The Circadian Regulation Of Glucocorticoid Release In Rodents

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Abstract

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Biology

Nearly all organisms living near the surface of the Earth contain circadian (*circa* – "about"; *diem*- "day") clocks functioning as an endogenous timekeeping mechanism by which the organism can coordinate biological processes with 24h cycles in the external environment (such as the daily light:dark cycle). In nature, the ~24h free-running oscillation of circadian clocks is synchronized or entrained with the precisely 24h solar day cycle. In mammals, the master circadian pacemaker is located in the hypothalamic suprachiasmatic nucleus (SCN) which regulates ~24h rhythms of biological activity. The master circadian pacemaker within the SCN modulates adrenal gland release of glucocorticoids (GCs).

GCs are a class of steroids critical for the mobilization of energy throughout the organism. GCs are released from the adrenal cortex and exhibit a circadian oscillation anticipating the onset of locomotor activity. In addition to humoral input to the adrenal cortex via adrenocorticotropic hormone (ACTH), the functional anatomy of the adrenal cortex demonstrates extensive sympathetic input. These signal transduction cascades are largely dependent on cyclic adenosine monophosphate (cAMP)-associated signaling. Recent studies demonstrate cAMP is a core clock component required for the maintenance of cellular circadian activity. Circadian GC release might be regulated by 2 independent pathways: 1) via circadian modulation of ACTH release from the pituitary gland in the hypothalamic-pituitary-adrenal (HPA) axis; 2) via a multisynaptic neural pathway between the SCN and adrenal cortex. The studies of this dissertation demonstrate behaviorally split golden hamsters exhibit a dissociation of circadian oscillations of cortisol (the major hamster GC) and ACTH, suggesting the circadian

release of GCs is regulated by modes independent of the HPA axis, such as multisynaptic innervation.

Additionally, phase coordination of the adrenal circadian clock with the external environment can be controlled by temporal fluxes in cAMP signaling in the adrenal gland, which is ultimately responsible for resetting the adrenal circadian clock, as well as the generation of ~24h oscillations in the concentration of circulating plasma GCs. Chapter 3 of this dissertation demonstrates phase specific responsiveness to temporary and reversible interference with rhythmic adrenal cAMP signaling, a hallmark of entrainment processes.

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DEDICATION

I dedicate my dissertation to my loving wife, Jodi. I would not have made it through school without her support and I want to thank her for all she has sacrificed to enable me to pursue this research.

CHAPTER 1

INTRODUCTION

The field of Chronobiology and the studies of this dissertation are concerned with mechanisms by which living organisms adapt their physiology and behavior to time. Through natural selection, nearly all organisms on Earth have developed biological clocks functioning as endogenous timekeeping mechanisms that are critical for coordinating rhythmic biological processes with rhythmic environmental cycles. The 24h rotation of the Earth drives numerous environmental cycles (such as the daily light:dark (LD) cycle) and endogenous circadian (circa- “about” ; diem- “day”) biological clocks coordinate rhythmic biological processes with these 24h environmental cycles. Circadian rhythms persist in constant conditions with a circa-24h period and are pervasive throughout life at the behavioral, physiological, and molecular levels of biological organization (Gachon et al., 2004). Single-cell circadian clocks generate ~24h oscillations in the activity of transcription/translation machinery, which act as a mechanism to optimize the function of a specific tissue to daily fluxes in metabolic demands (Hastings et al., 2007). In fact, in nearly every cell in the human body, there are molecular biological clocks coordinating bodily processes. At the organismal level of organization, the mammalian circadian system is hierarchical with a master circadian clock located in the hypothalamic suprachiasmatic nucleus (SCN) orchestrating the phase of circadian oscillatiors in the rest of the brain and in peripheral organs throughout the body (Stratmann and Schibler, 2006). Circadian biologists hypothesize the deleterious health consequences associated with occupations exposing personnel to chronic jet lag or rotational shift work are the manifestation of a disruption of the

cohesive alignment of these multiple circadian waveforms (Hastings et al., 2003). In other words, the net phase relationship among the collective circadian oscillations within the individual becomes temporally disrupted as all circadian oscillators are shifted at different rates in response to changes in cyclic environmental stimuli. The specific subject of this dissertation is an examination of the behavioral, physiological, and molecular regulators governing the circadian oscillation of plasma glucocorticoids (GCs).

Glucocorticoids

GCs are a class of steroid hormones which are a relatively old subject of study within the fields of medicine and endocrinology. In 1936, Tadeus Reichstein and co-investigators discovered and deposited a patent for the primary GC in rats, corticosterone (CORT), while near simulataneously Hans Seyle discovered their primary physiological role in the stress response (Selye, 1936). As several decades of additional research accumulated, it became apparent that GCs are vital and major players in allowing animals to cope with mental and physical stress or, in Seyle's words, "the syndrome produced by diverse nocuous agents." Additionally, this body of research collectively determined stress-induced GC release is regulated by the hypothalamic-pituitary-adrenal (HPA) axis.

Within the HPA axis, the hypothalamic paraventriular nucleus (PVN) contains parvocellular neurosecretory neurons which make corticotropin-releasing hormone (CRH) and regulate the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland into the systemic blood stream (Fig. 1A). Melanocortin 2 receptor (MC2R) is the receptor for ACTH and highly expressed in the *zona fasiculata* of the

adrenal cortex. MC2R is a membrane bound G-coupled protein receptor operating through adenylate cyclase (AC) activation and cyclic adenosine monophosphate (cAMP) second messenger signaling (Fig. 1B). In addition to the known humoral input of ACTH to the adrenal gland, it is known that the *zona fasiculata* receives input from a variety of neural stimuli including pre- and post-ganglionic sympathetic neurons and type I and II cell types in the adrenal medulla which are developmentally derived as heavily modified post-ganglionic neurons (Engeland and Arnhold, 2005). In the stress response, ACTH-triggered elevation of intracellular cAMP activates Protein Kinase A (PKA), which activates cAMP Response Element Binding (CREB) protein via phosphorylation (Fig. 1B). Phospho-CREB is a transcription factor activating a diverse range of genes and functions. During stress, this results in the increased expression of genes involved in producing GCs and releasing GCs into the systemic bloodstream. Free and transcortin carrier protein-bound GCs circulate throughout the system and bind 2 different types of intracellular GC receptors and poorly understood membrane-bound receptors (Bartholome et al., 2004). The intracellular receptors, mineralocorticoid receptor (MR) and glucocorticoid receptor (GR), bind GCs and act as transcription factors elevating the expression of genes associated with energy mobilization; however this specific subset of genes varies depending of the specific tissue type (Dickmeis, 2009). As a result of the body's global response to elevated circulation of GCs, basic building blocks of anabolic metabolism and energy production become elevated including but not limited to glucose, free fatty acids, and amino acids. Additionally, GCs exert anti-inflammatory effects and are implicated in lung maturation during fetal

development. Finally, plasma GCs also exhibit a circadian oscillation with a peak anticipating the onset of locomotor activity and wakefulness.

The mammalian circadian system

In the mammalian circadian system, the organism receives input from external environment stimuli such as the LD cycle. This information is received by non-image forming photoreceptors in the retina and relayed to the SCN (Hattar et al., 2002; Morin and Allen, 2006). The SCN is composed of a group of ~50,000 neurons in humans or ~20,000 rats and is responsible for regulating ~24h rhythms of behavior and physiology. This regulation is achieved by adjusting ~24h rhythms in the activity of cellular machinery in extra-SCN clocks within the brain and peripheral clocks throughout the body (Hastings et al., 2003). These clocks act as local modulators to optimize the function of a given tissue type with the external environment. In constant conditions, biological clocks and clock outputs are allowed to *free-run* or oscillate with an approximately 24h rhythmicity. In nature, these biological clocks coordinate or synchronize the ~ 24h endogenous oscillation of biological clocks with the precisely 24h photoperiod of the environment through a process called *entrainment*. All of these cellular clocks, whether in the SCN or periphery, rely on intracellular transcription/translation feedback loops of "clock genes." The core clock genes consist of *Brain And Muscle Aryl Hydrocarbon Receptor Nuclear Translocator* (*Bmal*)*, Clock* (*Clk*)*,* the *Period* genes (*Per1* and *Per2*) and *Cryptochromes* genes (*Cry1* and *Cry2*) (Fig. 3) (Ko and Takahashi, 2006)*.* In this gene network, BMAL and CLK proteins activate *Per* and *Cry* transcription. However, as PER and CRY proteins accumulate, they migrate to the nucleus and inhibit the ability of BMAL and CLK to induce *Per* and

Cry transcription, thus inhibiting their own transcription. The kinetics of the positive and negative limbs of these feedback loops generate ~24h oscillations in the products of clock genes, as well as “clock-controlled genes” (Fig 3). About 8–10 percent of all expressed genes in any tissue are under circadian regulation and only about 10 percent of cycling genes in any pair of tissues are the same, suggesting a tissue-specific function for the circadian clockwork (Storch et al., 2002). Recently, cAMP was determined to be a core-clock component (O'Neill et al., 2008). In other words, it has all the properties of a clock gene, except for not being a gene but a small signal-transduction molecule. A core clock component is a factor that is critical in determining the canonical properties of the circadian waveform, which is characterized by three basic waveform parameters: period, phase and amplitude.

At the organismal level of organization, the mammalian circadian system comprises of a 3-tiered hierarchy in which the master pacemaker within the SCN regulates all other circadian oscillators (including the adrenal gland); however, a subset of peripheral oscillators are also subordinate to the circadian oscillation of GCs from the adrenal gland (Fig 4) (Balsalobre et al., 2000). Interestingly, GCs have the ability to directly interact with the molecular clockwork, as it was recently found that the *Cry* core clock genes are tightly connected with the regulation of rhythmic repression of GC receptors (Lamia et al., 2011). Thus, GCs are potentially a critical endogenous signal by which the SCN relays circadian phase information throughout the body.

Circadian modulation of GC release

One critical process regulated by the SCN is the circadian release of glucocorticoids (GC) from the adrenal cortex (Moore and Eichler, 1972). Circadian GC

release exhibits a peak near the onset of locomotor activity, preparing the organism for the increased energetic demands of wake relative to sleep. One potential pathway regulating the circadian oscillation of GC release is the HPA axis (Fig. 1A; Fig. 2). Plasma ACTH concentrations exhibit a low amplitude circadian oscillation with an anticipatory rise in ACTH prior to the elevation of CORT and onset of locomotor activity (Dr. Bill Engeland unpublished observations).

Recent evidence suggests there is potentially another regulatory branch governing circadian GC release (Bornstein et al., 2008). In the mouse, light pulses during the early subjective night induce corticosterone release, without a corresponding rise of plasma ACTH and this light-induced CORT release is dependent on an intact SCN and Greater Thoracic Splanchnic Nerve (SPLN) integrity (Ishida et al., 2005). These observations of light-induced CORT release are also correlated with an upregulation of *Per1* clock gene expression in the adrenal gland. Additionally, light-induced corticosterone release is correlated with SPLN activity as measured through extracellular field potentials when mice are presented a light pulse during the subjective night, but not when an identical treatment is applied during the subjective day (Ishida et al., 2005). In line with these results, tract-tracing studies in the rat demonstrate a multisynaptic neural pathway between the SCN and the adrenal cortex (Buijs et al., 1999) (Fig. 2). Finally, as part of this dissertation I have shown that hypophysisectomized rats exhibit small but significantly elevated concentrations of plasma CORT relative to bilaterally adrenalectomized rats (Fig. 5; $p<0.001$).

The subject of this dissertation is to elucidate mechanisms and pathways regulating the circadian oscillation of plasma GCs and entraining the adrenal circadian clock.

CHAPTER 2 (Lilley et al (2012) *Endocrinology*)

Circadian Regulation of Cortisol Release in Behaviorally Split Golden Hamsters

INTRODUCTION

Virtually all organisms have circadian clocks (or pacemakers) functioning as endogenous timekeeping mechanisms that drive daily biological rhythms. Under natural conditions, circadian (~24 h) pacemakers entrain to 24-h exogenous cycles (such as the light-dark cycle), harmonizing daily physiological and behavioral processes with the external environment. In mammals, the master circadian clock within the hypothalamic suprachiasmatic nucleus (SCN) coordinates the precise timing of daily biological rhythms through neural and humoral outputs to other brain regions and extra-SCN circadian clocks (Hastings et al., 2003).

One critical process regulated by the SCN is the circadian release of glucocorticoids (GC) from the adrenal cortex (Moore and Eichler, 1972). Circadian GC release exhibits a peak near the onset of locomotor activity, preparing the organism for the increased energetic demands of wake relative to sleep.

When golden hamsters (*Mesocricetus auratus*) are housed under constant light conditions (LL), the circadian locomotor activity pattern of approximately 60% of the animals will split into two bouts of locomotor activity approximately 12 h apart. Each of these peaks presumably represents the independent outputs of the asymmetrically active bilaterally paired left and right SCN (de la Iglesia et al., 2000). This model has been used to delineate pathways underlying the circadian regulation of the preovulatory LH surge (Swann and Turek, 1985; de la Iglesia et al., 2003). To our knowledge,

however, no studies have used the split hamster model to elucidate regulatory pathways by which the circadian system controls GC release.

The first potential regulatory branch controlling circadian GC release is via the hypothalamic-pituitary-adrenal (HPA) axis. In this branch, SCN efferents directly (Vrang et al., 1995) and indirectly (Buijs et al., 1993) regulate CRH-containing cells in the paraventricular nucleus. These neurosecretory cells subsequently release CRH, which induces the release of ACTH from the anterior pituitary gland, which in turn, triggers the release of GC from the adrenal cortex.

Recent evidence suggests there is potentially another regulatory branch governing circadian GC release (Bornstein et al., 2008). In the mouse, light induces the release of corticosterone without a corresponding rise of plasma ACTH. This light-induced corticosterone release is dependent on an intact SCN and innervation of the adrenal by the thoracic splanchnic nerve (Ishida et al., 2005). In line with these results, tract tracing studies in the rat demonstrate a putative multisynaptic neural pathway between the SCN and the adrenal cortex (Buijs et al., 1999).

Finally, SCN coordination of the phase of a circadian clock within the adrenal gland is also implicated in circadian GC release. Adrenal gland-specific circadian clock knockdown mice exhibit dampened circadian oscillations of corticosterone (Son et al., 2008). Interestingly, circadian clocks of the left and right adrenal glands in split hamsters oscillate in antiphase, mirroring the activity of the SCN and suggesting the phase of the adrenal circadian clock is regulated through neural pathways instead of systemically released humoral factors such as ACTH (Mahoney et al., 2010).

The current study exploited the split hamster to test the hypothesis that the circadian release of GC is the result of the integration of SCN-adrenal communication via the following: 1) a multisynaptic neural pathway and 2) the HPA axis.

MATERIALS AND METHODS

Animals and monitoring of locomotor activity

Male golden hamsters were purchased from Charles River Laboratories (Wilmington, MA) and used in accordance with regulations established by the University of Washington Institutional Animal Care and Use Committee. Animals were 30 d old upon their arrival and individually housed under LL (~250 lux). Animals were provided with food and water ad libitum, which was restocked randomly, and wheel-running locomotor activity was continuously monitored via ClockLab software (Actimetrics, Wilmette, IL). After 2 months under LL, approximately 67% of the hamsters exhibited stable behavioral splitting of locomotor activity as determined by visual inspection of actograms. The remaining unsplit hamsters were used as control animals.

Surgeries

Animals were anesthetized and implanted with jugular catheters. Briefly, the right external jugular vein was exposed and SILASTIC brand (Dow Corning Corp., Midland, MI) tubing (inner diameter × outer diameter: 0.020 × 0.037) was inserted into the vessel until the end reached the right atrium. The tubing was stitched to the vein with two nylon sutures to hold its position. An exit site for the cannula was created in the scapular region of the animal by sc tunneling with a 16-gauge needle. The catheter was then

filled with heparinized saline (20 U/ml) and sealed with the tip of a 22-gauge needle that had been filled with SILASTIC glue (Dow Corning). Animals were allowed to recover from the surgery for 3–5 d before blood collection.

Blood collections

Blood was serially collected from unstressed animals to generate complete 24-h hormone profiles of plasma cortisol and ACTH. In the group of hamsters used for cortisol measurements (n = 8 control; n = 6 split), 25 µl of blood were collected every hour for a total of 23 h. In the group of animals used for ACTH measurements (n = 6 control; n = 8 split), 250 µl of blood was collected every 3 h for 21 h. After each blood draw, the volume was replaced 1:1 (25 and 250 µl for cortisol and ACTH collections, respectively) with warm (37 C) saline. Potential cortisol and ACTH release in response to the stress of the sampling procedure was controlled in several ways. First, the start of sampling for each individual hamster varied relative to the individual's onset of wheel-running activity; thus, peaks in hormone levels that correlated with activity onsets were not due to the initiation of the blood collections. Second, animals underwent mock collection procedures at least three times per day for 1–2 d before collection to acclimate the animals to frequent human handling. Third, all samples were collected within 3 min of removing the animals from their home cages. In split animals, the asymmetry between the length of each of the two activity bouts (if any) was not taken into account for the bleeding schedule because this asymmetry bears no relationship to which side of the SCN (left or right) is responsible for each bout (de la Iglesia et al., 2000). Blood was collected in cold EDTA-treated syringes for ACTH assays and heparin-treated syringes for cortisol assays. After blood collection, plasma was isolated

by centrifuging at 4 C and 2000 × g for 15 min and stored at −80 C until conducting hormone assays.

Determining plasma cortisol and ACTH concentrations

Although hamsters are dual secretors of both corticosterone and cortisol, the release of cortisol shows a higher amplitude circadian rhythm (Albers et al., 1985; Meyer-Bernstein et al., 1999), and cortisol is clearly a more potent regulator of energy homeostasis in this species (Solomon et al.). Plasma cortisol concentrations were determined in triplicate with a cortisol express enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI) in accordance with the manufacture's protocol. This assay has minimal cross reactivity (0.14%) with corticosterone. Samples were run at 1:50 dilution. Inter- and intraassay coefficients of variability were 11.57 and 11.86%, respectively. Plasma ACTH concentrations were determined by RIA according to previously established methodology (Jasper and Engeland, 1991). Antibody directed against rat ACTH (Rb7) was acquired as a generous gift from Dr. Bill Engeland (University of Minnesota, Minneapolis, MN). Briefly, plasma and a serial dilution of rat ACTH 1-39 standards (Bachem, Torrance, CA) were exposed to ACTH antibody (1:120,000 antibody in phosphate buffer with EDTA, Triton X-100, aprotinin, BSA, and blue food coloring, which does not interfere with assay and helps with keeping track of the pipetting sequence) overnight at 4 C. Then 125I-labeled ACTH 1-39 (DiaSorin, Stillwater, MN) was added to a final concentration of 25 cpm/µl per reaction and allowed to incubate overnight at 4 C. On the third day of the assay, goat antirabbit IgG secondary antibody and normal rabbit serum (Peninsula Laboratories) were added to the reaction and incubated for 4 h at 4 C, after which separation buffer (phosphate

buffer with EDTA and 99.9% protease free BSA) was added to the reaction and centrifuged at 4 C for 25 min at 2500 rpm. The supernatant was then aspirated and the remaining pellets were counted in duplicate on a γ-counter (Wizard 1470 automatic γ-counter; PerkinElmer, Waltham, MA) for 10 min each. Serially diluted ACTH standards were used to generate a four-parameter logistic standard curve via AssayZap software (Biosoft, Cambridge, UK) from which unknown concentrations were calculated. Inter- and intraassay coefficients of variability were 6.28 and 5.17%, respectively. Areas under the curve for complete cortisol and ACTH 24-h outputs were relatively quantified in Photoshop (Adobe, San Jose, CA) by using the magic wand tool, which returns a pixel number for each area.

Statistics

Plasma hormone concentrations were log transformed to obtain homogeneity of variance as determined by Fligner and Bartlett's tests, and statistical differences were then determined by ANOVA. For hormone profiles, the effect of time was determined by one-way ANOVA. Interaction of treatment (control or split) and time were determined by two-way ANOVA. Repeated-measure ANOVA was not used because a subset of hamsters lacked a complete 24-h profile (the minimum number of samples for any single time point was 4; see figure legends for details). Specific differences were determined by planned comparisons between split and controls for each individual time point.

RESULTS

Circadian release of cortisol exhibits two peaks in behaviorally split hamsters

After serially bleeding behaviorally split and unsplit hamsters housed in LL, plasma cortisol profiles were determined for each individual (Fig. 6A). Over a 24-h period, split hamsters exhibited two phases of elevated plasma cortisol, each preceding one of the two projected onsets of wheel-running activity. In contrast, control unsplit hamsters exhibited a single peak in plasma cortisol before the onset of wheel-running activity. Fig. 7A shows mean plasma cortisol for all split and unsplit hamsters. There was an effect of sample time on plasma cortisol levels in both groups as assessed by one-way ANOVA for control [$F_{(23)} = 5.39$, $P < 0.001$; n = 8 and 6–8 samples per time point] and split [$F_{(23)} = 3.36$, $P < 0.001$; n = 6 and 4–6 samples per time point] hamsters. Additionally, two-way ANOVA revealed an interaction of control/split state with sample time [$F_{(1,23)} = 3.13$, $P < 0.001$] and a marginally nonsignificant control/split group effect [$F_{(1)} = 3.07$, $P = 0.081$]. Post hoc analysis showed split hamsters had a lower cortisol peak before one of the two bouts of locomotor activity, compared with the single cortisol peak observed in control animals. Additionally, split hamsters exhibited a second significant elevation of plasma cortisol 10–12 h after one of the locomotor activity onsets, which was also just before the onset of the other locomotor activity bout. Furthermore, the area under the curve for hamsters with a complete 24-h profile demonstrates a significant reduction of the overall 24 h cortisol output in split hamsters relative to controls [$t_{(5.95)} = 2.47$, $P < 0.05$].

Plasma ACTH is not concomitantly elevated with cortisol in split hamsters

To test potential mechanisms regulating the dual peaks of cortisol in split hamsters, we repeated the above experiment but collected blood every 3 h for a total duration of 24 h to create plasma ACTH profiles from split (n = 8 and 6–8 samples per time point) and unsplit control (n = 6 animals and 4–6 samples per time point) hamsters (Figs. 6B and 7B). The sampling frequency was reduced to eight time points because a larger volume of plasma (100 µl) was required to assay ACTH. However, the 3-h sampling frequency is well within the minimum Nyquist frequency (one third of period) to detect an approximately 12 h oscillation (Marmarelis and Marmarelis, 1978). As illustrated in Fig. 6B, unsplit control hamsters displayed a single peak in plasma ACTH, occurring before the onset of locomotor activity, as seen for plasma cortisol in the first experiment. In contrast, split hamsters exhibited no significant elevation in plasma ACTH at any time (Fig. 6B). One-way ANOVA revealed an effect of time on plasma ACTH in control [$F_{(7)} = 4.61$, $P < 0.001$] but not in split [$F_{(7)} = 1.15$, $P = 0.346$] hamsters (Fig. 7B). Two-way ANOVA, with group (split/unsplit) and time as factors, revealed a significant interaction [$F_{(1,7)} = 2.47$, $P = 0.02$] but no significant effect of group [$F_{(1)} = 1.53$, $P = 0.21$]. Post hoc analysis revealed control hamsters had significantly higher ACTH levels at the onset of locomotor activity. Although the two-way ANOVA yielded no effect of group, analysis of area under the curve showed there was a decrease in the overall 24-h ACTH output of split relative to control hamsters [$t_{(8.18)} = 2.2611$, $P = 0.05$].

DISCUSSION

The circadian release of GC was characterized as an SCN-dependent rhythm 4 decades ago (Moore and Eichler, 1972), yet the pathways by which the master circadian clock regulates circadian GC release remain unknown. We show LL-housed hamsters that display a single approximately 24-h locomotor activity rhythm also display a single peak of cortisol release that is concomitant with ACTH release. Conversely, LL-housed hamsters that behaviorally split show two corresponding peaks of cortisol approximately 12 h apart, which are independent of circadian rhythmic ACTH release. These results suggest that the circadian release of cortisol is the result of the dual contribution of ACTH and non-ACTH regulatory pathways.

Two master clocks for physiology and behavior

Splitting in the hamster is the result of antiphase oscillations of the left and right SCN (de la Iglesia et al., 2000). Here we demonstrate that these antiphase circadian pacemakers can sustain two rhythms of cortisol release that are 180° out of phase. This finding is consistent with previous work in which behaviorally split female hamsters display LH surges approximately 12 h apart (Swann and Turek, 1985). Collectively, these data suggest circadian endocrine rhythms in general might show a dual oscillatory pattern in behaviorally split hamsters. Furthermore, core body temperature rhythms in the split hamster also show an approximately 12-h oscillation (Pickard et al., 1984). To our knowledge, no circadian outputs studied in behaviorally split hamsters have escaped the approximately 12-h modulation by the left and right SCN, indicating the neural and peripheral targets that produce these outputs remain responsive to ~12-h signals emerging from the master circadian pacemaker. Together, these results provide

strong evidence, from a neurologically, genetically, and pharmacologically intact animal model, for the unequivocal preeminence of the SCN in the regulation of rhythmic, physiological and behavioral processes and for an unparalleled localization of function in the brain. Importantly, work by Kalsbeek *et al.* (Yan et al., 2005; Kalsbeek et al., 2008) indicates the SCN may send signals that stimulate or inhibit GC release, depending on the time of the day. Thus, the two antiphase-oscillating SCN in the split hamster could respectively and simultaneously send inhibitory and stimulatory signals to the adrenal glands. This idea is supported by the fact that within each SCN of the split hamster two subsets of neurons also oscillate in antiphase (Tavakoli-Nezhad and Schwartz, 2005; Yan et al., 2005).

Although the split master circadian clocks can time a multiplicity of rhythms to an approximately 12-h period, the pathways by which they do so depend on the specific circadian rhythm examined. Locomotor activity rhythms rely on diffusible factors released by the SCN (Silver et al., 1996). In contrast, the circadian regulation of cortisol, corticosterone, LH, and melatonin release relies on synaptic connections of SCN efferent fibers on specific neural targets (Meyer-Bernstein et al., 1999). The regulation of the LH surge in split female hamsters is associated with the activation of the left- or right-sided GnRH network by ipsilateral SCN efferents, likely involving direct and indirect synaptic connections with GnRH neurons (de la Iglesia et al., 1995; Van der Beek et al., 1997; Vida et al., 2010). In the split female hamster, these connections presumably lead to the approximately 12-h activation of the hypothalamo-pituitary-gonadal axis. In contrast, the dual cortisol peaks we describe cannot be explained by the approximately 12-h activation of the HPA axis. Split hamsters show no significant

rise in plasma ACTH corresponding with the two elevations in cortisol. In the mouse, light-induced corticosterone release has been shown to occur without a concomitant increase in ACTH and is abolished by SCN lesions as well as by removing sympathetic input to the adrenal via splanchnic denervation (Ishida et al., 2005).

Our results in the split hamster suggest this neural pathway from the SCN to the adrenal could contribute to the circadian release of cortisol without the intervention of rhythmic ACTH input. Such a neural pathway could drive circadian cortisol release by direct stimulation of adrenal cortical cells or by modulating adrenal sensitivity to ACTH (Ulrich-Lai et al., 2006) (Fig. 8). Recent evidence indicates the adrenal peripheral clock locally regulates the amplitude of circadian GC release (Son et al., 2008), and it is conceivable that SCN signals relayed through the splanchnic nerve may set the phase of the adrenal clock, which in turn may sustain a rhythm in GC synthesis and release. In striking support of this model, circadian expression of Period1 (Per1), a genetic component of the molecular circadian clock, in the left and right adrenal cortex, and particularly the adrenal medulla, of the split hamster oscillate in antiphase, mirroring the expression of Per1 in the SCN (Mahoney et al.). Although it is not clear what the role of medullary Per1 expression could be, it may also modulate glucocorticoid synthesis and release by the cortex. In summary, circadian GC release is likely the result of combined SCN input to the adrenal through the HPA axis and the sympathetic nervous system (Fig. 8). This latter pathway could directly influence GC synthesis and release and/or impose a rhythm in sensitivity to ACTH. Although evidence for a role of sympathetic innervation on the regulation of GC release has been previously described (Jasper and Engeland, 1991; Ishida et al., 2005; Ulrich-Lai et al., 2006), our study is the first to

suggest its involvement in circadian GC release in the absence of rhythmic ACTH release. The dual regulation of circadian GC release by the HPA axis and sympathetic innervation could represent a mechanism that assures proper phase and amplitude of circadian GC release. Furthermore, each pathway's contribution could change under different conditions. For instance, sympathetic innervation could be more prominent in split than unsplit hamsters and therefore sufficient to sustain a circadian rhythm of GC release in the absence of an ACTH rhythm.

Cortisol negative feedback is likely altered in split hamsters

Our study demonstrates behaviorally split hamsters show two peaks of cortisol release; however, their overall 24-h cortisol output is reduced compared with unsplit control animals. Despite the observed overall reduction in plasma cortisol, we hypothesize the bimodal pattern of cortisol release leads to increased negative feedback, reducing the levels of ACTH and the general drive of the HPA axis. Because we observed a diminished total 24-h cortisol and ACTH output in split hamsters compared with unsplit animals, we hypothesize that the timing of release, not the absolute amount of cortisol *per se*, may represent a more critical signal to increase negative feedback. Alternatively, the antiphase oscillation of the left and right SCN in the split hamster could lead to changes in pulsatile release of ACTH that are undetected by our sampling frequency (Gudmundsson and Carnes, 1997); a recent study has shown that exogenously administered ACTH induces corticosterone release only if administered at a pulsatile but not at a constant rate (Spiga et al., 2011). Finally, we did not measure corticosterone in our study. Although the circadian regulation of both cortisol and corticosterone is similar in golden hamsters, the rhythm of cortisol secretion

has higher amplitude in intact animals (Albers et al., 1985; Meyer-Bernstein et al., 1999). On the other hand, chronic stress in the hamster has been shown to increase cortisol but not corticosterone (Ottenweller et al., 1985). We do not think this differential stress-induced regulation of both GCs is relevant to our results because unsplit hamsters in our study show similar peak levels of cortisol as hamsters housed under 14-h light, 10-h dark cycles (Albers et al., 1985); future studies should determine whether corticosterone is regulated similarly to cortisol in the split hamster and whether negative feedback is indeed increased by the bimodal secretion of GC.

Our results in a neurologically and genetically intact animal support a model in which the circadian release of GCs is the result of the dual contribution of ACTH and non-ACTH regulatory branches emerging from the master circadian pacemaker located in the SCN. Furthermore, our data suggest the negative feedback systems regulating GC release may be more sensitive to the timing of glucocorticoid release than to the absolute levels of circulating GCs.

CHAPTER 3

cAMP mediated phase shifting of the adrenal circadian clock

INTRODUCTION

Circadian Internal Synchronization

In the mammalian circadian system, the master circadian pacemaker within the SCN regulates the phase of circadian oscillators distributed throughout the organism through a variety of specific neural and humoral outputs. Additionally, it has been demonstrated that GCs are potentially a critical endogenous signal by which the SCN communicates time information to body peripheral oscillators (Dickmeis, 2009). It has been demonstrated that exogenous application of dexamethasone (a synthetic GC) induces phase shifts in fibroblast, kidney, liver, and heart (Balsalobre et al., 2000). Additionally, the SCN does not express MR or GR while the receptors are ubiquitously expressed throughout the rest of the body (Balsalobre et al., 2000). Thus, GCs represent a unidirectional signal in the context of organismal organization of the mammalian circadian system and an attractive candidate mechanism by which the SCN can coordinate circadian oscillations of peripheral clocks.

The Adrenal Peripheral Clock

Coordination of the phase of a circadian clock within the adrenal gland is implicated in circadian GC release (Son et al., 2008). Clock genes are rhythmically expressed in the adrenal gland peaking in expression at 1h before lights off when housed in a 12:12LD photoperiods or circadian time (CT) 11 when housed in constant dim red light (Fahrenkrug et al., 2008). Furthermore, adrenal gland-specific circadian

clock knockdown mice exhibit dampened circadian oscillations of corticosterone, supporting the notion that the adrenal circadian oscillator can locally time the synthesis and/or release of GCs (Son et al., 2008). Interestingly, clock gene oscillations of the left and right adrenal glands in behaviorally split hamsters oscillate in antiphase, mirroring the activity of the left- and right-sided SCN and suggesting the phase of the adrenal circadian clock is regulated through neural pathways instead of systemically released blood borne factors like ACTH (Mahoney et al., 2010). Finally, as shown in Chapter 2, this unilateral antiphase circadian oscillation of clock genes in the adrenal is associated with a dual (circa-12 h) pattern of GC release (Lilley et al., 2012).

Adrenal Gland Functional Anatomy

The adrenal cortex and medulla are highly innervated tissues receiving a diverse array of signals via the HPA axis and the SPLN nerve (Engeland and Arnhold, 2005). As outlined in the Introduction of this dissertation, the adrenal receives humoral input through the HPA axis via ACTH. This is achieved through the expression of MC2R (the ACTH receptor) in the adrenal cortex, whose activation leads to increases of cAMP within the *zona fasiculata*, the adrenal cortical region of GC synthesis and release. Additionally, the adrenal cortex is highly innervated by a number of transmitter systems associated with many metabotropic receptor types including: adrenergic α2, adrenergic β, neuropeptide Y (NPY), and vasoactive intestinal peptide (VIP) receptors (Fig. 9) (Engeland and Arnhold, 2005). A commonality among these receptor types is the usage of cAMP as a second messenger in their signal transduction cascade.

cAMP and adrenal clock entrainment

As previously described, cAMP represents a new class of core-clock component, possessing all the attributes of a core clock gene other than not being a gene but a small signal transduction molecule (O'Neill et al., 2008). Also, data suggests the relative levels of the P-CREB oscillate with circadian rhythmicity in the SCN (Obrietan et al., 1999) and the *Per1* clock gene contains cAMP response elements (CRE) regions within its regulatory sequence (Genbank Accession #AF223952). Furthermore, CREs drive *luciferase* bioluminescence in a circadian fashion (O'Neill et al., 2008). Based upon the adrenal functional anatomy and the molecular circadian clock, these data suggest intracellular cAMP might be a critical hub of information flow where extra-adrenal signals are received and integrated into the molecular circadian clock to coordinate its phase. In this Chapter, I test the hypothesis that cAMP represents a critical signal in phase shifting the adrenal clock. Using an *in vitro* assay that reports circadian clock-gene expression in the adrenal I show that the manipulation of cAMP levels leads to shifts in the adrenal clock that depend on the circadian phase of treatment.

MATERIALS AND METHODS

Animals

A breeding colony of *Per1-lucficerase* (*Per1-luc)* rats was maintained in a 12:12 LD photoperiod in a decentralized facility at the University of Washington (UW) campus in accordance to UW Institutional Animal Care and Use Committee regulations. All experiments were performed on male rats between 9 and 14 months old at time of experimentation.

Adrenal Culture Explant Preparation and Recording

Animals were sacrificed by CO_2 exposure and decapitation after which their abdomen was immediately cleaned with 70% ethanol. After opening the abdominal cavity, the adrenals were dissected out and immediately placed in Eppendorf tubes containing cold Hank's balanced salt solution (HBSS; Gibco 14170) with supplements of the following final concentrations: 4mM $NaHCO_3$, 10mM HEPES, and penicillin(100U/ml)/streptomycin(172mM) (all solution supplied by Gibco). Adrenals were then transferred from the tube containing HBSS to a new tube containing warm liquid-state 4% agarose solution which was immediately chilled on ice. After solidification of the agarose, a block of agarose containing the adrenal gland was cut out, blotted on filter paper, and superglued to a vibratome stage where it was sliced in chilled HBSS at 500μm sections. Each adrenal section was then cut into wedges containing cortex and medulla tissue (Fig. 11 inset) and placed in a single well of a 48 well culture plate (Corning 3548) containing filtered recording media composed of 1% (w/v) Dulbecco's Modified Eagle's Medium - low glucose (Sigma D2902), 0.35% (w/v) D-glucose (Sigma D2902), 4mM $NaHCO_3$ (Gibco 25080), 10mM HEPES (Gibco 15630),

penicillin(25U/ml)/streptomycin(43mM) (Gibco 15140), 1X B-27 supplement (Gibco 17504), and 0.1mM luciferin (Promega E1602) which was sealed with vacuum grease (Dow-Corning 14-635-5D). The 48 well plates were then transferred to a light-tight chamber equipped with a PIXIS Deep Cooled CCD camera (Princeton Instruments), fitted with a wide-angle lens, and programmed to take 29-min exposures every 30min. At the end of recording, a bright field image was taken for orientation of luminescence movies and histology of the adrenal slices was analyzed via hematoxylin-eosin (H&E) staining.

Pharmacological Treatment Experimental Paradigm

The circadian rhythm of *Per1* expression in tissue explants of the *Per1-luc* rat is reported by bioluminescence that results from *luciferase* interaction with its substrate luciferin. The left adrenal gland of all animals was collected from animals killed 1h before lights off and each slice culture was allowed at least 24h to equilibrate to the recording media prior to pharmacological treatment. Additionally, each time point contains multiple replicates of blank negative control slices and drug-treated cultures from an individual rat. To assess the ability of changes in cAMP levels to phase-shift the adrenal clock, a 2h pharmacological treatment was applied after the initial equilibration period (Fig. 10). Identical treatments were applied at 4 different circadian times (CT 20, 2, 8 and 14) with CT12 the extrapolated time of light off in the animal's housing chamber (Fig. 10). Acute elevation of intracellular cAMP concentrations were induced by the use of the following drugs, diluted in either water or dimethyl sulfoxide (DMSO; Fisher BP231) depending on solubility: Forskolin from *Coleus forskohlii* (Sigma F3917), inactive 1,9-Dideoxyforskolin from *Coleus forskohlii* (Sigma D3658) and 3-Isobutyl-1-

methylxanthine (IBMX; Sigma I7018). Forskolin and IBMX raise intracellular cAMP via activation of Adenylate Cyclase (AC) and inhibiting cAMP phosphodiesterases, respectively. 1,9-Dideoxyforskolin is a pharmacologically inactive molecule structurally similar to Forskolin.

Analysis

Raw data from each whole adrenal slice is subject to a local regression function to detrend drift of the 24h mean and enable normalization necessary to calculate the phase of circadian oscillations (Fig. 11A,B). The acrophase of each circadian waveform and the endogenous free-running period (FRP) was quantified with detrended datasets in El Temps software, using the serial analysis and periodogram analysis tools, respectively. For descriptive visual representation, detrended data calculated in R is exported to MATLAB to produce heatmaps of oscillations (Fig. 11C). This representation includes the 24h prior to treatment for every culture recording and at least 2 subsequent post-treatment peaks in the oscillation of all cultures. Additionally, adrenal cortex and medulla oscillations were characterized and each region of interest was determined by the H&E histology of single adrenal slices.

Statistics

Acrophases of peaks in the waveform were quantified in El Temps and subject to Analysis of Variance (ANOVA). To negate the effects of pseudoreplication and obtain biologically relevant degrees of freedom, statistical differences between control and treatments groups were fitted with a factorial ANOVA model to determine sources of variation and statistical interaction between individual animals (2 animals per treatment time point), individual adrenal slices (3 to 6 slices in each control or treatment group at

each time point), and experimental groups (control and Forskolin-IBMX (For-IBMX)). Differences between the endogenous FRP and complete solar LD cycle (24h) were determined by a 1-sample t-test.

RESULTS

A characterization of *Per-luc* circadian oscillations in the rat adrenal gland *in vitro*

The endogenous period of rat adrenal gland oscillations was calculated for the adrenals of 4 rats, each recorded for 7 days *in vitro*. Chi-squared periodogram analysis revealed an endogenous period of the rat adrenal gland oscillator to be 23.62h ± 0.02h (n=4). Additionally, a 1-sample t-test demonstrates a significant difference between the endogenous FRP (23.62h ± 0.02h) and the solar day period (24h) ($t = -17.705$, $p < 0.001$).

Differences in the oscillations of adrenal cortex and medulla within a single, histologically intact adrenal slice were also observed in these recordings (Fig. 12A). In every slice, the medulla showed a very low amplitude oscillation and its phase lagged that of the cortex by approximately 5h. Considering the low amplitude and robustness of medulla circadian oscillations, as well as the minimal surface area of each adrenal slice containing medulla tissue, it was determined it has a limited contribution to the adrenal oscillator as a whole, thus subsequent analysis was performed on the whole adrenal slice (combined adrenal cortex and medulla).

Manipulation of recording media does not induce phase shifts

A control pulse experiment was performed in order to confirm the validity of the experimental paradigm. Several negative controls where used to assess 1) if the recording medium could be changed mid-recording without inducing phase shifts in response to brief temperature changes (Buhr et al., 2010); 2) if the volume of solvents used to suspend the pharmacological agents would induce phase shifts via small dilutions of the recording media; 3) the effects of inactive 1,9 dideoxyforskolin as a negative control for Forskolin (Fig. 12B). The experimental treatment groups consisted of high and low concentrations of Forskolin (20μM and 100μM , respectively) and of IBMX (250μM and 500μM, respectively) (Fig. 12B). All treatments were applied from CT18 to CT20. The only treatments that induced a visible phase delay were 100μM Forskolin and 500μM IBMX, while the phase of all other groups remained unchanged by the experimental manipulation (Fig. 12B).

Circadian *Per1* expression exhibits a phase-specific response to temporary elevation of intracellular cAMP content

For-IBMX (20μM and 500μM, respectively) treatments were applied at 4 different time points distributed over 24h (Fig. 13). Concentrations of For-IBMX are based upon previous research that determined cAMP as a core clock component (O'Neill et al., 2008) and our dose-response (Fig. 12B). Calculated acrophase values were analyzed via a factorial ANOVA with rat (2 rats per time-point), treatment ((-) Control and For-IBMX), and slice (individual piece of adrenal) as factors.

Pulses at CT18-20 induced delays in the acrophase of the first waveform following pharmacological treatments of For-IBMX (Treatment $p < 0.001$, Rat $p < 0.001$,

Rat:Treatment p=0.64, Slice p=0.858, Rat:Slice p=0.516, Treatment:Slice p=0.991, Rat:Treatment:Slice p=0.242) (Fig. 13C; Table 1). Table 1 contains a complete description of F values and p values for all factors and interactions analyzed for all treatments. Each recording is internally controlled by measuring the acrophase of the pre-treatment day and no significant differences were observed between treatments (Treatment p=0.442, Rat p=0.121, Slice p=0.363, Rat:Treatment p=0.35, Rat:Slice p=0.275, Treatment:Slice p=0.1737, Rat:Treatment:Slice p=0.0971) (Fig. 13C; Table 1). The significant difference observed between rats is a result of truncation of the initial 30min-2h of post-treatment recording to removing experimental artifacts associated with the recording system. This small truncation is consistently performed for all control and For-IBMX treated slices in each internally controlled recording and results in the same relative effect on all slices (control and treated) for each rat. Additionally, the accompanying significant difference of Treatment ($p < 0.001$) in conjunction with a lack of a statistical interaction between rat and treatment ($p=0.35$) indicates the treatment is exerting a statistically significant delay in the acrophase of the waveforms of each internally controlled group of slices (Fig. 13C). Similar results were obtained when performing the same analysis on the 2nd post-treatment waveform of pulses applied from CT18-20 (Treatment $p < 0.001$, Rat $p < 0.001$, Rat:Treatment p=0.727, Slice p=0.539, Rat:Slice p=0.436, Treatment:Slice p=0.368, Rat:Treatment:Slice p=0.960) (Fig 13C; Table 1).

In contrast, when the identical For-IBMX drug cocktail treatment was applied from CT12 to CT14 (Fig. 13A) there was no significant difference observed between the phase of the oscillations of control and treatment groups during the pre-treatment or

post-treatment oscillations (Table 1). Similarly, pulses applied from CT 6-8 (Fig. 13D) also did not induce phase-shifts.

Finally, pulses from CT0 to CT2 lead to instability of the circadian oscillation of luciferase-reported *Per1*. Treatments at this time point inverted the expression of *Per1*; however, the reduction in rhythmicity prevented us from making reliable acrophase calculations.

DISCUSSION

Phase-resetting information can be communicated to the adrenal clock through cAMP signaling

My data demonstrate that acute, reversible disruption of cAMP content leads to phase shifts in the adrenal clock. This response is dependent on the circadian phase of stimulation, suggesting that cAMP signaling may represent a mechanism by which the adrenal gland can decode central temporal information to remain in phase with the external environment. The endocrine and/or neural signals that reset the adrenal clock are unknown, but an array of chemical signals that reach the adrenal cortex, including ACTH, norepinephrine, NPY, and VIP use cAMP as a second messenger. Additionally, the source of these transmitter systems include both SPLN nerve input directly to the adrenal cortex and signals to the cortex from Type I/II medulla cells, suggesting the possibility of intra-adrenal organization as well. Interestingly, the adrenal cortex and medulla display differences in the phase and amplitude of *Per1* expression suggesting

the presence of anatomically and functionally distinct circadian oscillators within the adrenal gland. Together, these results suggest cAMP may represent a key signal transduction molecule conveying phase information emanating from the SCN master circadian clock.

Model of rat adrenal gland entrainment

Based upon the adrenal functional anatomy (Engeland and Arnhold, 2005), the circadian molecular clockwork (O'Neill et al., 2008), and phase specific responses to disruption of oscillatory cAMP signaling, we hypothesize intracellular cAMP might be a critical information hub where entrainment signals from outside the adrenal gland are integrated into the molecular circadian clock during the subjective night (Fig. 14). Pulses applied during the subjective night induce delays in the oscillation of the adrenal clock and pulses applied during the subjective dawn induce what appear to be ~12h shifts in the oscillation. Interestingly, our adrenal cultures showed a shorter than 24h FRP, which suggests that the adrenal cortex contains a circadian oscillator that needs to synchronize by delays to the 24h cycle. Collectively, these data suggest a model in which signals emerging during the early subjective circadian night entrain the adrenal clock through daily delays of the circadian molecular clockwork (Fig. 14). This model is consistent with the discrete model of entrainment (Pittendrigh and Daan, 1976; Johnson et al., 2003).

Future Directions

Currently I am carrying out experiments to determine the phase of adrenal circadian oscillations *in vivo*. I have developed a working protocol for adrenal *Bmal1 in situ* hybridization and an experiment is underway to determine the relative phase

relationship of components of the molecular circadian clock and cAMP signaling cascades (Fig. 14).

CHAPTER 4

CONCLUSIONS

Consciousness Is Stressful

Awareness and the ability to feel and experience stimuli from the external environment is a stressful behavioral state relative to unconsciousness. In other words, diminished interaction with the external environment (i.e. sleep) results in a temporary break from decision making, judgment formation, and the exorbitant amount of information animals are required to perceive, integrate, and react to as a result of biological, psychological, and social demands. Despite the intuitive nature of this claim, the science of this dissertation demonstrates remarkable redundancy in the mechanisms by which energy is mobilized in response to stressful stimuli and increased metabolic demands of wake relative to sleep. How does the master circadian pacemaker specifically code temporal information to the adrenal in a manner that is not interpreted as a stress response? My results suggest mechanisms by which a major stress-associated output (GCs) is able to discriminate between the perceptions of stress and signals instigating daily energy mobilization (Chapter 2).

ACTH release in the unsplit hamster exhibits a subtle ~24h oscillation anticipating the peak of cortisol release and onset of locomotor activity (Figure 7B). This relatively diminutive rise of ACTH relative to the robust GC oscillation suggests the additive or synergistic involvement of alternative inputs to the adrenal gland regulating circadian GC release (Figure 7). Collectively, these data suggest temporal information is encoded by sympathetic input to the adrenal gland through a multisynaptic neural pathway that either modifies adrenal sensitivity or directly stimulates GC synthesis and

release, ultimately generating circadian oscillations of plasma GCs. The redundancy in the generation of a GC-release rhythm is directly supported by the fact that even in the absence of an ACTH rhythm in the split hamster, the SCN master circadian clock manages to convey circa-12h temporal information to the adrenal gland. Interestingly, this result also suggests ACTH output from the pituitary gland cannot resolve ~12h rhythmic signals arising from the hypothalamus despite bilateral uncoupling of the SCN, the ~12h rhythmicity of locomotor activity, and the presence of circa-12h oscillations of cortisol (Figure 7B).

More Than Masters And Slaves

Historically, the theoretical concept of the mammalian circadian system consisted of a 2 tiered hierarchy with a master circadian pacemaker in the SCN and all other circadian oscillators were categorized as slave oscillators. However, recent research suggests GCs likely represent a ***major internal harmonizer*** by which the SCN conveys temporal information to the rest of the body (Kalsbeek et al., 2012). Virtually all hormones exhibit a circadian oscillation in systemic circulation. However, currently GCs represent the only class of hormone with the capacity of coordinating circadian oscillations of other “slave” oscillators (Kalsbeek et al., 2012). This capacity has been demonstrated in nearly every tissue type studied thus far. Moreover, it is also widely known that there is a highly ubiquitous expression of GC receptors in a diverse array of tissue types. Thus, I propose that the mammalian circadian system is composed of a 3 tiered hierarchy with the master circadian pacemaker residing in the SCN at the top of the hierarchy, the adrenal glands in the middle tier, and all other peripheral clocks subordinate to the SCN, but a major subset also subordinate to the output of GCs from

the adrenal glands (Figure 4). I hypothesize the robustness of the circadian oscillation in GC release is not only adaptive in allowing an individual to cope with wake-associated stresses, but also in assuring circadian internal synchronization among peripheral oscillators in the organism as a whole. Importantly, internal desynchronization of circadian rhythms is a physiological signature of environmental challenges like nocturnal shiftwork (Sack et al., 1992; Haus and Smolensky, 2006) and understanding circadian GC secretion could provide access to novel treatments of circadian disorders associated with unusual temporal environments.

My dissertation suggests that the robust circadian oscillation of circulating GCs is the result of tight SCN control of GC release via the redundant pathways described in Chapter 2 and SCN synchronization of the adrenal circadian clock via the mechanisms described in Chapter 3. Additional factors such as body temperature (Buhr et al., 2010) and feeding (Sujino et al., 2012) have been also hypothesized to be involved in the coordination of peripheral clock oscillations. However, these processes represent emergent properties of overt physiological and behavioral phenomena, and depend on multiple factors which collectively generate circadian oscillations of body temperature and feeding behavior. For instance, temperature is likely not the sole result of a singular SCN output but rather dependent on a collection of outputs including activity-induced heat production, metabolism, and energy mobilization, which in turn may depend on GC release among other factors.

Negative Feedback Of The HPA Axis

My experiments shed new light on the nature of HPA axis negative feedback (Engeland and Yoder, 2011). It is widely known that exogenous application of natural or

synthetic GCs results in a large scale diminished drive of the HPA axis in rodents and humans. Traditionally, this has been conceptualized as a simplified, unidirectional, indirect relationship. As more GCs are released from the adrenal gland, less ACTH is released from the pituitary gland (Kalsbeek et al., 2012). In medicine, patients with hypercortisolism (Cushing's Syndrome) derived from adenoma or adenocarcinoma at a location in the HPA axis often receive surgery in combination with exogenous GCs to respectively remove the tumor and capitalize on suppressive negative feedback effects to maintain a physiologically relevant HPA activity. However, the data of Chapter 2 suggests that a previously unrecognized circadian component of HPA axis negative feedback. In this body of work, the drive of the HPA axis in the behaviorally split hamster becomes diminished as a result of temporal disruption of cortisol release, despite the fact that the overall output of cortisol is reduced. Additionally, preliminary unpublished data in the split hamster suggests this temporal disruption not only alters negative feedback of HPA axis drive, but also exerts similar negative feedback effects at the molecular level in the hippocampus via downregulation of MR and GR (data not shown).

Signal Integration Of Adrenal Inputs

As previously discussed, the adrenal gland is subject to a diverse array of neural and humoral inputs. This is mediated by ACTH, adrenergic α2, adrenergic β, neuropeptide Y (NPY), and vasoactive intestinal peptide (VIP) receptors (Figure 9) (Engeland and Arnhold, 2005). Given that each of these transmitter systems utilize cAMP as a second messenger, I hypothesize phase coordination of the adrenal circadian clock with the extra-adrenal environment can be controlled by temporal fluxes

in cAMP signaling in the adrenal gland (Chapter 3). I hypothesize cAMP signaling may be dually responsible for entraining the adrenal circadian clock, as well as the generation ~24h oscillations in the concentration of circulating plasma GCs. I hypothesize the proposed cAMP-associated molecular model of adrenal circadian clock entrainment (Figure 14) emerges from the collective input of the multiple physiological regulatory branches controlling the adrenal medulla and cortex, and it represents a potential mechanism by which the master circadian clock in the SCN can communicate temporal information to the adrenal clock and numerous peripheral oscillators.

Although the exact nature of adrenal gland inputs remains to be elucidated, it is possible circadian GC release could be regulated by a combinatorial input of ACTH and SPLN innervation. Exogenous application of ACTH induces GC release and this induction exhibits a morning/evening difference with a larger GC output during the subjective dusk as compared to an identical application of ACTH during the subjective dawn (Ulrich-Lai et al., 2006). Interestingly, the high level of GC release in response to the application of ACTH during the subjective dusk is abolished in SPLN-X animals, suggesting sympathetic innervation is critical for priming the adrenal to the ACTH challenge (Ulrich-Lai et al., 2006). However, the exact role or significance of this result in the regulation of circadian GC rhythmicity remains an open question.

Additionally, circulating ACTH oscillates in a circadian fashion in unsplit hamsters (Figure 7B), suggesting this classic HPA signal may itself be partially involved in resetting the phase of the adrenal circadian clock. If this is the case, why doesn't the circadian clock in the adrenal gland reset every time there is stress-induced ACTH release? Both ACTH and neural input to the adrenal gland operate through cAMP

signaling and my results show pharmacologically increased intracellular cAMP concentrations will phase delay the adrenal clock at specific locations within the endogenous circadian oscillation. In other words, these increases can shift the adrenal clock at some circadian phases but not others. This circadian phase specificity is a defining property of circadian systems that entrain to phase shifting stimuli according to the discrete model of entrainment (Pittendrigh and Daan, 1976; Johnson et al., 2003). Therefore, it is possible that this phase specificity allows the animal to avoid phase shifting by stressful stimuli presented at specific phases of the circadian cycle despite the fact that stressful and temporal information are both coded through cAMP signaling.

Also, it remains unknown if the adrenal can resolve ~12h signals emerging from the SCN in behaviorally split hamsters to generate ~12h rhythmicity of cortisol release. The work of this dissertation demonstrates the circadian oscillation of cortisol release is capable of generating ~12h oscillations in concert with dissociated locomotor activity. However, it remains unknown if each circa-12h peak is associated with both bilaterally paired adrenals oscillating in phase with a period of ~12h or if it is generated by both adrenals oscillating in antiphase with a period of ~24h and each peak of cortisol is associated with the activity of a single adrenal gland. Interestingly, it has been demonstrated that clock gene expression in the adrenal glands of behaviorally split hamsters becomes uncoupled from each other, oscillating in antiphase (Mahoney et al., 2010). This result suggests the likely scenario that each of the peaks of cortisol observed in Chapter 2 of this dissertation is the output of a single adrenal gland as opposed to both adrenals oscillating in unison with a period of ~12h. Furthermore, it has been demonstrated that ultradian pulses of ACTH release are a critical aspect of

stimulating GC release in rats (Spiga et al., 2011). Yet, it remains unknown if the putative circadian modulation of ultradian ACTH pulse frequency is also able to resolve ~12h signals.

Importantly, the functional role of any signal transduction molecules capable of entraining the adrenal circadian clock has not been shown until now. Chapter 3 of this dissertation provides the first evidence of a signal-transduction pathway (via cAMP as a second messenger) for the entrainment of the adrenal peripheral oscillator.

Intra-Adrenal Circadian Organization

Interestingly, the adrenal gland can be thought of as a developmentally derived interface between the nervous system and visceral organs where a single gland in the body cavity contains a mesoderm-derived cortex and ectoderm-derived medulla. Also, as briefly demonstrated in Chapter 3 of this dissertation (Figure 12A), the circadian clock within the adrenal gland is actually composed of 2 anatomically and functionally distinct oscillators, one within the cortex and the other within the medulla. Given that these functionally distinct portions of the adrenal oscillate out of phase and modes of cortex-medulla intercommunication are known to be present in the gland, it is conceivable that the adrenal circadian clock is also subject to intra-gland organization that is critical for the hormonogenesis of GCs, norepinephrine (NE), and epinerphrine (E). For example, type I and II ganglionic cells in the medulla project to the adrenal cortex (Engeland and Arnhold, 2005), thus sympathetic input to the cortex might be regulated by the medullar clock. Additionally, the vasculature of the adrenal gland results in blood flowing from the cortex to the medulla and the catalytic conversion of NE to E by Phenylethanolamine N-methyltransferase (PNMT) in the medulla is known to

be enhanced by GCs (Betito et al., 1992), which are released in a circadian fashion. Thus, there is potentially a critical functional role for the differences in the phase between the cortical and medullar clocks, namely an internal temporal organization of the regulation of GCs, NE, and E.

What It Means

Overall, this research not only reveals a novel mode of GC regulation via the circadian modulation of GC release, but also provides mechanistic insight to how the body maintains the harmonic balance of circadian oscillations within an individual at the organismal level of biological organization (Figure 4). Additionally, these data suggest the circadian rhythmicity of GCs is governed by the SCN through a complex set of multi-step regulatory branches from the brain to the adrenal gland involving a multisynaptic neural branch through the sympathetic nervous system which modulates adrenal gland responses to HPA axis activity or directly regulates GC synthesis and release (Lilley et al., 2012). Additionally, my results reveal cAMP-associated signaling is likely critical for entrainment of the molecular circadian clock in the adrenal gland (Chapter 3).

It is well established that the master circadian pacemaker is within the SCN and responsible for communicating daily temporal information throughout the body. My experiments provide insight into how the SCN regulates an oscillator that is in turn critical for coordinating oscillations of numerous oscillators throughout the body. In other words, the adrenal gland is likely a key interface between the SCN and peripheral clocks. Thus, understanding the phase coordination of the adrenal clock and circadian GC release is potentially paramount in understanding circadian internal synchronization as a whole and can enhance mechanistic understanding of how chronic challenges to

the circadian system can manifest in deleterious metabolic consequences associated with chronic jet lag and rotational shiftwork.

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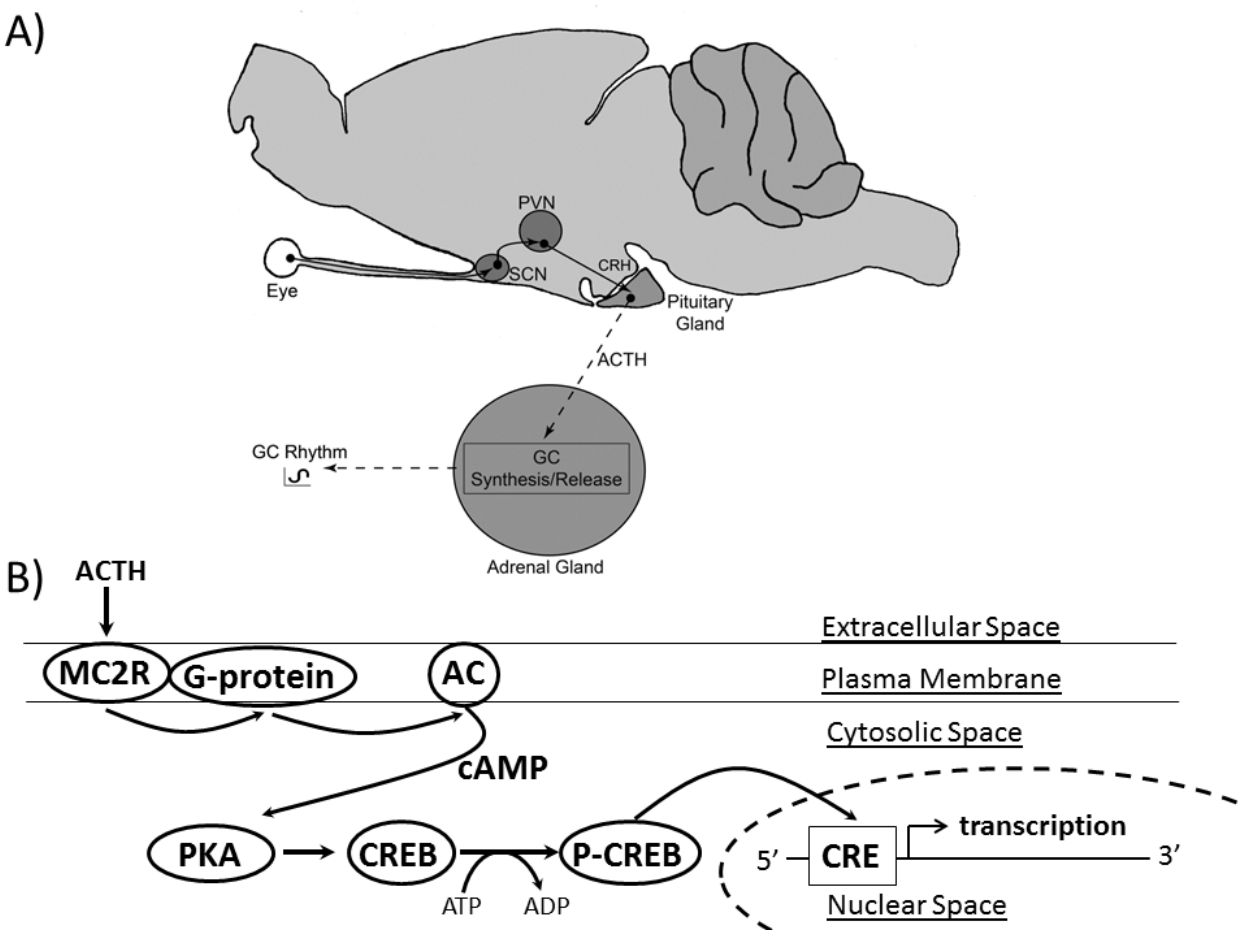

Figure 1

A) Schematic representation of circadian input to HPA axis. Entraining light information is perceived by the retina in the eye and relayed to the SCN. Efferent neurons from the SCN monosynaptically or multisynaptically project to CRH neurons in the hypothalamic PVN. Axons arising from these neurosecretory neurons project through the median eminence and release CRH into the portal blood system of the anterior pituitary. CRH receptive cells in the pituitary are stimulated to release ACTH into the systemic bloodstream, which in turn triggers the release of GCs from the adrenal gland.

B) ACTH triggered signal transduction cascade during stress. Binding of ACTH to MC2R triggers a G-protein conformational change that activates AC to synthesize cAMP. cAMP activates PKA which facilitates the phosphorylation of CREB from its inactive form to the active P-CREB form. P-CREB binds CRE regulatory sequences, upregulating the transcription of genes associated with GC synthesis and release during the stress response.

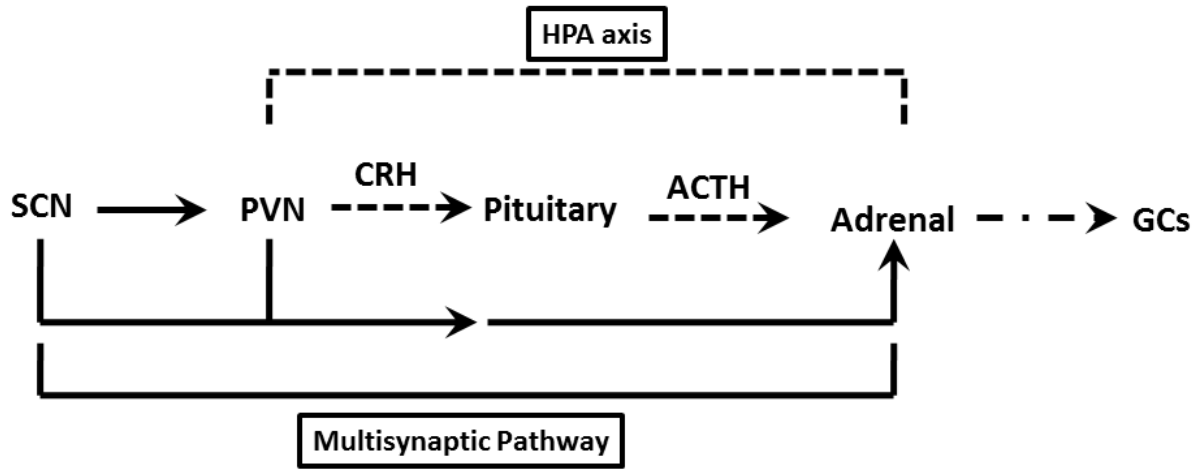

Figure 2

Schematic of potential input branches regulating circadian GC release from the adrenal gland. The circadian release of GC might be regulated by a neuroendocrine pathway through the HPA axis and/or a multisynaptic neural pathway through the sympathetic nervous system.

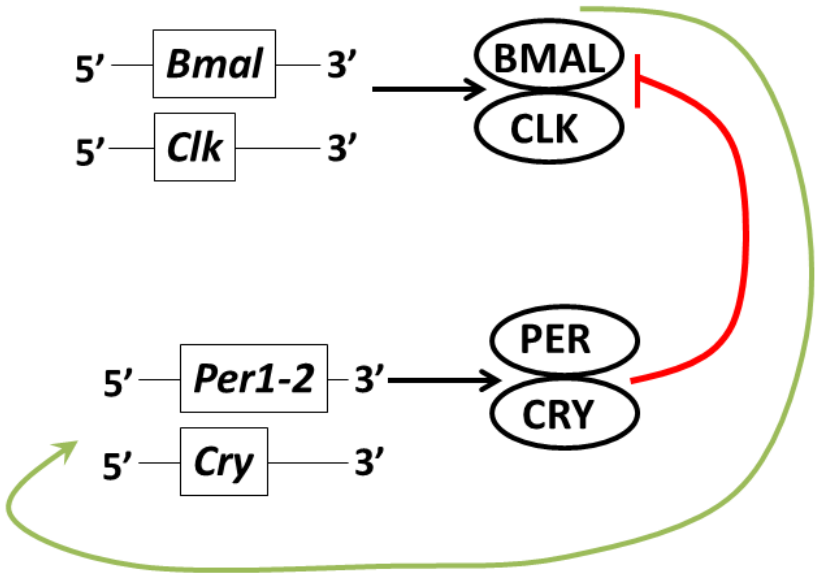

Figure 3

Simplified schematic of the mammalian molecular circadian clockwork. BMAL and CLK proteins form a heterodimer which induces transcription of *Per1-2* and *Cry1-2* via the positive limb (green) of the interconnected feedback loops. PER-CRY heterodimers, in turn, migrate to the nucleus and inhibit the ability of the BMAL-CLK to stimulate their transcription through the negative limb (red) of the interconnected loops.

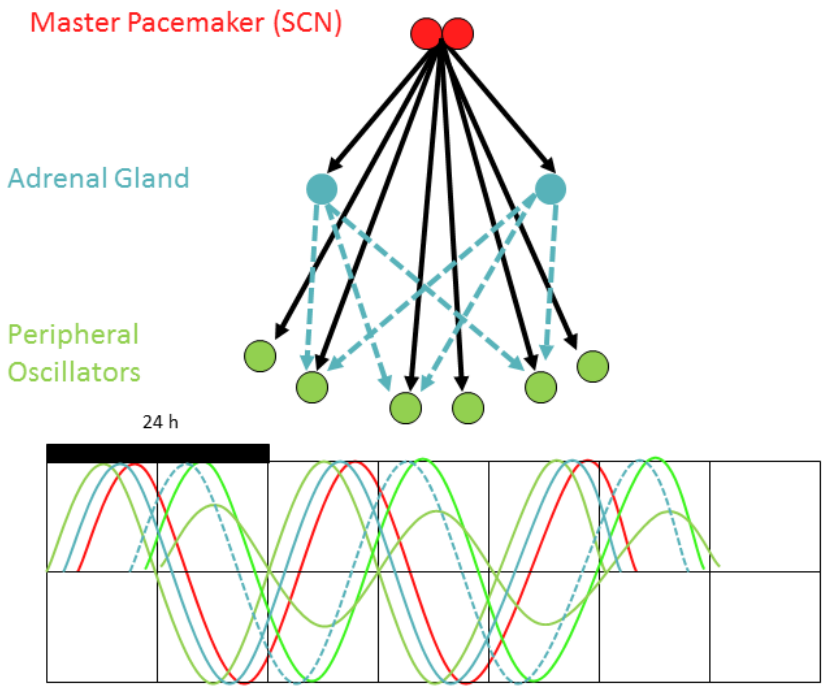

Figure 4

Three-tiered hierarchy of the mammalian circadian system at the organismal level of organization. The master circadian pacemaker within the SCN (red) regulates the phase of circadian oscillations within the adrenal glands (blue) and peripheral oscillators (green). Additionally, the majority of peripheral oscillators receive phase-resetting information from the adrenal glands via GCs (dashed blue line). Below: This hierarchical organization leads to finely orchestrated phase-relationship between the body's circadian oscillators with each waveform representing a hypothetical circadian oscillator.

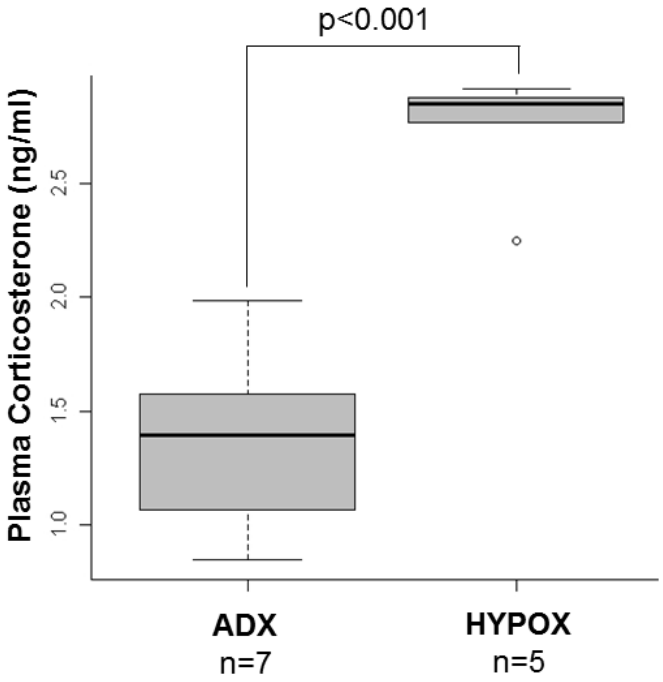

Figure 5

Plasma corticosterone in rats lacking adrenal glands or the pituitary gland. Bilaterally adrenalectomized rats (ADX) demonstrate statistically significant lower levels of corticosterone than hypophysisectomized (HYPOX) rats ($p<0.001$). Animals were serially bled throughout the light phase or throughout the dark phase, and the data for each phase pooled. In the boxplot, the horizontal line represents the median, the outer edges of the box represent the 1st quantile, the outer edges of the "whiskers" represent the 5-95% confidence interval, and the dot represents an outlier.

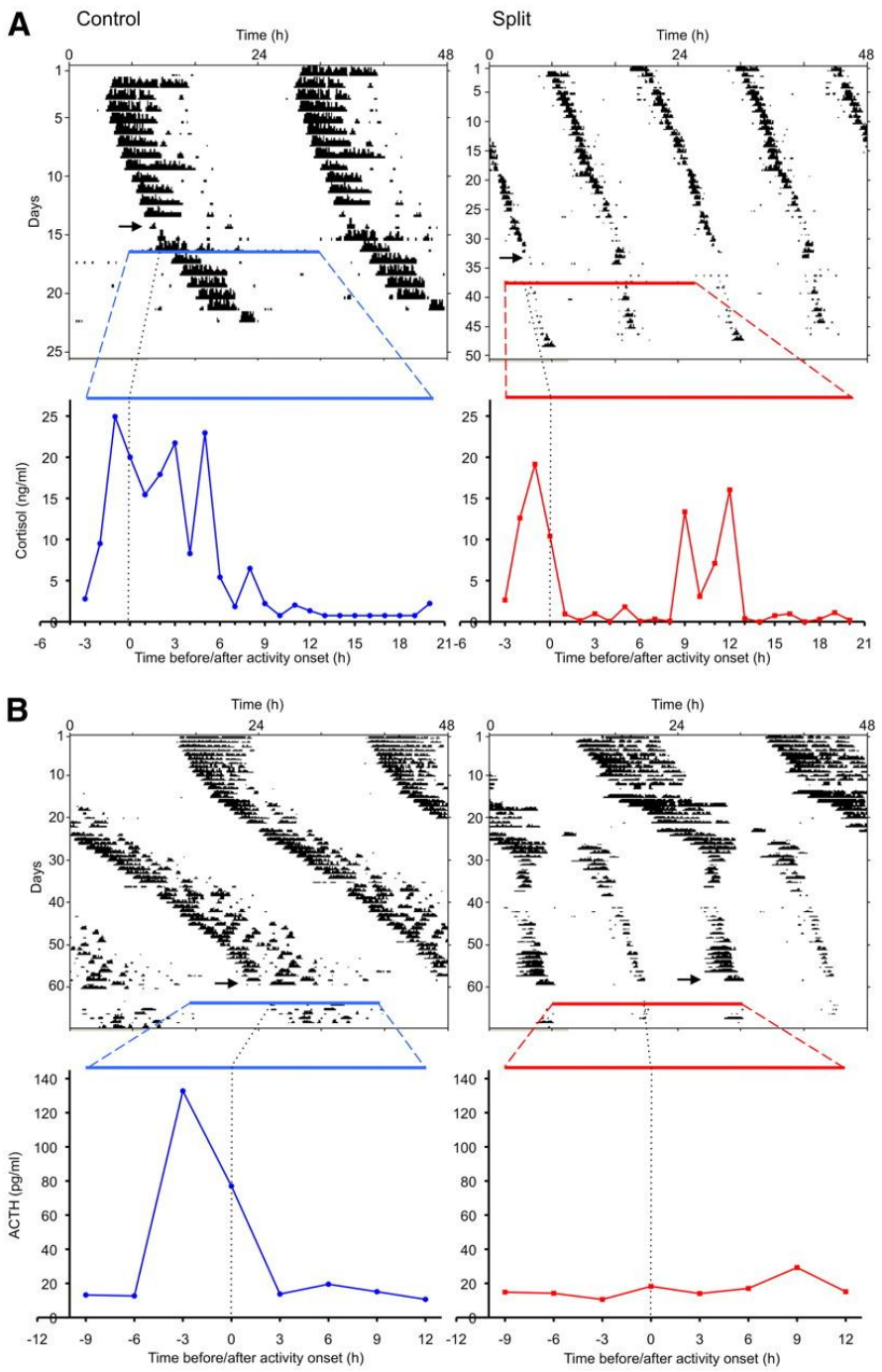

Figure 6

Representative double-plotted actograms of wheel-running activity and hormone profiles. Control (blue) and split (red) hamsters were surgically implanted with jugular catheters (arrows), and blood was collected for 24 h (colored horizontal lines on actograms). A, Plasma cortisol of representative control and split hamsters relative to wheel-running activity onset (dotted line). B, Plasma ACTH of representative control and split hamsters relative to wheel-running activity onset (dotted line). Dashed diagonal colored bands expand the 24-h scale on the day of bleeding for better visualization.

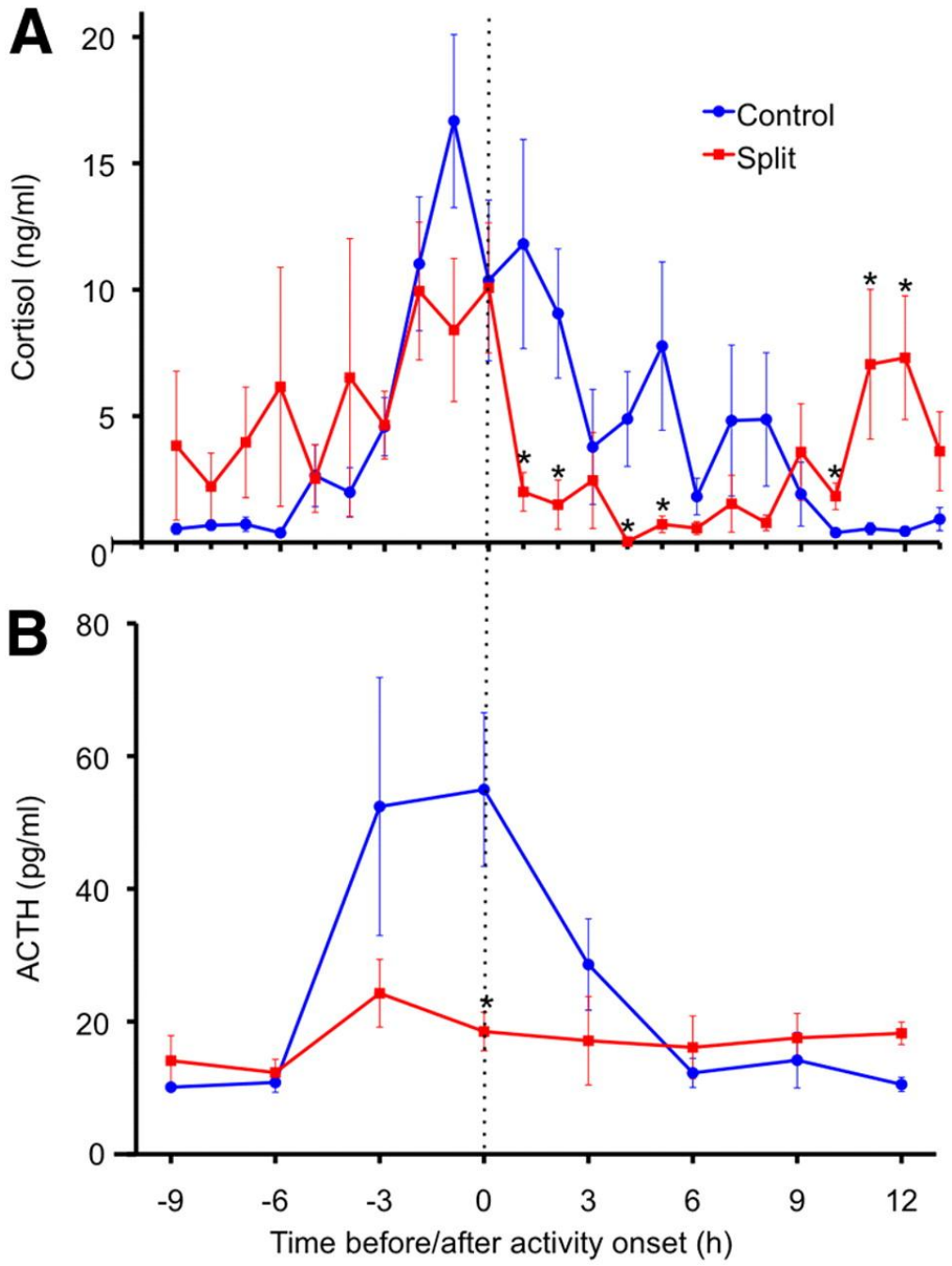

Figure 7

Mean plasma cortisol and ACTH 24-h profiles from control and split hamsters. A, Twenty-four-hour plasma cortisol of control (blue; n = 6–8) and split (red; n = 4–6) hamsters. B, Twenty-four-hour plasma ACTH of control (blue; n = 4–6) and split (red; n = 6–8) hamsters. Onset of wheel-running activity is indicated by dotted line. Data are shown as mean ± sem. *, $P < 0.05$ vs. control.

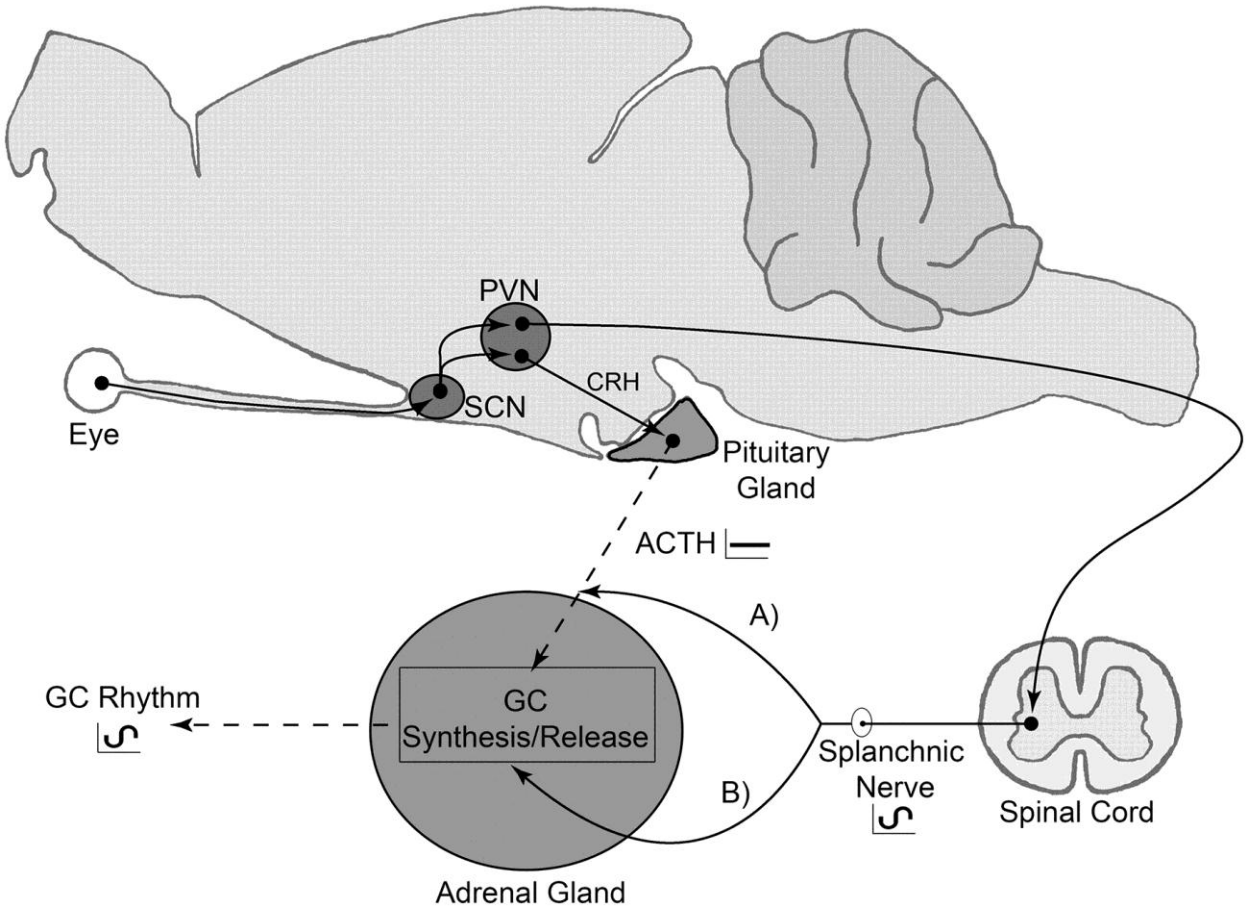

Figure 8

Circadian regulation of GC release. Light input from the retina entrains SCN master circadian clock, which in turn can time GC release through input to preautonomic or CRH-containing neurons in the paraventricular nucleus (PVN) and two distinct regulatory branches: one regulatory pathway dependent on ACTH release and the other through an autonomic multisynaptic pathway. Autonomic multisynaptic input to the adrenal either modulates adrenal sensitivity to ACTH (A) or GC synthesis/release directly (B).

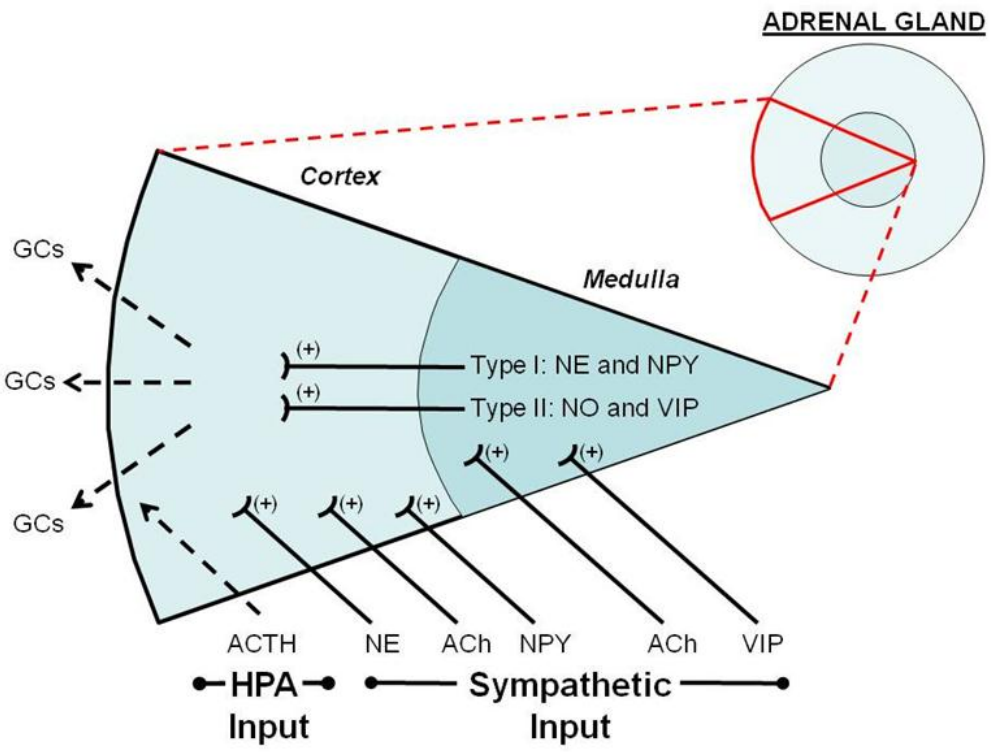

Figure 9

Functional anatomy of the adrenal gland. The adrenal cortex receives humoral input via ACTH as a part of the HPA axis, as well as sympathetic input via the SPLN nerve (Norepinephrine (NE), Acetylcholine (ACh), Neuropeptide Y (NPY), and Vasoactive Intestinal Peptide (VIP)) and adrenal medulla via Type I (NE and NPY) and Type II (Nitric oxide (NO) and VIP) cells.

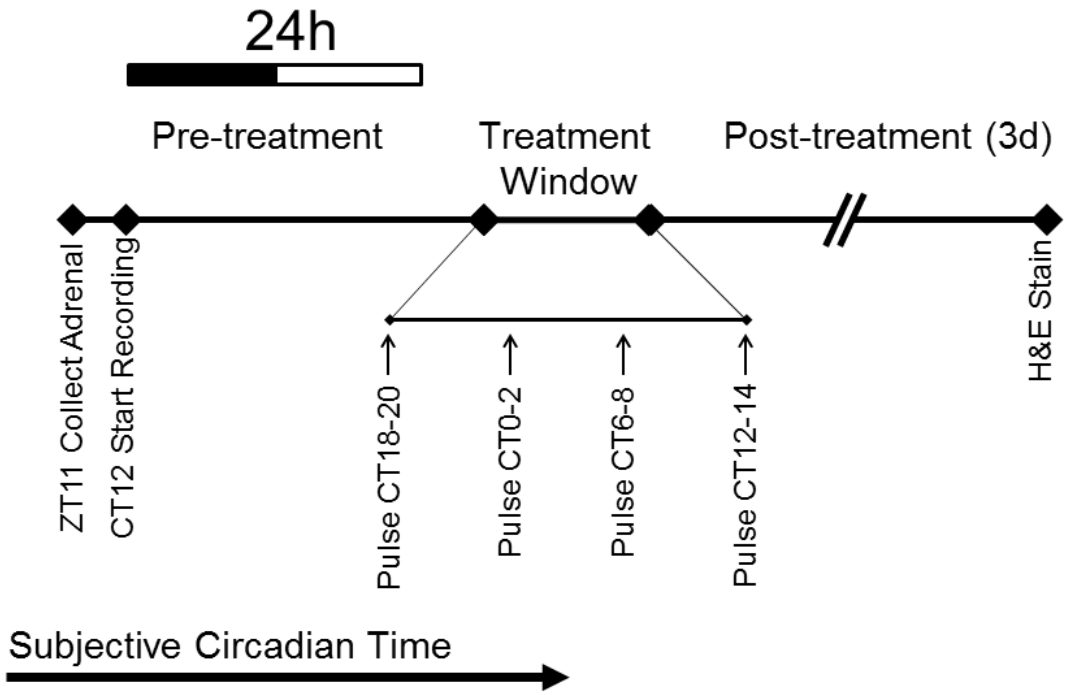

Figure 10

Schematic representation of pharmacological pulse experimental paradigm. Animals are killed at Zeitgeber Time (ZT11), the time of the *in vivo* peak of *Per1* expression in the rat adrenal gland. All cultures are allowed at least 24h to equilibrate to recording media. Then, 4 different groups of animals are exposed to For-IBMX and blank control pulses for 2h starting at circadian time (CT)18, 0, 6, or 12. After the pulse treatment, 3 days of post-treatment recording is acquired and adrenal histology is analyzed by hematoxylin and eosin (H&E) staining at conclusion of recording.

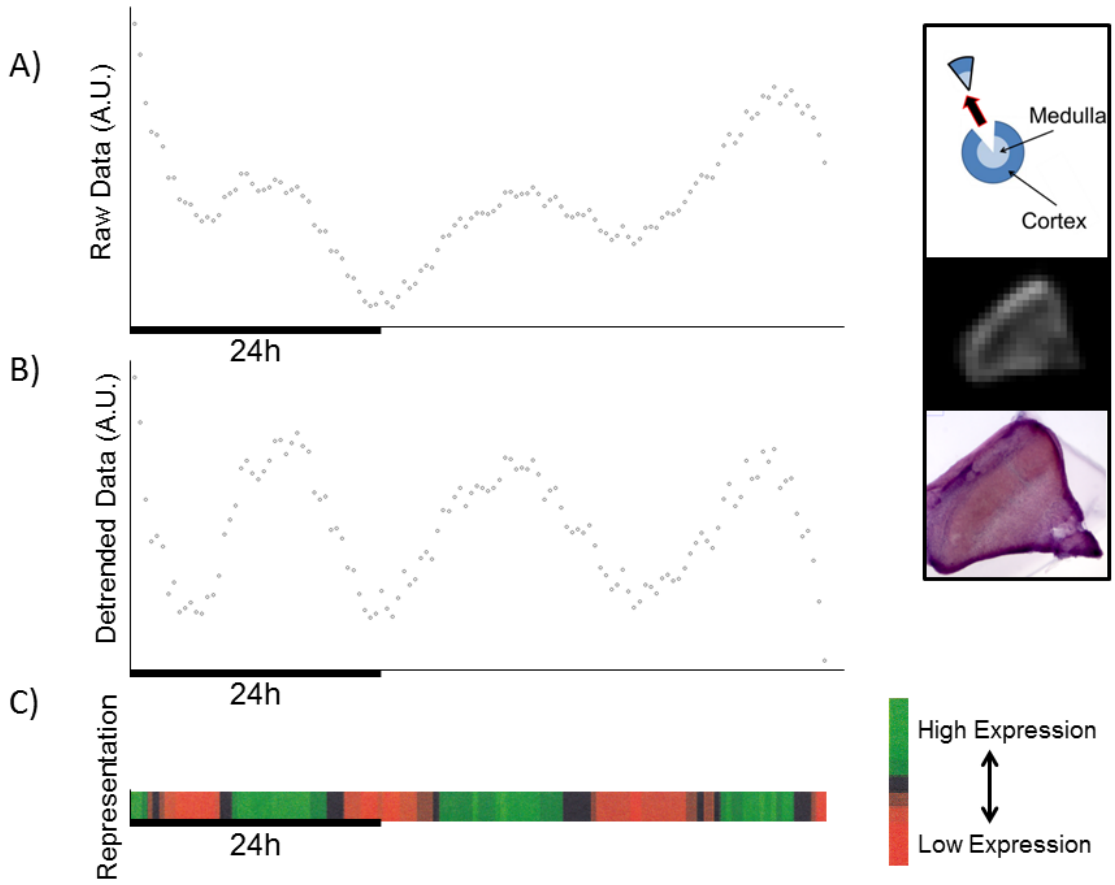

Figure 11

Analysis of *luciferase* bioluminescence. Raw data is obtained by scanning the optical density over each adrenal gland for each recording (A), normalized to detrend drift (B), and represented in heatmap form (C) for visualization. Representative adrenal slice images (inset) of adrenal dissection (top), single CCD camera image (middle), and H&E stain (bottom).

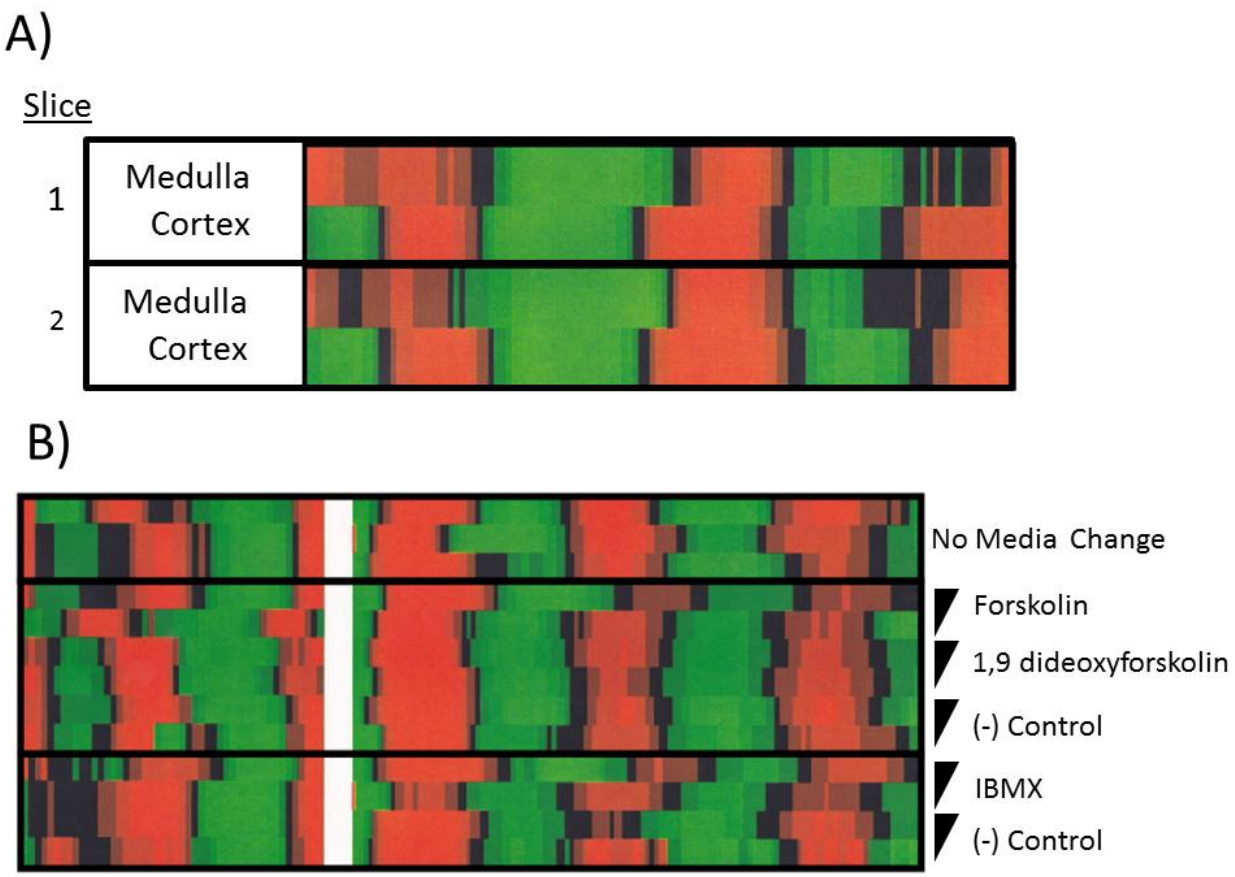

Figure 12

Differential phase of adrenal cortex and medulla oscillations within a single slice of 2 adrenal slices (A). Control pulse experiment with pharmacological manipulations applied from CT18 to 20. The treatment groups consisted of high and low concentrations (indicated by right triangles) of Forskolin (20µM and 100µM , respectively), 1,9 Dideoxyforskolin (20µM and 100µM , respectively) and IBMX (250µM and 500µM, respectively). In (B) (-) Controls consist of solvent volumes with the same volume as the dilutions used to obtain the high and low concentrations of each drug.

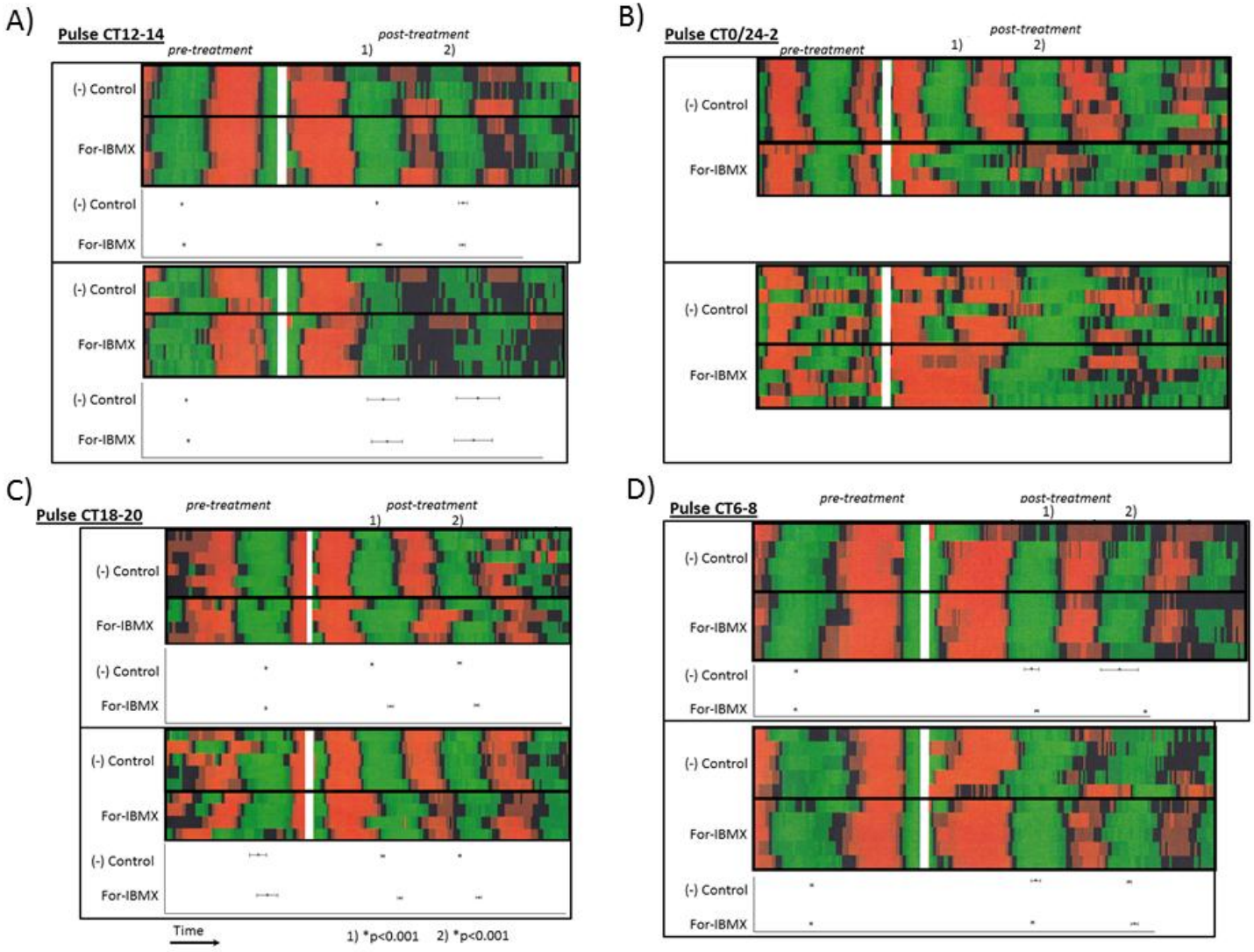

Figure 13

Identical 2h For-IBMX pulses induce delays in the adrenal clock when applied at CT18 (B), but not CT12 (A) or CT6 (D). Each time point consists of multiple control and treatment slices from 2 rats (top and bottom sets of heatmaps in each subsection) and each row represents a single slice. The pre-treatment time is 24h prior to pulse and each post-treatment section consists of at least 2 complete transient waves in control cultures. Beneath each heatmap is the quantified acrophase of each detrended data set for each rat. Acrophase calculations for CT0/24-2 pulses (B) are omitted due to the inability to make reliable calculations from severally disrupted oscillations.

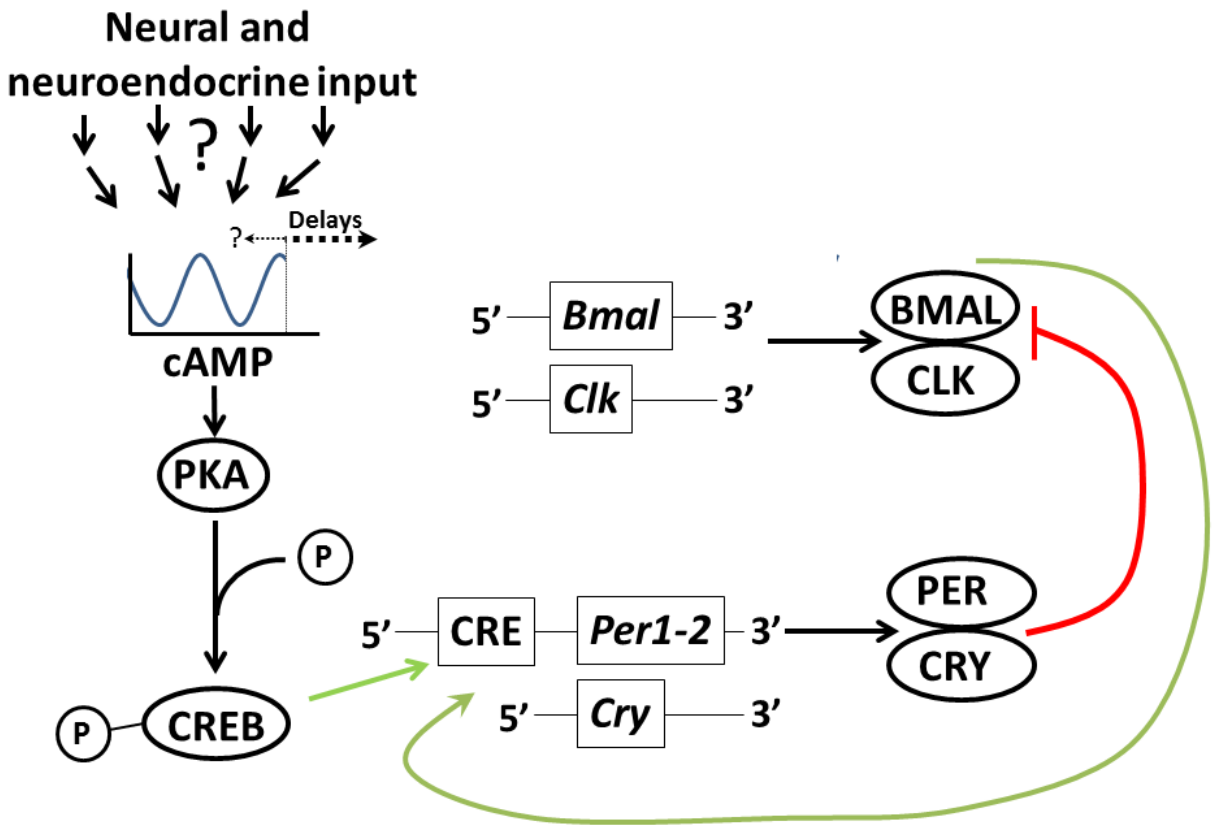

Figure 14

Model of adrenal gland entrainment. Extra-adrenal neural and/or neuroendocrine circadian input modulates intracellular cAMP and cAMP-signaling, regulating the expression of the core clock genes, *Per1-2*, and coordinating the phase of the molecular circadian clock with the external environment.

Table 1. Summary of Pre- and Post-Treatment Statistics

		Rat		Treatment		Slice		Rat:Treatment		Rat:Slice		Treatment:Slice		Treatment:Rat:Slice	
Pulse Time	Transient wave	F-value	p-value	F-value	p-value	F-value	p-value	F-value	p-value	F-value	p-value	F-value	p-value	F-value	p-value
CT12-14	pre-treatment	0.42	0.529	0.334	0.574	0.0061	0.939	0.22	0.647	0.246	0.629	0.539	0.477	0.103	0.753
CT12-14	post- treatment 1	2.185	0.163	0.0692	0.797	0.185	0.674	0.0033	0.955	0.0005	0.982	0.0281	0.869	0.0008	0.978
CT12-14	post-treatment 2	5.118	**0.0415**	0.033	0.859	0.232	0.638	0.0095	0.924	0.0177	0.896	0.0081	0.93	0.0567	0.815
CT18-20	pre-treatment	2.822	0.121	0.637	0.442	0.901	0.363	0.953	0.35	1.316	0.276	2.116	0.174	3.289	0.0971
CT18-20	post- treatment 1	30.362	**< 0.001**	20.268	**< 0.001**	0.0335	0.858	0.233	0.639	0.451	0.516	0.0001	0.991	1.532	0.242
CT18-20	post-treatment 2	26.553	**< 0.001**	75.419	**< 0.001**	0.402	0.539	0.128	0.728	0.654	0.436	0.879	0.368	0.0026	0.96
CT6-8	pre-treatment	3608.38	**< 0.001**	1.021	0.329	0.0019	0.966	0.407	0.5338	0.2	0.661	0.0549	0.818	3.695	0.0751
CT6-8	post- treatment 1	2.40	0.144	0.0378	0.849	14.235	**0.00206**	0.9645	0.343	1.433	0.251	7.537	**0.01579**	1.219	0.288
CT6-8	post-treatment 2	4.159	0.0608	3.092	0.101	1.0425	0.324	1.218	0.288	0.244	0.629	0.566	0.464	0.296	0.595

BOLD indicates p<0.05