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Effect of glucocorticoid status on clock gene expression in components of the hypothalamic-pituitary-adrenal axis

Sara Fardi
University of Colorado Boulder

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Sara Fardi
Department of Psychology and Neuroscience

Thesis Advisor:
Robert Spencer, Department of Psychology

Committee Members:
Ryan Bachtell, Department of Psychology
Michael Breed, Department of Ecology & Evolutionary Biology

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Abstract:

This thesis explores the role of glucocorticoids in entraining the rhythmicity of clock gene expression in components of the hypothalamic-pituitary-adrenal (HPA) axis, specifically the paraventricular nucleus (PVN) of the hypothalamus and the anterior pituitary. Physiological and behavioral circadian rhythms rely on molecular oscillators inside cells of living organisms. Rhythmic expression of associated clock genes is dependent on the hypothalamic suprachiasmatic nucleus (SCN), which is synchronized to the light/dark cycle with an intrinsic rhythm near 24 hours. Extra-SCN brain regions and peripheral tissues in the body have been shown to have molecular clocks; interestingly, the SCN has sparse direct neural input in these regions. Previous research has indicated that glucocorticoids (CORT) are able to entrain clock gene rhythmicity in peripheral tissues, suggesting the SCN may synchronize peripheral clocks through a diurnal expression of CORT. Little work has been done to explore this possible mechanism of entrainment in the brain but has been characterized in periphery where CORT has been shown to cause a phase shift, induce, or abolish clock gene expression in tissues such as liver and kidney. This thesis characterizes clock gene expression in response to three different CORT profiles: antiphasic CORT treated, phasic CORT treated, and vehicle treated in adrenalectomized rats. We found that expression of clock genes *Per1* and *Per2* varied with CORT treatment in both the PVN and anterior pituitary, but also that CORT influenced genes differently in the two regions. The results suggest that CORT may have a permissive effect in the periphery, necessary for clock gene expression, but has an entraining effect in the brain. This study was an important step in understanding the role of CORT in HPA axis regulation and disruption. Such disruptions and dysregulation of the HPA have been observed in numerous conditions such as Posttraumatic Stress Disorder, Major Depressive Disorder, and sleep disorders.

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Introduction:

The hypothalamus plays central roles in both the diurnal and stress systems. The diurnal system refers to the 24 hour cycle living organisms have functionally evolved to. Virtually all organisms express an internal clock system; the molecular read out of this clock can be found almost ubiquitously in tissue (Takahashi et al., 2008; Ko and Takahashi, 2006). These rhythms create an internal circadian rhythm under the influence of the light/dark cycle. The master clock is housed within the suprachiasmatic nucleus (SCN) of the hypothalamus; its primary input is light/dark information transferred from the retina to the hypothalamus via the retinohypothalamic tract, and is responsible for integrating light cues from the environment.

The stress system, also centrally controlled by the hypothalamus, helps organisms to adapt to aversive changes and stressors. Organisms' hormonal responses to aversive changes are controlled by the paraventricular nucleus (PVN) of the hypothalamus, the head of the hypothalamic-pituitary-adrenal (HPA) axis (Meerlo et al., 2002). The full HPA axis consists of parvocellular corticotropin-releasing-hormone (CRH) and arginine vasopressin (AVP)-secreting cells in the PVN, the adrenocorticotropic hormone (ACTH) secreting pituitary corticotrophs, and the glucocorticoids (CORT) released from the cortex of the adrenal gland. CORT is the final effector of the HPA axis' hormonal cascade. Receptors of CORT, glucocorticoid receptors (GR), are ubiquitously expressed in the body, facilitating the hormones wide spread influences. The stress system reestablishes homeostasis by regulating physiological activities including intermediary metabolism, immunity, and reproduction (Chrousos, 2009; Churousos and Kino, 2007). It also suppresses the PVN and pituitary gland in a closed negative feedback loop. Important to note, are the adverse effects hyperactivation of the HPA axis can have, such as Posttraumatic Stress Disorder, while chronic activation of the HPA axis and prolonged elevation

of CORT may induce obesity and insulin resistance (Chrousos, 2009; Churousos and Kino, 2007).

Without the influence of stress stimulation ACTH, a pituitary hormone released in response to CRH, and CORT show diurnal rhythmic activity, with plasma levels peaking in anticipation of the organism's active period. The SCN is primarily responsible for the cyclic, baseline functioning of the HPA axis (Fahrenkrug et al., 2008).

It is not just the diurnal activity of the HPA axis the SCN controls, it is also responsible for synchronizing the clocks in the extra-SCN brain and body (Girotti et al., 2009). The molecular mechanism for circadian rhythms, physiological and behavioral, depends on the oscillatory expression and autoregulation of clock genes. These rhythmically expressed clock genes have been characterized in the SCN, extra-SCN brain, and peripheral tissues (Amir and Stewart, 2009; Guilding and Piggins, 2007; Feillet et al., 2008) such as the pancreas, thymus, and lungs (Saper et al., 2005). Among the core clock genes are positive regulators, circadian locomotor output cycle kaput (*Clock*) and brain and muscle aryl hydrocarbon receptor nuclear traslocator-like (*Bmal*), which in turn induce the expression of their own negative regulators, the *Period* (*Per1*, *Per2*, *Per3*) and *Cryptochrome* (*Cry1*, *Cry2*) genes. CLOCK/BMAL1 proteins form a heterodimer which binds the E-box response elements located in the promoter region, stimulating the transcription of the negative regulating clock genes. PER and CRY proteins accumulate as dimers in the cytoplasm, which repress CLOCK/BMAL1 heterodimerization and subsequent induction of their own promoters (Kondratov et al. 2006). The time course for this standard negative feedback loop is 24 hours (Kiyohara et al., 2006).

These clock genes, while expressed in virtually all cells with an intrinsic rhythm near 24 hours, rely on normal SCN functioning to synchronize phasic expression with the light/dark

cycle. Interestingly, direct SCN innervation is sparse in extra-SCN tissues; the lack of direct input suggests an alternative mechanism of control. While the SCN lacks significant direct innervation of most extra-SCN tissue, GRs are ubiquitously expressed in every cell in the body with the noted exception of the SCN itself. Several studies have shown CORT plays a crucial role in regulating diurnal rhythms in peripheral tissue (Balsabore et al., 2000) and entraining autonomic clocks (Horseman and Ehret, 1982), in establishing and maintaining circadian behavioral patterns (Sage et al., 2004), and molecular circadian rhythms (Balsaobre et al., 2000; Pezuk et al., 2012).

The absence of GRs in the SCN leaves the master clock mostly unaffected by CORT fluctuations. The lack of GR in the SCN coupled with the SCN's control of the HPA axis, and therefore CORT release, is a potential mechanism where CORT is used to synchronize the peripheral clocks. Disrupting circadian CORT secretion can cause a phase-shift in clock gene expression in the periphery (Bur et al., 2010; Segall and Amir, 2010). GR acts as a transcription factor for numerous genes, significantly the canonical core clock gene *Period1* (*Per1*) which includes a sensitive glucocorticoid-response element (GRE) in its promoter region (Yamamoto et al., 2005). The GRs action as a transcription factor is a possible way glucocorticoids entrain peripheral clocks, especially for genes with a functional GRE in the promoter region as they may be induced by diurnal CORT and sensitive to disruptions in the normal expression of CORT.

While the effect of disrupted CORT on clock gene expression has been characterized in the periphery, little research has been done on characterizing clock gene expression and CORT-dependent influence within the HPA axis itself. Understanding the rhythmic expression of clock genes within the HPA axis, a potential regulatory mechanism for circadian rhythms, is essential as research on circadian rhythms expands into non-SCN areas of the brain. The wide spread

presence of rhythmically expressed clock genes in peripheral tissue and extra-SCN brain suggests functional relevance. In genetically mutant mice with knockdown of certain core clock genes present a range of behavioral phenotypes associated with impaired cognition and emotional function (Takahashi et al., 2008; Garcia et al., 2000; McClung, 2007). As well, interference in the rhythmic, baseline function of the HPA axis has been implicated in several diseases such as Major Depressive Disorder and sleep disorders insomnia and sleep apnea (Pariante and Miller, 2001; Buckley and Schatzberg, 2005; Gold et al., 1988).

The purpose of this thesis is to explore the extent to which glucocorticoids (CORT) regulate clock gene expression in the anatomical components of the HPA axis. In this study, we investigated the effect of three CORT profiles—antiphasic to the endogenous peak of CORT, in the early morning in nocturnal rodents; phasic CORT, where CORT was injected in the evening at the onset of the active period and normal peak time of endogenous CORT; and vehicle injected groups—on the rhythmic expression of core clock genes *Bmal1*, *Per1*, *Per2* in the SCN, PVN, and anterior pituitary of adrenalectomized male rats. Adrenals were surgical removed from rats to remove endogenous, circulating CORT to test the necessity of the hormone for rhythmic expression of clock genes. Injection times, AM and PM, were chose based on the diurnal rhythm of CORT in adrenal intact rats; CORT peaks at the onset of the dark, active period in rats and reaches its trough in the morning. The AM injection acts in opposition to input from the SCN allowing us to investigate if the influence of CORT can override photoperiod information. Core clock genes *Bmal1*, *Per1*, and *Per2* were selected as they are the positive and negative arms, respectively, of the molecular clock. As well, *Per1* has been characterized with a hyperactive GRE. It's also been suggested that *Per2* has a GRE (So et al., 2009); our lab has also demonstrated in the past that *Per2* is more rhythmically expressed in the anterior pituitary

(Girotti et al., 2009). The intermediate pituitary expresses no GRs and is vestigial in humans; the posterior pituitary consists mostly of magnocellular axons. As well these other regions are not central to the function of the HPA axis stress system. For these reasons they were not included in this examination. It is important to address that adrenalectomy removed the final component of the HPA axis from our experiment. However, the adrenals have been shown to have an autonomous molecular clock that is not sensitive to fluctuations in CORT because of saturation, making present GR's functional insignificant (Son et al., 2008).

Given that there are no GRs in the SCN, it is unlikely that there will be an effect of CORT status on clock gene expression within the nucleus. Based on preliminary research in our lab, *Bmal1* expression in the PVN will not be influenced, but *Per1* expression, given its sensitivity to CORT, may display a blunted amplitude due to antiphasic CORT treatment (Woodruff, Fardi & Spencer 2013, unpublished data). Studies of the pituitary have provided varied results. While *Bmal1* has been repeatedly shown as rhythmic, *Per1* rhythmicity is varied based on experimental conditions (Bur et al., 2010; Girotti et al., 2009). We anticipate that antiphasic CORT treatment would disrupt the normal expression of *Per1*, but based on past observations of gene expression in the PVN, rats treated with phasic (PM) CORT and vehicle would display rhythmicity. *Per2* expression, however, has been shown to be rhythmic except in instances where feeding was disrupted (Bur et al., 2010; Girotti et al., 2009). Given these results and the presence of continuously occupied GRE by dimerized GR in the promoter region of *Per2* (So et al., 2009), we expect *Per2* gene expression to by rhythmic in both CORT treated groups, possibly with a difference in phases between AM and PM injected groups, but not in the vehicle group.

Materials and Methods:

Animals

Male Sprague-Dawley rats (250-280g; Harlan Laboratories, Indianapolis, IN) were given a 2-week acclimation period prior to surgery. Rats were housed two per cage (polycarbonate tubs, 47 cm x 23 cm x 20 cm), given food (Teklad Rodent Diet 8640; Harlan) and water pre-adrenalectomy, 0.9% saline post-adrenalectomy, *ad libitum*. All rats were maintained on a 12:12-h light/dark cycle. Experimental procedures were approved and adhered to the guidelines put forth by the University of Colorado Institutional Animal Care and Use Committee.

Surgery

Bilateral adrenalectomy was performed on all rats. Halothane was used to anesthetize animals during surgery. Incisions were made bilaterally through the skin and peritoneal wall near the kidney; adrenal glands of all rats were removed. Following surgery, rats were given a 2-day recovery period before administration of injections. Adrenalectomized rats were given 0.9% saline water *ad libitum*.

Injections

After the post-surgery recovery period, all rats received a 0.3 ml intraperitoneal (i.p) injection of either vehicle (60% saline, 30% propylene glycol, 10% ethanol) or 2.5 mg/kg CORT (Steraloids, Newport, RI). Injections were administered daily for 13 days. The adrenalectomized rats were divided into four treatment groups: CORT AM which received a CORT injection at Zeitgeber Time (ZT) 1, one hour after lights on, CORT PM which received a CORT injection at ZT 11, one hour before lights off, and Vehicle groups which received injections either at ZT 1 or ZT 11 (Fig. 1).

Experimental Procedure

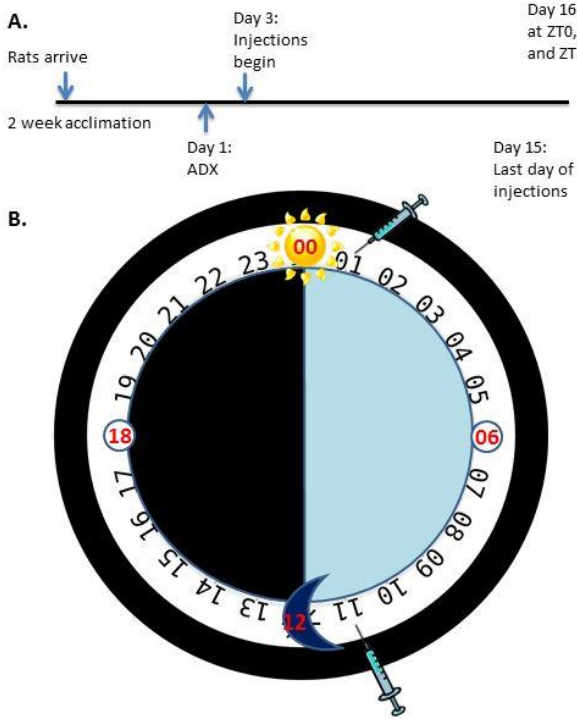

Figure 1: Experimental design. **A**: a timeline of the experiment. **B**: 24 hour experimental design. Red text denotes ZT of sacrifice (ZT0, ZT6, ZT12, ZT18). Needles mark the times injections were administered [ZT1 (AM injection, one hour after lights on) and ZT11 (PM injection, one hour before lights off).

Following 13 days of consecutive injections, groups of rats (for CORT-injected groups: n=6; for vehicle-injected: n=4; total number of animals: N=80), were decapitated at four time points [ZT 0 (lights on), ZT 6 (6 hours after lights on), ZT 12 (lights off), and ZT 18 (6 hours after lights off), Fig. 1]. To avoid acute effects of transitioning between lights on/off, decapitations for ZT 0 and ZT 12 were performed approximately 15 minutes prior to the target times. Brains and pituitaries were extracted and flash-frozen in a dry-ice isopentane at -30˚C. Collected tissue was later stored at -80˚C.

In Situ Hybridization and Image Analysis

Using a Leica Microsystems cryostat (model 1850, Bannockburn, IL USA) coronal brain slices (12 µm) were collected from the suprachiasmatic nucleus (SCN) and paraventricular nucleus of the hypothalamus (PVN) [~1.30 and 1.80 mm posterior to bregma] or transversely (12 µm) through the middle of the pituitaries. Tissue was thaw mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) and stored at -80˚C.

In situ hybridization for *Bmal1*, *Per1*, and *Per2* mRNA and analysis of digitized X-ray films were performed as previously described (Girotti et al. 2009), with some provisions that should be noted. Slides were fixed 4% paraformaldehyde, washed in 2X saline-sodium citrate buffer, and treated with triethanolamine (TEA). Acetic anhydride was added to the TEA to

decrease background and strengthen signal specificity by inactivating ribonucleases. Following this treatment, slides were dehydrated in graded ethanol washes (50%, 75%, 95%, 95%, 100%, and 100%) and allowed to air dry. Slides were then cover-slipped with 65µl of radioactive hybridization buffer, which contains a riboprobe made early in the day through a series of 37˚C incubations and separated using a G50/50 sephadex column. This applied ~$1.5x10^6$ cpm of radioactivity per slide. The ^{35}S-UTP labeled antisense probes were generated within the lab using transcription-buffer, DNASE, T7 (*Per1* and *Per2* RNA polymerase) and T3 (*Bmal1* polymerase) polymerases, and GCA nucleotide (PROMEGA, Madison, WI, USA). The identity of the DNA was verified by DNA sequencing (University of Colorado Boulder Molecular Cellular and Developmental Biology sequencing facility).

Radioactive slides were exposed to film for 2 or 3 weeks depending on signal strength before developed (auto-developer, Konica Minolta Medical and Graphic, Inc). Tissue film was digitized using Scion Image (Scion Corporation). Images were analyzed at the level of SCN, PVN, and anterior pituitary; brain regions of interest were determined using rat brain atlas (Paxinos and Watson, 1998). Optical density was performed with Image J (NIH analysis software) on eight sections were averaged per subject. Measurements were made within regions of interest with a standardized size. Examples of in situ hybridization signals at peak expression levels for each gene tested in various tissues are presented in Figure 2.

Statistical Analysis

Diurnal changes of hormone secretion and clock gene expression were fitted to a basic cosinusoid function $\{y_i = M + A\cos(\omega t_i + \phi) + e_i \quad i=1,\ldots, N$; where M is the MESOR, A is amplitude, ω is frequency, t is time, and ϕ is acrophase$\}$. Acrophase, confidence interval, and time of decapitation were expressed as ZT. The program Chronolab was used to estimate the

parameters of best fit for each treatment group with 95% confidence intervals. To examine the primary effects and interactions of time of day and CORT status, two way ANOVAs were performed. It is important to note that the ZT 1 and ZT 11 vehicle injected groups were combined into one ("Vehicle") as there was not a significant difference in the clock gene expression data between the two groups. Post hoc pairwise comparisons (Fisher's least-significant difference) were examined only in instances where the ANOVA yielded a significant interaction between time of day and CORT status. SPSS (Statistical Package for the Social Sciences) was used in these analyses and a $p<0.05$ was deemed significant.

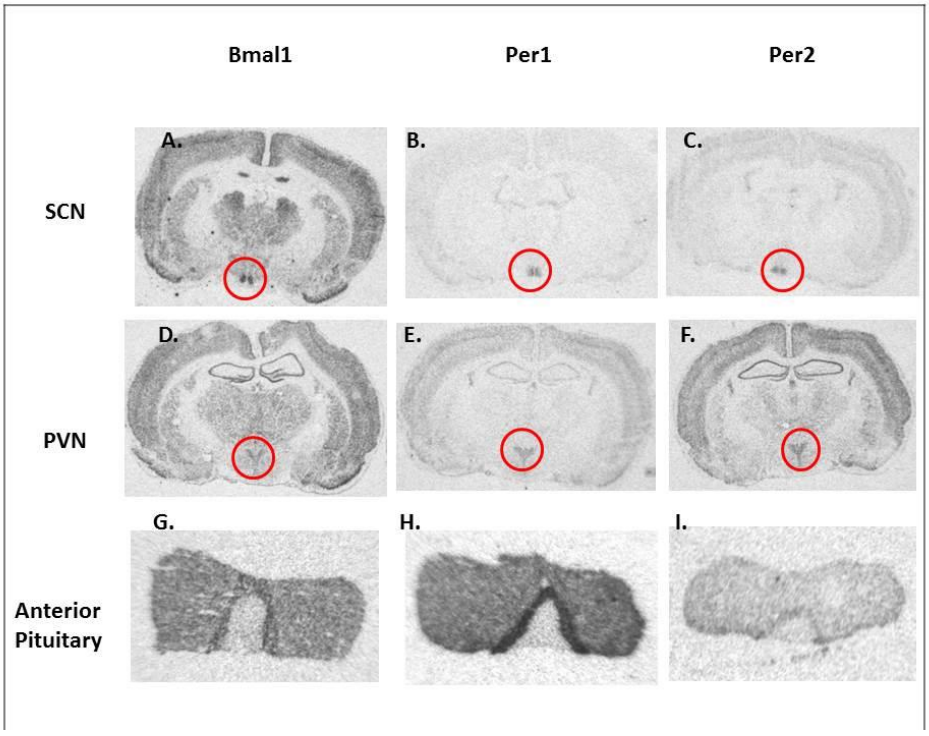

Figure 2: Representative audioradiogram images from *Bmal1* , *Per1*, *Per2* in PM CORT rats, images selected to closest peak ZT times. Regions of interest are within the red circle on the coronal brain images. **SCN (A-C)**: *Bmal1* peak at ZT 12. *Per1* peak at ZT6 and *Per2* peak at ZT12. **PVN(D-F)**: *Bmal1* peak at ZT0. *Per1* peak at ZT6 and *Per2* peak at ZT18. **Anterior Pituitary (G-I)**: *Bmal1* peak at ZT18. *Per1* and *Per2* peak at ZT 12.

Results:

Suprachiasmatic Nucleus (SCN):

Bmal1 mRNA expression in all treatment groups showed a significant 24 hour rhythm ($p<0.001$) with peaks in the early dark phase (acrophase: ZT12.31, confidence interval: 11.1-13.5 for AM CORT; acrophase: ZT12.53, confidence interval: 11.4-13.7 for PM CORT; acrophase:

ZT13.28, confidence interval: 12.1-14.5 for Vehicle; Table 1). The rhythms presented with similar mesor (M) values (M= 130.54 AM CORT; M=133.72 PM CORT; M=137.95 for Vehicle; Table 1). There was a significant time of day effect ($F_{(3,77)}$=52.83, p<0.001) but no CORT effect. A significant interaction was found between the two factors ($F_{(6,77)}$=2.84, p=.016), and while posthoc analysis revealed significant difference at ZT12 in AM CORT *Bmal1* expression when compared to PM CORT (p=0.025). Likely, this difference is not functionally relevant as the amplitudes of all treatment groups overlap (amplitude: 43.34, confidence interval: 30.5-56.2 for AM CORT; amplitude: 30.91, confidence interval: 21.7-40.1 for Vehicle; amplitude: 30.83, confidence interval: 21.6-40.1 for PM CORT) (Fig. 3A).

Per1 mRNA expression displayed a significant 24 hour rhythm for all CORT statuses (p<0.001) with acrophases in the light phase (acrophase: ZT4.13, confidence interval: 3.3-5 for AM CORT; acrophase: ZT4.52, confidence interval: 3.7-5.3 for PM CORT; acrophase: ZT3.72, confidence interval 3.3-4.1 for Vehicle; Table 1). The mesor values were similar despite CORT status (M=87.57 for AM CORT; M=84 for PM CORT; M=82.47 for Vehicles; Table 1). There was a significant time of day effect ($F_{(3,77)}$=165.19, p<0.001) but no CORT effect. As with *Bmal1*, there was a significant interaction between the two factors ($F_{(6,77)}$=2.86, p=.015), with a difference in expression at ZT12 of the vehicle group (p=0.038) compared to PM CORT. Likely, there is no functional relevance as the confidence intervals of AM CORT and Vehicle treated groups' amplitudes overlap with that of PM CORT (amplitude: 29.21, confidence interval: 22.8-35.6 for AM CORT; amplitude: 40.53, confidence interval 36.2-44.9 for Vehicle; amplitude: 35.08, confidence interval: 27.7-42.5 for PM CORT) (Fig. 3B).

Regardless of CORT status, *Per2* mRNA was expressed with significant 24 hour rhythms (p=0.005 for AM CORT; p<0.001 for PM CORT; p=0.022 for Vehicle). Peak mRNA expression

occurred in the light phase (acrophase: ZT7.82, confidence interval 7.3-8.2 for AM CORT; acrophase: ZT8.1, confidence interval 7.5-8.7 for PM CORT; acrophase: ZT8.45, confidence interval 7.9-9.1; Table 1). All treatment groups presented with similar mesor values (M=150.89 for AM CORT; M=150.14 for PM CORT; M=151.97 for Vehicle; Table 1). Time of day had a significant effect ($F_{(3,77)}$=194.41, p<0.001), but there was no CORT effect or interaction between the two factors (Fig. 3C).

Paraventricular Nucleus (PVN):

Bmal1 mRNA expression displayed significant 24 hour rhythms for all treatment groups (p<0.001) with peaks occurring in the early light phase (acrophase: ZT0.68, confidence interval 23.5-1.9 for AM CORT; acrophase: ZT1.87, confidence interval 0.6-3.1 for PM CORT; acrophase: ZT0.87, confidence interval 23.5-2.2 for Vehicle; Table 1), antiphasic to the expression profile of *Bmal1* in the SCN. Mesor values were similar for all treatment groups (M=65.27 for AM CORT; M=69.91 for PM CORT; M=63.76 for Vehicle; Table 1). There was a significant time of day effect ($F_{(3,77)}$=73.65, p<0.001), but no CORT effect and no interaction between factors (Fig. 3D).

Expression of *Per1* mRNA displayed significant 24 hour rhythms regardless of CORT status (p<0.001) with acrophases in the early dark phase (acrophase: ZT13.62, confidence interval 12.1-12.6 for AM CORT; acrophase: ZT13.33, confidence interval 12.3-14.4 for PM CORT; acrophase: ZT12.55, confidence interval 11.5-13.6 for Vehicle; Table 1). Importantly, *per1* expression in the PVN is antiphasic to its SCN counterpart which demonstrates the PVN's responsiveness to different input than photoperiod, here the rat's nocturnal activity pattern. All treatment groups presented with similar mesor values, though there is lower average mRNA expression of *Per1* mRNA in the antiphasic treated group likely as a result of blunted amplitude

(M=120.1 for AM CORT; M=136.09 for PM CORT; M=131.15 for Vehicle; Table 1). There was a significant time of day effect ($F_{(3,77)}$=78.92, p<0.001) and CORT effect ($F_{(2,76)}$=5.21, p=0.008), and interaction between the two ($F_{(6,77)}$=5.7, p<0.001). This effect can be seen in the blunting of *per1* expression in antiphasic treated animals compared to the PM CORT group at ZT12 (p<0.001) (Fig. 3E).

Per2 mRNA expression was expressed with significant 24 hour rhythms regardless of CORT status (p=0.002 for AM CORT; p<0.002 for PM CORT and Vehicle). Acrophases for all treatment groups occurred in the dark phase (acrophase: ZT18.61, confidence interval 16.6-20.6 for AM CORT; acrophase: ZT15.56, confidence interval 14.5-16.6 for PM CORT; acrophase: ZT15.46, confidence interval 14.7-16.2 for Vehicle; Table 1). It is important to note that there is a shift in the *Per2* mRNA expression further into the dark phase and is not truly antiphasic to its SCN counterpart. Mesor values for PM CORT and Vehicle groups were similar, while average expression of *Per2* mRNA in the antiphasic group was lower, a likely result of blunted amplitude (M=119.98 for AM CORT; M=132.45 for PM CORT; M=128.94 for Vehicle; Table 1). There was a significant time of day effect ($F_{(3,75)}$=194.41, p<0.001), no CORT effect, but interaction between the two factors ($F_{(6,75)}$=4.55, p=0.001). This effect manifested as blunted *Per2* mRNA expression at ZT12 in the antiphasic CORT treated group compared to PM CORT (p<0.001) (Fig. 3F).

Anterior Pituitary:

Bmal1 mRNA expression was shown to have a significant 24 hour rhythm in all treatment groups (p<0.001). All three groups had peak values which occurred in the dark period regardless of CORT status (acrophase: ZT 19.79, confidence interval: 18.9-22.5 for AM CORT; acrophase: ZT 21.85, confidence interval: 19.0-20.3 for PM CORT; acrophase: ZT 20.73,

confidence interval: 18.9-22.5 for Vehicle; Table 1), in sync with *Bmal1* mRNA expression in the PVN. The mesor values of treatment groups were similar (M=77.61 for AM CORT; M=74.5 for PM CORT; M=68.36 for Vehicle; Table 1). While there was a significant time of day effect ($F_{(3,79)}$=28.05, p<0.001) there was no significant effect of CORT status or interaction between the two factors (Fig. 3G).

In both AM and PM CORT groups, *Per1* mRNA presented with significant rhythmicity (p=0.036 for AM CORT; p=.006 for PM CORT). Despite visual appearance, the Vehicle group did not have a significant 24 hour rhythm. Both AM and PM CORT groups peaked near the end of the light period (acrophase: ZT 9.29, confidence interval: 6.1-12.5 for AM CORT; acrophase: ZT 10.48, confidence interval: 8.1-12.7 for PM CORT; Table 1). This expression pattern of *Per1* mRNA is similar to that displayed in the PVN. Treatment groups displayed similar mesor values (M=87.57 for AM CORT; M=84 for PM CORT; M=82.47 for Vehicle; Table 1). Similarly to *Bmal1* expression, there was a significant time of day effect ($F_{(3,79)}$=6.27, p=0.001) but no significant effect of CORT status or interaction between factors. This may be because, despite a lack of rhythmicity in Vehicle group, *Per1* mRNA expression has amplitude similar to other treatment groups (Fig. 3H).

Per2 mRNA expression had a significant 24 hour rhythm in all treatment groups (p=0.005 for AM CORT; p<0.001 for PM CORT; p=0.022 for Vehicle). It is important to note that while the Vehicle group was rhythmic its amplitude was significantly blunted (amplitude: 6.89, confidence interval: 2.1-11.7 for Vehicle compared to amplitude: 56.75, confidence interval: 25.0-88.5 for AM CORT and amplitude: 48.44, confidence interval 31.6-65.2 for PM CORT; Table 1). Similar to *Per1* mRNA, *Per2* mRNA expression in all groups tended to peak in the hours prior to the dark period (acrophase: ZT 9.65, confidence interval: 7.3-11.9 for AM

CORT; acrophase: ZT 9.56, confidence interval: 8.2-10.9 for PM CORT; acrophase: ZT 12.00, confidence interval: 9.2-14.8 for Vehicle; Table 1), phasic to *Per2* expression in the PVN. The mesor value of the AM CORT group is elevated compared to both PM CORT and Vehicle groups (M=178.36 for AM CORT; M=120.22 for PM CORT; M=83.94; Table 1). There was a significant interaction between time of day and CORT status ($F_{(6,77)}$=6.37, p<0.001). This was seen in the Vehicle group's blunted amplitude, such that *Per2* mRNA expression was lower at ZT 6 (p=0.009) and ZT 12 (p<0.001) compared to PM CORT (Fig. 3I).

It is important to note that rhythmicity of *Per1* and *Per2* are antiphasic to *Bmal1* both within the PVN and anterior pituitary and in comparison to the SCN.

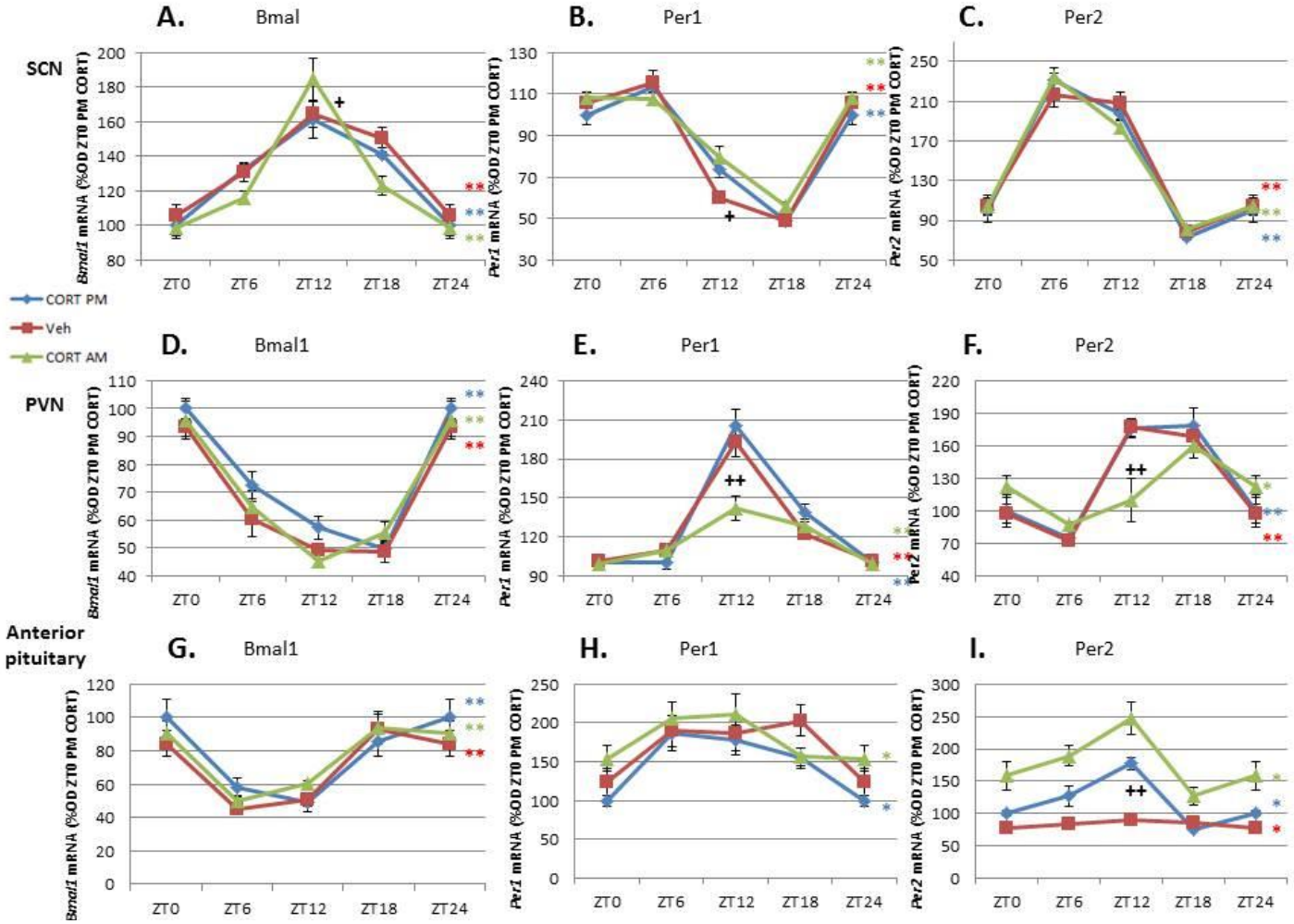

Figure 3: Temporal patterns of clock gene expression in SCN and individual components of the HPA axis. Levels of *Bmal1*, *Per1*, *Per2* mRNAs were determined by in situ hybridization in the **SCN (A-C)**, **PVN (D-F)**, and **anterior pituitary (G-I)**. The ZT 0 time point was plotted twice (ZT 0 and ZT 24) for graphical visualization. The period of lights off is denoted by a dark bar above the x-axis. The blue line represents the PM CORT group, red line represents the Vehicle group, and the green represents the AM CORT group. * denotes significant rhythmicity p<0.05. ** denotes significant rhythmicity p<0.001. + denotes Fisher's Least Square Difference (FLSD) significance p<0.05. ++ denotes FLSD significance p<0.001.

Table 1:

Measure	Treatment Group	PR	p-value	Mesor	ϕ	Confidence Interval
Bmal1						
SCN	CORT AM	70.1	<0.001	130.54	12.30666667	11.1-13.5
	CORT PM	69.7	<0.001	133.72	12.52666667	11.4-13.7
	Vehicle AM/PM	62.8	<0.001	137.95	13.28	12.1-14.5
PVN	CORT AM	67.3	<0.001	65.27	0.68	23.5-1.9
	CORT PM	66.2	<0.001	69.91	1.873333333	0.6-3.1
	Vehicle AM/PM	62.4	<0.001	63.76	0.873333333	23.5-2.2
Anterior Pituitary	CORT AM	73.3	<0.001	77.61	19.79333333	18.9-22.5
	CORT PM	65.2	<0.001	74.5	21.84666667	19.0-20.3
	Vehicle AM/PM	39.1	<0.001	68.36	20.73333333	18.9-22.5
Per1						
SCN	CORT AM	82.1	<0.001	87.57	4.133333333	3.3-5
	CORT PM	82.1	<0.001	84	4.52	3.7-5.3
	Vehicle AM/PM	92.7	<0.001	82.47	3.72	3.3-4.1
PVN	CORT AM	59	<0.001	120.09	13.62	12.1-12.6
	CORT PM	72.5	<0.001	136.09	13.32666667	12.3-14.4
	Vehicle AM/PM	66	<0.001	131.15	12.55333333	11.5-13.6
Anterior Pituitary	CORT AM	27.1	0.036	181.92	9.293333333	6.1-12.5
	CORT PM	38.3	0.006	155.51	10.48	8.1-12.7
	Vehicle AM/PM	9.2	0.224	177.34	13.28666667	--
Per2						
SCN	CORT AM	93.3	<0.001	150.89	7.82	7.3-8.2
	CORT PM	90.9	0.001	150.14	8.1	7.5-8.7
	Vehicle AM/PM	85.5	<0.001	151.97	8.446666667	7.9-9.1
PVN	CORT AM	44.9	0.002	119.89	18.61333333	16.6-20.6
	CORT PM	74.4	<0.001	132.45	15.56	14.5-16.6
	Vehicle AM/PM	81.6	<0.001	128.94	15.46	14.7-16.2
Anterior Pituitary	CORT AM	41	0.005	178.36	9.653333333	7.3-11.9
	CORT PM	63.2	<0.001	120.22	9.56	8.2-10.9
	Vehicle AM/PM	21.9	0.022	83.94	12.00666667	9.2-14.8

Table 1: Estimates of measured acrophase (ϕ) for *Bmal1*, *Per1*, and *Per2* mRNA in the SCN, PVN, and anterior pituitary are summarized above. Associated confidence intervals (C.I.) are given only where rhythmicity was significant. Percent rhythm (PR), p values, and mesor values are also listed. Estimated acrophases and C.I. are given as ZT.

Discussion:

Clock Gene Expression in the SCN:

In this study we investigated the influence of three CORT profiles (no CORT, antiphasic AM CORT, and phasic PM CORT) on the molecular clocks in the PVN and anterior pituitary, as well as the SCN. Core clock genes *Bmal1*, *Per1*, and *Per2* mRNA exhibited rhythmic expression in the investigated components of the HPA axis and the SCN in phasic CORT treated groups. *Per1* and/or *Per2* behaved in an oscillatory way to *Bmal1* mRNA, displaying antiphasic rhythmicity. This has been previously shown (Woodruff, Fardi, and Spencer 2013, unpublished data; Girotti et al., 2009) and indicates that there is a functional molecular clock in these cells. Moreover, gene expression in the SCN presented with a different phase relationship than the components of the HPA axis, which replicated findings in Girotti et al.'s 2009 study. We found that CORT treatment did effect the expression of *Per1* and *Per2* in both the PVN and anterior pituitary.

As expected, due to the lack of GRs in the SCN, *Bmal1*, *Per1*, and *Per2* gene expression showed robust 24 hour rhythms largely unaffected by CORT status. In line with previous reports *Bmal1* and *Per1* displayed an oscillatory rhythm with peaks and troughs in antiphase to each other (Girotti et al., 2009). *Per2* gene expression peaked between the acrophases of *Bmal1* and *Per1*, overlapping with the acrophase of *Per1* expression. While statistical difference was found among treatment groups, such that the antiphasic CORT treated group was elevated at ZT12 for both *Bmal1* and *Per1* expression, the overlapping confidence intervals of amplitudes likely means there is not a functionally significant difference. The oscillatory behavior of these genes is support of the presence of an autonomous molecular clock within the SCN.

*Clock Gene Expression in the PVN***:**

Within the PVN clock genes were also rhythmically expressed in all treatment groups. *Bmal1* and *Per1* expression were antiphasic to each other and their SCN counterparts. This may be a reflection of the primary source of input to these areas: the SCN is attuned to the light/dark cycle and photoperiod; where the PVN is sensitive to activity pattern, nocturnal in rats.

Interestingly, *Per2* expression in vehicle and PM CORT treated groups again peaked at the same time as *Per1*, though the peak is extended, reaching into the dark phase. Antiphasic CORT treated rats presented with blunted *Per2* expression at ZT 12, peaking solely at ZT 18. While removal of CORT did not abolish the rhythms of the clock genes, antiphasic CORT treatment caused significant blunting of the amplitudes of *Per1* and *Per2* expression at ZT 12 compared to vehicle and PM CORT groups. The antiphasic CORT injection may have caused competing input, acting opposite the light/dark cycle input from the SCN, and decreasing the acrophase of *Per1* expression. This result was expected given the presence of a GRE in its promoter region and the ubiquitous expression of GR in the PVN. The functional, highly responsive GRE and the blunting influence of antiphasic CORT on *Per1* expression in the PVN is indicative of its ability to override the entraining effect of photoperiod on clock gene expression in the PVN.

Per2 rhythmicity in PM CORT and Vehicle groups was unaffected by adrenalectomy when compared to the adrenal intact rats in Girotti et al.'s 2009 study. AM CORT treated rats displayed rhythmic but blunted expression of *Per2*. The GRE in the promoter region of *Per2* has been described as continuously occupied, meaning that as long as GR is bound to it, expression of PER2 protein is rhythmically expressed (So et al., 2009). It is not clear what the effect of adrenalectomy and antiphasic CORT would be on such a mechanism, as the injected CORT

dissipates more rapidly in the body than an endogenous peak. Adrenalectomy has been shown to blunt the expression of PER2 protein in the oval nucleus of the bed nucleus of the stria terminalis and central nucleus of the amygdala (Amir and Stewart, 2009). However, an effect in mRNA expression was found only in the antiphasic CORT treated group of our study. The idea of competing inputs, light/dark information from the SCN and the antiphasic CORT injection, may be responsible for the blunted rhythm of *Per2* mRNA.

Conversely, a functional GRE in the promoter region of *Bmal1* has not been identified, and consequently this may be why it was unaffected. As well, the PM CORT treated rats showed rhythmic expression which matched rhythmicity of adrenal-intact rats (Woodruff, Fardi & Spencer 2013, unpublished data). This CORT profile enables a normal rhythm of *Per1* that may facilitate the maintenance of *Bmal1* expression through the negative influence of its protein product.

Clock Gene Expression in the Anterior Pituitary:

In the pituitary *Bmal1* expression showed a robust 24 hour rhythm in all treatment groups, however in Vehicle groups *Per1* expression was not rhythmic and *Per2* expression was extremely blunted at the acrophase. The timing of peaks and troughs of *Bmal1* and *Per2* of rhythmically expressed groups match previous reports (Bur et al., 2010; Girotti et al., 2009) and are loosely in phase with the PVN.

Studies have reported different results regarding *Per1*: either not significantly rhythmic (Girotti et al., 2009) or rhythmic with a peak at ZT 12 (Bur et al., 2010). Where these studies characterized baseline clock gene expression and response to restricted feeding, we investigated the influence of different CORT profiles. We found that in the pituitary both CORT-treated groups *Per1* displayed a 24 hour rhythm with an acrophase at ZT 12; however, the vehicle group

did not. Given that there was no interaction between CORT status and time of day, it is unlikely that there is an effect of effect on *Per1* mRNA expression. It may be that CORT's presence was necessary for rhythmicity, but the time of the peak did not matter.

Per2 expression in the pituitary, despite the statistical rhythmicity, was nearly a flat line, with gene expression effectively abolished when compared to other treatment groups. While this effect needs to be replicated, the elevated mean expression may suggest that *Per2* gene expression is up-regulated in response to antiphasic CORT treatment. It is possible that *Per2,* which has a continuously bound GRE (So et al., 2009), is up-regulated due to a lack of circulating, endogenous CORT and the influence of a high dosage, antiphasic injection of CORT. The rhythm of *Per2* expression was robust when CORT was present suggesting that CORT has a permissive effect in the pituitary.

Bmal1 seemed unaffected by CORT status in the anterior pituitary; this may be because even in the vehicle group, where *Per1* expression was not rhythmic in the pituitary, there were still high levels of mRNA and the possibility that PER1 protein was still present and able to regulate BMAL1 through negative feedback.

The pituitary may be integrating multiple time cues from the periphery and brain, causing it to have a rhythm Bur et al. described as flexible (2010). They investigated the effect of restricted feeding on clock gene expression in the pituitary. Day time restricted feeding causes a shift in CORT expression and activity in rats, in some ways acting as an antiphasic CORT treatment. They found that *Per2* mRNA expression was blunted in adrenal intact and adrenalectomized rats in the anterior pituitary, which suggests presence of CORT may not be crucial to synchronizing the oscillator in the gland. Unfortunately, *Per1* was not investigated in

that experiment. Our findings suggest differently, that CORT may have a permissive effect in the anterior pituitary, specifically for expression of *Per2*, meaning it must be present for rhythmicity.

As the mammalian anterior pituitary is not innervated, other hormonal factors must come into play and enable synchronization (Yoo et al., 2004). The anterior pituitary, which is not homogenized tissue, but composed of numerous types of endocrine cells, may require a multi-input system of synchronization sensitive to multiple entraining factors, such as CORT, photoperiod, and may be further coordinated by pituitary hormones in lieu of conflicting input. The latter types of coordination and synchronization may be especially important for expression of *Per1* which does not seem to be primary in the pituitary's molecular clock, as *Per2* and *Bmal1* have been repeatedly shown to express antiphasic rhythms (Girotti et al., 2009; Bur et al., 2010). *Per1* may also be more sensitive to the light/dark cycle as Bur et al. found mRNA expression shifted in response to varied photoperiods (2010. Differential expression in endocrine cell type of the anterior pituitary may explain why Girotti et al. did not find *Per1* mRNA rhythmic (2009); in our experiment, the administered dosage of CORT, 2.5 kg/mg, is higher than the endogenous peak and could have entrained the *Per1* expression, creating an artificial rhythm. The possibility that *Per1* is differentially expressed in different endocrine cells of the anterior pituitary requires further investigation and may clarify part of the complexity of characterizing clock gene expression in the gland.

Conclusion:

In this study we demonstrate that the molecular clocks in various components of the HPA axis are differentially responsive to various CORT profiles, influencing specific clock genes and that CORT may have a different role in the periphery than the brain. Presence and CORT status affects the amplitude and robustness of rhythmicity of clock genes in both the PVN and anterior

pituitary. Antiphasic CORT treatment influenced the phase and amplitude of clock genes, namely *Per1* and *Per2* in the PVN. This suggests that CORT may be able to override the influence of photoperiod and input from the SCN, at least enough to compete with the input and alter molecular functioning. In the anterior pituitary, the absence of CORT, abolished *Per2* mRNA expression, suggesting that CORT may have a permissive role in the clock gene's expression. This means that CORT must be present for there to be rhythmic clock gene expression of *Per2* in the anterior pituitary but may not be responsible for entraining their rhythms. Further exploration into the complex integration of time cues in the pituitary are needed to understand the role of CORT on *Per1* gene expression, and the possibility of multiple time cues being integrated.

Dysregulation of normal HPA axis functioning has been linked with psychiatric disorders (Pariante and Lightman, 2008). Depending on the kind of disruption or disorder, hypercortisolemia, seen in Major Depressive Disorder (Ahiara et al., 2007; Pariante & Miller, 2001), or hypocortisolemia, observed in Depersonalization Disorder and Posttraumatic Stress Disorder (Heim et al., 2000; Simeon et al., 2001; Schuder, 2006). Both profiles have the potential to created metabolic and immune disorders given the normal role CORT in the body. Phase shifts in CORT have been implicated in manic-depressive disorders (Wehr et al., 1980). Given CORT's role in regulating and entraining molecular clocks, disruption in the circadian and HPA axis systems and interactions may cause dysynchronization in molecular clocks in the periphery and non-SCN brain region. People who experience jet lag often and shift workers experience internal desynchrony and are at increased for depression and cardiovascular disorders (Penev et al., 1998; Knutsson, 2003). In particular the effect of antiphasic CORT which impacted *Per1* and *Per2* gene expression PVN, caused variation in clock gene expression in the pituitary,

and potentially disengaging the gland from the PVN. Characterizing and understanding the mechanisms underlying regulation of the molecular clocks in the HPA axis and the wide spread consequences of disruption are crucial steps to developing effective treatments for these disorders.

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