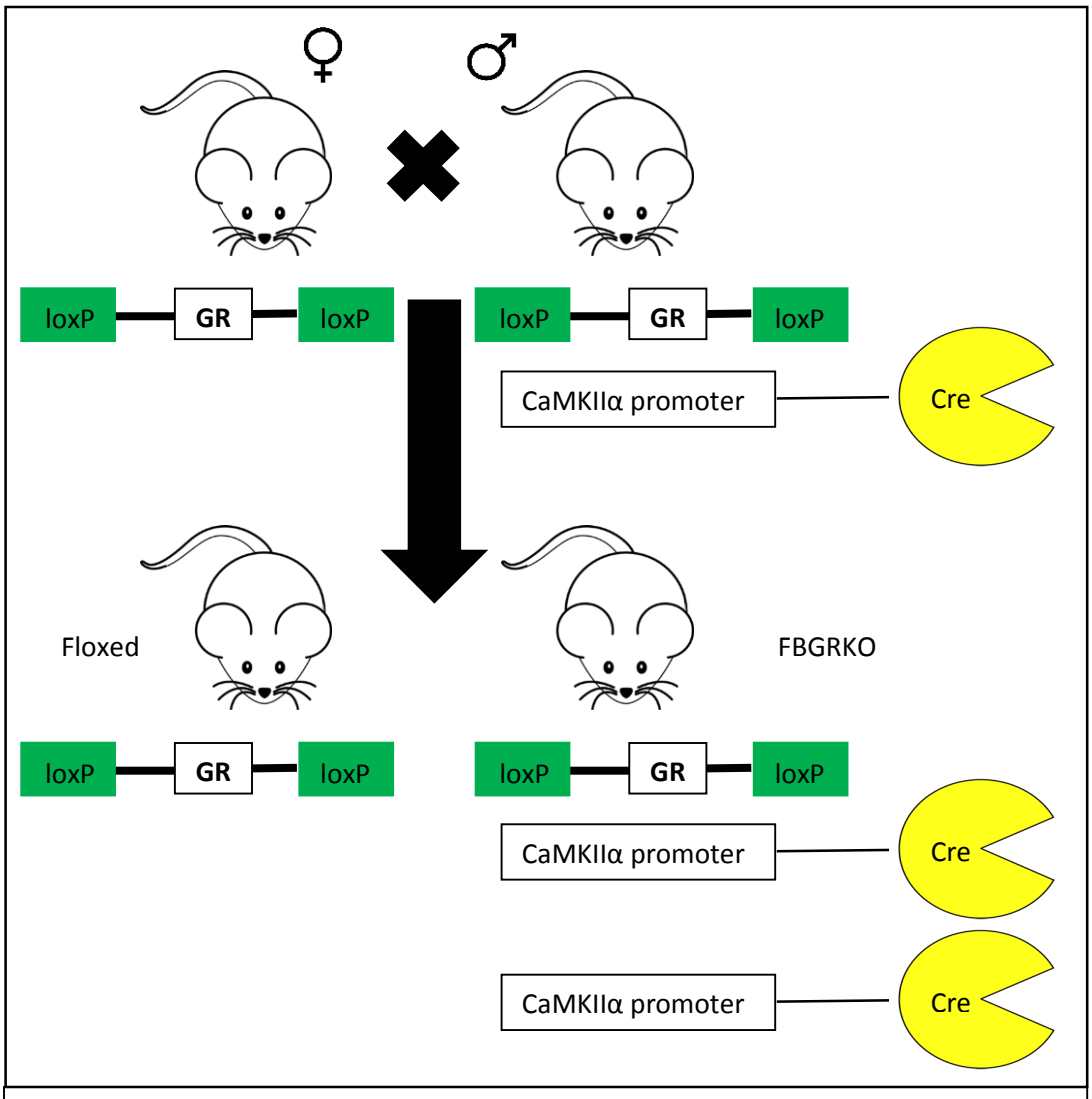


Figure 8. Representative image of FBGRKO mice. GR floxed female is crossed with GR floxed male hemizygous for the CaMKIIα-Cre transgene (represented by the single CaMKIIα-Cre transgene in image). Offspring are GR floxed or GR floxed with CaMKIIα-Cre transgene (FBGRKO). The offspring are homozygous for the CaMKIIα-Cre transgene which creates the complete GR knockout (represented by the two CaMKIIα-Cre transgenes in image).

and will serve as the controls, while those that contained both the GR floxed region and the CaMKIIα-Cre transgene will be referred to as the forebrain glucocorticoid receptor knockout (FBGRKO) mice.

The CaMKIIα-Cre transgene is ideal for this study as it is not fully active until 3 weeks of age. This makes it a good mechanism for manipulation because it reduces the possibilities of developmental defects or compensatory mechanisms caused by the early stage gene knockout (Tsien et al., 1996). The initial expression of CaMKIIα mRNA begins postnatally in the forebrain and floxed GR regions are progressively deleted from age 3-6 months in the hippocampus (HPC), prefrontal cortex (PFC), and some subregions of the amygdala (AMY) (Burgin et al., 1990). The hippocampus, cerebral cortex, striatum, nucleus accumbens, and dentate gyrus had nearly a complete deletion of GRs. The CEA had a 50% deletion of GRs while the basolateral nucleus and medial nucleus of the amygdala had a nearly complete deletion. The PVN and SCN were not affected because they do not express CaMKIIα (Vincent et al., 2013; Boyle et al., 2005; Kolber et al., 2008). This knockout of glucocorticoid receptors in the forebrain was specific to pyramidal cells which are primarily glutamatergic neurons and therefore, mostly excitatory cells (https://www.jax.org/strain/005359').

We investigated the FBGRKO effect on clock gene expression on many brain regions including the SCN, PVN, AMY, HPC, PFC, and insula. The SCN is essential to investigate because it is the master clock of circadian rhythms in the brain and body. The SCN is one of the only known regions in the brain and body that lacks GRs (Balsalobre et al., 2000). Therefore, with the FBGRKO manipulation, we expected to see no changes in clock gene expression in the SCN between the FBGRKO and floxed mice because this region already lacks GRs. We do expect a time of day difference in the clock gene expression in the SCN of the animals sacrificed in the morning compared to the evening, because *Per1*, *Per2*, and *Bmal1* mRNA all have robust rhythms in the SCN. The PVN is the head of the HPA axis, and thus has an important role in CORT production. Since the PVN does not express CaMKIIα, we also did not expect to see any effects from the FBGRKO manipulations. However, we expected to see significant

effects in all other regions that received a knockout of GRs. Subregions of the AMY (central nucleus of the amygdala (CEA), basolateral nucleus of the amygdala (BLA), medial nucleus of the amygdala (MEA)), subregions of the HPC (CA1, CA3, superior blade of the dentate gyrus (Supra DG), inferior blade of the dentate gyrus (Infra DG)), subregions of the PFC (anterior cingulate (AC), prelimbic (PL), infralimbic (IL), ventral orbital (VO)), and insula were analyzed for this study because of their essential role in stress, emotion, learning, and memory (Rawashdeh et al., 2014; Buijs et al., 1993; Herman et al., 2005; Logan et al., 2015; McClung et al., 2007). These brain regions are important in the exploration of the role of GRs on the molecular clock under basal conditions because they are known to have robust rhythmicity in clock gene expression and therefore we expect to see a time of day difference between the animals sacrificed in the morning and the evening for all clock genes examined. Many psychiatric disorders associated with disruptions in circadian rhythms and stress are also associated with disruptions in function and activity of these brain regions. Human postmortem subjects with major depressive disorder show altered core clock gene diurnal rhythms in extra-SCN brain regions including the PFC, HPC, AMY, and nucleus accumbens, and chronic stress can disrupt normal clock gene expression in many relevant brain areas (Li et al., 2013; Logan et al., 2015). We hypothesized that the knockout of GRs in these brain regions would alter clock genes mRNA expression in the AMY, HPC, PFC, and insula due to the disruption of CORT's ability to bind to GRs.

This study investigated *Per1*, *Per2*, and *Bmal1* mRNA clock gene expression in SCN and extra-SCN tissues of FBGRKO and floxed mice sacrificed at ZT1.5 or ZT13 to investigate the necessity of GRs for differences in clock gene expression. These genes were examined in order to see clock gene expression in both the positive and negative components of the molecular clock after the FBGRKO manipulation. Due to the given sacrifice times of the animals, a significant time of day effect is expected in some but not all of the tissues investigated because the peak and trough times of these genes are not all at the sacrifice times of ZT1.5 and ZT13 in all the examined tissues. Although we do expect to see a significant

difference in clock gene expression between the FBGRKO and floxed mice for all clock genes examined, we hypothesize that due to the knockout of GRs in the forebrain, the GRE will not be able to induce *Per1* clock gene expression, which will then affect the feedback onto the other core clock genes and alter the rhythms of clock gene expression in the forebrain. Furthermore, comparing findings of the present study to previous reports of rats will help broaden the knowledge of core clock gene expression in mice. This study provides an initial understanding of the role of GRs in diurnal core clock gene expression in mice.

Materials and Methods

The following were completed by Dr. Jacobson's lab

Animals

The initial transgenic mice used to breed the mice for the current study were characterized and provided by Dr. Louis Muglia (Cincinnati, Ohio) and given to Dr. Lauren Jacobson (Albany, NY). Dr. Jacobson's lab then bred the mice for the current study, sacrificed the animals, froze the brain tissue, and delivered them to Dr. Spencer's lab (Boulder, CO) where clock gene expression was analyzed. All animal use was approved by the Institutional Animal Care and Use Committee of Albany Medical College and followed the standards of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 2010) and European Commission Directive 2010/63/EU. Male mice were from the T-29 founder line aged ~2-5 months at time of experimentation were group-housed on 12:12 hour light:dark cycles (lights on at 06:30) with ad libitum access to rodent chow and water. Animals were individually housed 24 hours before experimental testing to reduce disturbances during testing. FBGRKO and floxed mice were littermate- or age-matched for all experiments.

Forebrain Glucocorticoid Receptor Knockout Mice

Calcium calmodulin kinase II α (CaMKIIα) is a gene that is selectively expressed in glutamatergic neurons in the forebrain of mice. T-29 mice are transgenic for the Cre recombinase gene under control of the CaMKIIα gene promoter. Therefore, since CaMKIIα gene expression is specific for the forebrain, the excision actions of Cre recombinase also only occur in the forebrain. C57BL/6 female mice homozygous for the floxed GR exon 2 (provided by Dr. Louis Muglia, University of Cincinnati; Brewer et al., 2003) were crossed with C57BL/6 male mice from the T-29-1 founder line with the CaMKIIα-Cre transgene under CaMKIIα promoter control (Jackson Laboratories stock number 005359; Bar Harbor, ME; Vincent et al., 2013). This breeding resulted in C57BL/6 floxed GR mice which were either Cre+ (FBGRKO) or Cre- (floxed GR) mice (Figure 8). The Cre+ (FBGRKO) mice littermates had a forebrain-specific disruption of GR that has been documented in previous reports (Vincent et al., 2013; Boyle et al., 2005; Kolber et al., 2008; Brewer et al., 2003; Tsein et al., 1996; https://www.jax.org/strain/005359.; http://www.informatics.jax.org/allele/MGI:4946944?recomRibbon=open).

Tail DNA was obtained (Invitrogen PureLink Genomic DNA kit; Carlsbad, CA) and screened by Polymerase Chain Reaction (PCR) for the presence or absence of the Cre recombinase transgene and for homozygosity of the floxed GR allele (Vincent et al., 2013).

Tissue Collection

Animals were taken from their home cage and individually housed for 24 hours prior to sacrifice. 38 animals were taken from these cages and immediately sacrificed at 1.5 hours after lights on (ZT1.5) or at 1 hour after lights off (ZT13). Number of animals in each condition combination of genotype and sacrifice time ranged from 7-10. Brains were then extracted and fresh-frozen in molds with Optimal

Cutting Temperature (OCT) compound and stored frozen at -70° C until further use. Frozen brains were shipped to the University of Colorado Boulder for subsequent processing.

The following were completed by Dr. Spencer's lab

Processing the mice brains

Coronal sections (12µm thick) were cut at -24° C using a cryostat (Leica CM 1850) at the levels of the prefrontal cortex (Bregma ~1.98 to 1.54 mm anterior to Bregma), suprachiasmatic nucleus (Bregma ~0.34 to 0.94mm posterior to Bregma), paraventricular nucleus (Bregma ~0.7 to 0.94 mm posterior to Bregma), and hippocampus and amygdala (Bregma ~1.34 to 2.06mm posterior to Bregma). The *Paxinos and Franklin* (second edition, 2001) Mouse Brain Atlas was used for guidance. Brain slices were thaw-mounted on Colorfrost Plus microscope glass slides where they were then stored at -70° C until use in assays.

In Situ Hybridization

In situ hybridization protocols used a radiolabeled riboprobe which was complementary to the mRNA for the gene of interest. The riboprobe binds to the gene of interest and the radioactively labeled nucleotide in the probe shows how much of that gene is being expressed. This two-day assay was performed as previously described by Girotti et al. (2009) and Ginsberg et al. (2003). Briefly, the hybridization buffer was mixed with the previously made ^{35}S radiolabeled riboprobe (specific to the gene of interest) using a series of incubations at 37 ° C and further separated in a G50/50 sephadex column. Glass slides were taken from storage in -70° C and put immediately into 4% paraformaldehyde (PFA) in phosphate buffer and then washed in 2 x standard sodium citrate buffer (SSC). Slides were then placed

in a solution containing 0.1M triethanolamine (TEA) and 0.25M acetic anhydride and dehydrated in increasing levels of ethanol before being allowed to air dry. Once slides were dry, 98 µl of the probe/hybridization buffer combination were pipetted onto each slide and coverslipped, giving each slide ~1,000,000 counts of radioactivity. Slides were incubated for 16-22 hours overnight in trays containing a 50% formamide humidified atmosphere at 54 ^{o}C.

The second day of the in situ hybridization involved floating off the coverslips from the slides in each tray using 2 x SSC, then incubating slides in a 200 µg/mL RNAse solution (cat No. R5503; Sigma, St. Louis, MO) for 1 hour at 37 ^{o}C. Slides were then rinsed with decreasing concentrations of SSC washes (2x, 1x, 0.5x, 0.1x) and then incubated for an hour in 0.1 x SSC at 65 ^{o}C. Slides were dehydrated in increasing levels of ethanol and allowed to air dry before being exposed to x-ray film for 2-4 weeks.

Densitometry

Autoradiographs are the brain images produced on the x-ray films by decay emissions of the radioactive ^{35}S from the in situ hybridization assay. X-ray films were developed using an auto-developer (Konica Minolta Medical and Graphic, Inc.). Digitized brain images were obtained using a Northern Light lightbox model B95 (Imaging Res Inc., St. Catharines, Ontario, Canada) and a Sony CCD video camera model XC-ST70 fitted with a Navitar 7000 zoom lens (Rochester, NY) connected to an LG3-01 frame grabber (Scion Corp., Frederick, MD) inside a Dell Dimension 500, and captured with Scion Image beta rel. 4.0.2. Images were opened in ImageJ64 (NIH shareware), where the specific brain regions of interest for both hemispheres were selected for quantification based upon anatomical landmarks using a circular outline and converted into uncalibrated optical densities. Regions of interest include the suprachiasmatic nucleus (SCN), paraventricular nucleus (PVN), agranular insular cortex (insula), subregions of the prefrontal cortex: anterior cingulate (AC), prelimbic (PL), infralimbic (IL), ventral orbital

(VO), subregions of the hippocampus: CA1, CA3, superior blade of the dentate gyrus (Supra DG), inferior blade of the dentate gyrus (Infra DG), and subregions of the amygdala: central nucleus (CEA), basolateral nucleus (BLA), and medial nucleus (MEA). Regions of interest were determined using the *Paxinos and Franklin* (second edition, 2001) Mouse Brain Atlas.

Statistical Analysis

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS, Mac version 21, 2012). Two-way analyses of variance (ANOVA) were performed to determine if there were significant main effects of time of day (ZT1.5 vs. ZT13) or genotype (FBGRKO vs. floxed) and significant interactions for each brain region and gene of interest. Significance was set at $p<.05$. Fischer's least significant difference (FLSD) post hoc analysis was then completed for brain regions of each gene showing significant differences.

Results

Table 1. Statistical analysis of *Per1*, *Per2*, and *Bmal1* mRNA expression comparing genotype (FBGRKO vs floxed) and time of day (ZT1.5 vs ZT13) differences throughout the brain.

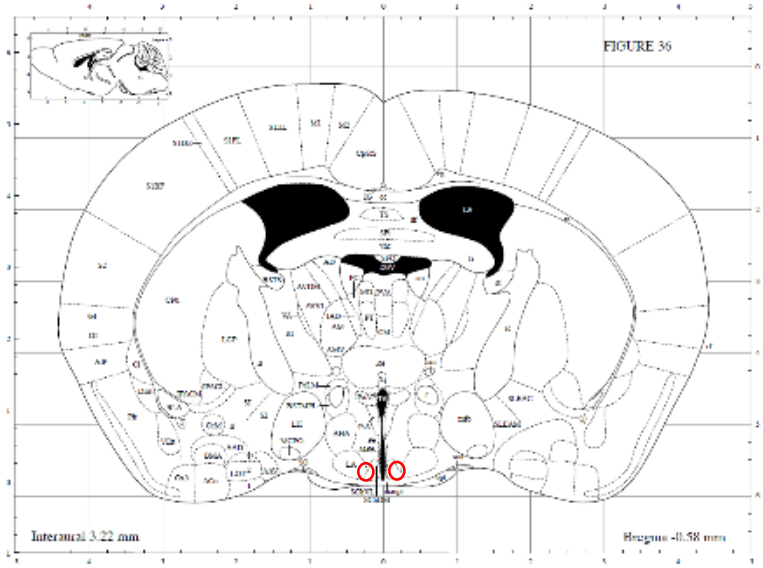
Brain region	Gene of interest	Genotype	ZT	Interaction
SCN	*Per1*	$F_{(1,32)}$=0.01	$F_{(1,32)}$=4.33*	$F_{(1,32)}$=0.00
	Per2	$F_{(1,33)}$=0.02	$F_{(1,33)}$=96.81**	$F_{(1,33)}$=0.83
	Bmal1	$F_{(1,33)}$=1.02	$F_{(1,33)}$=7.47*	$F_{(1,33)}$=0.14
PVN	*Per1*	$F_{(1,32)}$=0.54	$F_{(1,32)}$=46.67**	$F_{(1,32)}$=0.01
	Per2	$F_{(1,33)}$=0.03	$F_{(1,33)}$=2.54	$F_{(1,33)}$=0.53
	Bmal1	$F_{(1,33)}$=2.06	$F_{(1,33)}$=23.75**	$F_{(1,33)}$=0.13
CEA	*Per1*	$F_{(1,30)}$=0.00	$F_{(1,30)}$=3.92	$F_{(1,30)}$=0.56
	Per2	$F_{(1,33)}$=0.28	$F_{(1,33)}$=0.11	$F_{(1,33)}$=0.71
	Bmal1	$F_{(1,32)}$=0.28	$F_{(1,32)}$=12.71**	$F_{(1,32)}$=0.10
BLA	*Per1*	$F_{(1,30)}$=0.37	$F_{(1,30)}$=3.24	$F_{(1,30)}$=0.37
	Per2	$F_{(1,33)}$=0.00	$F_{(1,33)}$=0.14	$F_{(1,33)}$=0.16
	Bmal1	$F_{(1,32)}$=0.09	$F_{(1,32)}$=16.24**	$F_{(1,32)}$=0.00
MEA	*Per1*	$F_{(1,30)}$=0.00	$F_{(1,30)}$=5.19*	$F_{(1,30)}$=0.54
	Per2	$F_{(1,33)}$=0.48	$F_{(1,33)}$=0.14	$F_{(1,33)}$=0.70
	Bmal1	$F_{(1,32)}$=0.76	$F_{(1,32)}$=12.70**	$F_{(1,32)}$=0.12
CA1	*Per1*	$F_{(1,30)}$=0.64	$F_{(1,30)}$=0.41	$F_{(1,30)}$=0.40
	Per2	$F_{(1,32)}$=0.01	$F_{(1,32)}$=1.47	$F_{(1,32)}$=0.47
	Bmal1	$F_{(1,32)}$=0.40	$F_{(1,32)}$=2.55	$F_{(1,32)}$=0.30
CA3	*Per1*	$F_{(1,30)}$=0.11	$F_{(1,30)}$=0.02	$F_{(1,30)}$=0.41
	Per2	$F_{(1,32)}$=0.00	$F_{(1,32)}$=5.87*	$F_{(1,32)}$=0.38
	Bmal1	$F_{(1,32)}$=1.37	$F_{(1,32)}$=8.72*	$F_{(1,32)}$=1.42
Supra DG	*Per1*	$F_{(1,30)}$=0.02	$F_{(1,30)}$=0.12	$F_{(1,30)}$=0.03
	Per2	$F_{(1,32)}$=0.01	$F_{(1,32)}$=1.25	$F_{(1,32)}$=0.24
	Bmal1	$F_{(1,32)}$=0.42	$F_{(1,32)}$=5.80*	$F_{(1,32)}$=0.02
Infra DG	*Per1*	$F_{(1,30)}$=0.14	$F_{(1,30)}$=0.81	$F_{(1,30)}$=0.12
	Per2	$F_{(1,32)}$=0.02	$F_{(1,32)}$=0.87	$F_{(1,32)}$=1.03
	Bmal1	$F_{(1,32)}$=0.54	$F_{(1,32)}$=7.56*	$F_{(1,32)}$=0.17
AC	*Per1*	$F_{(1,32)}$=1.69	$F_{(1,32)}$=0.00	$F_{(1,32)}$=0.29
	Per2	$F_{(1,32)}$=0.58	$F_{(1,32)}$=1.23	$F_{(1,32)}$=0.30
	Bmal1	$F_{(1,33)}$=0.33	$F_{(1,33)}$=16.66**	$F_{(1,33)}$=0.23
PL	*Per1*	$F_{(1,32)}$=0.63	$F_{(1,32)}$=0.45	$F_{(1,32)}$=0.02
	Per2	$F_{(1,32)}$=0.27	$F_{(1,32)}$=0.25	$F_{(1,32)}$=0.15
	Bmal1	$F_{(1,33)}$=0.13	$F_{(1,33)}$=8.12*	$F_{(1,33)}$=0.48
IL	*Per1*	$F_{(1,32)}$=1.13	$F_{(1,32)}$=1.32	$F_{(1,32)}$=0.22
	Per2	$F_{(1,32)}$=0.58	$F_{(1,32)}$=0.00	$F_{(1,32)}$=0.02
	Bmal1	$F_{(1,33)}$=0.04	$F_{(1,33)}$=5.37*	$F_{(1,33)}$=0.07
VO	*Per1*	$F_{(1,32)}$=1.64	$F_{(1,32)}$=0.48	$F_{(1,32)}$=0.74
	Per2	$F_{(1,32)}$=1.23	$F_{(1,32)}$=0.42	$F_{(1,32)}$=0.39
	Bmal1	$F_{(1,33)}$=0.50	$F_{(1,33)}$=36.21**	$F_{(1,33)}$=0.41
Insula	*Per1*	$F_{(1,32)}$=1.56	$F_{(1,32)}$=0.23	$F_{(1,32)}$=0.16
	Per2	$F_{(1,32)}$=0.26	$F_{(1,32)}$=0.07	$F_{(1,32)}$=0.00
	Bmal1	$F_{(1,33)}$=1.09	$F_{(1,33)}$=26.86**	$F_{(1,33)}$=1.99

Two-way analysis of variance results for main effect of genotype, time of day, and interaction, *$p<0.05$, **$p\leq0.001$. SCN – suprachiasmatic nucleus, PVN – paraventricular nucleus, CEA – central nucleus of the amygdala, BLA – basolateral nucleus of the amygdala, MEA – medial nucleus of the amygdala, Supra DG – superior blade of the dentate gyrus, Infra DG – inferior blade of the dentate gyrus, AC – anterior cingulate, PL – prelimbic, IL – infralimbic, VO – ventral orbital, insula – agranular insular cortex

Hypothalamus

SCN

9A.



9B.

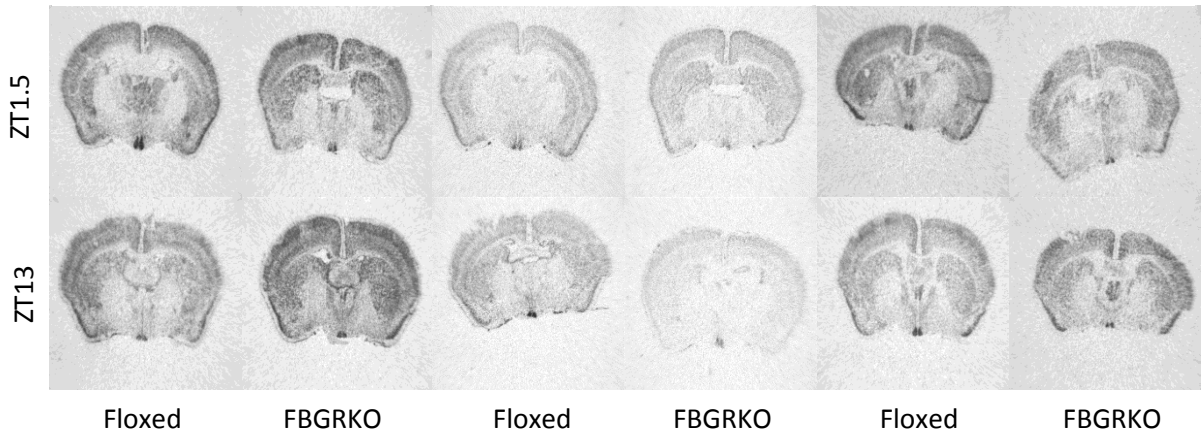


Figure 9. Representative images of the SCN. (A) *Paxinos and Franklin* Mouse Brain Atlas image highlights the SCN. (B) Representative autoradiographs for *Per1*, *Per2*, and *Bmal1* mRNA clock genes at the level of the SCN at ZT1.5 and ZT13 for both floxed and FBGRKO mice.

Mouse Brain Atlas image and representative autoradiographs at the level of the SCN for FBGRKO and floxed brains for all clock genes are shown in Figure 9. There were no significant genotype effects in the SCN for *Per1*, *Per2*, or *Bmal1* mRNA. There was a significant time of day effect for *Per1*, *Per2*, and *Bmal1* mRNA (Table 1). Post hoc analysis revealed ZT13 had higher optical density values than ZT1.5 in *Per2* mRNA for both floxed (p=0.000) and FBGRKO (p=0.000) mice as well as in *Bmal1* mRNA for floxed mice (p=0.030) (Figure 10).

10.

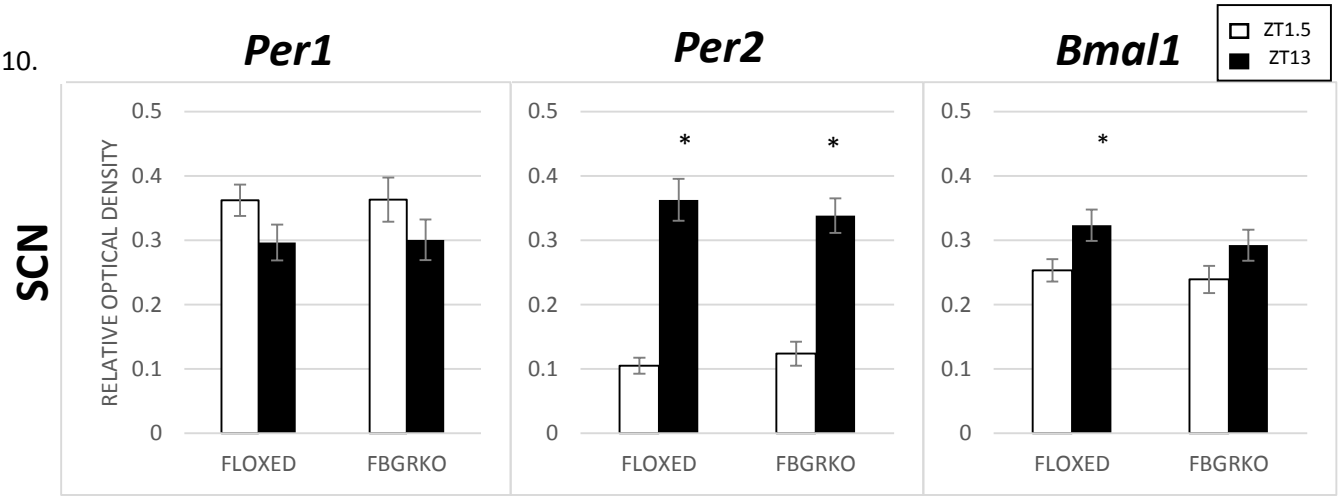
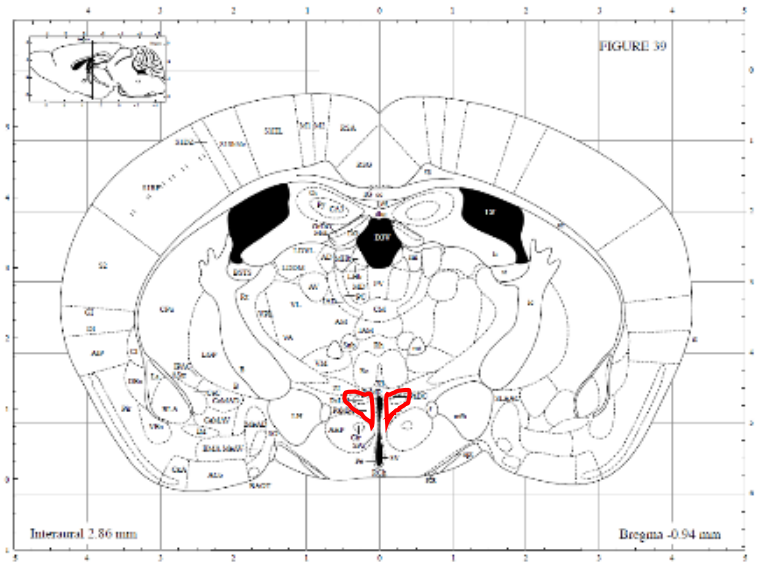


Figure 10. Relative optical density levels for *Per1*, *Per2*, and *Bmal1* mRNA clock gene expression in the SCN. *p<0.05 represents significant statistical difference from AM values.

PVN

11A.



11B.

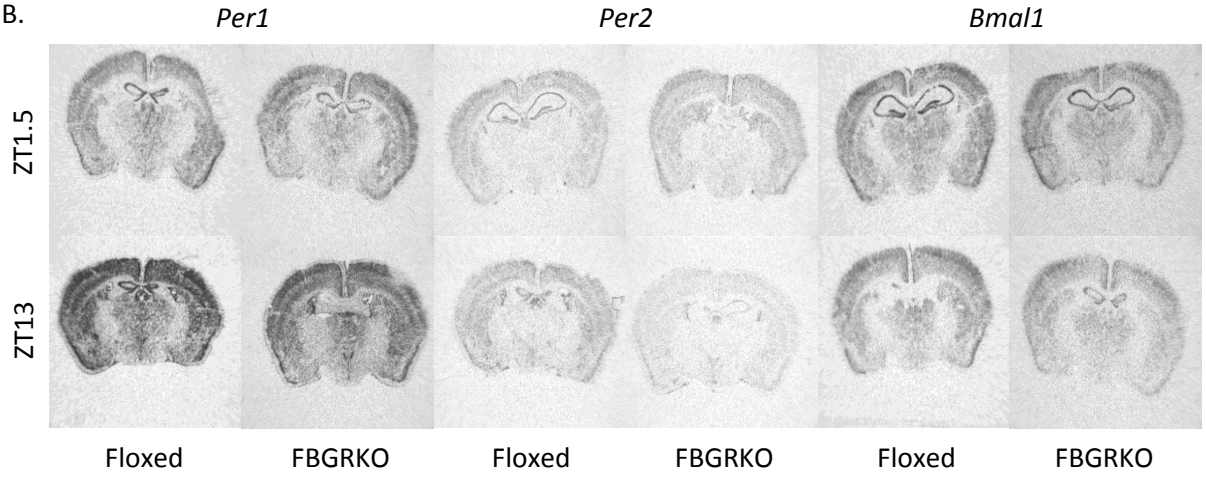


Figure 11. Representative images of the PVN. (A) *Paxinos and Franklin* Mouse Brain Atlas image highlights the PVN. (B) Representative autoradiographs for *Per1*, *Per2*, and *Bmal1* mRNA clock genes at the level of the PVN at ZT1.5 and ZT13 for both floxed and FBGRKO mice.

Mouse Brain Atlas image and representative autoradiographs at the level of the PVN for FBGRKO and floxed brains for all clock genes are shown in Figure 11. There were no significant genotype effects in the PVN for *Per1*, *Per2*, or *Bmal1* mRNA. However, there was a significant time of day effect for *Per1* and *Bmal1* mRNA, but not *Per2* mRNA. Post hoc analysis revealed that *Per1* mRNA had higher clock gene expression at ZT13 for both floxed (p=0.000) and FBGRKO (p=0.000) mice while *Bmal1* mRNA had higher clock gene expression in the PVN at ZT1.5 for both floxed (p=0.001) and FBGRKO (p=0.004) mice (Figure 12).

12.

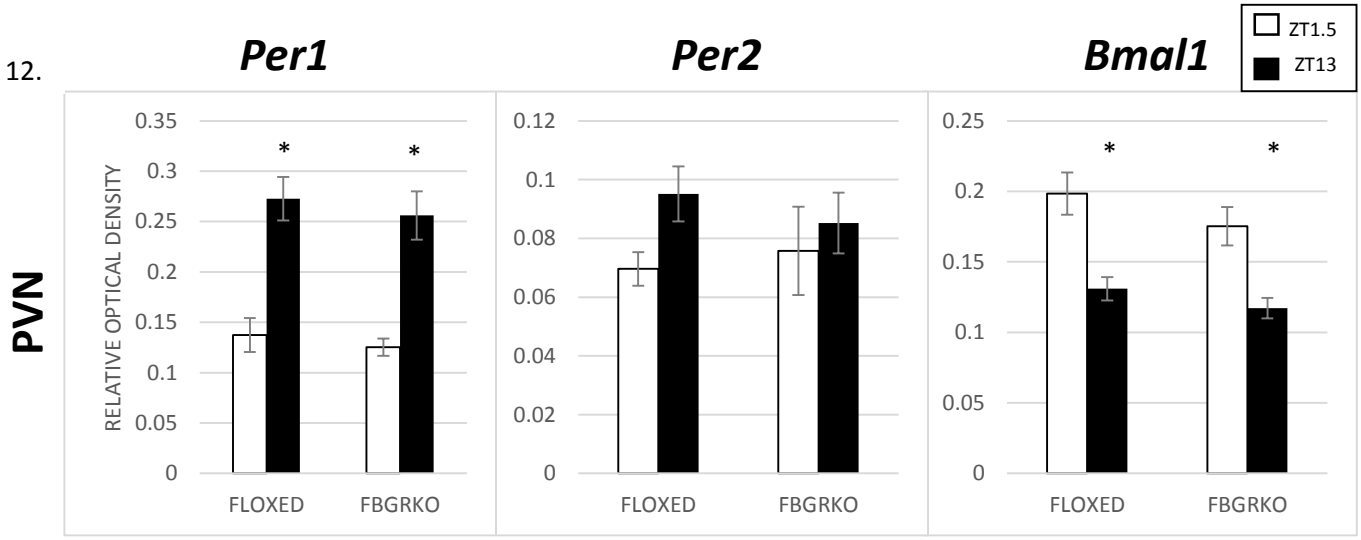
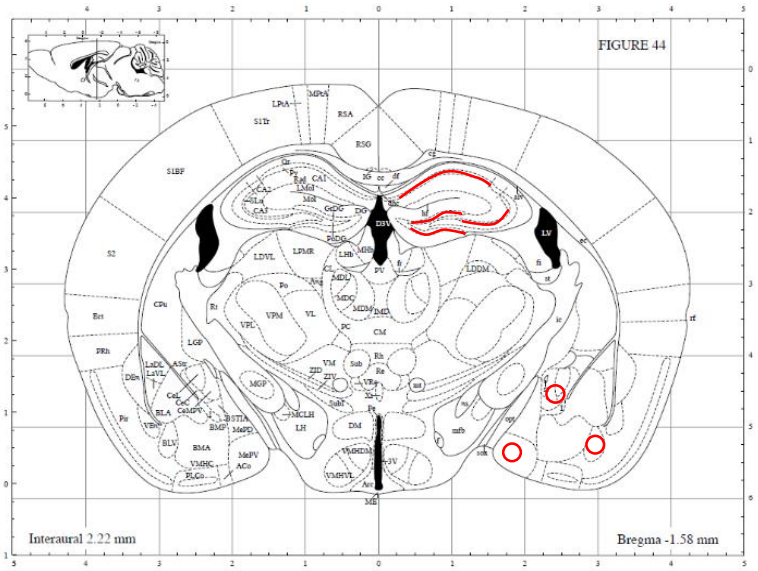


Figure 12. Relative optical density levels for *Per1*, *Per2*, and *Bmal1* mRNA clock gene expression in the PVN. *p<0.05 represents significant statistical difference from AM values.

Limbic System

Right hemisphere of subregions of the hippocampus (CA1, CA3, Supra DG, Infra DG) and amygdala (CEA, BLA, MEA) are highlighted in the *Paxinos and Franklin* Mouse Brain Atlas image of Figure 13A with representative images from each time and gene in Figure 13B.

13A.



13B.

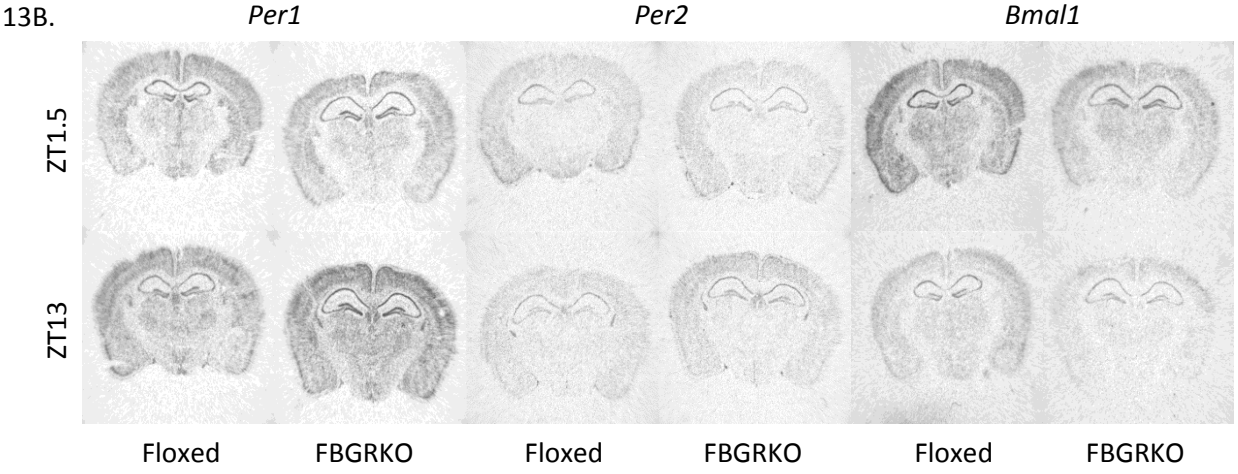


Figure 13. Representative images of subregions of the HPC and AMY. (A) *Paxinos and Franklin* Mouse Brain Atlas images of the HPC and AMY. Lines define right hemisphere regions of the HPC: CA1 (top), CA3 (middle, right), Supra DG (middle, left), and Infra DG (bottom). Circles define right hemisphere regions of the AMY: CEA (top), BLA (right), MEA (left). (B) Representative autoradiographs for *Per1*, *Per2*, and *Bmal1* mRNA clock genes at the level of the HPC and AMY at ZT1.5 and ZT13 for both floxed and FBGRKO mice.

Amygdala

There were no significant genotype differences for *Per1*, *Per2*, or *Bmal1* clock gene mRNA expression in the CEA, BLA, or MEA. *Bmal1* mRNA, however, did show a significant time of day effect in the CEA, BLA, and MEA. *Per1* mRNA also had a significant time of day effect in the MEA and showed a trend in the CEA. Post hoc analysis revealed that *Bmal1* mRNA levels were higher at ZT1.5 than ZT13 for both floxed (p=0.007) and FBGRKO (p=0.035) mice in the CEA, floxed (p=0.005) and FBGRKO (p=0.011) mice in the BLA, and floxed (p=0.007) and FBGRKO (p=0.036) mice in the MEA (Figure 14).

Hippocampus

There were no significant genotype effects for *Per1*, *Per2*, or *Bmal1* mRNA. The CA1 of the HPC showed no significant time of day differences for any of the clock genes examined. However, *Bmal1* mRNA had a significant time of day difference in the CA3, Supra DG, and Infra DG. The CA3 also had a significant time of day effect for *Per2* mRNA. Post hoc analysis revealed that *Per2* (p=0.048) and *Bmal1* (0.008) clock gene mRNA expression was significantly higher at ZT1.5 in the CA3 for FBGRKO mice. The Infra DG showed significantly higher *Bmal1* clock gene mRNA expression at ZT1.5 than ZT13 in FBGRKO mice (Figure 15). The Supra DG showed similar results to the infra DG, but time of day effect did not reach significance (p=0.418) (data not shown).

14.

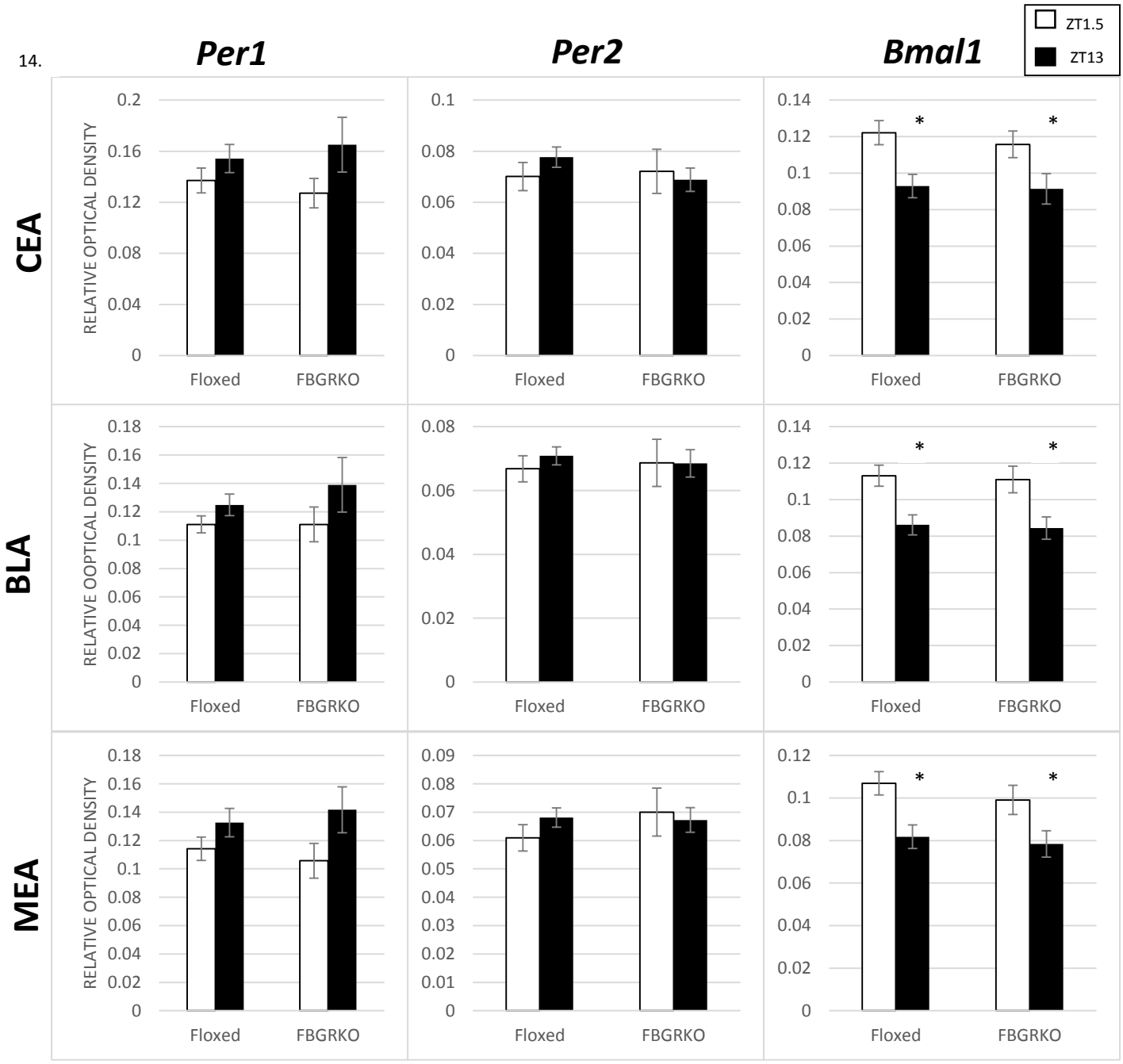


Figure 14. Relative optical density for *Per1*, *Per2*, and *Bmal1* mRNA clock gene expression for subregions of the AMY: CEA, BLA, and MEA. *p<0.05 represents significant statistical difference from AM values.

15.

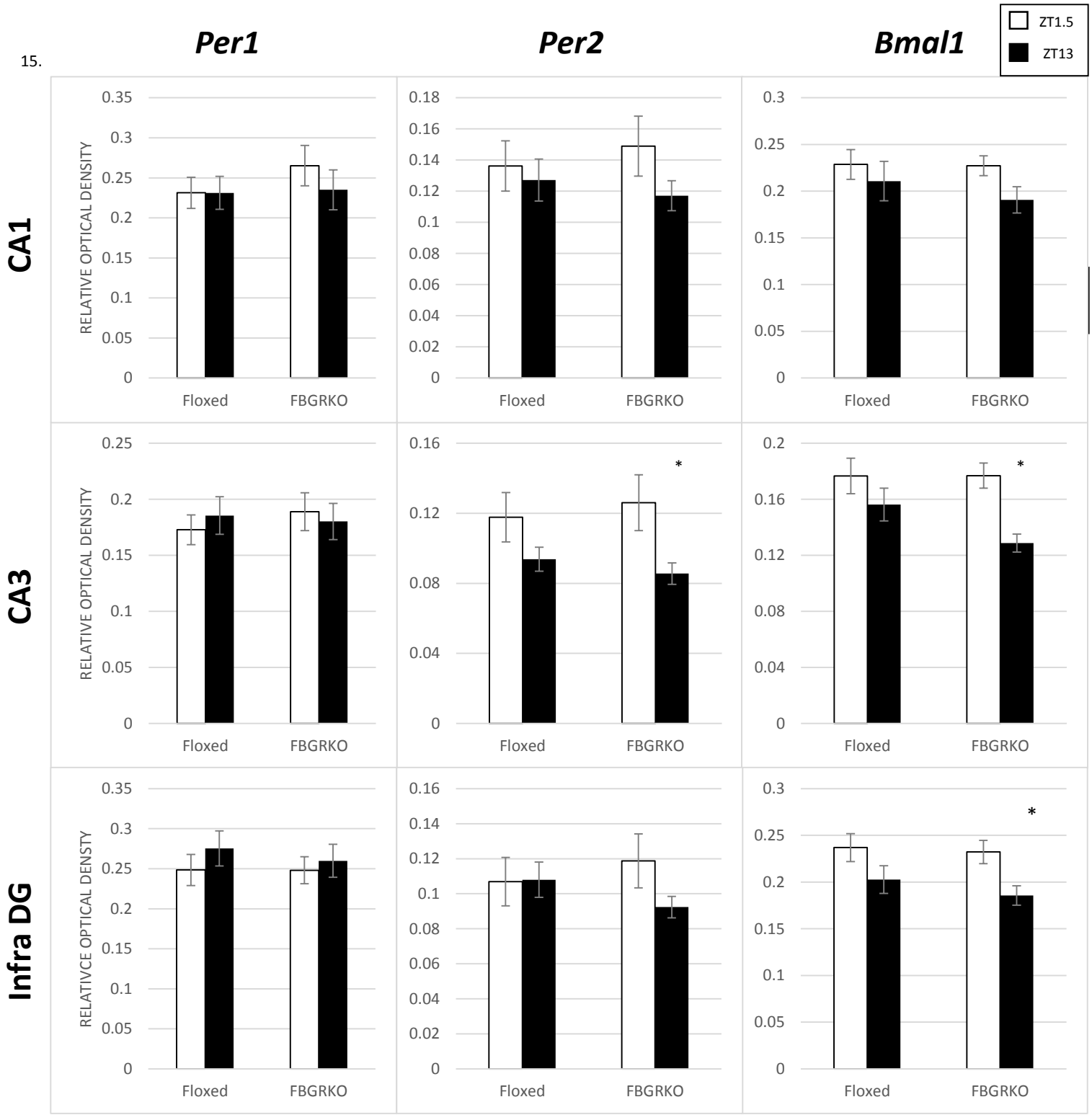
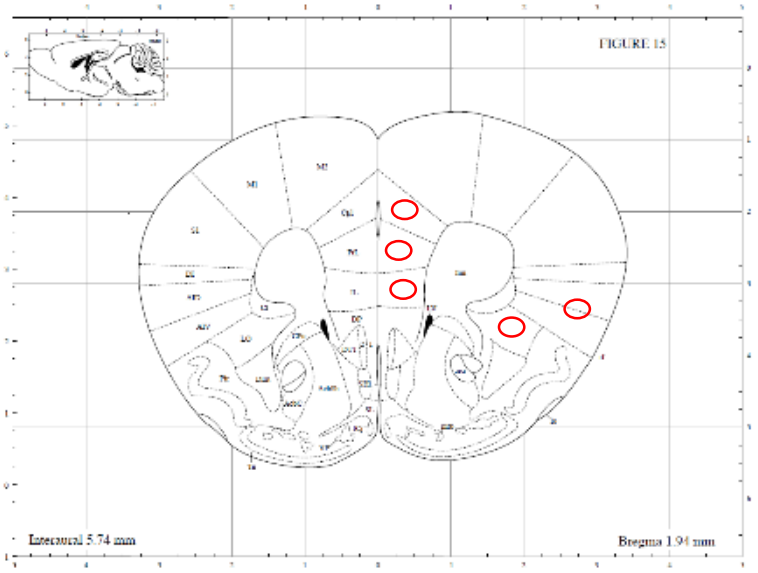


Figure 15. Relative optical density for *Per1*, *Per2*, and *Bmal1* mRNA clock gene expression for subregions of the HPC: CA1, CA3, and Infra DG. *p<0.05 represents significant statistical difference from AM values.

Prefrontal Cortex and Insula

16A.



16B.

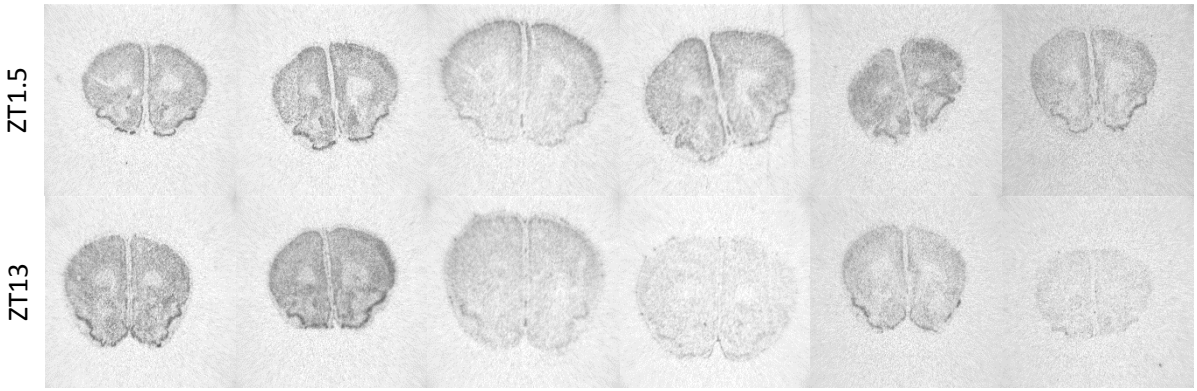


Figure 16. Representative images of subregions of the PFC and insula. (A) *Paxinos and Franklin* Mouse Brain Atlas images of PFC. Circles defining right hemisphere regions starting top left in counterclockwise direction: AC (top left), PL (middle left), IL (bottom left), VO (bottom right), and insula (top right). (B) Representative autoradiographic images taken for *Per1*, *Per2*, and *Bmal1* mRNA clock genes at the level of the PFC at ZT1.5 and ZT13 for both floxed and FBGRKO mice.

Right hemisphere subregions of the PFC and insula are highlighted in the Mouse Brain Atlas image and representative autoradiographs at the level of the PFC for FBGRKO and floxed mouse brains for all clock genes are shown in Figure 16. There were no significant genotype differences in any subregion of the PFC (AC, PL, IL, VO) or insula. *Bmal1* mRNA, however, had a significant time of day effect for all regions examined. Post hoc analysis revealed ZT1.5 had more *Bmal1* mRNA clock gene expression than ZT13 in floxed mice in the AC ($p=0.013$), PL ($p=0.015$), VO ($p<0.001$), and insula ($p=0.000$), as well as in FBGRKO mice in the AC ($p=0.004$), VO ($p=0.001$), and insula ($p=0.014$) (Figure 17).

17.

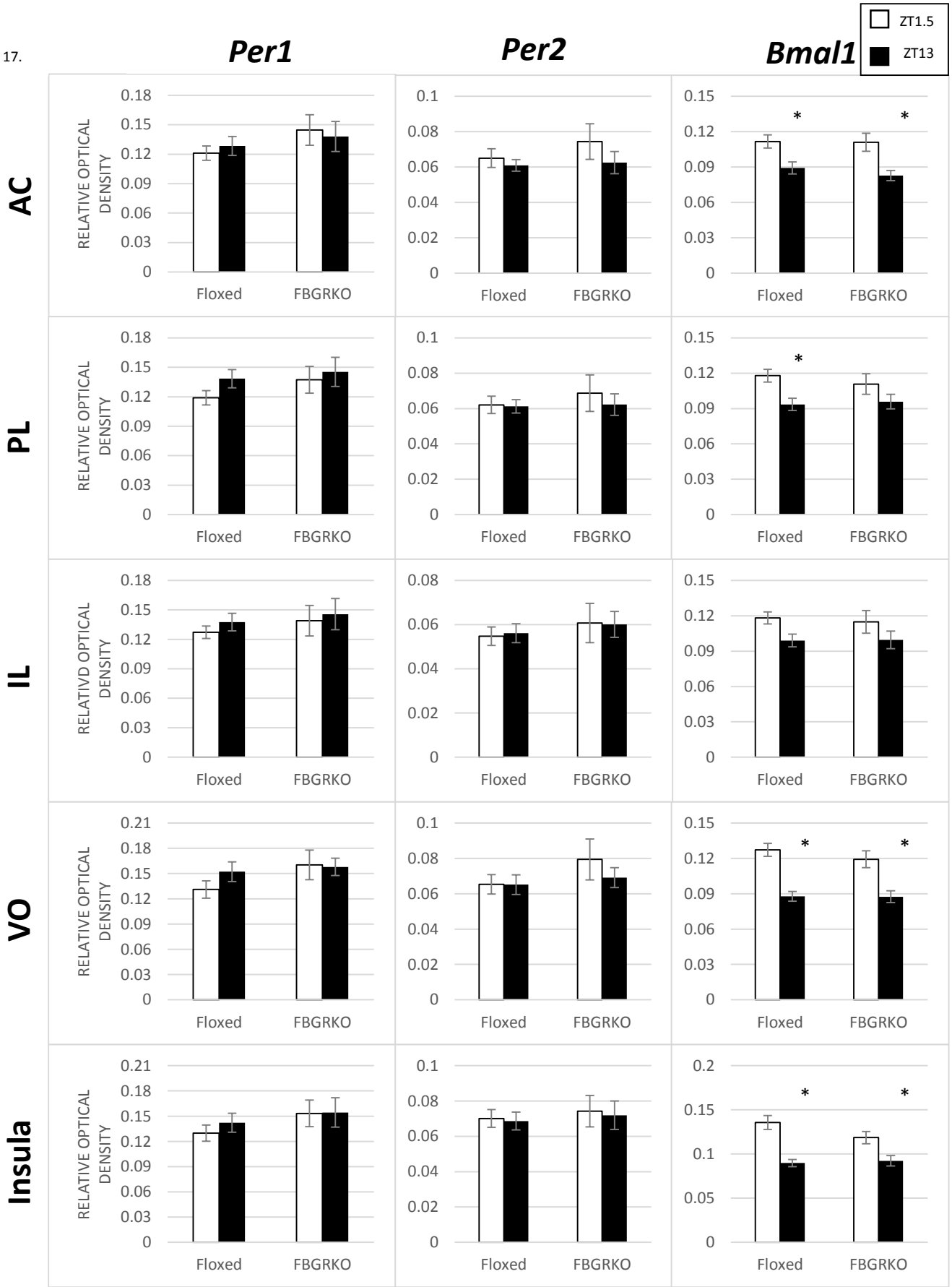


Figure 17. Relative optical density for *Per1*, *Per2*, and *Bmal1* mRNA clock gene expression for subregions of the PFC: AC, PL, IL, VO and insula. *p<0.05 represents statistical difference from AM values.

Discussion

The SCN houses the master clock and synchronizes circadian rhythms throughout the brain and body. The SCN has few direct projections to other brain and body regions and it is still unknown how it communicates with extra-SCN clocks. CORT may play an essential role in entraining the molecular clock as there is a circadian rhythm of CORT under SCN-control, and GR is expressed throughout the brain and body. Furthermore, there is a GRE in the promoter region of the *Per1* clock gene, which may be the mechanism in which CORT can entrain the molecular clock. With transgenic mice that had a forebrain-specific glucocorticoid receptor knockout (FBGRKO), we were able to investigate the importance of GRs in entraining the molecular clock and inducing day-night differences in clock gene expression throughout the body. Analysis of in situ hybridization assays revealed no genotype differences between FBGRKO mice and floxed mice in *Per1*, *Per2*, and *Bmal1* mRNA or in the SCN, PVN, AMY, HPC, PFC, and insula. However, there were significant time of day differences in *Bmal1* mRNA expression for most brain regions examined. Results from this study, may allow us to better understand CORT's role as an entrainment factor and possible compensatory mechanisms that may explain the lack of genotype effect between the FBGRKO and floxed mice.

Time of Day Effects

As hypothesized, there was a significant main effect of time for *Per1*, *Per2*, and *Bmal1* mRNA in the SCN as well as for *Per1* and *Bmal1* mRNA in the PVN. Overall time of day trends in the data match those of previous studies seen in male rats and mice (Chun et al., 2015; Rath et al., 2014; Albrecht et al., 1997; Shearman et al., 1997; Tei et al., 1997; Sun et al., 1997). In general, *Per1* and *Bmal1* mRNA expression in the SCN were antiphasic to each other, as well as antiphasic between the SCN and PVN. Although limited by only two time points, mRNA expression of the current study matched previous

reports of clock gene expression in mice and rats in the SCN in terms of acrophase (Rath et al., 2014; Albrecht et al., 1997; Bae et al., 2001; Sun et al., 1997; Hastings et al., 1999; Chun et al., 2015). Previous studies have shown that the SCN is the only notable brain region with rhythmic differences of gene expression between *Per1* and *Per2* genes (Chun et al., 2015; Albrecht et al., 1997; Bae et al., 2001). Specifically, Chun et al. (2015) found that, in rats, *Per1* mRNA expression in the SCN peaks around ZT4 and reaches trough values around ZT16 while *Per2* mRNA expression peaks around ZT12 and troughs around ZT0. The present study shows a significant main effect of time for both *Per1* and *Per2* mRNA expression in the SCN; however, possibly due to this time of day difference, post hoc analysis only revealed a significant difference between ZT1.5 and ZT13 in the floxed and FBGRKO mice of *Per2* mRNA. Therefore, mice appear to match previous results found in rats having differential timing in clock gene expression of *Per1* and *Per2* genes in the SCN. This study also supports previous results of diurnal differences in clock gene expression in SCN and extra-SCN tissues.

Clock gene expression in the PVN of this study also closely match previously published data on clock gene expression in rats (Chun et al., 2015; Takahashi et al., 2001; Girotti et al., 2009). We found no significant time of day differences in *Per2* expression in the PVN for either the floxed or FBGRKO mice. This finding is interesting as there was a significant time of day difference for both *Per1* and *Bmal1* mRNA in floxed and FBGRKO in the PVN. Other studies have shown significant time of day differences in clock gene expression in the PVN with peak *Per1* and *Per2* mRNA levels around ZT12 and trough levels around ZT0. In contrast, it has been shown that *Bmal1* mRNA peak levels in the PVN occur around ZT4 and trough levels occur around ZT16 (Chun et al., 2015; Takahashi et al., 2001; Girotti et al., 2009). The HPC, AMY, PFC, and insula all showed similar clock gene mRNA expression to the PVN. All regions examined had significant time of day effects in the FBGRKO of *Bmal1* mRNA except CA1, PL, and IL. Floxed mice also had significant time of day differences in *Bmal1* mRNA in all regions except CA1, CA3,

Supra DG, Infra DG, and IL. Although there is a limited time of day effect in all brain regions outside of the hypothalamus, the results support previous reports of mRNA expression levels in extra-SCN tissues.

It has been well established in rats that clock gene expression in the CEA and SCN are in phase with each other, an effect that has not been seen with any other brain regions (Chun et al., 2015, Amir et al., 2004,). Most extra-SCN brain regions are antiphasic to the SCN or have a shifted phase compared to the SCN. This is true for *Per1*, *Per2*, and *Bmal1* mRNA and PER2 protein (Harbour et al., 2013; Lamont et al., 2005; Chun et al., 2015). It has been speculated that this relationship of clock gene expression in the CEA and SCN may be due to the fact that both brain regions are comprised predominantly of GABAergic neurons but the functional importance is yet to be determined. Our results of clock gene expression in mice do not support the same conclusions as in rats. However, other studies in mice have reported results similar to those found in the current study (Moriya et al., 2015). The SCN and CEA appear to have different circadian timings, with *Per1* and *Per2* mRNA appearing out of phase and *Bmal1* mRNA expression appearing antiphasic between the SCN and CEA in these mice. The reasons behind the differences in results seen in mice versus rats are yet to be determined.

Male rats have been shown to have a blunted circadian rhythm of core clock genes in the HPC compared to females (Chun et al., 2015). The HPC is comprised of the CA1, CA3, Supra DG and Infra DG. This blunting in rhythmicity of *Per1*, *Per2*, and *Bmal1* mRNA in the HPC in male rats may also occur in male mice which could be a contributing factor to why there were only a few time of day differences in the HPC of mice in this study. There were no differences in overall mRNA levels between the floxed and FBGRKO mice at either ZT, but if male mice do have a blunted rhythm and there are only two time points to analyze, it would be very difficult to detect a genotype effect in rhythmicity. It is also very difficult to determine if there are any differences in clock gene expression, especially when the times we have are not the peak and trough levels of clock gene expression.

Overall, the time of day differences seen in the SCN, PVN, and other brain regions analyzed match previously reported clock gene expression; however, there is a significant limitation of the present study with having only two time of day points for reference. Having only two time points of reference, ZT1.5 and ZT13, is not optimal for investigating clock gene expression for several genes in the many regions of interest. ZT1.5 and ZT13 are not peak and trough times in which we would expect to see the maximal differences in gene expression for *Per1* and *Per2* mRNA. *Bmal1* mRNA has an acrophase and trough at times closer to ZT1.5 and ZT13 which is why we saw more significant time of day differences between clock gene expression for *Bmal1* mRNA compared to *Per1* and *Per2* mRNA in many brain regions (Chun et al., 2015; Bae et al., 2001; Moriya et al., 2015). Previous studies have shown that in nocturnal rodents *Bmal1* mRNA peaks around ZT0 in most extra-SCN tissues and troughs around ZT12, while *Per1* and *Per2* mRNA peak around ZT16 and trough around ZT4 (Chun et al., 2015; Moriya et al., 2015). The times of day in the present study, ZT1.5 and ZT13, are much closer to the peak and trough times of *Bmal1* mRNA than either *Per* gene. Given this, the time of day differences seen in the *Bmal1* mRNA results are likely seen near peak and trough mRNA expression levels increasing the likelihood of noticing a time of day effect. In contrast, *Per1* and *Per2* mRNA may be in the middle of their rhythms and therefore at similar mRNA levels, making it difficult to observe any differences between the two times of day. With only two times for comparison, it is difficult to see changes in the rhythmicity of clock gene expression because it is nearly impossible to conclude blunted or shifted rhythms. Therefore, the lack of time of day effects in the *Per1* and *Per2* mRNA expression in SCN, PVN, AMY, HPC, PFC, and insula are explained by the limited temporal resolution.

Genotype Effects

The SCN and PVN are both nuclei within the hypothalamus. This portion of the forebrain does not express CaMKIIα, and therefore is not subject to conditional GR knockout. Considering that the mice in the present study had a CaMKIIα promoter-dependent glucocorticoid receptor knockout, it is not surprising that we did not see a genotype effect in these regions. Earlier studies documented that there is no conditional knockout of GRs in the PVN and the SCN is one of the only known brain or body region that lacks GRs (Vincent et al., 2013; Balsalobre et al., 2000). Even if the FBGRKO did affect the hypothalamus, we would not expect to see a genotype effect in the SCN due to the fact that the SCN lacks GRs and would not be affected by this manipulation.

Given that there are no GRs to bind to the GRE in the promoter region of the *Per1* gene after the FBGRKO manipulation, we predicted *Per1* mRNA expression may have been impaired, thereby potentially altering clock gene expression in the molecular clock. Thus, we expected to see differences in genotype between the FBGRKO and floxed mice in regions of the forebrain with the FBGRKO manipulation, suggesting that GRs may be involved in the synchronization of circadian rhythms from the SCN to extra-SCN tissues. The AMY, HPC, PFC, and insula are all forebrain regions and therefore the forebrain-specific glucocorticoid receptor knockout should act strongly on these areas, deleting all GRs. The CEA is an exception as it is documented to have only a 50% knockout of GRs, while most other brain regions had a nearly complete knockout. With this, we expected to see only a partial difference between the FBGRKO and floxed mice in the CEA from the FBGRKO manipulation. Surprisingly, we found no genotype effect in any brain region examined for any core clock gene analyzed. It is believed that CORT entrains the molecular clock by binding to GRs which dimerize and bind to a GRE in *Per1*'s promoter region. Therefore, we expected that the FBGRKO manipulation would have the greatest effect on Per1 mRNA expression but other clock genes would also be impacted. In addition to the *Per1* gene, recent studies have shown there is a GRE in the promoter region of other core clock genes, including *Per2*,

Bmal1, and *Cry1*. These findings suggest that other clock genes may be influenced by CORT; however, there is less evidence that these GREs are functional across a variety of tissues and conditions (Cheon et al., 2013; So et al., 2009; Reddy et al., 2007). The unexpected lack of significant genotype effects in FBGRKO mice requires further investigation to determine the role of GRs in the characterization of the molecular clock.

The CEA is also a unique case, as it only has about a 50% deletion of GRs in the FBGRKO condition (Vincent et al., 2013). Given this, we expected only a partial genotype effect in the FBGRKO mice. However, similar to other brain areas, we saw no effects or even trends for a significant genotype effect in the CEA. The lack of a genotype effect seen in the CEA may be due to the fact that it only had a 50% GR knockout in FBGRKO mice; however, considering the genotype and time of day effects seen in the CEA are similar to those found in the other forebrain regions, the expected partial knockout effect is likely irrelevant.

The lack of genotype effect found in all brain regions for all clock genes examined may be due to a multitude of factors. First, it is possible that CORT does not play a role in the entrainment of the circadian clock. Other studies have investigated the effect of adrenalectomy (ADX) in rats. This procedure removes the adrenal glands, the main organ for releasing CORT, from the animal. This procedure is known to eliminate endogenous CORT. Under basal conditions, ADX rats have been shown to maintain core clock gene rhythmicity; however, the circadian rhythm of these clock genes was altered in a gene-specific manner in the SCN and subregions of the PFC (Woodruff, submitted). Another study has investigated the role of ADX rats maintained in constant dark conditions to eliminate the influence of light on the rhythm of the circadian clock. In the absence of light cues, rats housed in dark:dark conditions maintained a synchronized PER2 protein expression between the SCN and bed nucleus of the stria terminalis. Similar results were found between rats housed in dark:dark and blind rats (Amir et al., 2004). The results of these studies show that even in the absence of CORT and light cues, the SCN is able

to maintain its circadian rhythm and continue to synchronize the rhythms of other extra-SCN and peripheral clocks. Thus, CORT may not be necessary for diurnal expression of core clock genes in all extra-SCN tissues.

It is also possible that GRs do not have a necessary role in the diurnal expression of core clock genes in extra-SCN tissues. The lack of a genotype effect seen between the FBGRKO and floxed mice suggest that GRs may not have a primary role in diurnal differences of core clock gene expression. If CORT, but not GRs, does have a necessary role in core clock gene expression, it is possible that CORT modulates diurnal rhythms of *Per1*, *Per2*, and *Bmal1* mRNA in the forebrain regions through a series of non-GR mediated neuronal projections. For example, vasopressin is a clock-controlled gene that is released from the SCN in a circadian manner and is used as a measurement of circadian rhythms, especially in human studies (Kretschmannova et al., 2005; Van der Veen et al., 2005). Vasopressin has been shown to amplify circadian rhythms and may synchronize neurons in the SCN to the environment or each other (Liu et al., 2005; Kalsbeek et al., 2010). Liu et al., (2005) showed that human subjects exposed to CORT prior to death had a suppressed vasopressin gene expression in the SCN compared to subjects that were not exposed to CORT. Depressed subjects frequently show increased vasopressin while subjects with Alzheimer's disease have decreased vasopressin. These studies suggest that CORT and vasopressin may be acting together in a mechanism that does not involve GRs to influence circadian rhythms and possibly clock gene expression. It is also possible that the effects of CORT act via GRs in brain regions that are not included in the forebrain. The dorsal raphe nuclei (DRN) is not a part of the forebrain but has strong serotonergic projections to the forebrain (Herman et al., 2005; Lowry et al., 2002). CORT is necessary for diurnal activity in the DRN; therefore, the FBGRKO would have no effect on GR expression in the DRN allowing CORT to entrain the DRN via GRs. This entrainment signal would then project to the forebrain region and provide an entraining neural input (Lowry et al., 2002).

Another possible explanation for the lack of genotype differences in the present study is that other zeitgebers are strong enough to entrain the molecular clock even in the absence of GRs. Zeitgebers are external cues that may influence the molecular clock with food intake and light exposure being the most potent. Although the FBGRKO mice did not have GRs and therefore *Per1* mRNA could not be induced by the binding of CORT, it is possible that eating and light exposure at the right time of day were strong enough factors to entrain the molecular clock. Food intake and light exposure at the appropriate circadian time may be enough to keep the molecular clock timing synchronized so that the FBGRKO effect was not substantial enough to have a detrimental impact. Mice in the current study were given food and water ad libitum but the 12:12 hour light:dark cycle may have been sufficient to entrain these mice to the correct circadian time. It would be interesting to see whether there would be a significant effect on FBGRKO animals kept in a dark:dark cycle with ad libitum and/or restricted feeding. Then, if food intake and light exposure were not present to maintain synchronization of the molecular clock, there may be a significant effect of the FBGRKO condition. Amir et al., (2004), showed that rats kept in constant darkness still maintained a rhythmic PER2 protein synchronization in the SCN suggesting that there are many possible zeitgebers that influence the molecular clock.

Another theory to explain the lack of FBGRKO effect involves compensation by mineralocorticoids receptors (MRs). CORT has the ability to bind to both MRs and GRs, a process that may be influenced by the amount of circulating CORT. CORT has approximately a 10-fold higher affinity for MRs to which it binds under normal, basal conditions. MRs are the primary regulator of negative feedback to the HPA axis when CORT levels are low, such as during the early light phase in rodents when circadian rhythms are at their trough. GRs are the primary regulator of HPA axis activity when CORT levels are high, such as during the onset of the active phase. However, there is growing research showing the importance of MRs during peak times as well (Jacobson et al., 2005; Pace and Spencer, 2005). One study shows that MRs may be necessary for feedback to the HPA axis during a mild stressor,

but not during a stressor that has a more robust CORT response (Pace and Spencer, 2005). The initiation of the T-50 founder line of FBGRKO mice begins in the embryo of the offspring and thus, there may be sufficient time for the offspring to compensate for the lack of GRs. This compensation may be a characterization of the mice that is passed down in all future generations of FBGRKO mice. Initial characterization of the T-29-1 FBGRKO mice found that along with the decrease in GRs throughout the forebrain, there was an increase of MR mRNA expression in the dentate gyrus, CA1, and CA2 of the HPC, but not CA3 (Vincent et al., 2013). This increase in MR expression combined with the decrease in GR expression may have an essential role in the molecular outcomes when analyzing FBGRKO mice. Even in high CORT situations when CORT would naturally bind to GRs, the absence of GRs may encourage CORT to bind to MRs in order to continue the cascade of effects and avoid completely stopping the feedback process. Thus, when mice lack GRs as in the present study, it is possible that CORT always binds to MRs in order to maintain its effects. Interestingly, MRs also have the ability to bind to the GRE (Arriza et al., 1987; Trapp et al., 1994; Fuller et al., 2000). Therefore, it is possible that even though GRs were knocked out of the FBGRKO mice, MRs may have established a compensatory mechanism to avoid the detrimental effects of not having GRs. To further investigate this, the current FBGRKO brains will undergo GR and MR in situ hybridization assays in order to validate the lack of GRs in the forebrain and the possible upregulation of MRs due to compensation.

A final consideration when analyzing the results of these FBGRKO mice is that this knockout did not delete GR expression from every cell in the forebrain. CaMKIIα is a promoter that only affects pyramidal cells (https://www.jax.org/strain/005359). Pyramidal cells are primary glutamatergic cells; however, oligodendrocytes and GABAergic interneurons are other cells in the forebrain that express GRs. Serum and glucocorticoid regulating kinase 1 (*Sgk1)* is an enzyme that is highly GR mediated and is also influenced by CORT. *Sgk1* expression is primarily found in white matter, such as oligodendrocytes, as well as the hippocampus (specifically CA3). An in situ hybridization assay has been completed for

(*Sgk1*) mRNA which will validate that the hippocampus was affected by the FBGRKO manipulation and to validate that oligodendrocytes were not affected by the FBGRKO. Results from this assay will elucidate the effects of the FBGRKO on other cell types in the forebrain and may help explain the lack of genotype effect seen in all tissues examined. Since there were no genotype differences from the FBGRKO, pyramidal cells may not be the primary regulator of core clock gene expression. Analysis of this tissue is still in process and it may provide insights towards discovering the functional impairments of the FBGRKO and the resulting consequences on various cell types.

It would also be pertinent to investigate the FBGRKO effect in GABAergic interneurons. Considering that CaMKIIα mainly affects glutamatergic neurons and the resulting genotype effects were nonsignificant, clock gene expression may be primarily expressed in GABAergic neurons. Fluorescence in situ hybridization (FISH) protocols are commonly used to double label mRNA in one tissue. Future studies should use a FISH protocol to double label cells for GR and GAD65, a marker for GABAergic neurons, mRNA in order to see if they are expressed in the same cells. If GRs and GAD65 are expressed in the same cells, then GABAergic neurons may be the primary regulator of the role of GRs on core clock gene expression. This information will allow us to make stronger conclusions as to which cell types are the primary regulators of core clock gene expression. The lack of genotype effect from the FBGRKO manipulation could indicate that core clock gene expression in that pyramidal cells is entrained by neuronal input from other cell types that do express GR; and therefore, the FBGRKO was insufficient to have detrimental effects. Interneurons may express core clock genes even in the absence of GRs from pyramidal cells, which would suggest that interneurons may be the principle cell for coordinating core clock gene expression, even in pyramidal cells. If this is the case, CORT may still be important for entraining the molecular clock of GABAergic cells, and indirectly pyramidal cells. The results of the FISH assay may reveal a genotype difference between the FBGRKO and floxed mice, which will provide more information on which cell types are necessary for CORT modulation of core clock gene expression.

Concluding Summary

We found no significant genotype differences between mice that had a conditional forebrain-specific knockout of glucocorticoid receptors and control floxed mice that had no conditional knockout. We did find significant main effects of time, mostly in *Bmal1* clock gene mRNA expression in many brain regions. With our limitations in temporal resolution, it is difficult to demonstrate alterations in circadian rhythm of *Per1*, *Per2,* and *Bmal1* mRNA (e.g., phase shifting or blunted rhythm expression) in these brains regions. The similarities between core clock gene expression in mice and rats suggest there are robust rhythms that appear to be replicable between nocturnal species. The lack of effect of the FBGRKO in the AMY, HPC, PFC, and insula suggests many questions requiring further investigation. First, clock gene expression may be independent of CORT and/or GRs may not be necessary for circadian clock gene expression. Compensatory mechanisms to explain the lack of a genotype effect are also possible, including the upregulation and increased functional importance of MRs, effects of GR presence in non-pyramidal cell types, and other zeitgebers. GR and MR in situ hybridizations will be completed to confirm the knockout of GRs and to investigate the possible upregulation of MRs. It is also possible that the FBGRKO mice had a knockout in a type of cell (e.g., pyramidal) that has no role in entraining the molecular clock. Therefore, the completion of the *Sgk1* in situ hybridization and GR/GAD65 FISH assays will be an essential step in investigating which cells received the conditional FBGRKO and which other cells may be possible regulators of the molecular clock. Overall, this knowledge may lead to a better understanding of the entrainment of the molecular clock, which could be beneficial to those suffering from mood and psychiatric disorders that are associated with disrupted clock genes and an altered circadian clock.

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