A users guide to HPA axis research

Robert L Spencer, Terrence Deak

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A USERS GUIDE TO HPA AXIS RESEARCH[1]

Robert L Spencer[a] and Terrence Deak[b]

[a]Department of Psychology and Neuroscience, University of Colorado Boulder, Boulder, Colorado, USA

[b]Department of Psychology, Binghamton University - SUNY, Binghamton, New York, USA

Corresponding Author: Robert L. Spencer, Robert.spencer@colorado.edu

[1] This manuscript is submitted in fond memory of our colleague and friend, Dr. Randall Sakai, who continually strived to improve the quality of experiments being conducted, the impact of the work being published, and the training models we use to foster the next generation. One of us (RLS) worked closely with Randall as fellow postdoctoral trainees at Rockefeller University, and Randall was a significant force in shaping my scientific development. He enlisted me in his mission to establish rigorous conditions for each of our scientific measurements and to adopt thoughtful experimental procedures that would provide meaningful insights into the physiology of the research subject. Randall's impeccably high standards continue to drive us forward, and we hope this article will provide useful guidance for those who find themselves pursuing and interpreting studies in HPA axis regulation.

ABSTRACT

Glucocorticoid hormones (cortisol and corticosterone - CORT) are the effector hormones of the hypothalamic-pituitary-adrenal (HPA) axis neuroendocrine system. CORT is a systemic intercellular signal whose level predictably varies with time of day and dynamically increases with environmental and psychological stressors. This hormonal signal is utilized by virtually every cell and physiological system of the body to optimize performance according to circadian, environmental and physiological demands. Disturbances in normal HPA axis activity profiles are associated with a wide variety of physiological and mental health disorders. Despite numerous studies to date that have identified molecular, cellular and systems-level glucocorticoid actions, new glucocorticoid actions and clinical status associations continue to be revealed at a brisk pace in the scientific literature. However, the breadth of investigators working in this area poses distinct challenges in ensuring common practices across investigators, and a full appreciation for the complexity of a system that is often reduced to a single dependent measure. This Users Guide is intended to provide a fundamental overview of conceptual, technical and practical knowledge that will assist individuals who engage in and evaluate HPA axis research. We begin with examination of the anatomical and hormonal components of the HPA axis and their physiological range of operation. We then examine strategies and best practices for systematic manipulation and accurate measurement of HPA axis activity. We feature use of experimental methods that will assist with better understanding of CORT's physiological actions, especially as those actions impact subsequent brain function. This research approach is instrumental for determining the mechanisms by which alterations of HPA axis function may contribute to pathophysiology.

Keywords: glucocorticoid; HPA axis; cortisol; corticosterone; stress; ACTH

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1. INTRODUCTION

Glucocorticoid hormones are powerful regulators of all mammalian physiological systems including the central nervous system (1-3). These regulatory effects depend on a dynamic and complex profile of ultradian, circadian and stress reactive glucocorticoid hormone circulating levels (4,5). Glucocorticoid hormone production and secretion is controlled by the hypothalamic-pituitary-adrenal (HPA) axis neuroendocrine system (Fig1A). A number of pathological biomedical conditions are associated with altered HPA axis activity profiles. Dysregulation of HPA axis activity is strongly associated with some mental health disorders (e.g. depression, PTSD, schizophrenia) (6-8) and other biomedical disorders (e.g. Type II diabetes, hypertension, chronic fatigue syndrome, fibromyalgia and chronic facial pain) (9-13). Altered glucocorticoid hormone profiles also contribute to the adverse health effects of persistent psychological or physiological stress (14-16). These altered profiles may be manifest by changes in basal glucocorticoid hormone secretion patterns and/or alterations in the response to acute stressor challenge (7,17). There is also considerable interest in the prospect that signs of HPA axis dysregulation serve as valuable biomarkers in the clinical setting. For example, HPA axis dysregulation is a significant covariate for various subgroups of certain disorders, and it has been associated with differential treatment responsiveness, recovery rate, and probability of disease relapse (18-22). Consequently, study of HPA axis and glucocorticoid physiology continues to be an important scientific biomedical endeavor.

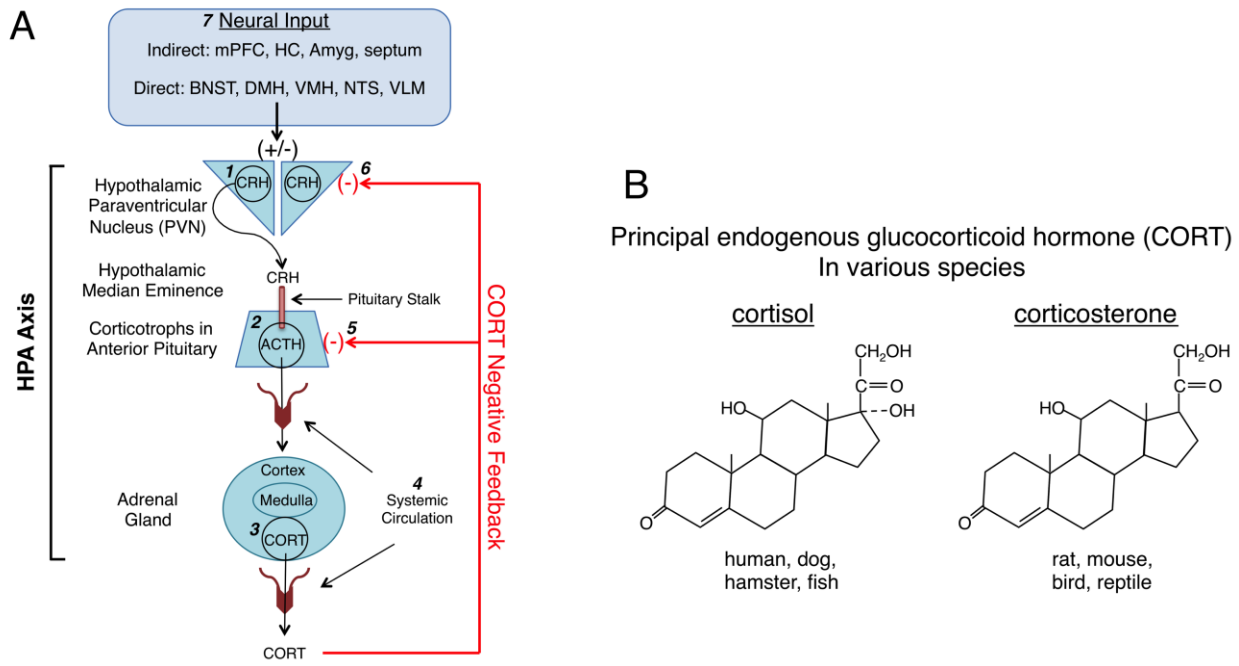

Figure 1. HPA axis and endogenous glucocorticoid hormones (CORT). **A**. The HPA Axis consists of 3 populations of cells and the specialized hormones that each secretes: *1*] neurons in the medial parvocellular portion of the hypothalamic paraventricular nucleus (PVN) secrete corticotropin releasing hormone (CRH), *2*] endocrine cells (corticotrophs) in the anterior pituitary secrete adrenocorticotropic hormone (ACTH), and *3*] endocrine cells primarily in the zona fasciculata of the adrenal cortex secrete the glucocorticoid hormones cortisol and/or corticosterone (CORT). CORT is secreted into the systemic circulation [*4*] and affects cells throughout the body, including the brain. CORT produces direct negative feedback inhibition of corticotrophs in the anterior pituitary [*5*] and CRH neurons in the PVN [*6*]. Activity of the HPA axis is directly and indirectly controlled by various neural activity present throughout the forebrain and brainstem [*7*]. Direct innervation of the CRH neurons in the PVN includes afferents from the bed nucleus of the stria terminalis (BNST), the dorsomedial hypothalamus (DMH), the ventromedial hypothalamus (VMH), the nucleus tractus solitarius (NTS), and the ventrolateral medulla (VLM). Indirect control includes the medial prefrontal cortex (mPFC), hippocampus (HC), amygdala (Amyg) and septum. **B**. The principal endogenous glucocorticoid hormones in vertebrates are the closely related steroid molecules (4 carbon ring based structure) cortisol and corticosterone.

There are a number of excellent reviews of glucocorticoid physiology and its importance for health (e.g. (4,23-27)). Most of these reviews, however, only touch on some of the conceptual and logistical aspects that one must consider when conducting and evaluating HPA axis-related research. In this paper, our primary objective is to provide a rudimentary Users Guide that may help assist others with the design and interpretation of research that includes HPA axis manipulations and measurements, especially within the realm of in vivo stress neurobiology. We will begin by reviewing the components and functional operation of the HPA axis. This knowledge is essential in order to manipulate and measure the HPA axis in a meaningful fashion. We will then examine strategies for manipulating the HPA axis, and discuss considerations for measuring aspects of HPA axis function. We acknowledge that this guide is rodent

centric, as that reflects both our first hand expertise, and to some extent the species bias of biomedical research. However, the molecular, cellular and systems-level components and function of the HPA axis are highly conserved in mammals. Consequently, many of the principles and considerations outlined here can be readily applied to study of other species including humans.

2. OVERVIEW OF THE HPA AXIS

Glucocorticoid hormones are the systemic effector hormone of the HPA axis. The principal circulating glucocorticoid hormone in humans, many other mammals (e.g. dogs and hamsters) and most fish is the steroid molecule cortisol. In rats, mice, birds and most reptiles it is the closely related molecule, corticosterone (Fig 1B) (28). In this Users Guide we will use CORT as an abbreviation for both of these molecules except in cases where the distinction is important.

Although we do not have voluntary control over HPA axis activity and CORT secretion, that secretion is tightly coupled to environmental/experiential events. Experiences considered to be stressful, for example, are often an effective stimulus for CORT secretion, and this hormone is therefore commonly referred to as a stress hormone. However, other stimuli that many would not consider stressful can also elicit CORT secretion (e.g. exercise, anxiolytic drugs, sexual experience, or shift in operant appetitive reward schedule) (29-34), whereas other circumstances considered to be stressful are not necessarily associated with acute CORT secretion (e.g. chronic neuropathic pain, anxiety and self reports of stress) (35-38). We caution the user of this guide to not simply equate elevated CORT with stress. Thoughtful and provocative discussions of the stress concept can be found elsewhere (e.g. (29,39-46)). CORT secretion also has a very prominent diurnal rhythm and CORT is a key mediator of circadian regulation of physiological function (47). Because the CORT molecule is lipid soluble, it readily crosses the blood brain barrier. Consequently the brain is a major direct target for this hormone, in contrast to many other hormones, including the other major stress-related hormone, epinephrine (also known as adrenaline) (48).

2.1. Components of the HPA axis

The cellular components of the HPA axis have been well characterized and consist of three populations of cells located within the hypothalamus, pituitary and adrenal gland. Each of these cell populations secretes a hormonal signal that provides for the functional interaction of the system (Fig 1A).

2.1.1. Hypothalamic PVN CRF neurons.

In most cases the primary determinant of CORT secretion is the ongoing activity of a population of neurons whose cell bodies are located in the medial parvocellular portion of the paraventricular nucleus of the hypothalamus (PVN). These neurons project to the hypothalamic median eminence ("hypophysiotropic" neurons), and they produce the neurohormone corticotropin releasing factor (CRF) (also known as corticotropin releasing hormone[2]). The mammalian CRF is a 41 amino acid peptide, and

[2] Andrew Schally, in his Nobel Prize acceptance paper (http://www.nobelprize.org/nobel_prizes/medicine/laureates/1977/schally-lecture.pdf) stated that after the structure of corticotropin releasing factor had been determined that henceforth it should

its sequence is identical in rats, mice and humans (Fig. 2A). CRF is a potent and necessary releasing hormone for ACTH secretion from the anterior pituitary. Many, if not all of the hypophysiotropic CRF neurons also produce the neurohormone arginine vasopressin (AVP), especially after repeated or chronic stimulation, which can then be co-secreted with CRF (51). These CRF neurons are the most integrative component of the HPA axis as they receive direct and indirect neural input from a number of brain regions (52). Increased activity of these neurons is necessary for normal stress-induced CORT secretion (53). It is less clear to what extent these neurons are necessary for circadian variation in basal CORT secretion (47). These neurons are also directly regulated by glucocorticoids and are a primary site of action for glucocorticoid negative feedback (4,54-56). Because only very small quantities of CRF are released in the median eminence, hypophysiotropic CRF levels cannot be measured in the systemic circulation.

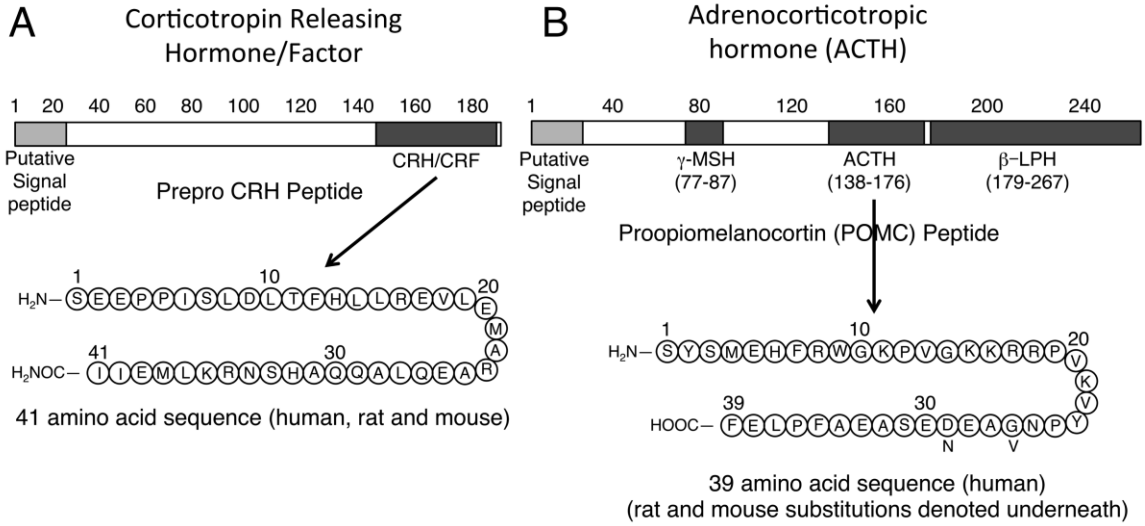

Figure 2. Basic structure of CRF and ACTH hormones. Diagrams illustrate the basic precursor protein structure and the final cleavage product peptide hormone amino acid sequence (single letter code) for (**A**) corticotropin releasing factor (also known as corticotropin releasing hormone) and (**B**) adrenocorticopic hormone found in human, mouse and rat.

2.1.2. Anterior pituitary corticotrophs.

The corticotrophs of the anterior pituitary are endocrine cells that synthesize and secrete the 39 amino acid peptide hormone adrenocorticotropic hormone (ACTH) (Fig. 2B). The amino acid sequence of this hormone in rats and mice is identical and has two minor amino acid substitutions compared to the human sequence. The ACTH peptide is

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be called corticotropin releasing hormone. Wylie Vale, who first determined the structure and amino acid sequence of corticotropin releasing factor (49), however, insisted on the continued designation of the peptide as CRF, because the peptide's "physiological function extends far beyond the biology of a hormone" (50). The International Union of Pharmacology (IUPHAR) has adopted this nomenclature for the peptide (CRF), and recommends CRF_1 receptor and CRF_2 receptor designation for the two known mammalian receptors for CRF; whereas the corresponding receptor gene names are CRHR1/Crhr and CRHR2/Crhr2, and the gene encoding the CRF preprohormone is CRH/Crh (50).

a cell type specific cleavage product of the proopiomelanocortin (POMC) prohormone (57). The mature peptide is stored in secretory vesicles, and therefore is available for rapid release. Corticotrophs under normal conditions have very low intrinsic activity (4). Corticotroph exocytosis of ACTH is primarily controlled by CRF acting at CRF_1 receptors. AVP acting at V1bR receptors may be an important co-factor (58). Other factors may also directly regulate corticotroph activity, such as circulating cytokines during inflammation/infection (59). ACTH levels in the systemic circulation are typically expressed as pg/ml in humans and rodents.

2.1.3. Adrenal cortical CORT producing cells.

CORT is synthesized in cells located primarily in the zona fasciculata layer of the adrenal cortex. CORT synthesis is triggered by ACTH stimulation of the melanocortin 2 receptor, which then initiates a series of enzyme-mediated reactions that convert cholesterol into CORT (60). Because CORT is lipid soluble, it cannot be stored within vesicles, but rather passively diffuses out of cells as it is formed. Similar to corticotrophs, CORT producing cells have minimal intrinsic activity in the absence of stimulation by ACTH (4). Thus, both ACTH and CORT secretion ultimately depends on upstream CRF neuron activity. However, whereas vesicular ACTH is rapidly released into the circulation after HPA axis activation, there is a time lag of ~3-5 min before an increase in circulating CORT levels (61). This is due to the time necessary for de novo CORT synthesis. This differential time-lag for ACTH and CORT secretion after HPA axis activation is a key consideration for measurement of these circulating hormone levels in experimental conditions (see section 4.1.). CORT units in human studies are typically expressed either as µg CORT/100ml (also known as µg/deciliter or µg%) or as nmoles/liter (1 µg CORT/100 ml = 27.6 nmoles/liter cortisol). These same units are also widely used in rodent studies (1 µg CORT/100ml = 28.9 nmoles/liter), as a means for direct comparison to human CORT levels. Some researchers prefer to express CORT levels in the rodent as ng/ml (1 µg CORT/100 ml = 10 ng/ml), given that a rat's total blood volume is much less than 100 ml (~10 ml). Because there are several conventions for units of CORT expression, careful attention to units is essential when establishing normative ranges for CORT across experimental conditions. For consistency, the discussion that follows will express all CORT data in common units of µg/100ml.

2.2. **HPA axis function**

Activity of the HPA axis and associated CORT secretion can be divided into 3 distinct temporal patterns: basal ultradian pulses, basal circadian fluctuation and stimulus (e.g. stressor)-induced activity (Fig. 3). Each of these different components of CORT secretion may individually or in combination be necessary for optimal molecular, cellular and systems-level function. In addition, each of these components depends on separate regulatory control factors exerted at each of the anatomical elements of the HPA axis.

2.2.1. Basal activity

Basal HPA axis activity of the laboratory rodent corresponds to the activity observed when the rodent is left undisturbed throughout the day in its home cage. Thus,

this activity can be considered to reflect the intrinsic activity of the HPA axis in the absence of changing environmental stimuli. This basal activity has both an ultradian and circadian rhythmic component (and in some species also a circannual component) (47).

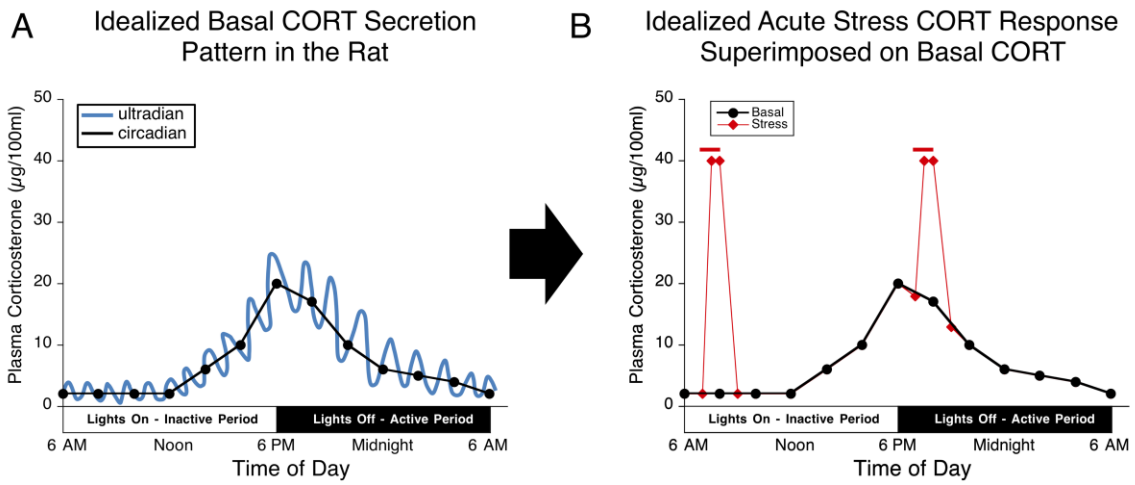

Figure 3. Idealized basal and stress-induced CORT secretion pattern in the rat. **A**. Basal corticosterone (CORT) secretion in the rat has an ultradian (hourly pulses) and circadian rhythm. The basal peak CORT secretion of this nocturnal species coincides with the beginning of the rat's active period. Relative ultradian and circadian basal cortisol levels are similar in humans except that peak basal levels occur at the opposite time of day (around dawn). **B**. Stimulation of the HPA axis, such as occurs with acute stress, produces similar maximal CORT levels regardless of the time of day. Red horizontal lines above both acute stress CORT responses depict the time of stressor onset and duration. Typically CORT levels reach their maximum within 30 min after stressor onset, and return to basal levels 60-90 min after stressor termination.

2.2.1.1. *Ultradian*. A number of mammalian species including rats and humans have an ultradian rhythm of basal CORT and ACTH secretion that is reflected by pulses of circulating hormone levels that occur approximately every 60 min (5). The pulsatility of basal hormone secretion depends primarily on a pituitary-adrenal closed loop oscillation of ACTH feedforward stimulation of adrenocortical CORT production, followed by subsequent CORT feedback inhibition of corticotroph ACTH secretion (62). However, the robust presence of this oscillation appears to require intermediate levels of hypothalamic CRF drive to corticotrophs. The frequency and amplitude of each pulse is modulated by a number of factors that includes circadian phase (increased during circadian active phase), sex (greater in female than male rats), strain of rat (greater in F344 than Sprague-Dawley rats) and age (dampened with advanced age) (5). For example, male Sprague-Dawley rats lack CORT pulses during the first half of their inactive circadian phase, whereas female F344 rats have large regular CORT pulses throughout the day (5,63). Interestingly, CORT pulsatility can modulate the magnitude of a CORT response to acute stress. A larger stress-induced CORT response will be produced If stressor stimulation occurs during the rising phase rather than the falling phase of an ultradian CORT pulse (63,64). The presence of CORT pulses has functional relevance for a variety of behavioral, physiological and molecular measures including aggressive behavior in response to an intruder rat and glucocorticoid receptor

(GR) target gene expression level (65-67). Thus, the alteration of CORT pulsatility and its possible functional consequences should be considered in any HPA axis manipulation experiment.

Though functionally important, ultradian rhythms of CORT secretion are an under-appreciated facet of HPA axis regulation. This is in large part due to the fact that ultradian rhythms can only be assessed through the use of serial blood sampling procedures such as venous catheterization and microdialysis, which allow for detailed within subject time course analyses in a manner that precludes handling of the animal to acquire the sample. Furthermore, averaging data across subjects tends to have a smoothing effect on CORT secretion patterns that obfuscates the natural ultradian rhythm that occurs within subjects. However, visual inspection of raw ACTH and CORT values within a given data set will often reveal occasional deviations from the group average (e.g. higher than average baseline; lower than expected peak). Though often attributed to individual differences in reactivity to the experimental context, these individual differences are perhaps as likely to reflect individual differences in the timing and amplitude of ultradian oscillations across subjects. In this way, ultradian rhythms may be a valid alternative hypothesis to explain apparent “individual differences” observed in a given dataset.

2.2.1.2. *Circadian.* Most mammals have a prominent daily fluctuation of basal CORT levels, with maximal levels (basal peak) corresponding to the onset of the circadian active period. As a result, humans have basal peak CORT levels during the early morning around the habitual time of awakening, whereas nocturnal animals, such as rats and mice, have basal peak levels at the beginning of the evening (Fig. 3). In male rats and mice the circadian trough of basal CORT levels is very low (< 5 µg/100 ml) and in some animals it cannot be discriminated from the non-specific signal present in ADX plasma (4). Basal peak CORT levels in rodents is routinely between 15-20 µg/100 ml. In humans trough and peak basal CORT are typically 4 µg/100ml and 16 µg/100ml, respectively (68) (Table 1).

In the laboratory under controlled light:dark cycles, the basal peak CORT levels for most strains of rats and mice coincides with the daily time of lights-off. The daily peak in CORT exhibits a steady anticipatory rise that begins several hours before lights-off, and the daily peak persists, even if animals are maintained under constant dark or low light illumination (69). Combined, these observations indicate that the generation of this diurnal CORT rhythm depends on endogenous circadian control. The Master Clock operation located in the hypothalamic suprachiasmatic nucleus (SCN) is ultimately responsible for this circadian control (70). However, the means by which the SCN exerts this control is not completely understood and it involves separate multisynaptic neural input to the PVN and the adrenal cortex (47,69). Control at the level of the adrenal cortex is especially critical for the prominent circadian CORT rhythm in rodents as there is a much larger circadian amplitude of CORT secretion compared to ACTH secretion (4) (Table 1). Both SCN descending neural input to the adrenal cortex and intrinsic adrenal cellular clock function contribute to the large circadian amplitude of CORT secretion (47).

Although the normal daily timing (circadian phase) of basal peak CORT is determined by the light:dark cycle entrained SCN, the circadian phase of CORT

secretion can become uncoupled from the Master Clock in response to restricted daily access to food. For example, if rats and mice are given daily access to food only during the early period after lights-on (a time of day when they are normally inactive, engage in very little feeding, and have trough levels of basal CORT) then the phase of CORT secretion will shift so that basal CORT levels peak at the new anticipated time of daily food availability (69,71). This suggests that the circadian rhythm of CORT is not solely controlled by the SCN, but that a separate central neural system, referred to by some as a food entrainable oscillator, can also control diurnal CORT secretion (72).

Humans have not only a pronounced daily anticipatory rise of CORT that peaks around dawn, but also an additional sharp surge of CORT secretion immediately upon awakening. This cortisol awakening response (CAR) typically peaks 30 min after awakening (73,74). There is growing research interest in the CAR. CAR is likely a reactive response of the HPA axis to awakening, rather than an intrinsic component of basal circadian CORT secretion (75). Regardless of its basis, it may be a useful biomarker for differential HPA axis activity in various clinically relevant groups or individuals (76). Whether other species also exhibit CAR is undetermined.

2.2.2. Stress-induced activity

With the caveat in mind that not all stimuli that activate the HPA axis may be considered stressful, we will refer to this aspect of HPA axis activity as stress-induced activity. PVN CRF neurons can be considered as serving as a final common neuron for one of the brain-controlled effector response systems. Considerable progress has been made in determining the neural networks within the brain that are stressor-reactive, and how those networks control PVN CRF neuron activity (52,77,78). One take away from that functional neuroanatomy is that CRF neurons receive direct neural innervation from a limited number of brain structures, with the majority of direct inputs arising from other hypothalamic sites, the bed nucleus of the stria terminalis and brainstem nuclei. The CRF neurons, for example, lack direct input from cortex, and therefore CRF neurons lack the sensory information necessary to discriminate between various environmental stimuli (e.g. context) and complex situations (e.g. stimulus predictability and controllability). Thus, CRF neurons are not the brain's stressfullness detector. Instead, the magnitude and duration of an HPA axis response to acute or repeated stressor challenge largely depends on neural processing external to the HPA axis and the subsequent integration by PVN CRF neurons of converging net excitatory drive.

2.2.2.1. *Acute stress.* The magnitude of an endogenous CORT response evoked by an acute stressor or other classes of stimuli can vary substantially depending on the stressor, species, strain of species, sex and age. However, despite the large diurnal variation in basal CORT levels, the maximum stimulated activity level appears to be fairly constant over the course of the day (Fig. 3). The dynamic range of a CORT response to HPA axis stimulation is somewhat limited due to a relatively modest level of maximum CORT production rate that can be achieved within the adrenal cortex (4). Nevertheless, the magnitude of the CORT response does vary reliably with different stressors, and the differential response level may be considered an indicator of the intensity of the stressor, at least in terms of HPA axis responsiveness (79,80).

In the male rat maximal stress-induced CORT levels are approximately 40-60

µg/100 ml depending on strain (for example, higher levels in F344 than Sprague-Dawley or Lewis rats) (81). For Sprague-Dawley rats this is a 10-30 fold increase from low circadian trough CORT levels, and only a 2-4 fold increase from high circadian peak CORT levels (Fig. 3). We find that transfer of a rat to a housing tub that lacks bedding or placement in an open field produces a small but significant CORT and ACTH response, placement on an elevated pedestal or tube restraint produces a moderate response, and forced swim or footshock produces a somewhat higher response (82,83) (Fig. 4). Vehicle injection also produces a relatively low but reliable HPA axis response, even if the rats have been habituated to the procedure. In fact, we find that virtually any disturbance of a lab rat, such as brief handling, movement of its home cage or even entry into the housing room elicits some HPA axis response. The low reactivity threshold of the HPA axis makes it difficult to measure actual basal CORT levels (see section 4.1.1.). However, it should be noted that typically the effect of these minor disturbances are examined during the rat's inactive phase (lights-on phase) when basal HPA axis activity is very low, and similar manipulations during the rat's active phase (lights-off phase) may have less of an effect on the ongoing HPA axis activity.

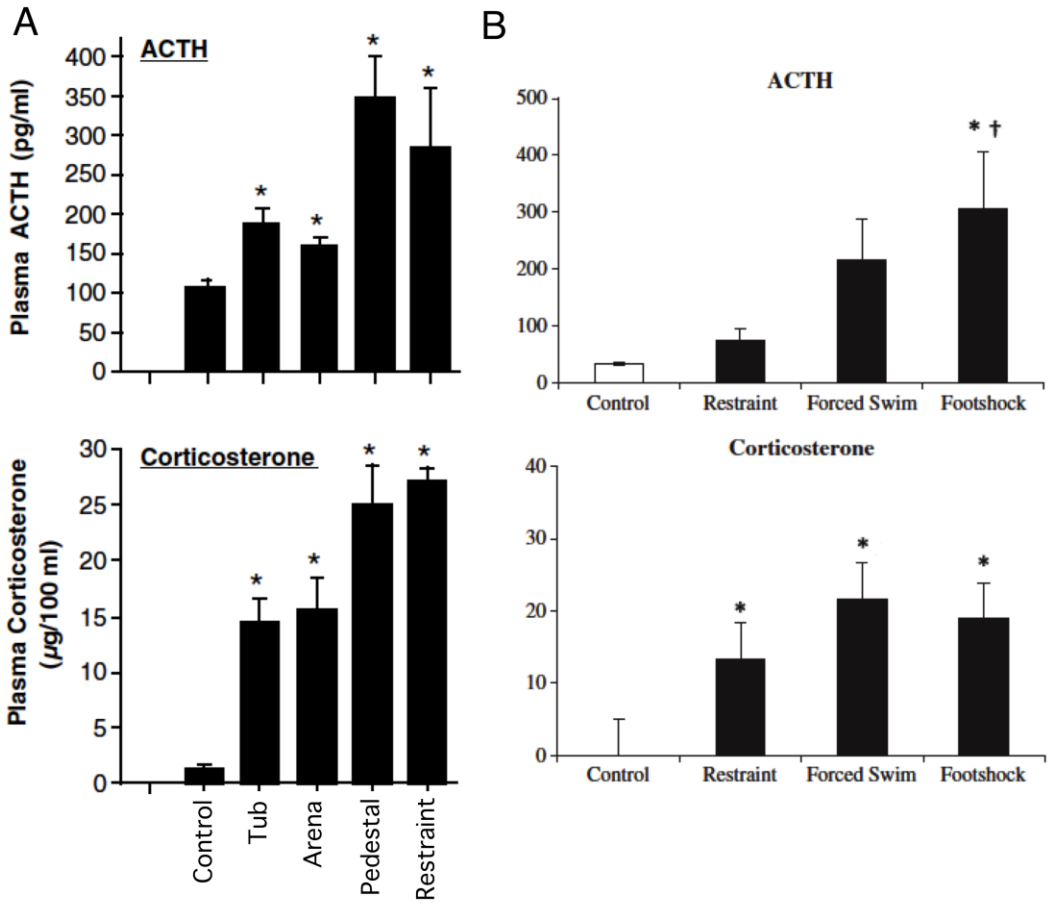

Figure 4. Representative ACTH and CORT plasma levels present in rats 30 min after onset of various stressors. **A**. Blood samples (tail clip method) of adult male Sprague-Dawley rats were collected immediately after 30 min of first time exposure to a housing tub lacking bedding, circular arena, elevated pedestal or restraint tube. Control samples were collected at the same time of day from rats that had been left undisturbed in their home cage. *significantly different

from control levels ($p < 0.05$) (adapted from (82)). **B**. Blood samples (trunk blood) of adult male Sprague-Dawley rats were collected immediately after 30 min of first time exposure to restraint tube, forced swim at 25 °C, or intermittent footshock. Control samples were collected at the same time of day from rats that had been left undisturbed in their home cage. *significantly different from control levels ($p < 0.05$); †significantly different from restraint ($p < 0.05$) (adapted from (83)).

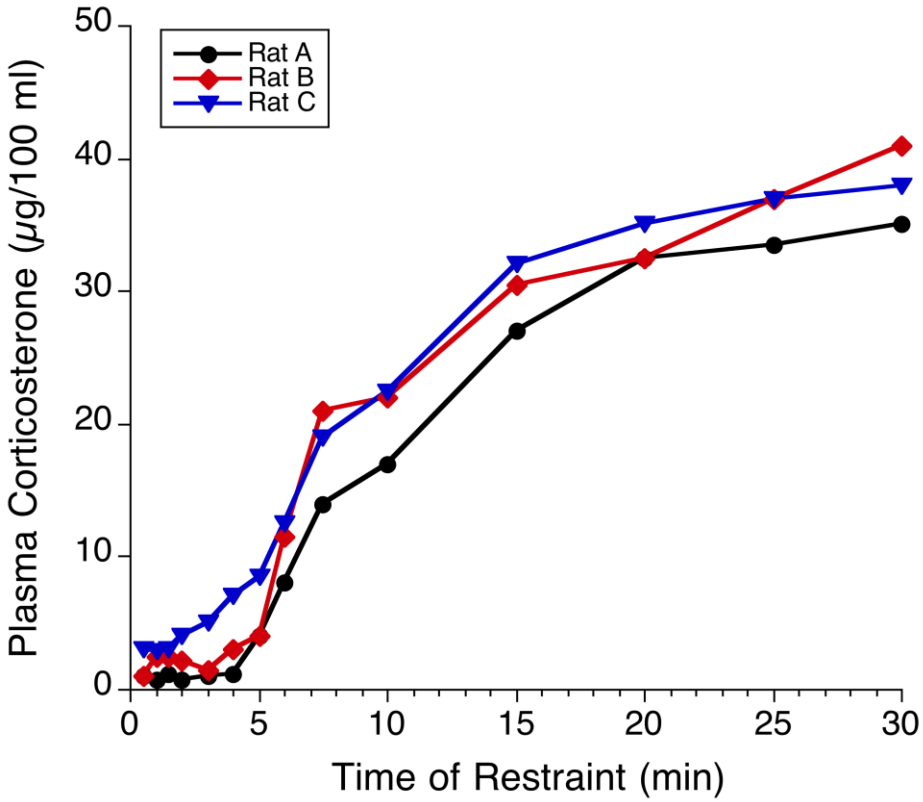

Figure 5. Time-course for increased plasma CORT levels in 3 rats during restraint. Rats (adult male Sprague-Dawley) were placed in a restraint tube within 30 seconds after experimenter entry into the animal housing room, and serial blood samples (~50 µl/sample) were collected from the tail vein (tail clip method) (RL Spencer, previously unpublished data).

The duration of a CORT response to an acute stressor depends to some extent on the intensity and duration of the stressor. As described above (section 2.1.3), there is a time-lag of 3-5 minutes before circulating CORT levels increase after stressor onset, due to the de novo synthesis rate of CORT (Fig. 5). Peak CORT levels are usually attained within 30 min after stressor onset, whereas peak ACTH levels are typically attained sooner (e.g. (84)). In rodents, CORT has a very short half-life in blood (≤ 15 min) (Section 2.4.2.), resulting in a somewhat tight temporal relationship between elevated CORT levels and ongoing HPA axis activation. Due to this short half-life, CORT levels typically return to basal levels within 60-90 min after termination of acute stressor exposure. If the duration of acute stressor presentation is relatively long (> 30 min), a decline in CORT levels may be observed before the termination of stressor challenge. For example, CORT levels decline precipitously in some strains of rats that are restrained continuously for 4 hours (81) (Fig. 6). However, if the rats are then

immediately challenged with a novel stressor, CORT levels again rise. Consequently the decline in CORT levels during 4 hours of restraint does not reflect exhaustion of HPA axis response capability (e.g. depletion of ACTH vesicular stores) or glucocorticoid negative feedback suppression of the axis. Instead, there appears to be some within-session habituation that is likely a result of short-term neural adaptation central to the HPA axis. This same pattern of within-session habituation has been observed during intermittent footshock delivered across a 2 hr session, suggesting that within session habituation may not be unique to moderate, passive stressors such as restraint, but instead may reflect a more general adaptation to sustained periods of stress (85).

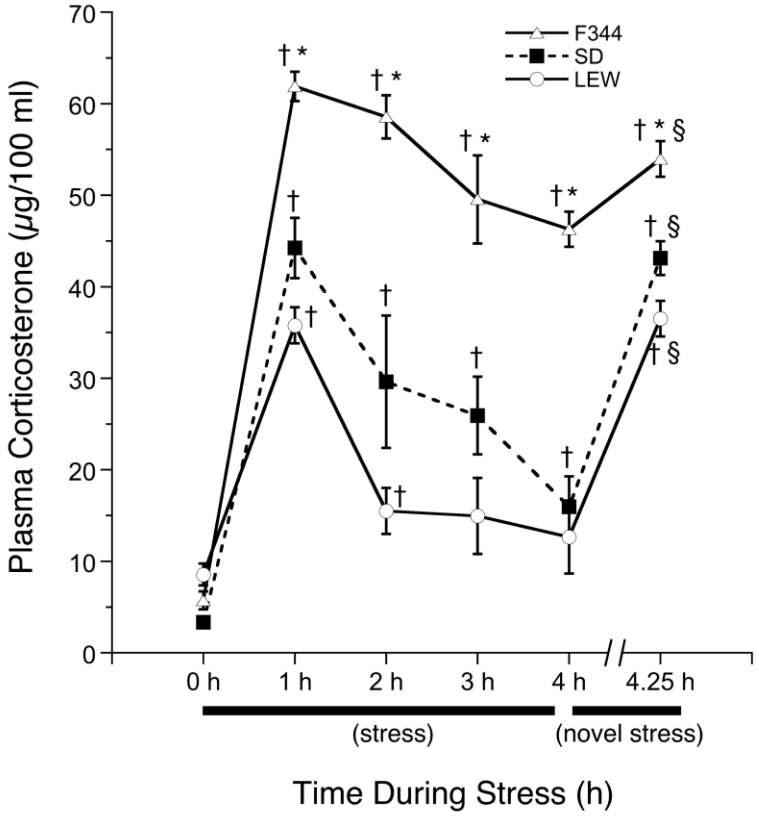

Figure 6. Rat strain comparison of CORT response to long session of restraint (4 hr) followed immediately by novel stressor challenge. Adult male rats of 3 different rat strains (Fischer 344 – F344, Sprague-Dawley – SD, and Lewis – LEW) were exposed to restraint (Plexiglas tube) for 4 h, and were then immediately exposed to a different novel stressor (wire mesh tube restraint) for an additional 15 min. Serial blood samples were collected from the tail vein (tail clip method). †significantly different from baseline (0 h) ($p < 0.05$), §significantly different from 4 hr time-point for the same strain ($p < 0.05$), *significantly different from corresponding SD and LEW value ($p < 0.05$). (reprinted from (81))

2.2.2.2. *Repeated/Chronic Stress*. There is considerable evidence that exposure to an acute stressor elicits some adaptive changes both intrinsic and extrinsic to the

HPA axis. These adaptive changes include a wide range of different cellular processes, and the duration of expression for each of these processes can range from very brief (< min) to very long (many days) (86-89). Consequently, the HPA axis response to an acute stressor may vary depending on prior stressor exposure and the state of those adaptive changes present at the time of subsequent stressor exposure.

One of the most reliable phenomenon of stress neurobiology research is that the HPA axis response declines (habituates) to repeated exposure to the same stressor (homotypic stressor). However, if the HPA axis is then challenged with a novel stressor (heterotypic stressor) it then displays either a normal or often a facilitated response to that heterotypic stressor challenge (87,90). A parallel habituation and facilitation of the central neural response (as evident by immediate early gene expression) is present in various stressor-reactive brain regions indicating that the stress response adaptation (stressor specific habituation, and generalized stressor response facilitation) is primarily extrinsic to the HPA axis (91).

One strategy to study the effects of repeated stress that elicits persistently strong HPA axis responses is to expose rats to a variety of different stressors that are presented in an unpredictable manner (chronic variable stress). Chronic variable stress regimens do not elicit constant high CORT levels, but instead produce episodic acute CORT surges, although it is not uncommon for these repeated stressors to result in significantly elevated basal CORT levels (92). These stress regimens are also effective at producing physiological signs of HPA axis hyperactivity, such as adrenal hypertrophy and shrinkage of the thymus (93) (see section 4.6.).

One chronic social stress model that produces a more persistent level of sustained stress is the visible burrow system (VBS) developed by Robert and Caroline Blanchard (94-96), and subsequently refined by Randall Sakai and colleagues (97). Groups of 4 male rats and 2 female rats are housed in a semi-naturalistic setting that includes an open surface area with access to food and water, and adjacent enclosed chambers and connecting tunnels. Whereas groups of male rats housed together in conventional large vivarium cages exhibit minimal aggression, in the VBS, the males establish very strong dominant-subordinate hierarchies in which the dominant male controls access to the females, food and water, through aggressive attacks of subordinate rats. The subordinate rats over several weeks exhibit signs of substantial chronic stress, including weight loss, adrenal hypertrophy, thymus involution, reduced corticosteroid binding globulin (CBG), and elevated basal CORT (96,98). Although basal CORT levels of subordinate rats are elevated in this extreme situation, they are not nearly as high as acute stress levels of CORT (95). This has implications for the interpretation of the physiological relevance of experiments that use exogenous CORT treatment to maintain sustained high stress-levels of CORT (see section 3.1.)

2.2.3. Sex differences

Female rats typically have higher basal CORT levels than males, and this difference is even greater during the circadian peak than trough, resulting in a greater circadian amplitude in basal CORT levels (99,100). Female rats also typically have a higher HPA axis response than male rats when challenged with the same stressor. However, the sex difference varies with the type of stressor (101). Interestingly, bioactive CORT levels (see Section 2.4) in the brain may not differ much in female and

male rats (102), for reasons not fully understood. The sex difference in basal and stress-induced HPA axis activity depends on both organizational and activational effects of gonadal steroids (103-105). An illustration of the important activational role of gonadal steroids is that female rats have a greater HPA axis response to restraint stress during proestrous than during other phases of their estrous cycle (106). There is also some evidence for activational effects of gonadal steroids on basal and stress-induced CORT levels in women (107). In general women and men have similar basal CORT levels (108). Men, however, tend to have a greater increase in CORT levels after challenge with the trier social stress test compared to women in their follicular phase (107).

2.2.4. Developmental/Life Span

Although the vast majority of preclinical stress studies have been performed in young adult rodents (rats and mice), HPA axis reactivity varies substantially across the lifespan in ways that appear to reflect the underlying developmental neurobiology rather than experiential factors such as the individual's prior stress history. For instance, infant rats typically undergo a stress hyporesponsive period (SHRP) that persists from about postnatal day (PND) 4 through PND18 (109). The effect is observed in both plasma ACTH as well as CORT responses evoked by stress, and the hyporesponsiveness appears to reflect a general hyporeactivity to most stress challenges (110). The exact age range for the SHRP varies by a few days (on either end) depending upon the species, strain, and rearing conditions, and may reflect an influence of maternal care on negative feedback regulation of the axis (111). It is notable, however, that the HPA axis at these early ages is not strictly unresponsive, but instead just subdued, relative to conspecifics whose ages are outside the hyporesponsive period. Though the functional significance of the SHRP remains unclear, investigators have argued that HPA hyporeactivity is probably an adaptive measure that protects the infant brain (and perhaps other end organs) from the potentially deleterious action of high CORT activity during critical periods of infant development (112).

When compared to adult conspecifics, adolescent rats (typically PND28-42 for early adolescence, or P42-P56 for late adolescence) differ markedly in both their ACTH and CORT responses to restraint (113). The most consistent finding among adolescents is a more protracted HPA axis response that is suggestive of delayed recovery (or impaired shutoff) of the axis as well as impaired habituation to repeated stressor exposure (114,115). These effects are most pronounced during the week prior to puberty onset (116). Intriguingly, recent studies have shown that this deficit in HPA axis shutoff among adolescents is not likely due to problems with the canonical corticosteroid receptor mediated negative feedback regulation of the axis, and suggests an alternative mechanism underlying maturation of the HPA axis shutoff mechanism (117).

The HPA axis response appears to display other unique characteristics and adaptations during late aging. For instance, one recurring finding in aged rats is an increase in basal levels of CORT present in plasma or brain relative to younger adults (118-120). Aged rats also show an increase in stress-evoked CORT release, an effect that may be due to impaired negative feedback regulation arising from failing prefrontal cortex mediated inhibition in these animals (121,122). Consistent with this, other studies

have shown that centrally-mediated negative feedback regulation of the axis is disrupted during aging, whereas systemic (eg. pituitary) negative feedback inhibition may be enhanced in aging (123). Finally, the potentiated CORT response observed in the plasma of aged rats may not translate into differences in bioactive CORT levels in the brain at the peak of the stress response (119). Although not meant to be a comprehensive summary, the section above provides a general framework for understanding key features of how HPA axis reactivity and intrinsic regulation are shaped across both early development and into late aging.

2.3. Corticosteroid Receptors

As for any hormone, a critical aspect of CORT's physiology depends on receptor mediated signal transduction. The classically characterized CORT receptors are especially complex in their biochemistry. This complexity includes ligand-dependent activation dynamics, intracellular localization, post-translational modification, protein-protein interactions, protein-DNA interactions, protein-RNA interactions, recycling and degradation. All of these factors contribute to CORT's specific cellular actions. The details of these glucocorticoid receptor features are reviewed elsewhere (124-129). We provide here only a brief overview of these receptors.

2.3.1. Receptor mediated regulation of gene expression ("genomic effects")

Because CORT is a lipid soluble molecule that can passively diffuse across phospholipid bilayers, it allows for the fact that CORT receptor proteins are intracellular rather than embedded in the outer cell membrane. Two intracellular receptors for endogenous glucocorticoids have been isolated and characterized—the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR) (130,131). Prior to the isolation and sequencing of the MR and GR genes, the presence of two separate intracellular receptors were inferred by pharmacological studies (132). What was initially designated as the Type I corticosteroid receptor corresponds to MR (which is also the primary receptor for the mineralocorticoid hormone aldosterone), whereas the Type II receptor corresponds to GR. Both receptors are members of the nuclear hormone receptor gene family and they function as hormone-dependent transcription factors (133). In the absence of ligand these receptors are part of a multiprotein complex that includes heat shock protein 90 (hsp90). The unliganded form of these receptors are found predominantly in the cytoplasm. Upon binding ligand, MR and GR dissociate from the hsp90 containing multi-protein complex (receptor activation), thereby revealing a nuclear localization domain which allows for nuclear import, accumulation and retention of the receptor within the nucleus. As classically characterized, the activated forms of MR and GR form homodimers or heterodimers and bind to a palindromic 15 DNA base pair consensus sequence (glucocorticoid response element, GRE) often located in the vicinity of the promoter region of certain target genes. MR/GR binding to a GRE can then enhance or repress gene transcription. More recent characterizations suggest that MR and GR may also regulate gene transcription by acting as monomers that bind GRE half-sites, or through protein-protein interactions with other transcription factors (e.g. fos/jun or CREB) or transcription factor co-regulators (e.g. CREB binding protein) (134-136). These monomer DNA binding or protein-protein interactions may facilitate or hinder the transactivational effects of other transcription factors. Although both MR and

GR are closely related in structure, they differ in their affinity for glucocorticoids, their phenotype expression levels, and transactivational properties (see Sections 2.3.2. and 2.3.3.)

A large proportion of CORT's effects depend on MR/GR mediated alteration of gene expression and subsequent changes in gene product functional levels ("genomic effects"). This is a mechanism of action that can result in relatively long-lasting and sometimes dramatic changes in cellular function (hours to days), but also requires a significant time delay after receptor activation before alteration of cellular function is observed (typically greater than 60 min). This temporal profile of CORT action must then be taken into account when designing and interpreting experiments in terms of the timing between HPA axis manipulations and various response measures.

2.3.2. MR/GR expression

The extensive range of CORT effects is consistent with the fact that GR is widely expressed in most cell types throughout the body (137). There are only a few notable exceptions, such as the suprachiasmatic nucleus of the hypothalamus (138) and melanotrophs of the pituitary intermediate lobe (139,140). In both cases the lack of GR expression appears to be necessary to protect those cells from CORT regulation of a particular target gene (*Per1* in SCN, *Pomc* in melanotrophs) that is readily regulated by CORT in other cell types. MR expression is more restricted, with high expression in the collecting ducts of the kidney and the hippocampus, and lower expression elsewhere (141). The thymus may be one tissue that selectively lacks any MR expression (142).

MR and GR expression at both the mRNA and protein level can vary with different experiential conditions. The receptors are regulated in an autoregulatory negative fashion, such that the receptors upregulate in the absence of glucocorticoids, and downregulate in the presence of high glucocorticoids (143,144). However, the receptor levels in the brain tend to be quite stable in the face of chronic stress (95,145).

Because these two receptors have different effects on gene expression there is the likelihood that the relative proportion of MR and GR present in a particular target cell is an important factor for CORT's effect. Ron de Kloet (146) has proposed that this MR/GR balance is especially important in mediating some of CORT's effects in the brain, and that disturbance of the appropriate receptor balance can contribute to certain pathological conditions.

In human tissue a number of alternate spice variants of GR have been identified (124). The best characterized of these is GRβ, which in contrast to the predominant form of GR (GRα), has an altered carboxy terminus amino acid sequence that interferes with the ability of the expressed protein to bind CORT. GRβ, therefore, may function as an in vivo dominant negative form of GR, although its expression in general is low relative to GRα (124,147). Rats and mice lack the specific GRβ alternate splice form found in humans (148). However, recent studies have identified unique alternate splice forms of the carboxy terminus portion of GR found in mice (149) and rats (150). These alternate splice forms are expressed in relatively low levels in peripheral tissue, and their possible neural expression and physiological relevance remains to be determined.

2.3.3. Relative MR/GR occupancy by physiological CORT levels

MR and GR bind natural and synthetic glucocorticoids with different affinities. MR binds cortisol, corticosterone, and aldosterone with high affinity (Kd ~ 0.5 nM) and most synthetic glucocorticoids with very low affinity. GR on the other hand, binds synthetic glucocorticoids such as dexamethasone and RU28362 with a high affinity (Kd ~ 0.1 nM), cortisol and corticosterone with a lower affinity (Kd ~3-5 nM), and aldosterone with a much lower affinity (151,152).

The differential affinity of MR and GR for CORT has important significance for their relative role in mediating the effects of varying basal and stress-induced circulating CORT levels. Because MR has a 10 fold higher affinity for CORT than GR, it is occupied to a greater extent than GR by a given circulating level of hormone. Initial estimates of MR and GR occupancy by CORT in the rat determined that the majority of MR (90% or more) are occupied even during low basal levels of hormone secretion, whereas GR does not become significantly occupied until CORT levels are elevated by acute stress or at the peak of the circadian cycle (153-155). Some subsequent studies indicate that MR can contribute to the functional effects of acute stress-induced CORT levels, such as CORT negative feedback (156). MR protein levels rapidly upregulate in the rat brain after adrenalectomy (157). This upregulation is likely to have led to an overestimation of the proportion of MR that are occupied by low basal CORT levels, since those estimates were based on comparisons of available MR binding levels in adrenal-intact and adrenalectomized rats (see Section 4.4.).

2.3.4. Receptor mediated rapid effects (“non-genomic effects”)

It has long been recognized that glucocorticoids can produce rapid cellular effects (within seconds to a few minutes) that are too fast to be dependent on alterations in gene transcription and subsequent protein translation/maturation. These rapid effects are often referred to as “non-genomic” effects of glucocorticoids. The fast negative feedback effects of CORT on HPA axis activity and the CORT-dependent rapid enhancement of hippocampal glutamate release are two examples of these non-genomic effects (84,158). These rapid effects may be mediated by protein-protein interactions of MR and GR with certain signaling molecules (158,159). However, there is some evidence for a separate integral membrane receptor for glucocorticoids that may be coupled to a G-protein (160,161).

2.4. Key modulatory factors of CORT bioavailability

Because CORT is able to passively diffuse across cellular membranes, it is present in virtually all cells of the body, and those intracellular levels fluctuate in close relationship to the fluctuating CORT levels in the systemic circulation. However, there are several important factors that regulate the concentration of CORT present inside a particular cell, i.e. the CORT that is bioavailable to interact with MR and GR.

2.4.1. Corticosteroid Binding Globulin (CBG)

CORT’s lack of water solubility requires that it associate with blood proteins in order to circulate throughout the body. CORT has a weak association with some blood proteins, such as serum albumin. But, it also has a very high affinity (~1-10 nM in the rat and human) for the carrier protein CBG (also known as transcortin), which is produced by the liver (162,163). Given the high affinity of CORT for CBG, the majority (>90%) of CORT is bound to CBG, and is not able (i.e. “free”) to cross the blood brain barrier, or

diffuse into target cells (164). The proportion of total CORT that is free (bioavailable) increases some with high stress-induced circulating levels of CORT as the CBG buffering capacity becomes saturated. However, even then less than 10% of total CORT is free (36,165). CBG levels are especially high in the anterior pituitary, thereby resulting in a lower occupancy of MR and GR in the anterior pituitary than in other tissues for a given circulating CORT level (155,166). CBG levels are regulated by various factors that then impact CORT bioavailability (162,167). For example, liver production of CBG is suppressed by acute inflammation (168,169), whereas sustained high glucocorticoid levels also suppress CBG production (170). Consequently, both conditions (inflammation, sustained high CORT) have the capacity to increase free CORT through an indirect mechanism involving reduced carrier protein in blood. Conversely, estrogens increase CBG production, and this can be an important factor for sex comparisons of CORT levels or assessment of CORT levels in females across the menstrual cycle, different stages of pregnancy, or pre or post menopause (162,167,171). In those cases a difference in total CORT levels may not reflect to the same extent differences in free CORT levels. Interestingly, enzymatic cleavage of CBG within specific tissue has been demonstrated that results in reduced CBG affinity for CORT. This localized CBG cleavage has been hypothesized as a mechanism by which bound CORT is "liberated" for use by immune cells within local tissue sites of inflammation and infection (163).

Most synthetic glucocorticoids do not bind CBG (172). As a consequence, even if they have a similar affinity for GR as CORT, their bioavailability will be substantially higher for a given systemic concentration. Also, given the repressive effect of glucocorticoids on CBG production, long-term synthetic glucocorticoid treatment will down regulate CBG levels. CBG is also a carrier protein for progesterone, and its downregulation may also impact progesterone bioavailability (162).

2.4.2. CORT metabolism

The very short-half life (< 15 min in rodents and humans) of circulating CORT (84,173-176) is due primarily to the rapid metabolism of CORT to biologically inactive water-soluble forms in the liver, and subsequent excretion through the urine (Table 1). CORT can also be metabolized within various target cells by the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD). There are 2 isoforms of this enzyme, 11β-HSD1 and 11β-HSD2. 11β-HSD2 converts corticosterone to deoxycorticosterone and converts cortisol to 11-deoxycortisol (also known as cortisone), both of which have low affinity for MR and GR. This enzyme is expressed in high levels in the kidney collecting duct and ensures that aldosterone rather than CORT has preferential access to MR (177). Interestingly, 11β-HSD1 preferentially regenerates CORT from its metabolites in a tissue-specific manner (178). For instance, there is a relatively high expression level of 11β–HSD1 in the rodent hippocampus, and genetic reduction of these levels or pharmacological inhibition of 11β-HSD1 activity has been shown to protect hippocampal function from some of the effects of chronic or acute CORT elevation (179,180). In addition, the 11β-HSD enzymes are richly expressed in the placenta (181), and thereby regulate glucocorticoid access to the developing fetus, which can have enduring influences on the trajectory of health and disease of the offspring (182). In this way, local tissue expression of the 11β-HSD enzymes exert a level of fine control over

glucocorticoid action that may not be detected by examination of only circulating levels of CORT.

2.4.3. Blood brain barrier and multi-drug resistance P-glycoprotein

CORT and other steroids diffuse across cell membranes, including the endothelial cells that form the tight junctions of the blood vessels in the brain (i.e. blood brain barrier). However, endothelial cells express a multi-drug resistance P-glycoprotein on their luminal surface (183). This protein serves to actively transport various molecules out of the endothelial cells back into the lumen of the blood vessel. In this way, P-glycoprotein serves as a gatekeeper to protect the brain from certain chemical substances. Some synthetic glucocorticoids, such as dexamethasone and prednisolone are bound by this protein and excluded from endothelial cells (184). Although this efflux pump can be overwhelmed by high levels of dexamethasone, relatively low levels are prevented from entering brain parenchyma (185). Thus, the relative access to centrally-located CORT receptors can vary substantially between different glucocorticoids depending on their blood levels.

3. EXPERIMENTAL MANIPULATION OF THE HPA AXIS

Because CORT is the primary effector hormone of the HPA axis, the general research objective of HPA axis physiology research is to test whether a particular aspect of circulating CORT (ultradian, circadian or reactive) is necessary or sufficient for dependent measures of interest. This general objective is typically achieved by either directly or indirectly manipulating CORT levels, or by treating the subject with MR/GR agonists or antagonists.

3.1. Acute glucocorticoid treatment

The most frequent experimental manipulation of the HPA axis is to treat subjects with exogenous glucocorticoids. Scientists may have a range of research objectives and practical considerations that guide their adopted glucocorticoid treatment strategy. These strategic considerations begin with the choice of glucocorticoid, followed by choice of dose, dosing regimen, vehicle and route of administration. If the study is physiological rather than pharmacological in nature, then a primary research objective is to simulate a glucocorticoid condition in the research subject that may occur under normal or pathophysiological conditions. A common research objective is to simulate the glucocorticoid profiles associated with various aspects of stress, such as acute stress, repeated acute stress, or chronic stress. Typically the rationale of these studies is to see if stress levels of CORT are sufficient to produce certain outcomes associated with a particular stressor exposure. The most straightforward approach then is to treat the subject with the same glucocorticoid that is the principal endogenous glucocorticoid of that species (corticosterone or cortisol). This ensures similar pharmacodynamic and pharmacokinetic actions as would occur with endogenous glucocorticoid secretion. As outlined below (Section 3.5.), there are no synthetic glucocorticoids that have similar relative affinities for MR, GR and CBG as does CORT, and likely none that have the very short half-life of CORT. Another advantage of using the natural glucocorticoid is that one can then directly measure the resultant blood levels and know how those levels compare to stress-induced endogenous CORT levels in that particular subject.

It is possible to approximate fairly closely the absolute blood levels and temporal profile of an acute CORT response to acute stress as illustrated above. Fig 7 shows time-response curves for the concentration of plasma CORT levels present in young adult male rats after intraperitoneal (i.p.) or subcutaneous (s.c.) injection of a range of CORT doses (186,187). Note that the route of administration makes a significant difference in the temporal profile. Whereas similar plasma CORT levels are attained 30 min after administration of the same dose of CORT given i.p. or s.c., the s.c. administration results in more sustained circulating levels. The i.p. dose is more rapidly absorbed into the systemic circulation and it likely produces higher peak levels that occur prior to 30 min. The i.p. dose is also more rapidly cleared from the system, likely due to first pass clearance by the liver (188). Although intravenous CORT infusions are less common in rodent studies, more than a 10-fold lower dose of CORT (~0.3 mg/kg) is appropriate if given intravenously (66,84). The choice of dose and route of CORT administration may depend on the acute stressor response one desires to simulate. But if the route of administration is i.p. or s.c., comparison of Figures 4 and 7 suggests that a dose of 5 mg/kg likely recapitulates maximum physiological levels of CORT that can be achieved under stressful circumstances. A dose of 2.5 mg/kg of CORT is more representative of typical acute stress CORT responses in the rat.

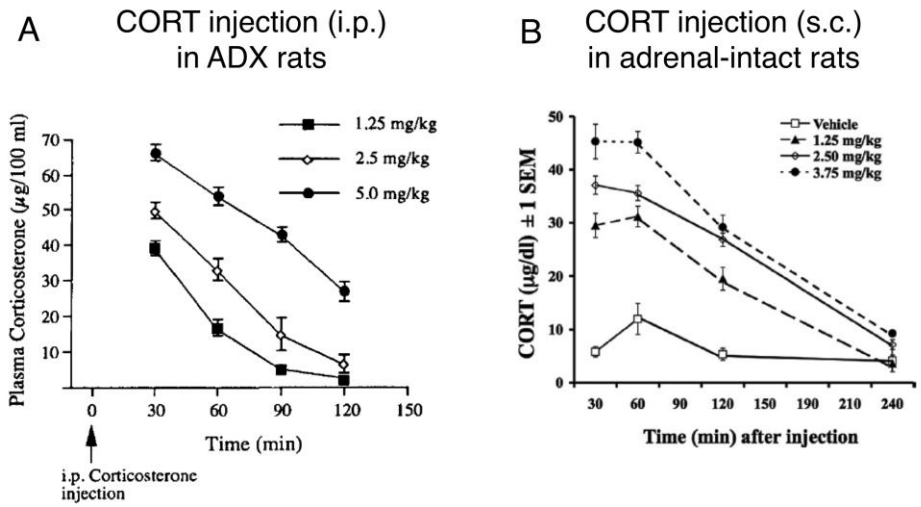

Figure 7. Plasma CORT levels produced by systemic injection of rats with different CORT doses. **A**. Plasma CORT levels in serial blood samples (tail clip method) from adrenalectomized (ADX) adult male Sprague-Dawley rats after injection with 3 different doses of corticosterone (1.25, 2.5 or 5.0 mg/kg, i.p.) (reprinted from (186)). **B**. Plasma CORT levels in serial blood samples (tail clip method) from adrenal-intact adult male Sprague-Dawley rats after injection with 3 different doses of corticosterone (1.25, 2.5 or 3.75 mg/kg, s.c.) or vehicle (16% EtOH, 44% propylene glycol, 40% phosphate buffer saline, s.c.) (reprinted from (187)).

Consistent with this dose recommendation, Figure 8 shows the results of the use of in vivo microdialysis (see Section 4.3.) to measure extracellular CORT in the hippocampus during a single session of intermittent footshock, a stress challenge which produces near-maximal plasma concentrations of CORT in the male Sprague Dawley rat. A second comparator group was injected with 2.5 mg/kg (s.c.) of CORT, a dose that has previously been shown to mimic the footshock-induced rise in plasma CORT.

Groups were studied simultaneously and samples were analyzed within a single assay to ensure appropriateness of comparisons. The primary question of interest was whether exogenous CORT would produce comparable extracellular CORT in the hippocampus as the natural stress challenge (footshock) it was intended to simulate. As illustrated in Figure 8, injection of 2.5 mg/kg CORT led to an identical peak in hippocampal CORT, but the exogenous CORT peaked slightly earlier (~30 min) prior to the peak observed in rats exposed to footshock. These data provide additional guidance on the selection of appropriate doses to mimic high stress levels of CORT through an exogenous injection approach, and allow for the conclusion that specific target tissues (hippocampus, in this case) likely receive comparable glucocorticoid exposure regardless of whether the CORT is derived endogenously or exogenously.

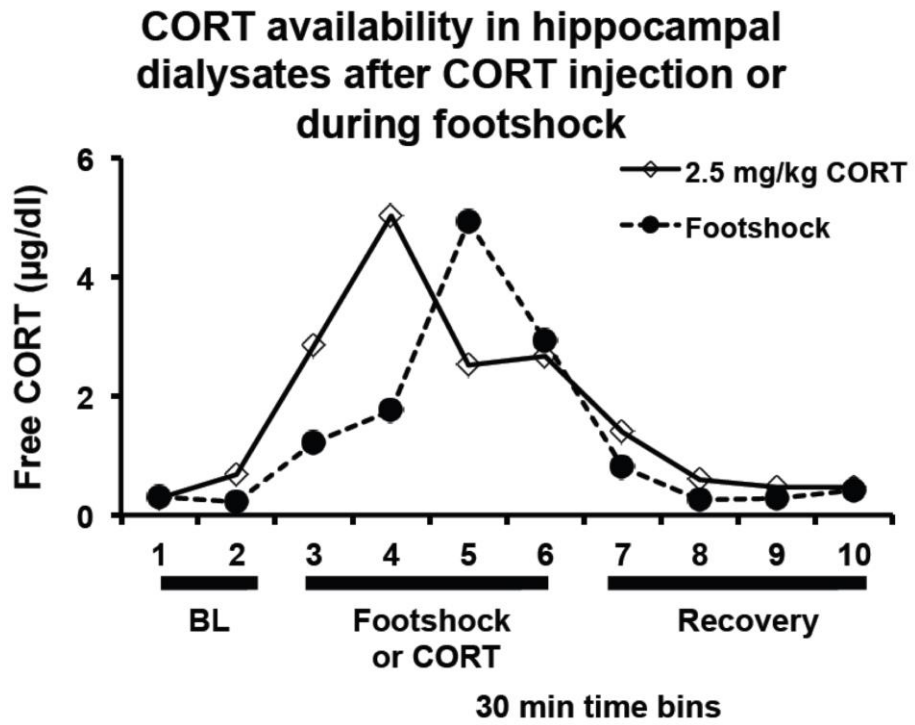

Figure 8. CORT (2.5 mg/kg s.c.) produces hippocampal free CORT levels comparable to that seen after footshock stress. After collection of two baseline samples using microdialysis probes targeting the hippocampus, adult male Sprague-Dawley rats (n=3-4 per group) were exposed to 80 footshocks (1.0 mA, 5 secs each, 90 sec variable ITI) or were injected with 2.5 mg/kg (s.c.) and placed into a standard microdialysis bowl. Sampling interval was 30 min epochs and perfusion rate was 2 µl/min. Corticosterone was measured in dialysates utilizing standard RIA procedures. Peak concentrations of CORT in dialysates were identical between experimental groups, although CORT-treated rats exhibited an earlier peak (T Deak, previously unpublished data).

A disconcertingly large number of published studies have used much higher acute or repeated doses of CORT. For example, treatment of rats or mice with ~30-40 mg/kg CORT is fairly common, and in most cases circulating concentrations of CORT achieved under these dose conditions are not reported. As a result, the mechanism of action of these supraphysiological glucocorticoid levels is difficult to interpret at this time.

The primary difference in GR occupancy profile after injection of very high CORT levels (compared to lower, more physiologically-relevant levels) is the duration of maximal GR occupancy. Probably all doses of CORT above 5 mg/kg produce near maximal occupancy of GR within 30 min after injection. Importantly, in the case of high pharmacological levels of CORT, the duration of maximal GR occupancy is probably much longer than can occur with an endogenous CORT response to most laboratory-based stressors (e.g. see Fig 6). Not only will it take substantially longer for enzymatic degradation of CORT to occur with supraphysiological concentrations, the ability of locally-expressed enzymes to protect specific tissue types and/or target cells from excessive glucocorticoid exposure may be overwhelmed. Further concerns about the lack of physiological relevance associated with repeated high dose CORT treatment is illustrated by one study that acknowledged that daily CORT treatment (15-20 mg/kg, s.c.) produced 50% mortality after 3 months (189). Consequently, one must carefully consider the dose and means of exogenous CORT delivery in order to identify a tractable approach for achieving the intended experimental goals.

3.2. **Vehicle and route of administration considerations**

Corticosterone and cortisol, as well as most synthetic glucocorticoids are not soluble in water due to their non-polar steroid structure. These compounds are soluble in 100% ethanol, various oils and glycols, with an upper limit of solubility in 100% ethanol around 25 mg/ml. There is a trade off between choosing a vehicle that adequately dissolves the glucocorticoid, but does not itself produce a physiological response. For systemic treatment via the subcutaneous route of administration, sesame oil, peanut oil and propylene glycol have been used with good success. None of these vehicles in full concentration, however, are advisable for use with intraperitoneal injection due to adverse effects when administered in relatively large volume (> 0.1 ml) into the peritoneum. We find that a vehicle of 10% ethanol, 30% propylene glycol, and 60% sterile saline is well tolerated when injected i.p. (186). This vehicle permits CORT solubility upwards of 5 mg/ml if the solution is bath sonicated and heated to 50 °C within 1 hr prior to use. Hydroxypropyl-β-cyclodextrin can also be used to dramatically increase CORT solubility in saline (66,190), but it may produce some side effects (191).

3.3. **Absence of stress-induced CORT while manipulating basal CORT**

Another approach that some stress glucocorticoid physiology studies have taken is to determine whether stress-induced glucocorticoid elevations are necessary for the various responses to a particular stressor. In this case, the research strategy is to prevent the elevation of glucocorticoids associated with stressor challenge. Because virtually all of the circulating CORT in mammals originates from the adrenal cortex, circulating CORT can be eliminated by removing the adrenal glands (i.e. adrenalectomy, ADX). This classic endocrinology extirpation strategy has been widely used in the study of glucocorticoid physiology. Such studies are straightforward in the laboratory rat because the adrenal is a discrete fully ensheathed gland that can be removed by a relatively simple surgical procedure. The rat tolerates ADX well for at least two weeks if they are maintained on a 0.5-0.9% saline drinking water solution in order to compensate for the absence of the mineralocorticoid hormone aldosterone, which is crucial for reuptake and maintenance of physiological concentrations of sodium. In other species

(including some mice), adrenal cortical tissue can be somewhat more dispersed in the extra-renal adipose tissue, making it difficult to execute a complete surgical ADX. The spared adrenal cortical tissue can over time regenerate and produce significant CORT levels.

Although ADX is an effective strategy for eliminating circulating CORT, the procedure also eliminates a number of other adrenal hormones including the adrenal medullary hormones (e.g. epinephrine and norepinephrine) and other adrenal cortical steroid hormones (e.g. aldosterone, progesterone, and DHEA). Thus, ADX studies should include ADX animals given CORT replacement in order to assess if an effect of ADX is truly CORT dependent. It is important to also remember, however, that ADX not only eliminates the presence of stress-induced CORT levels, but also the presence of ultradian and circadian basal CORT. There are many physiological processes that depend on the “permissive” effects of basal CORT for their normal operation (3,192). In some cases, the presence of constant low levels of CORT is sufficient to maintain these permissive glucocorticoid effects, but other physiological effects require the ongoing presence of the diurnal fluctuation of basal CORT (193-197).

To tease apart which aspects of CORT secretion may be necessary to restore normal physiological function (“normalization”) in ADX animals, researchers have devised several different CORT replacement strategies. Fairly constant levels of systemic CORT can be produced by implanting in rats and mice subcutaneous CORT-containing pellets. The target level of circulating CORT can be achieved by varying the percent of CORT present in the pellets, as well as varying pellet size and number (198,199). Typically, the steroid precursor molecule, cholesterol, is used to make control pellets, or to dilute the total amount of CORT present in individual pellets. Treatment of rats with CORT pellets that produce circulating CORT levels higher than 4.5-7.5 µg/100 ml (the approximate average daily CORT level) simulates a higher than normal physiological level of CORT, and is accompanied by signs of CORT over exposure, such as thymus involution and diminished body weight gain (199,200).

The use of osmotic mini-pumps to maintain constant circulating CORT levels is not viable except for maintenance of very low CORT levels, due to the limited reservoir capacity of the pumps. Only relatively low concentrations of CORT can be attained in aqueous solutions (see Section 3.2.). This limited reservoir capacity also precludes use of programmable subcutaneous pumps (iPrecio pumps) to deliver constant or fluctuating levels of systemic CORT. On the other hand, these programmable pumps may be an attractive means to deliver CORT with a controlled temporal profile directly into a specific brain region (although the pumps cannot accurately deliver their payload bilaterally) (201). These pumps may also be useful in delivering systemically functional levels of low concentrations of synthetic glucocorticoids that are more potent than CORT and are not buffered by CBG.

An effective and non-invasive strategy to maintain diurnal fluctuations of circulating CORT in ADX rats is to provide them with CORT in their saline drinking water (194,199). Nocturnal rats and mice engage in a majority of their daily feeding and drinking during the first few hours after the dark phase onset. This oral consumption of CORT results in relatively large increases in circulating CORT around the time of daily feeding and drinking. A CORT concentration of 25 µg/ml in drinking water appears to be appropriate to normalize many of the permissive physiological effects of CORT absent

in ADX rats (194,199). Higher doses of CORT in the drinking water have been used to test the effects of chronic CORT elevation such as may occur with chronic stress (202). As described above (Section 2.2.1.2.), the basal peak in endogenous CORT coincides with the onset of the dark phase, and this peak is anticipatory in that the increase begins several hours before the dark phase onset. Consequently, the daily elevation of CORT that is generated by CORT in the drinking water lags by several hours the endogenous CORT circadian peak (203) (Fig 9). Whether the several hour time lag in daily CORT levels produced by CORT in the drinking water compromises some of the normal physiological actions of circadian CORT secretion has not been examined. There is growing interest in the physiological role of circadian CORT secretion. One consideration is that it is not only important that there are daily peaks and troughs of basal CORT secretion, but that the daily timing of the circadian peak also matters. We have recently found that a daily injection of CORT in ADX rats that occurred just before the onset of the dark period normalized clock gene expression profiles in the prefrontal cortex, whereas the same CORT treatment regimen administered first thing in the morning completely disrupted prefrontal cortex clock gene expression (204).

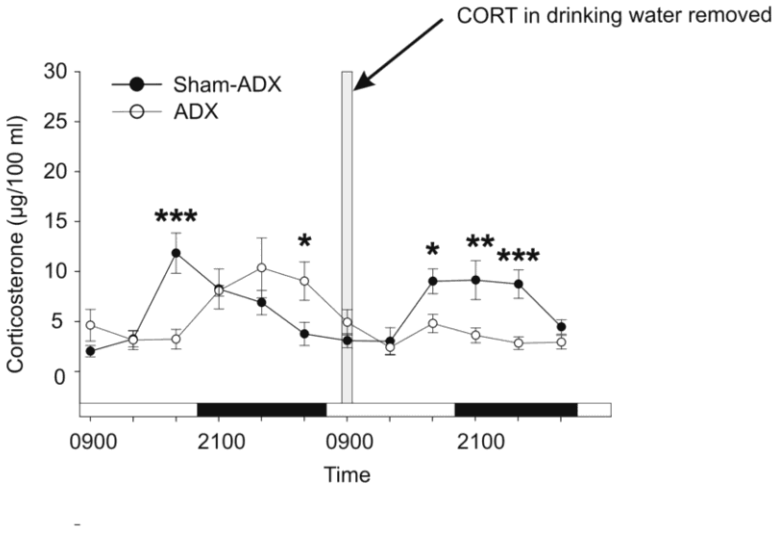

Figure 9. CORT in drinking water restores daily basal peak in circulating CORT levels, but with some time-lag compared to endogenous CORT secretion. Plasma CORT levels in serial blood samples (jugular catheter automated blood sampling system) from adrenalectomized (ADX) or adrenal-intact (Sham-ADX) adult male Sprague-Dawely rats over the course of 2 days (solid black bar above x-axis indicates dark phase). ADX rats had CORT in the drinking water (25 µg/ml in 0.9% saline containing 0.45% hydroxyl-propyl-beta-cyclodextrin) for the first day shown. Between-group comparisons: *p < 0.05, **p < 0.01, ***p < 0.001. (adapted from data presented in (203), courtesy of CA Lowry).

Another issue of consideration when using CORT in the drinking water concerns the acquired CORT levels present during the rat's inactive period. Given the very short half-life of CORT, circulating CORT levels decline rapidly after cessation of drinking. It is

not unusual for CORT levels to fall to undetectable levels during the rat's inactive period (199). We have found, however, that procedures such as vehicle injections during the inactive period can stimulate day-time bouts of drinking that results in short-term CORT elevations that can be substantially higher than low basal levels (unpublished observations). Thus, it may be advisable to replace CORT containing water bottles with saline only bottles during the day (205). The challenge of maintaining sufficient ambient CORT concentrations during the inactive period when using the CORT drinking water method has led some researchers to utilize a combined CORT drinking water plus low-dose CORT pellet procedure to restore normal physiological concentrations of CORT in ADX rats (206). Although this should theoretically provide appropriate basal CORT replacement during the inactive period while at the same time mimicking the natural circadian rise in CORT, an ongoing limitation of these basal CORT replacement strategies is that they do not recapitulate the ultradian CORT rhythm. Chronic indwelling i.v. catheters have been used to deliver hourly pulses of CORT to rats in order to study ultradian CORT actions (66). As illustrated above, a range of options for basal CORT replacement are readily available, and the strengths/limitations of each approach require careful consideration prior to implementation.

3.4. **Steroid synthesis inhibition**

An alternative strategy to eliminate stress-induced CORT secretion is to treat subjects with steroid synthesis inhibitors. This has been used in clinical research as well as in rodent studies. Certain doses of steroid synthesis inhibitors will effectively blunt or eliminate stress-induced endogenous CORT secretion without eliminating basal secretion. The extent to which only CORT is primarily affected by these drugs depends on the specific drug and its target steroidogenic enzyme. Aminoglutethimide, ketoconazole and trilostane inhibit the synthesis of both gonadal steroids and adrenal steroids. Widely used for CORT synthesis inhibition is metyrapone, which inhibits 11β-hydroxylase and aldosterone synthase, thereby affecting both CORT and aldosterone synthesis. A logistical drawback to the use of steroid synthesis inhibitors is that longer-term studies may require frequent injections to ensure sustained inhibition of CORT production. Moreover, higher doses of metyrapone can produce side effects, curiously even in ADX rats (207,208). This latter finding suggests that pharmacological ablation of CORT may have additional off-target effects such as altered neurosteroid synthesis, which is not directly influenced by surgical ADX procedures (209). To avoid these side effects, some studies have used a combination of low-dose metyrapone (~50 mg/kg) in combination with amino-glutethimide (100 mg/kg) to effectively suppress stress-induced CORT secretion (210,211). Although this strategy is decidedly imperfect, use of a pharmacological approach to block CORT release often has advantages where surgical manipulations such as ADX might be otherwise contraindicated.

3.5. **Pharmacological manipulation of MR and GR**

There are times when it may be advantageous to use an MR or GR selective ligand, to assess the receptor mediation of a particular CORT effect. Unfortunately, there are no pure selective MR or GR agonists or antagonists. A general tenet of pharmacology is that most drugs are "promiscuous", in that they cross-react with more than one type of cellular receptor. This promiscuity is definitely a feature of synthetic

ligands for MR and GR. As described above, MR and GR are members of a nuclear hormone receptor super family. Gonadal steroid receptors are other members of this super family and they have fairly high homology with MR and GR within the ligand-binding domain of the receptor (212). Consequently, many synthetic ligands that bind MR and/or GR also interact with some of these other steroid hormone receptors. Another reason for the lack of a pure selective MR or GR agonist or antagonist is the inherent biochemical complexity of steroid receptor function. As described above, steroid receptors are complex proteins with multiple functional domains that participate in various aspects of signal transduction. Thus, each ligand likely induces a unique receptor protein conformation that results in a unique profile of cellular actions. This phenomenon of unique ligand-receptor functional profiles has been well characterized for estrogen receptors and their selective estrogen receptor modulators (SERMs) (213). Keeping this limitation of a pharmacological approach in mind, there are some useful pharmacological tools for selectively manipulating MR and GR function (Table 2).

In the 1970's and 1980's a French pharmaceutical company Roussel Uclaf developed and characterized a number of steroid receptor synthetic ligands. At the time, the company would share small quantities of these compounds with researchers for basic non-clinical research. The original company is no longer in existence, and most of their steroid receptor selective ligands are not commercially available for research or clinical use. One exception is RU 486 (also known as RU 38486, or mifepristone) which is used clinically because of its antiprogesterone receptor properties. This compound, however, is also a fairly potent GR antagonist, with little affinity for MR. A number of studies, however, have found that RU486 has some partial agonist effects for certain glucocorticoid cellular actions (214). A couple of other RU compounds have been very useful for basic research. RU28362 is a highly selective GR agonist, and RU28318 is a selective MR antagonist (190,215). Sigma-Aldrich provides both of these compounds to early discovery researchers in small quantities for a rather high price. Sigma-Aldrich does not guarantee the purity or identity of these preparations. Spironolactone is used clinically as an antimineralocorticoid, however, it is also an androgen receptor antagonist (152). Eplerenone, is a more recently available selective, but less potent, MR antagonist (216).

Glucocorticoids are one of the most widely prescribed class of drugs in all of medicine. There are a number of synthetic glucocorticoids used in medicine because of their high potency and relatively long half-life. Some of the most commonly prescribed synthetic glucocorticoids arranged in order from least to greatest selectivity of glucocorticoid vs mineralocorticoid actions are cortisone < prednisone, prednisolone, < methylprednisolone < triamcinolone < dexamethasone, betamethasone (152). Various preparations of cortisol (also known as hydrocortisone) are also prescribed widely. Fludrocortisone has a 12.5-fold greater mineralocorticoid action than glucocorticoid action, and is prescribed as a mineralocorticoid drug. Each of these compounds is commercially available for preclinical studies.

Dexamethasone is a highly selective agonist for GR, and it is often used in research for this purpose. Researchers should be aware, however, that systemic treatment with this and other selective GR agonists for longer than an acute time frame produces a non-physiological condition of high GR activation simultaneously with unusually low MR activation. This is the consequence of the near complete shut down

of endogenous glucocorticoid secretion (the high affinity MR ligand) through the GR-dependent negative feedback actions of potent GR agonists. Treatment of rats with a relatively low dose of dexamethasone produces signs of high GR activation throughout the periphery, but very little MR or GR activation in the brain ("CNS hypocorticosteroid state") (217). This is due to the fact that: 1) a low dose of dexamethasone can potently shut-down ACTH secretion at the level of the anterior pituitary (and subsequent downstream CORT secretion), and 2) this dose of dexamethasone is largely excluded from brain tissue due to its interaction with the multidrug resistance P-glycoprotein localized in endothelial cells of the blood brain barrier (see section 2.4.3.).

3.6. **Probing different levels of HPA axis function**

Although differences in plasma CORT secretion are often attributed to differential CNS regulation of the HPA axis, group differences in CORT levels may also reflect differential function at the level of the anterior pituitary or adrenal cortex (218). For example, a variety of factors lead to alteration of CRF_1 receptor expression and function in the anterior pituitary (219). There may also be an up or down regulation of MR/GR in the anterior pituitary resulting in altered glucocorticoid negative feedback function. Alternatively, adaptation can take place at the level of the adrenal. For example, ACTH not only stimulates CORT production, but it has tropic/trophic effects on the steroid producing cells of the zona fasciculata such that chronic elevation of ACTH results in adrenal cortical hyperplasia and hypertrophy (220). As a consequence, subsequent CORT production is elevated in response to a given amount of ACTH. There is also evidence for adrenal CORT production to be regulated by adrenal endocannabinoid function (221).

The in vivo operation of the pituitary and adrenal components of the HPA axis can be assessed by CRF and ACTH challenge studies (85,222). By comparing the ACTH and CORT response to an acute challenge with CRF and by comparing the CORT response to an acute challenge with ACTH, it is possible to deduce whether comparison groups of interest have similar functioning of their pituitary response to CRF and adrenal response to ACTH. If one tests several different doses of CRF or ACTH one can also make inferences about whether there is an overall group difference in the maximal hormone response (i.e. pharmacological efficacy) to CRF or ACTH or whether there is a shift in the tissue sensitivity to these hormones.

One caveat for interpretation of CRF and ACTH challenge studies is that the stress of the injection of CRF or ACTH may lead to some additional endogenous CRF secretion and downstream ACTH and CORT secretion. Thus, a group difference in reactivity to the stress of injection could result in an apparent differential CORT response to exogenous CRF or ACTH challenge. One strategy for limiting this potentially confounding factor when examining adrenal responsiveness to exogenous ACTH may be to pretreat the subject with a high dose of a glucocorticoid, such as dexamethasone in order to clamp down endogenous ACTH secretion via glucocorticoid negative feedback. Group differences in the absorption, distribution or clearance of exogenous CRF or ACTH could also be a confounding factor in these challenge studies. For example, these pharmacokinetic factors are likely to vary with age, sex, or body weight. Measurement of the subsequent plasma levels of CRF or ACTH present after CRF or ACTH challenge, respectively, would provide validation

that these levels are comparable between comparison groups of interest.

3.7. **Dexamethasone challenge**

A number of medical conditions are associated with altered CORT secretion profiles. One prospect is that the altered CORT profile reflects HPA axis dysregulation as a result of altered glucocorticoid negative feedback function. A widely used test of glucocorticoid negative feedback function in human studies is the dexamethasone suppression test (DST) (223). Some conditions, such as major depressive disorder, are associated with an impaired DST result in which many individuals display an attenuated suppressive effect of dexamethasone on the subsequent early morning rise in basal CORT levels (224). Although this impaired DST response may reflect a fundamental decrement in normal glucocorticoid negative feedback function, it may also reflect greater than normal drive to the HPA axis that overcomes the tested level of glucocorticoid negative feedback. Another important issue is that low doses of dexamethasone, such as those used in human DST tests, are largely excluded from the brain by the multidrug resistant pump, as discussed above (Section 2.4.3.). Thus, the DST may primarily assess glucocorticoid negative feedback function at the level of the anterior pituitary (222,225). Although higher doses of dexamethasone or other glucocorticoids can be given systemically to produce central negative feedback, these will invariably also produce potent negative feedback at the level of the anterior pituitary. In such a case, use of ACTH or CORT as an endpoint will not allow for assessment of peripheral versus central negative feedback function. More recently, there has been good support for a combined dexamethasone and CRF challenge test to provide better sensitivity than the DST as a potential biomarker for neuropsychiatric disorders (20,226). The extent to which the test assesses both central drive to the HPA axis and central and peripheral glucocorticoid negative feedback sensitivity is unknown. Some other studies have used prednisolone as a glucocorticoid challenge drug because it has the ability to activate both MR and GR, and therefore probes both MR and GR glucocorticoid negative feedback function (227,228). However, in contrast to CORT, prednisolone has higher affinity for GR than MR (Table 2).

3.8. **Transgenic mouse models**

An increasing number of genetic mouse models have been developed in which GR or MR expression is knocked out, knocked down or over-expressed (229,230). Some of these genetic mouse models include conditional temporal or phenotype alteration of GR/MR expression (231-235). There are a number of issues and considerations that are inherent to the general use of genetic mouse models, as well as more specific issues for each of the MR/GR alteration models developed to date (236). However, discussion of these genetic issues and considerations is beyond the scope of this Users Guide.

4. MEASUREMENT OF HPA AXIS

4.1. **HPA axis hormone blood measures**

There are three important objectives necessary for accurate measurement of HPA axis hormonal activity: 1) to obtain blood samples that reflect the actual circulating hormone levels at a point in time of interest that are not confounded by the sampling

method itself, 2) to collect and process blood samples in a manner that preserves the hormone molecule present within the plasma sample, and 3) to use a hormone analysis method that is reliable and sensitive. We discuss these objectives below and their specific implications for the measurement of CORT and ACTH.

4.1.1. CORT

The most widely used HPA axis measure is the measurement of CORT in blood. As the effector hormone of the HPA axis, CORT levels represent the key functional output of HPA axis activity. Adopting the appropriate blood sampling procedure is critical for obtaining meaningful CORT measures. Because the HPA axis of laboratory animals are highly reactive to any disturbance including cage movement, brief handling and increased activity in their home cage room, it is very difficult to obtain a blood sample without triggering some activation of the HPA axis. Perhaps the one means by which this is possible is by obtaining a blood sample through a chronic indwelling venous catheter. That approach, however, may have its own limitations in terms of surgical stress, stress from the presence of the chronic indwelling catheter and missing data due to loss of catheter patency. Fortunately, because there is a several minute time lag after HPA axis activation before newly synthesized CORT levels begin to enter the systemic circulation, many researchers capitalize on this time window of opportunity to obtain a blood sample. If a blood sample can be obtained within several minutes after entering the animal's home room, then there is well-validated evidence that the CORT levels present in the blood sample reflect the circulating CORT levels that were present prior to the disturbance associated with the blood sample procedure (Fig 5). This window of opportunity has been effectively used for obtaining blood samples from the tail vein of rats (this is much more difficult in mice), from a retro-orbital bleed or from trunk blood after decapitation. Note that any use of anesthetic prior to these blood sample methods will lead to artifactually elevated HPA axis activity (237).

Tail vein sampling, however, may not permit serial blood samples of short interval (<30 min) without the confounding influence of some residual CORT elevations present in subsequent blood samples triggered by the stress associated with the prior blood sample procedure. With that said, some studies compared the impact of repeated tail blood sampling every 30 min during restraint and found that the impact of the sampling procedure above and beyond the CORT response to restraint itself was negligible (237,238). Regardless of the methodology used to collect serial blood samples, it is essential to make sure that the volume of blood removed within a given time frame does not lead to HPA axis activation as a result of hypovolemia. Well-established guidelines are that 10% of the subject's total blood volume can be safely removed on a single occasion with minimal adverse physiological consequences (239,240). Replacement with saline can assist with recovery and reduce signs of acute physiological adjustments. This volume of blood should only be removed once every 3-4 weeks. If blood is removed across consecutive days, removal of no more than 1% of the blood volume per day is recommended. A healthy young adult mouse or rat is estimated to have a blood volume that is 5-7% of its body weight.

As important as how one will obtain a blood sample from an experimental subject is when one will obtain that blood sample. Given the dynamic nature of CORT secretion that includes ultradian, circadian and stress-reactive components, a single blood sample

provides a very limited snap shot of an individual's HPA axis activity. Carefully controlling for time of day is especially important. This is a critical factor for studies of various experimental manipulations on daytime CORT levels in human subjects in whom basal CORT levels steadily decline throughout the morning and afternoon (241). In contrast, no predictable method for designing studies around ultradian oscillations currently exists.

Logistically, measuring CORT levels in blood samples is relatively straightforward. Total CORT levels in the circulation are high relative to standard CORT assay sensitivities. Thus, CORT can be accurately measured in very small plasma or serum samples (<10 µl). In addition, steroid molecules are very stable, thereby lending considerable latitude to the acceptable temperature and temporal conditions for sample processing and storage. Measurement of CORT in whole blood may not be advisable due to assay interference from blood cells. But, CORT can be readily measured directly in either plasma or serum, without the requirement for CORT extraction. Moreover, given the stability of steroid molecules, it is not necessary that blood samples be kept cold prior to or during processing. Post processing CORT may be stable for days in plasma or serum stored at room temperature (242), and may be stable for more than a year in plasma or serum stored at -20 C.

Commercial immunoassay kits are most widely used in research laboratories to measure CORT. These assays have good sensitivity for detecting low levels of CORT and they are very effective at determining relative CORT levels present across samples within an assay. They however, are not as reliable as liquid chromatography tandem mass spectrometry in determining absolute CORT levels, which may be important for some clinical diagnostic assessment (243). Both commercial radioimmunoassay (RIA) kits and enzyme immunoassay/enzyme-linked immunosorbent assay (EIA/ELISA) kits are available and effective for measuring CORT. One very important issue to recognize when using these commercial kits is that they are designed to measure total CORT, not free CORT. However, these assay kits only partially detect CORT when it is bound to CBG. Consequently, each kit requires some form of CBG inactivation or dissociation of CORT from CBG. CBG can be denatured by heating diluted plasma/serum samples for 60 min at 75 °C without adversely influencing endogenous CORT in the samples. Other kits use a proprietary displacement reagent (e.g. an enzyme or a detergent) to promote dissociation of CORT from CBG. When assaying plasma from ADX rats spiked with known amounts of CORT, we find that the use of the steroid displacement reagent in accordance with manufacturer instructions routinely underestimates total CORT levels (unpublished observations). Furthermore, use of the steroid displacement reagent can produce unwanted variability across samples due to differing efficacy in each tube/well, whereas heat inactivation of samples is more uniform and consistent across samples. We obtain the expected total CORT values in our spiked samples if we instead heat inactivate diluted plasma (1:50) with assay buffer provided in the commercial kit, and then run heat inactivated diluted sample in the kit without using the steroid displacement reagent. Note that it is not feasible to omit heat inactivation or omit use of the steroid displacement reagent in order to measure free CORT in a plasma/serum sample. The proportion of CORT bound by CBG under assay conditions will not reflect the same CORT-CBG interaction equilibrium present in the circulation. Valid measurement of free CORT levels in plasma depends on a dialysis/ultrafiltration strategy that requires

relatively large plasma volumes (244,245). These free CORT assays are notorious for their methodological difficulty and lack of precision (243). Attractive alternative strategies for obtaining estimates of free CORT are provided by measurement of CORT in saliva or microdialysates (see Sections 4.2.1. and 4.3).

Despite the fact that CORT can be measured using a reliable commercial kit, it is still important that internal quality control measures are used. We find it helpful to include in every assay at least 3 replicates of two different concentrations of CORT spiked ADX plasma distributed throughout the assay. This allows us to determine within and between assay coefficients of variation (CV = standard deviation/mean*100) for our assays. We use in every assay frozen aliquots of plasma from ADX rats that has been spiked with a relatively low concentration of CORT (e.g. 5 µg/100 ml) and a moderately high concentration of CORT (e.g. 25 µg/100 ml). This helps us to assess the quality of our assay for determining both low basal CORT levels as well as levels within the stress-responsive range. It is preferable for these quality control measures to use CORT spiked ADX plasma rather than CORT-spiked assay buffer in order to control for the possible effect of various proteins in plasma samples on assay specific and non-specific binding. The use of CORT spiked ADX plasma also permits us to assess the efficacy of our CBG inactivation procedure. By using these quality controls we find that despite our best efforts there can be small shifts in absolute CORT values across the assay from the samples placed in the first microtiter plate wells or centrifuge tubes to the last wells/tubes. Consequently, we also recommend that the position of samples from all comparison groups of interest be counterbalanced throughout the assay. If it is not possible to assay all experimental samples within the same assay, then it is also absolutely essential that comparison groups of interest be counterbalanced across assays. Despite the use of standard curves and all other precautionary measures, it is typical to see some small shifts in absolute CORT values across assays, which, without careful attention to counterbalancing, could be inadvertently interpreted as experimental effects.

4.1.2. ACTH

It is often desirable to measure ACTH in addition to CORT. Without examining ACTH levels it is not possible to definitely conclude that a treatment effect on CORT levels is due to an alteration of HPA axis activity rather than a direct effect at the level of the adrenal. Another advantage of measuring ACTH levels is that stress-induced ACTH secretion has a greater dynamic range of stimulus-intensity secretion capacity compared to CORT. It is possible for two treatments to produce different levels of HPA axis activity that is reflected in different peak ACTH levels, but not in peak levels of CORT secretion due to a ceiling effect on CORT secretion. Although the different magnitude of ACTH response should be reflected in different durations of maximum CORT secretion (e.g. area under the curve), this would not be detected without examining a time-course of the CORT response.

In contrast to measuring CORT, the measurement of blood levels of ACTH is not so straightforward. The objective of obtaining a blood sample that accurately reflects ongoing circulating ACTH levels is especially challenging. The rapid stress-induced bolus release of ACTH into the systemic circulation requires either the relatively stress-free collection of blood that is possible through an indwelling intravascular catheter, or

another rapid means of blood collection. Although small quantities of blood can be obtained fairly quickly from the tail vein, in contrast to CORT assays, ACTH assays require a much larger sample size (50-200 µl of plasma or serum). Thus, it is very difficult to obtain the blood volume necessary within an acceptable timeframe to permit accurate measurement of the ACTH levels that were present immediately prior to the onset of the blood sample procedure (237). With careful staging, however, it is possible to obtain trunk blood from decapitation of an unanesthetized rat or mouse in less than 30 sec after removal from its home cage.

Another difficulty in measuring ACTH is that is a labile peptide. Peptidases present in the blood probably contribute to its instability. It is important that blood samples be immediately chilled to 4 °C after collection, that they be promptly centrifuged at 4 °C, and then plasma aliquots snap frozen as soon as possible. Samples cannot be re-assayed after one-time thaw due to the extensive degradation of ACTH (unlike the continued integrity of CORT) as a result of a repeated freeze-thaw cycle. Our rule of thumb is that plasma samples for subsequent ACTH measure should be frozen within 30 min after blood collection. Some protocols for measurement of plasma ACTH recommend adding a protease inhibitor such as aprotinin to the plasma samples prior to freezing. In our experience we find this unnecessary if we can uphold our target 30 min processing time. There is also a general belief that the presence of heparin in blood samples produces artifactually high ACTH levels (i.e. false positives), possibly through alteration of ACTH-antibody interactions. Although we cannot find a published paper that documents this problem, it is generally accepted that one should use an anticoagulant other than heparin (e.g. EDTA) for collection of blood samples destined for subsequent ACTH measure. The possibility of false positives or other forms of heparin-induced interference with conventional ELISA/EIA procedures is often noted within ACTH kit instructions. Another frequent methodological recommendation for ACTH measurement is to not store samples in glass tubes due to risk of ACTH adsorption to glass surfaces. A study that systematically compared ACTH levels in samples stored in plastic, glass, or siliconized glass tubes did not find a difference in ACTH values between tube type, although the study confirmed the instability of ACTH in each type of tube if stored at 4 °C (246).

As described above for CORT, there are commercial immunoassays available for the measurement of plasma ACTH, and many of the same considerations for assay quality control should be applied to ACTH assays. ACTH does not have a high affinity carrier protein in the blood, so the ACTH levels measured in these assays reflect total ACTH levels. There is not a relatively easy way to obtain plasma samples that completely lack ACTH, so it is not feasible to generate plasma samples that are spiked with known quantities of ACTH to use for assay quality control. Instead, we collect and aliquot a large pool of plasma from rats killed under basal or acute stress conditions in order to have relatively low and high ACTH containing samples that we can include in each assay for determining our within and between assay variability.

4.2. **CORT measure in non-blood based samples**

Due to the peptide structure of ACTH and its rapid degradation in the circulation (~4.5 min half-life) (247), it is not viable to obtain meaningful measures of ACTH in other fluid compartments of the body, such as saliva or urine. On the other hand, the lipid

soluble CORT molecule distributes across all cellular and fluid compartments in the body.

4.2.1. Saliva

Saliva contains CORT that largely reflects free CORT levels present in tissue (248). The measurement of free CORT in human saliva is an attractive capability for human HPA axis research. Collection of saliva is noninvasive and can be performed by subjects outside the laboratory setting, including in the comfort of their home. This can be an excellent strategy for assessing basal night-time and morning CORT levels of individuals without the confounding influence of laboratory stress. Excellent reviews and guidelines for collecting salivary CORT are available (36,242,243).

4.2.2. Urine and feces

Although most CORT (>95%) is secreted in the urine as sulfate and glucuronide conjugated metabolites (243), assay of CORT and these metabolites in urine has been useful clinically as a general indication of relative daily circulating CORT levels. In humans a small percent of metabolized CORT is excreted in the feces, however in rats a majority of metabolized CORT appears to be excreted in the feces (249). Recently, there has been increased interest among field biologists in the use of fecal CORT levels as a marker of general HPA axis activity present within specific individuals or a group of animals under study (250,251). This is particularly common in avian species where access to blood samples is both difficult and invasive, whereas fecal droppings are readily accessible (252,253).

4.2.3. Hair

Researchers have found that small but reliable quantities of CORT can be measured in hair samples. There is intriguing support for these hair samples to reflect long-term accumulation of CORT that can then be related to the long-term integrated levels of HPA axis activity. Thus, CORT levels in hair may be a useful biomarker of chronic stress level (224,254).

4.3. **Microdialysis**

Some researchers have used microdialysis to measure CORT levels in dialysate fractions taken from various tissues of the body as well as the vasculature (see Fig. 8). As a result of the exclusion of CBG by most dialysis membranes, the CORT levels present in the dialysates are believed to represent the relative free levels of CORT. Due to the difficulty in determining the appropriate dilution factor present in the dialysates, these CORT measures cannot be used to assess absolute tissue concentration of CORT. However, this microdialysis approach can be very effective for obtaining stress free serial measures of relative free CORT levels in various tissues of interest (165,255).

4.4. **MR/GR occupancy**

Due to the unique properties of MR and GR, it is possible to estimate the extent to which endogenous or exogenous ligands occupy MR and/or GR in vivo. This is an attractive feature of glucocorticoid hormone research that is not an option for the study of most other intercellular signals. This capability is due to the distinct biochemical

differences between the unactivated and activated states of these receptors. An especially useful property of these receptors is their predominantly differential intracellular localization between the cytoplasm (unactivated state) and nucleus (activated state). The intracellular localization of the receptors can be directly visualized with immunohistochemistry, or indirectly by western blot analysis of receptor levels within different cell fractions (157,256,257). Another difference between the unactivated and activated state of the receptors is that only the unactivated state of the receptor is able to participate in a competitive ligand-receptor binding assay (153,154). Prior to the availability of reliable antibodies for measuring MR and GR, this differential ligand binding capability of the receptors was used to infer relative occupancy of the receptors (decrease in available receptors) in postmortem tissue. A limitation of this approach was that it required knowledge of the total number of receptors, which could only be obtained by measuring the available receptors in tissue that lacked the presence of glucocorticoids (i.e. adrenalectomized animals). Immunohistochemical examination of MR and GR intracellular localization in brain regions of ADX rats treated with microinfusions of putative MR and GR receptor ligands has been an effective means for validating the tissue spread and receptor selectivity of treatment with these ligands (258). Interestingly, we have found that intracerebroventricular injection of glucocorticoids results in very limited diffusion into brain tissue outside the ventricular borders (259). This is likely due to the rapid clearance of the steroid molecule from brain tissue as it encounters and diffuses into the rich microcapillary bed present throughout the brain.

4.5. **CRH and POMC gene expression**

Although it is not possible to directly sample and measure CRF release into the infundibular portal blood system of an unanesthetized small animal, it is possible to obtain an indirect assessment of the relative recent activity of PVN CRF neurons by observing relative levels of CRH gene expression in postmortem tissue. In hypophysiotropic CRF neurons, the CRH gene is rapidly induced by cellular activation (196,260-262). This rapid gene induction can be detected by monitoring the levels of CRH gene primary transcript (heteronuclear RNA), due to CRH hnRNA rapid turnover rate (263). For example, a large increase in PVN CRH hnRNA can be observed within 15 min after stressor challenge (196). Thus, CRH hnRNA serves as a CRF neuron specific immediate early gene. Sustained activation of CRF neurons will also result in an increase in CRH mRNA levels, but due to the relatively high constitutive pool of this molecule, such increases may not be observed until 60 min or more after cellular activation. POMC hnRNA serves as a similar phenotype-specific immediate early gene for corticotrophs in the anterior pituitary (140,264). Monitoring the expression of these cell-type specific immediate early genes instead of a more ubiquitous immediate early gene expression, such as c-fos, can be very useful given that both CRF neurons and corticotrophs are intermixed with other cell types.

4.6. **Adrenal and thymus weight**

Two of the three hallmark signs of general stress as identified and featured by Hans Selye, the “father” of stress research, are adrenal hypertrophy and thymic involution (46). Relative adrenal and thymus size/weight continue to serve as useful

biomarkers of general levels of HPA axis and glucocorticoid activity within the individual experienced across several days to several months. Higher than normal daily levels of ACTH lead to hypertrophy and hyperplasia of steroid producing cells in the adrenal fasciculata (220). Higher than normal daily levels of endogenous or exogenous glucocorticoid levels lead to shrinkage of the thymus due to thymocyte apoptosis and suppressed proliferation (265,266). High chronic levels of exogenous glucocorticoids will also lead to shrinkage of the adrenal cortex due to negative feedback suppression of ACTH secretion. Increased adrenal weight and thymic involution is often observed in rats and mice exposed to various chronic stress regimens (267). Increased adrenal size in humans may also be associated with stress as an increase is observed during a depressive episode (268). Thymus size has also been used as an index of the effectiveness of various glucocorticoid replacement strategies to normalize the general physiological state of adrenalectomized rats (199).

4.7. **Shipment and vivarium considerations in laboratory rodent studies**

Although most laboratories have standard protocols they employ in the execution of studies, careful attention to details at the experimental planning stage can have a significant influence on experimental outcomes. Some often over looked factors may contribute significant variance in basal and stress hormone concentrations across experiments and across laboratories. While such inter-experiment variability is largely to be expected, standardization of certain practices can help normalize outcomes and thereby improve reproducibility of findings. For instance, the majority of studies utilize rats and mice purchased from commercial vendors that ship these animals by truck or plane over substantial distances. Shipping stress not only results in decreased feeding and possible dehydration, but animals lack stable light:dark cycles during shipping. Moreover, some vendors have multiple animal colony sites distributed across the country in different time zones. Unless delivery from a specific colony site is requested by the customer, the vendor will fill orders from any of their colony sites depending on various factors such as overall census. Consequently, separate shipments of rats and mice from the same vendor can vary substantially in transit time (2-5 days) and light:dark cycle shifts, depending on the origination site of a particular animal shipment. By working with the vendor we are able to hold constant the vendor colony site from which our animals are shipped. We allow a minimum of one week for our animals to recover from shipping and acclimate to our animal housing conditions before the start of experimental procedures. Differential shipping-related stress may not only increase variability observed between different cohorts of animals, but it may also be a confounding factor if comparison groups of interest (e.g. sex or age) vary in their sensitivity to shipping. For example, stress associated with shipping of female adolescent mice can lead to long-term changes in neuroendocrine sensitivity (269). The season in which shipment occurs may also lead to notably different shipping experiences (extreme temperatures, shipping delays due to inclement weather, etc). Although many of these issues are seemingly beyond our control, we have on occasion delayed ordering rats due to impending weather concerns, as many shippers do not consider weather at the time of shipping. In one notable instance, we had a shipment of rats held up by a severe snowstorm and the rats were in transit under uncontrolled conditions for 5 days. We had to repeat the experiment at a later time due to aberrant

stress-related outcomes and unstable controls.

A related consideration is that mice and rats of a given strain can show considerable variation in stress reactivity and sensitivity depending upon the vendor from which they are bought (270). For instance, a recent study ((271)) systematically compared neuroendocrine and inflammatory responses in Sprague Dawley rats purchased from two different vendors widely used in the United States. Not only did HPA axis reactivity differ between rats from the two different vendors, but susceptibility to adjuvant-induced arthritis also differed. Although it is not clear whether these vendor-specific differences relate to unique rearing experiences, minor genetic drift, or differential shipping experiences, they underscore the notion that rodents of a given strain cannot be presumed to be identical across vendors. In our hands, we have observed similar differences in stress reactivity depending upon whether rats are purchased and shipped from a commercial vendor or bred on site for developmentally-themed experiments. Consequently, investigators should carefully consider sourcing of animals and how such choices might influence experimental outcomes.

Once animals are in the facility and acclimated, a second consideration is the use of enrichment devices, now required by most IACUCs. Of the many devices available for enrichment, we tend to avoid use of tunnels or tubes because they closely resemble devices used for restraint. For improved access to subjects and rapid acquisition of blood samples, it might be advantageous to avoid complicated climbing devices for enrichment. Finally, one must consider whether the availability of chew blocks in the home cage could be utilized by subjects as a coping measure (e.g. displacement behavior) that might facilitate stress recovery upon return to the home cage. To be clear, we are not arguing against the use of enrichment conditions to improve animal welfare. Instead, this should be viewed as a suggestion to thoughtfully consider what forms of enrichment are utilized, with a preference for enumerating the chosen conditions in the methods sections of published articles.

When it comes to final preparations for experimentation, several final points warrant discussion. First, we work closely with our Animal Care staff on scheduling of cage changes and daily tending of the room to ensure that cage changes/checks do not occur on the day of experimentation until after experimentation is complete. Otherwise, we find that ambient concentrations of stress hormones are typically elevated. Related to this, we routinely perform 2 days of pre-experimental handling, where each rat is handled briefly for 2-3 min on each of 2 consecutive days prior to experimentation. This allows us to conduct experiments with rats that are appropriately acclimated to human handling. In addition, one of those handling experiences allows us to capture baseline animal weights, and aids in careful monitoring of individual animal health. Here again, local traditions on pre-experimental handling can vary substantially across labs and we do not argue that our approach is the best or only choice. Instead, we simply suggest that pre-experimental handling should be both standardized across experiments, and procedures should be appropriately reported in methods sections. Finally, don't underestimate the potential confounding influence that noise and vibrations that emanate form nearby construction or building maintenance projects can have on HPA axis and other physiological measures (270,272,273).

5. CONCLUDING PERSPECTIVES

The vast scientific research literature examining glucocorticoid biological actions does not reflect a peculiar tunnel vision of scientists. Rather it reflects the remarkably widespread actions of glucocorticoid hormones and the diverse range of physiological processes affected by them. The information that is conveyed by this systemic hormonal signal – time of day and presence of environmental challenges — is critical for survival. So it is not surprising that virtually every cell and system of the body has evolved specific mechanisms to respond to the various aspects of this hormonal signal in a way that optimizes function. Similarly, cells have evolved numerous ways to capitalize on all aspects of CORT receptors' dynamically changing structure and intracellular location, rendering those receptors as premier examples of multifunctional intracellular proteins (i.e. "molecular swiss army knives").

The principles and practices outlined in this Users Guide have been instrumental in assisting scientists to determine the underlying mechanisms of glucocorticoid actions. A key prevailing finding of HPA axis research is that the physiology of endogenous glucocorticoid actions in the body is much more elaborate and complex than is revealed by strictly pharmacological studies of glucocorticoid actions. For example, whereas glucocorticoids are used extensively as pharmacological agents to suppress all aspects of the immune system, in vivo glucocorticoids modulate and fine tune immunity by promoting as well as constraining various components of immunity and inflammation (274-276). In the brain, endogenous glucocorticoids both facilitate and suppress memory processes and neurogenesis, depending on circulating hormones levels, recent and long-term secretion patterns and stimulus-response temporal relationships (277-280). Further understanding of HPA axis physiology (all elements of its control, adaptation, and effector hormone action) will be extremely valuable for the design and development of behavioral and pharmacological treatment strategies that will be of benefit for an extensive array of biomedical conditions.

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Table 1. Normal HPA axis hormone parameters in human and rat plasma.

		Basal Levels			
Species	**Hormone**	**Trough**	**Peak**	**Acute Stress**	**Plasma ½ Life**
Human	ACTH	5-15 pg/ml	10-50 pg/ml	40-80 pg/ml	19 min
Rat	ACTH	50 pg/ml	100 pg/ml	150-800 pg/ml	4 min
Human	Cortisol	4 µg/100 ml	16 µg/100 ml	20-35 µg/100 ml	49 min
Rat	Corticosterone	2 µg/100 ml	20 µg/100 ml	30-60 µg/100 ml	5-15 min

Sources: (4,82-84,107,108,152,173-176,227,247,281,282)

Table 2. Relative Potency/Affinity of some mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) agonists and antagonists.

Agonists	MR:GR Relative Potency	Notes
MR Agonist		
aldosterone	600:1	
fludrocortisone	12.5:1	Used clinically as mineralocorticoid
GR Agonist		
RU28362	1:800	
dexamethasone	1:25	Used clinically as glucocorticoid
MR/GR Agonist		
Cortisol/hydrocortisone	5:1	
corticosterone	5:1	
prednisolone	1:5	Used clinically as glucocorticoid
Antagonists	MR:GR Relative Affinity	
MR Antagonist		
spironolactone	25:1	Also blocks androgen receptor and stimulates progesterone receptor
eplerenone	>100:1	Less androgen and progesterone receptor cross reactivity than spironolactone, but also lower MR affinity
RU28318	?	Less androgen receptor cross reactivity than spironolactone
GR Antagonist		
RU486	1:200	Also blocks progesterone receptor; has partial GR agonist effects

Sources: (152,215,283-285)

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Highlights

- Glucocorticoid hormones dynamically regulate all mammalian physiological systems.
- Understanding glucocorticoid actions is important for biology and health research.
- The HPA axis controls circadian and stress-dependent glucocorticoid secretion.
- Strategies and best practices for study of HPA axis physiology are examined.