Negative feedback control over the hypothalamic-pituitary-adrenal axis response to stress is a function of temporal, spatial and physiological actions of corticosterone

by

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Abstract

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Negative feedback control over the hypothalamic-pituitary-adrenal axis response to stress is a function of temporal, spatial and physiological actions of corticosterone
Thesis directed by Professor Robert L. Spencer

Hypothalamic-pituitary-adrenal (HPA) axis activation leads a tightly coordinated cascade release of secretagogue pro-hormones that ultimately result in the production and secretion of glucocorticoids. Glucocorticoids in turn provide important regulatory control over the HPA axis. Stress-stimulated HPA axis hormone secretion requires alteration in paraventricular nucleus (PVN) signaling activity and recruitment of signaling proteins to mediate the excitatory coupling mechanism that lead to hormone release. These responses are mediated by the extracellular signal-regulated kinase (ERK). We examined if glucocorticoid regulation of HPA hormone output corresponded with alteration in the phosphorylated (active) form of ERK (pERK) within the PVN. Dephosphorylation of pERK within in peripheral cell types has been shown to be dependent on glucocorticoid up-regulation of the mitogen-activated protein kinase phosphatase-1 (*mkp-1*) gene. Thus, we also explored whether glucocorticoid regulation of the HPA axis was associated with PVN or anterior pituitary *mkp-1* gene upregulation. The last series of experiments in chapter 4 challenge the widely accepted model of glucocorticoid negative feedback. These experiments demonstrate a number of interesting novel characteristics about the interplay between stress, the HPA axis and glucocorticoids. First, stress-induced HPA axis activity has a differential hormone response which is determined by the timing of glucocorticoid exposure relative to the onset of stress. Second, stress can transiently evoke HPA axis resistance to the suppressive effects produced by glucocorticoid exposure during stress. The cumulative results of these studies demonstrate that negative feedback control of the HPA axis is functionally dependent on distinct temporal, spatial and physiological glucocorticoid actions.

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Thanks to my wife Lindsey for endless support, unwavering belief in my abilities and showing me love for who I am while still encouraging me to grow. Lastly I would like to dedicate this work to my father Dan Sundre whom I will remember not just by who he was but how he lived, respectfully and honestly. I am forever grateful to him for teaching me that joy can be found in even the most heart wrenching situations.

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Abbreviations

ADX	adrenalectomy
ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
BSA	bovine serum albumin
Dex	dexamethasone
CREB	cAMP response element binding protein
CRH	corticotropin-releasing-hormone
CORT	corticosterone
ERK	extracellular-regulated kinase
FLSD	Fisher's Least Significant Difference
GABA	g-aminobutric acid
GR	glucocorticoid receptor
GRE	glucocorticoid response element
IL	infralimbic
MAPK	mitogen-activated protein kinases
MKP-1	mitogen-activated kinase phosphatase 1
MR	mineralcorticoid receptor
nGRE	negative glucocorticoid response element
pERK	phosphorylated-extracellular-regulated kinase
PFC	prefrontal cortex
POMC	pro-opiomelanocortin
PrL	prelimbic

Chapter I

General Introduction

Exogenous glucocorticoid therapy and associated risks

Glucocorticoids (cortisol in humans and corticosterone in rats) are essential steroid hormones secreted by the adrenal cortex. Glucocorticoids regulate a variety of important immunologic processes (e.g. up-regulate anti-inflammatory proteins and down-regulate proinflammatory proteins), metabolic processes (increase blood glucose by several catabolic means), developmental processes (terminal maturation, axonal and dendritic remodeling and cell survival) as well as memory processes (acquisition and consolidation) [1-6]. Exogenous glucocorticoid related drug agonists are widely used in clinical application for the treatment of inflammatory and immunologically related diseases. Typically, high dose short-term glucocorticoid treatment is used to suppress acute and severe inflammatory reactions in emergency situations (e.g. allergic reactions, autoimmune disorders and asthma attacks); while long-term (low and high dose) glucocorticoid-suppressive-therapies are commonly used to treat adrenal insufficiency (inadequate adrenal production of cortisol) and autoimmune diseases such as uveitis (inflammation disorder of the eyes) [7,8].

Glucocorticoid therapy is accompanied by a host of clinical problems. General complications resulting from most glucocorticoid therapies include muscle weakness, reduced bone mass, latent diabetes mellitus, sodium retention, elevated arterial blood pressure, adverse psychiatric reactions and latent tuberculosis infection [7]. Long-term clinical use of glucocorticoids leads to the diminished ability to mount proper immune defenses in prevention of infections and disrupts reparative tissue processes [9]. Other common side effects of long-term (>2 weeks) glucocorticoid treatment are weight gain, systemic hypertension, dermatitis, myopathy, thinning of blood vessels and connective tissue, avascular necrosis of bones, ulceration in the gastrointestinal tract, and long-term psychiatric disturbances [10]. Furthermore,

glucocorticoid treatment increases the risk of glucose intolerance in normal adults, and leads to further collapse in glycemic control in diabetic patients [11]. Extensive long-term glucocorticoid treatment is considered a major risk factor associated with development and exacerbation of cardiovascular events by contributing to the development of hyperglycemia and hypertension [11,12]. Glucocorticoids have also been shown to have a dose dependent increase in the risk of heart failure [13]. High-dose and short-term (<7 days) glucocorticoid use in the prevention of high-altitude sickness has been shown to cause significant side effects (e.g. fatigue, depression, transient euphoria, irritability, lightheadedness, hyperglycemia, mania, delirium, insomnia, headache, leg cramps, and heartburn) [10]. Due to the morbidity associated with the widespread glucocorticoid therapies, further study, examination and targeted pharmacological development of glucocorticoids that produce beneficial treatment with corresponding decrease in adverse side effects is warranted and necessary.

Clinical implication of abnormal endogenous glucocorticoid activity

Abnormal secretion of glucocorticoids is a prime contributor to the onset and/or exacerbation of several physiologic and psychiatric disorders [14-18]. For example, hypercortisolemia (an abnormal increase in endogenous glucocorticoid activity) is characteristic of Cushing's disease, depressive episodes, bipolar disorder, panic disorder, schizophrenia, type II diabetes, hypertension, and obesity [19-21]. Severe depressive episodes are associated with impaired glucocorticoid (cortisol) negative feedback control of the HPA axis and significantly increased levels of cortisol [16]. Depressed patients display a disturbance in the circadian rhythm in adrenal cortisol secretion, typified by a consistently elevated and relatively flat cortisol secretion pattern [22]. Typically a single treatment with the glucocorticoid receptor agonist

dexamethasone (Dex) suppresses blood plasma cortisol concentrations for 24 hours, however, about 46% of depressed patients fail to respond to this treatment (i.e. Dex fails to prevent cortisol production over a 24 hour period) [23-25]. Furthermore, the decreased sensitivity to immediate glucocorticoid exposure exhibited by some depressed patients appears to be related to impairment at the hypothalamic neuronal anatomical sites that participate in control of glucocorticoid secretion [26]. Constantly high circulating levels of glucocorticoids have been shown to lead to hippocampal neuronal atrophy and a significant down regulation in brain derived neurotropic factor (BDNF) [19,27-29]. Interestingly, BDNF up-regulation and neurogenesis is somewhat restored by several antidepressants, suggesting that these clinical drugs reverse the atrophy of neurons in depressed patients [28].

On the other hand, hypocortisolemia (decreased glucocorticoid circulation or adrenocortical insufficiency) is characteristic of both mental disorders and systemic diseases such as PTSD, chronic fatigue syndrome, fibromyalgia (musculoskeletal pain), and chronic myogenous (facial pain). PTSD is typically associated with a distinctive profile relatively low plasma cortisol levels [18,30-32]. PTSD patients also display increased and prolonged glucocorticoid receptor responsiveness to Dex treatment, which can result in enhanced negative feedback control of the HPA axis [18,31]. Interestingly, PTSD is also associated with greater sympathetic nervous system arousal as reflected by catecholamine levels [33]. Preexisting high glucocorticoid receptor (GR) number in leukocytes before combat deployment of soldiers has been shown to be a predictive and vulnerability factor for subsequent PTSD onset [34]. However, it is unknown whether and how the observed higher pre-deployment GR number is involved in the pathophysiology of PTSD. Addison's disease is a rare and idiopathic chronic

endocrine disorder in which the adrenal glands do not produce sufficient glucocorticoids. It is often accompanied by fatigue, suppressed appetite, and hypotensive states. Since individuals with Addison's disease have deficient adrenal glucocorticoid production, they tend to be unable to react appropriately to the homeostatic challenges which are characteristic of addisonian crises [35]. This condition is also marked by sudden back and leg pain, low blood pressure, vomiting, and unconsciousness. It is increasingly evident that the circulating concentration of glucocorticoids must be tightly regulated when considering the pathological conditions associated with hypercortisolemia and hypocortisolemia as well as the mobility linked to glucocorticoid therapies.

Stress (a homeostatic game changer) and glucocorticoids

Endocrinologist Hans Selye originally used the term "general adaptation syndrome" to describe and characterize physiologic changes that were common to a wide variety of noxious stimuli [36]. Selye noted that initial responses to these stimuli would evoke activation of several physiologic systems, while persistent noxious conditions would eventually result in dysfunction of these systems accompanied by pathological changes (e.g. weight loss, immune suppression, and gastrointestinal ulcers etc.). The changes associated with general adaptation syndrome were eventually described as "stress" and the evoking stimuli known as "stressors." Selye proposed that the general adaptation syndrome involved two major systems of the body, the nervous system and the endocrine system. Today we know that the stress response is a multi-systemic reaction caused by the perception (psychological) or detection (physiological) of aversive or threatening stimuli. Stress evokes a comprehensive set of stress-sensitive neurological and endocrine systems that lead to activation of the sympathoadrenal medullary system and the

adrenocortical system (a.k.a hypothalamus-pituitary-adrenal axis) [37-40]. The coordinated activation of these stress response systems culminate in the release of their associated hormones, norepinephrine, epinephrine and glucocorticoids.

The hypothalamus-pituitary-adrenal (HPA) axis is the primary neuroendocrine system that mediates the secretion of glucocorticoids in response to stress. Glucocorticoids influence virtually every tissue and cell type in the peripheral and central nervous system and thus have extensive influence on all physiological, neurological, and endocrine systems [41,42]. Therefore, is not unexpected or surprising that the adverse effects of stress on health that stem from impaired regulation of the HPA axis by abnormal glucocorticoid activity is a major clinical issue. Consequently, a thorough understanding of the necessary conditions and mechanisms through which glucocorticoid activity regulates HPA response to stress is necessary. An important aspect of glucocorticoid activity is its ability to regulate the HPA axis [3,37,38,43-45]. However, the interplay between stress, the HPA axis, and glucocorticoids is complex.

The anatomical organization and secretagogue cascade of the HPA axis

To appreciate the importance of glucocorticoid negative feedback regulation of stress-stimulated HPA axis activity it is necessary to understand the functional neuronal and cellular organization of the system responsible for the physiological release of glucocorticoids. Neuroendocrine corticotropin-releasing-hormone (CRH) neurons located in the medial hypothalamic region of the paraventricular nucleus (PVN) (figure 1.1) release CRH through the same conventional stimulus-dependent mechanisms employed by all neurons [46,47]. The release of CRH requires alteration of membrane potentials which leads to initiation of action potentials and subsequent release of hormones [47]. Changes in stress-dependent gene induction,

membrane excitability and peptide release is mediated by an astonishing number of intracellular signaling molecules (e.g. PKA, PKC, MAPK, CaMKII, etc.). Stimulation of CRH neurons such as that from stress responsive inputs or light sensitive suprachiasmatic nucleus inputs, leads to the release of CRH peptide into the hypophyseal portal vasculature system located in the median eminence (inferior hypothalamus). This vasculature system transports CRH into the anterior pituitary. Once in the anterior pituitary, CRH activates endocrine corticotroph cells (figure 1.1), which leads to secretion of adrenocorticotropic hormone (ACTH) into the general circulatory system via the adenohypophysis. Stress leads to a rapid increase and somewhat sustained secretion of ACTH, which has an extremely high clearance rate (~3-4 minute half-life) [48]. Systemic circulation of ACTH ultimately causes the activation of the adrenal cortex to produce and secrete glucocorticoids (corticosterone or CORT). One of the most dynamic characteristics of CORT is its negative feedback control of the HPA axis (figure 1.1). CORT regulates HPA axis through direct negative feedback action at the PVN, anterior pituitary, and the excitatory neural inputs (prefrontal cortex, hippocampus and amygdala) that converge onto the PVN (figure 1.1). CORT negative feedback regulation of the HPA axis is functionally dependent on several distinct CORT secretion profiles [49,50].

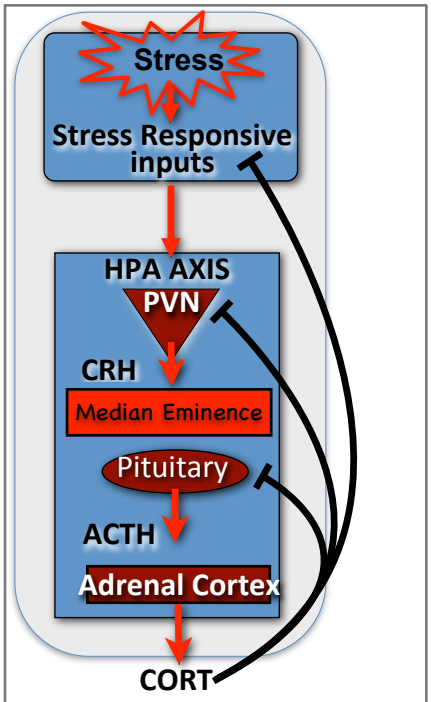

Figure 1.1 Components of HPA axis.

Physiological secretion patterns of CORT and impact on HPA axis functionality

CORT is secreted in several different physiological patterns, i.e. ultradian, tonic, and phasic. Ultradian CORT is secreted in a rhythmic, pulsatile fashion and is important in maintaining a 'background' responsiveness of various physiological systems involving corticosteroid receptors [51], however, we are primarily concerned with tonic and phasic CORT secretion patterns. The importance of tonic and phasic regulation of CORT over HPA function was established almost five decades ago [45,52-55]. These pioneering researchers clearly demonstrated that both tonic and phasic glucocorticoids modulate the HPA axis response to stress. Tonic CORT actions can be generally attributed to the effects that stem from the presence of basal levels of circulating CORT (figure 1.2). Tonic CORT secretion pattern is dependent on suprachiasmatic nucleus (SCN) control of the PVN which produces a circadian circulation pattern [45]. Tonic CORT secretion reaches its peak at the onset of the organism's active period and steadily declines as this stage progresses toward its trough, which is near the onset of sleep (inactive period). Most importantly, tonic CORT produce a proactive and constant modulation over HPA axis hormone secretion [44].

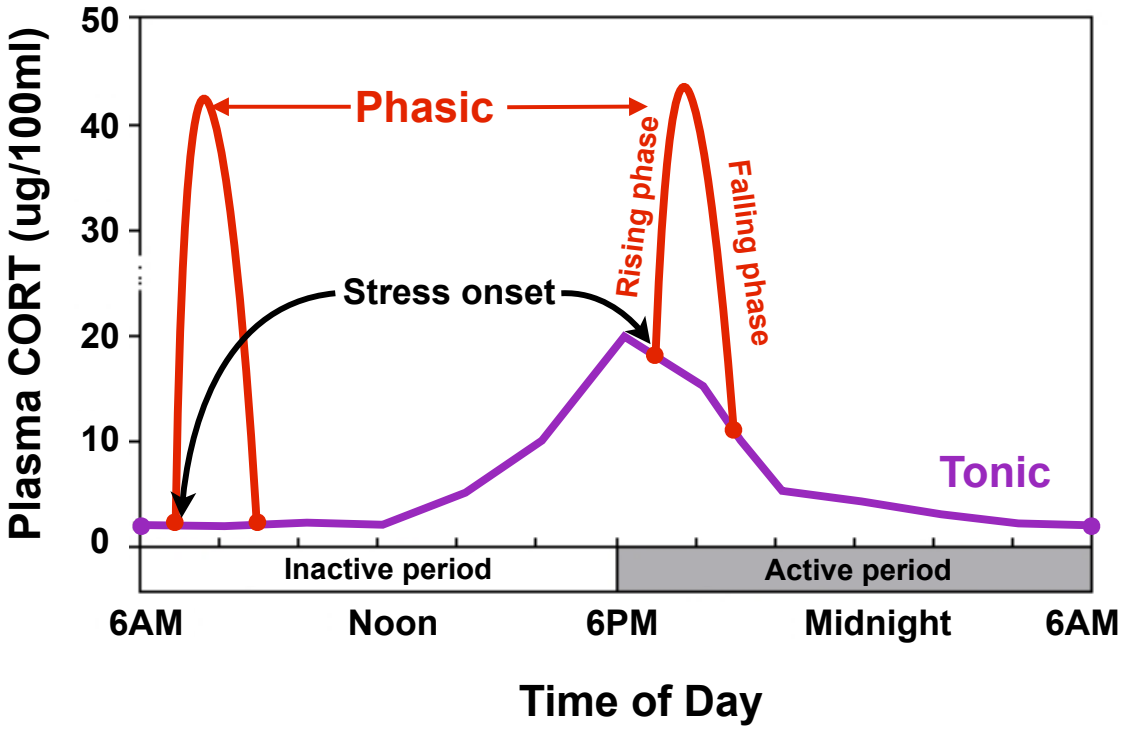

Figure 1.2 Idealized CORT secretion pattern in the rat.
Normal physiological secretion profile of endogenous tonic (purple) CORT and phasic CORT (red). Stress evokes phasic CORT secretion typified by a transient increase in CORT. Tonic is controlled by the SCN, which produces a circadian pattern.

Typically, tonic CORT negative feedback effects have been studied by adrenalectomy (ADX) combined with various replacement regimens of exogenous tonic CORT [56-58]. Removing the adrenal glands results in an augmentation of basal and stimulated HPA axis gene expression, protein activity, and hormone secretion [56-60]. Alternatively, pharmacological blockade with metyrapone (inhibits CORT production) or glucocorticoid receptors (GR) antagonist treatment, similarly produce an amplification in the ACTH response to stress [61-63]. These studies indicate that endogenous diurnal tonic CORT activity functionally constrains HPA axis activity.

Phasic CORT actions (sometimes referred to as *suppressive* or *reactive* actions) can be attributed to the effects of a rapid and transient increases in CORT, such as that elicited by an acute exposure to stress (figure 1.2). Stress-stimulated phasic increases in endogenous CORT begin within 3-5 minutes of stress onset [64] and quickly produce a greater concentration of CORT, regardless of the diurnal state of tonic activity. The rate of increase in endogenous phasic CORT secretion is transient [49]. Both acute and chronic stress states potently stimulate CORT secretion [65,66]. Phasic CORT actions produce a suppressive modulation over HPA axis activity [44,67,68]. Unlike tonic CORT activity which constrains the magnitude of the HPA axis reactivity to stress, phasic CORT has been shown to substantially suppress the subsequent stress-stimulated HPA axis activity.

The widely accepted model of phasic glucocorticoid negative feedback

The first detailed and widely accepted model of phasic glucocorticoid negative feedback function was proposed by Dallman and Yates [45,52-55] in the early 1960's. This model suggested that glucocorticoids exert multiple cellular effects that vary in their time course and

mechanism. Typically, to study phasic glucocorticoid negative feedback effects researchers pretreated subjects with an exogenous dose of CORT prior to HPA axis stimulation [45,49,52,67-72]. This particular pretreatment experimental paradigm has churned out a number of important characterizations about phasic glucocorticoid effects. Dallman and colleagues were the first researchers that contributed a significant amount of evidence indicating that negative feedback actions of glucocorticoids basically operate in categorically discrete timeframes (temporal periods).

The first temporal period is regarded as fast negative feedback, which has a rapid induction phase and a transient duration phase that is followed by an inactive period. Dallman and Yates believed that fast feedback was followed by an inactive period because this fast glucocorticoid effect only persisted for several minutes or as long as there was a significant increase in the concentration of glucocorticoids [45,49]. Others have also shown that fast glucocorticoid inhibition of ACTH release only occurred during the brief rising phase of CORT secretion as opposed to when the magnitude of CORT concentrations leveled (within minutes) or began to clear (falling phase), suggesting that fast feedback is rate-sensitive [73,74]. Fast glucocorticoid actions depend on non-genomic cellular alterations (protein-protein interactions), which are responsible for the immediate suppressive action over stimulated HPA axis activity [72,75-78]. Fast negative feedback does not require altered gene transcription and *de novo* protein synthesis; it depends on glucocorticoid receptor (GR) interactions with other signaling proteins that result in alterations in functional cellular responses within PVN neurons [72,75-78] as well as within the anterior pituitary [79].

Intermediate glucocorticoid negative feedback has a delayed induction phase (~1 hour), is dependent on high concentration of glucocorticoids and occurs when glucocorticoid levels are decreasing or have completely returned to basal levels [3]. Intermediate negative feedback effects provide suppressive action over stimulated HPA axis activity that can persist for hours [45,67,69]. I have shown in a recent *in vivo* study that the underlying mechanisms of intermediate CORT negative feedback control of HPA hormone response to restraint stress depends on CORT-induced *de novo* protein synthesis [67]. Certain *in vitro* studies support a protein synthesis-dependent glucocorticoid inhibitory effect on corticotroph function within a similar timeframe [80-83]. Some studies also suggest that glucocorticoid suppression of stimulated ACTH secretion depends on glucocorticoid induction of a protein that reduces membrane depolarization in corticotrophs [84,85]. Additionally, some genes have been implicated in glucocorticoid negative feedback, such as the calmodulin gene and various phosphatase and potassium-channel genes [86-89]. The identity of glucocorticoid induced protein(s) responsible for intermediate negative feedback remains inconclusive [90].

Fast and intermediate negative feedback are separated not only by their time-course and mechanism, but also by an inactivate period, respectively. As mentioned previously, fast glucocorticoid negative feedback is thought to be followed by an inactive period. The initial stress literature from the early 1960s-1980s provided some evidence that fast (<10 minutes) and intermediate (>1 hour) glucocorticoid negative feedback are separated by a strict timeframe (~45 minute) in which glucocorticoid administration fails to produce a functional negative feedback effect over stimulated HPA hormone secretion [45,49,52,91]. These early studies documented a glucocorticoid negative feedback "silent period." This notion was further supported by the

observation that fast glucocorticoid negative feedback is transient (i.e. rate-dependent or rate-sensitive) and intermediate negative feedback requires time to manifest (i.e. *de novo* protein synthesis dependent).

Regardless of ingenuity and fundamental groundwork describing the phenomenon of temporal phasic glucocorticoid negative-feedback that was detailed over 30 years ago, several problems have limited the relevance and application of those initial studies. For instance, many of those studies were not carried out under optimal experimental conditions. In many cases animals were anesthetized, and/or ADX, while a wide range of physiological and psychological stressors were used. Furthermore, a number of the initial studies that set the foundation detailing temporal glucocorticoid restrictions (silent period and rate-sensitivity) and requirements (induction and duration phases) of negative feedback were conceptually based on studies that relied on indirect hormone measurements and assays that typically produced variable and unreliable results [45,49,52]. Additionally, most of the phasic glucocorticoid research (current and classic) has focused on isolating the effects of glucocorticoids from the producing stressor through pretreatment paradigms. This particular focus has produced insufficient experimental examination and characterization of phasic glucocorticoid negative feedback effects during stress (i.e. when a phasic increase in CORT occurs).

Intrinsic spatial effects of glucocorticoids as indicated by stress-dependent gene expression

The HPA axis is significantly reactive to stress, i.e. increase in hormone release (CRH, ACTH and CORT). Additionally, the application of CORT typically produces a general suppression over HPA axis hormone output. However, measuring HPA hormone activity only communicates what state (activated or suppressed) the HPA axis is in. Since stress and the

suppressive actions of phasic CORT produce distinct HPA cellular alterations that involve changes in gene expression and hormone release we can compare functional HPA hormone output to experience-dependent gene expression within hypothalamic PVN neuroendocrine neurons to make inferences about the anatomical location of CORT negative feedback [3,67,68,72,92].

We routinely use experience-dependent reporter genes (e.g. *c-fos* mRNA and *crh* hnRNA) to study the intercellular and intracellular effects of CORT negative feedback. First, the magnitude of PVN experience-dependent gene induction (e.g. *c-fos*) is significantly correlated with the magnitude of the HPA axis ACTH and CORT response to different psychological stressors (e.g. tub, arena or restraint) [93]. As stress escalates a corresponding increase in a both *c-fos* gene induction and HPA hormone output occurs. Second, experience-dependent PVN and anterior pituitary genes (e.g. *c-fos*, *crh*, *pomc* and *zif268* etc.) are also differently responsive to fast and intermediate temporal glucocorticoid treatments [67,69]. Experience-dependent genes like *c-fos*, *crh* and *pomc* can be used as reporters about specific anatomical sites of stress activation and CORT negative feedback.

Glucocorticoids potently suppress stimulated HPA activity by acting on PVN CRH neurons. For example, phasic glucocorticoid negative feedback is evident within minutes when exogenous glucocorticoids are administered directly into the PVN (fast negative feedback) or in ~1 hour or more (intermediate negative feedback) prior to restraint challenge [68,72,94]. We recently determined that phasic CORT pretreatment (1 hour) administered both systemically and via PVN microinfusion inhibited subsequent stress-stimulated ACTH release and stress-induced PVN *crh* gene expression and corticotroph *pomc* hnRNA levels, but not stress-induced PVN *c-*

fos gene expression [67,68]. This pattern of intermediate phasic CORT-induced inhibition indicates that CORT can produce independent suppressive actions at the level of the PVN. This intermediate negative feedback action appears to alter stress-stimulated excitatory coupling mechanisms responsible for PVN CRH release and *crh* gene induction, without altering stress-associated excitatory inputs to the PVN and intracellular signaling proteins that are responsible for PVN *c-fos* gene induction (figure 1.3, compartment 2 and/or 3). Moreover, immediate early genes do not contain a known glucocorticoid response element, suggesting that glucocorticoids indirectly modulate these genes via alteration in stress responsive inputs and/or intracellular singling process that lead to induction of the *c-fos* gene.

Others have shown that fast (within several minutes) negative feedback is at least in part mediated by glucocorticoid-induced cannabinoid activity that subsequently reduces presynaptic glutamate stimulation of PVN CRH neurons, which alters the stress-stimulated inputs to the PVN (figure 1.3, compartment 1) [72,75,76,95]. However, these studies do not rule out fast CORT suppression of other intracellular PVN signaling pathways coupled to the excitatory mechanism responsible for CRH hormone release (figure 1.3, compartment 2 and 3) or similar suppressive effects in the anterior pituitary. Collectively, observations from intermediate and fast negative feedback studies indicate that the PVN is sensitive to different temporal phasic CORT profiles, and in response produces a PVN CRH output (increase or decrease) that matches the demands of homeostatic challenges that is reflected at the molecular (gene expression) and systems level (ACTH and CORT secretion).

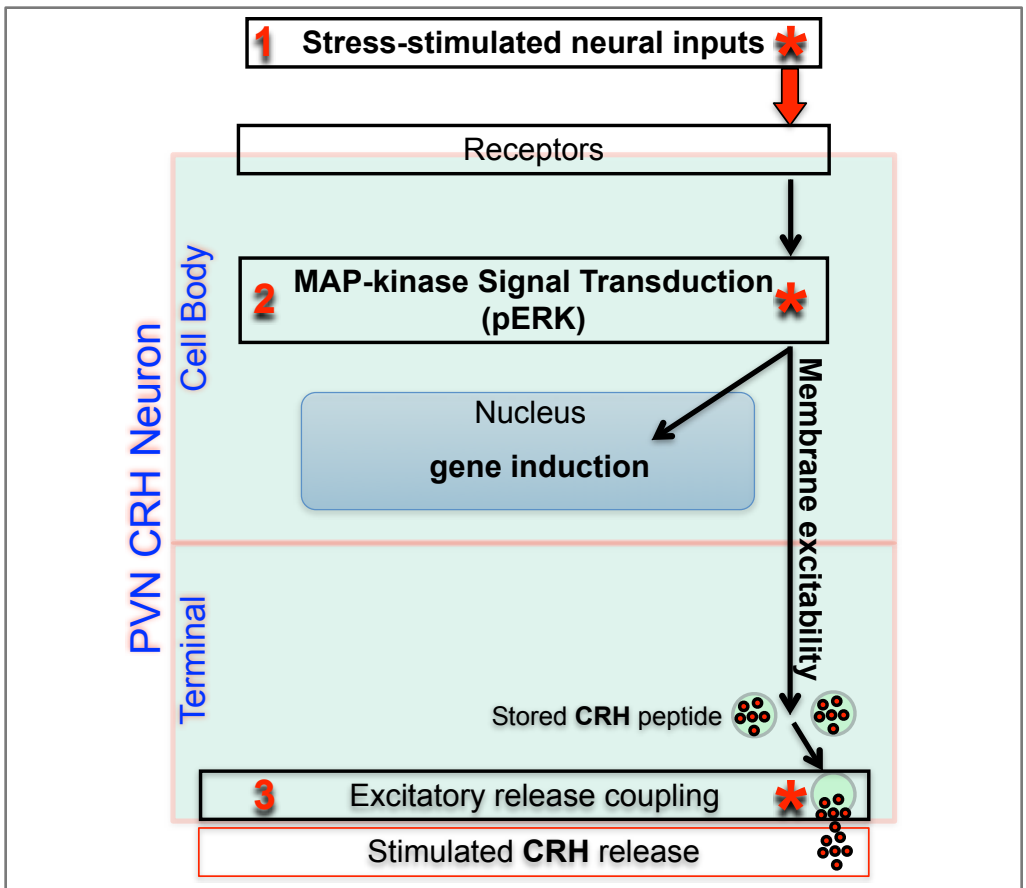

Figure 1.3 Conceptual framework of PVN CRH neuron.
PVN neuronal processes induced by stress (excitatory coupling of gene induction and exocytosis of CRH peptide). Both of these PVN CRH processes are dependent on signaling proteins. Red numbers (1-3) indicate extracellular or intracellular location for direct CORT negative feedback actions to occur (Adapted from Watts et al. 2005).

Glucocorticoids also potently suppress stimulated HPA activity by acting on the anterior pituitary. Using transformed corticotroph cell lines (AtT20 cells), the Shipston group has shown that within 30 minutes glucocorticoids induce a protein that interferes with CRH-stimulated ACTH secretion by altering activity of a large conductance calcium-activated potassium channel [86,96]. Also fast (within minutes) glucocorticoid suppression of stimulated HPA axis hormone activity depends on direct actions within the anterior pituitary. This temporal glucocorticoid negative feedback effect occurs regardless of transcriptional inhibition indicating it is non-genomic [79].

Our lab has also demonstrated that glucocorticoids produce time dependent alterations in stress-induced pro-opiomelanocortin (*pomc*) hnRNA, *c-fos* mRNA and *zif268* mRNA in the anterior pituitary [67,69]. These studies indicate that stress-induced increases in anterior pituitary *pomc* hnRNA are completely blunted by a 10 minute, 1 and 3 hour glucocorticoid pretreatment. However, the 10 minute glucocorticoid pretreatment did not suppress stress-dependent increases in *c-fos* and *zif268* gene induction. These temporal observations reflect direct glucocorticoid suppression of *pomc* gene transcription and possibly not a decrease in stress-stimulated CRH activation of the corticotrophs 10 minutes after treatment. A 3 hour CORT pretreatment is able to suppress stress-induced *c-fos* and *zif268* gene induction in the anterior pituitary, which reflects a time-dependent CORT induced decrease in PVN CRH release (figure 1.3 compartment 1-3). In support of these observations, the *c-fos* gene and other immediately early genes in the HPA axis are not known to have a GRE, thus are not transcriptionally regulated by glucocorticoids. Collectively, these studies [67-70] strongly indicate that each phasic glucocorticoid condition

(fast or intermediate) produces inhibitory effects on stress-induced gene expression that varies for each gene depending on the HPA axis anatomical site (PVN and anterior pituitary).

Stress-dependent signaling activity

There are a number of intracellular signaling proteins that participate in both stress-induced gene induction as well as stress-induced excitation-exocytosis coupling mechanism associated with PVN hormone release. The following signaling proteins have been implicated in these stress-induced PVN cellular processes (gene induction and/or hormone release): extracellular signaling-regulated kinase (ERK), cAMP-dependent protein kinase (PKA), and calcium/ calmodulin-dependent protein kinases II (CaMKII). PKA leads to the induction of *crh* gene transcription [97] and has been implicated in CRH peptide release in hypothalamic PVN explants [98]. Additionally, PKA triggers calcium-independent and CaMKII-dependent release of other HPA axis hormones [97,99]. PVN CaMKII activity is also differentially responsive to acute and repeated restraint exposure and is important for regulating calcium signaling in synaptic transmission by phosphorylating various proteins [100]. Both PKA and CaMKII may partially mediate stimulated intracellular responses that lead to both gene induction and hormone release within the HPA axis.

ERK is downstream protein member within the mitogen-activated protein kinases (MAPK) family. The active (phosphorylated) form of ERK (pERK) mediates a number of important cellular processes such as cellular proliferation, differentiation, survival, and cell-death [101]. Within the PVN, pERK can activate the transcription factor cAMP response element binding protein (CREB), which promotes *crh* gene induction [102,103]. Moreover, pERK indirectly participates in the induction of several experience-dependent genes within the HPA axis (e.g.

pomc, *zif268* and *c-fos*) [104-106]. Importantly, recent evidence shows that inhibition of ERK phosphorylation reduces the firing rate of CRH neurons in response to norepinephrine indicating that pERK is an important intracellular mediator of stress-stimulated alterations of CRH neuronal responses that lead to excitatory neurohormone release [47]. These numerous characteristics make pERK an ideal experience-dependent intracellular marker of recent neuronal PVN activation [107]. Given the extensive role pERK plays in the PVN, it is likely that the suppressive actions of CORT activity may also produce distinct alterations in stress-induced pERK activity. Consequently, the changes in PVN pERK immunoreactivity observed in chapter 2 provided specific information about the stress-induced state of the PVN and about the suppressive actions of CORT (e.g. by localizing those effects to intracellular actions specific to signaling activity, figure 1.3, compartment 1,2 and/or 3).

MKP-1 regulation of pERK

Mitogen-activated kinase phosphatase 1 (MKP-1) is part of a larger family of phosphatases that dephosphorylate MAP kinases. Importantly, MKP-1 regulates ERK activity by binding to pERK causing a conformational change in MKP-1, which then allows the MKP-1 protein to remove the phosphate groups on pERK rendering it inactive [108]. The two proteins then lose binding affinity and separate, whereby MKP-1 is then rapidly degraded. Interestingly, glucocorticoids inhibit pERK by upregulating *mkp-1* gene expression and decreasing MKP-1 protein degradation in fibroblast cells [87,109]. Likewise, glucocorticoids were also shown to induce *mkp-1* gene expression and dephosphorylate pERK in osteoblast cells [88], also mediating anti-inflammatory effects in immune cells by directly enhancing the induction of *mkp-1* gene [110,111]. These results suggest that CORT may up-regulate MKP-1 expression in

the cellular elements of the HPA axis and thereby reduce the levels and/or duration of stress-induced ERK activation, thus limiting ERK's ability to contribute to CRH release and production of new neurohormone peptide (figure 1.3 compartment 2 and 3). We examined this particular hypothesis in chapter 3.

Stress-dependent prolactin release—another important secretagogue

In conjunction with pairing HPA hormone output with changes in gene expression, we have also used stress stimulated prolactin secretion to examine possible anatomical locations where CORT exerts its negative feedback effects. Prolactin is a protein hormone secreted from lactotroph cells of the anterior pituitary most commonly known for lactogenesis [112]. Apart from lactogenesis, prolactin is also implicated in the maintenance of water and electrolyte homeostasis, metabolism, osmoregulation, and the promotion of growth in both females and males [113,114]. Prolactin release is controlled by an extra-HPA axis set of stress-reactive hypothalamic neuroendocrine neurons found in the arcuate nucleus. Interestingly, psychological stress increases prolactin secretion in both female and male rats [115-118].

We have recently shown that restraint increased prolactin secretion in male rats was unaffected by a 1 hour micro-infusion of CORT to the PVN, but the characteristic suppression over ACTH secretion was still observed [68]. This observation indicate that stress-stimulated lactotroph prolactin secretion is not dependent on stress-stimulated HPA axis activity. This further indicates that intermediate CORT actions do not appear to regulate extrinsic stress reactive systems as a whole, rather have independent suppressive action at the level of the PVN (i.e. regulate PVN CRH release, figure 1.3). By fundamentally comparing HPA hormone output with hypothalamic PVN and anterior pituitary experience-dependent gene expression as well as

other stress-simulated hormones like prolactin we can make further inferences about the anatomical cellular location of CORT negative feedback actions.

The corticosteroid receptors that mediate glucocorticoid actions

Glucocorticoid actions are mediated by two different corticosteroid receptors. CORT binds two closely related receptors, the mineralcorticoid receptor (MR) and the glucocorticoid receptor (GR). Although CORT readily binds to both MR and GR, it has a greater affinity for the MR [3]. Both corticosteroid receptors can produce markedly different physiological actions. For example, MR is activated in the absence of stress (primarily by tonic CORT) and is important for maintaining homeostatic function [38,44,119]. In contrast, GR is primarily activated during the exposure to stress and during diurnal peaks [3]. GR facilitates suppression of and/or recovery from the stress response [38,71,119]. It is important to note that while GRs and MRs are widely distributed throughout the central nervous system and periphery, the PVN does not express MR and pituitary has a notably high MR expression [3,120-122].

By convention, corticosteroid receptors are found in the cytoplasm and act as hormone-dependent transcription factors when bound to their associated ligands. GRs occupy the cytosol and MRs are populated in both the cytosol and nucleus [123]. When unbound both MRs and GRs are sequestered in a multi-protein complex which dissociates when these corticosteroid receptors are activated by glucocorticoids. Upon ligand binding and protein complex dissociation, a nuclear localization signal is exposed on MR/GR and the CORT bound receptor subsequently translocates to the nucleus [124]. Glucocorticoid activated corticosteroid receptors can assemble into homodimers or heterodimers and bind to glucocorticoid response elements in the promoter region of certain target genes and promote (upregulate) or inhibit (down-regulate) gene

transcription [125-128]. In addition to directly altering gene transcription, GR and MR can associate with other transcription factors (e.g. FOS or CREB) or transcription factor ligands (e.g. CREB binding protein) to regulate gene transcription indirectly [103,129]. We have recently shown that intermediate negative feedback is dependent on CORT-dependent *de novo* protein synthesis, however, what gene(s) and which corticosteroid receptor (GR or MR) are responsible for this protein induction remains to be determined.

There is some disagreement over the receptor type and/or cellular location that mediates fast glucocorticoid negative feedback signaling. Since fast feedback does not require altered gene transcription or *de novo* protein synthesis, we can rule out classic transcriptional corticosteroid receptor actions that are characteristic of intermediate negative feedback [49,79,80,130]. Tasker and colleagues used hypothalamic slices to demonstrate that fast glucocorticoid negative feedback actions are not blocked by GR antagonists (RU486), concluding that these actions are mediated by a novel membrane G-protein receptor and not classic GRs [78]. Ginsberg and Dallman showed that RU486 given to rats minutes prior to stress onset augments stress-stimulated ACTH release [71]. However, this RU486 augmentation of ACTH was transient and failed to influence subsequent stress-stimulated CORT activity. Together, these studies indicate that RU486 may be ineffective at blocking GR activity or have ineffective antagonistic effect over GRs. Interestingly, GR knockout mice are unable to produce a functional fast glucocorticoid negative feedback effect [76], indicating that fast glucocorticoid suppression is mediated by GRs. Furthermore, fast feedback is produced by pretreatment with synthetic selective GR agonists (e.g.RU28362 and dexamethasone or Dex), clearly demonstrating a role for GRs in mediating fast negative feedback effects prior to the on set of stress [71,72].

The Herman group used conjugated Dex-bovine serum albumin (which renders Dex impermeable to cell membranes) to demonstrate that fast glucocorticoid inhibition of stress-stimulated ACTH secretion is dependent on membrane-associated GRs [72]. However, it is important to note that BSA-Dex and BSA-CORT can dissociate from the BSA molecule. When dissociated from BSA, free Dex or CORT molecules can pass through the plasma membrane and activate intracellular GRs [1]. Also, both Dex-BSA and CORT-BSA have lower binding effectiveness compared to free dissociated glucocorticoids like CORT and cortisol (i.e. Conjagated forms of Dex and CORT have altered affinities for the GRs) [131]. Thus, any interpretations from studies using steroids conjugated to BSA are problematic.

Others have shown that glucocorticoids exert fast negative feedback at the pituitary level through a mechanism that is independent of GRs [79]. Some evidence has also indicated that GR activity in the forebrain suppresses HPA hormone secretion during stress, however, these studies failed to demonstrate that GR activity in the PVN or pituitary does not also independently provide suppressive actions as well [132,133]. Clearly, more research is needed to determine the receptor type and location involved in fast glucocorticoid negative feedback.

Experimental studies Chapters 2-4

Glucocorticoid research conducted over the last 4 decades has provided clear evidence that the negative feedback effects of glucocorticoids operate through a complex set of categorically distinct physiological (secretion patterns), spatial (inter- and intra- cellular effects) and temporal (phasic and tonic) actions. Chapter 2-5 demonstrate that the collective status of these three glucocorticoid actions (physiological, spatial and temporal) determine how the HPA axis responds to stress [46]. Glucocorticoids act directly at the hypothalamic anatomical level of the

HPA axis to suppresses both neurohormone secretion and gene induction (figure 1.3, compartment 2 and 3) [68,134]. We also know that stress-stimulated HPA axis neurohormone secretion and gene induction are facilitated by changes in protein signaling activity, such as those seen in pERK. However, it is unclear whether tonic or phasic CORT has suppressive action over signal transduction events within PVN CRH neurons and if these suppressive actions correspond with alterations in stress-induced PVN transcriptional processes and neurohormone secretion. Thus chapter 2 illustrates a series of experiments that examined the ability of tonic and phasic CORT to modulate levels of the active (phosphorylated) form of ERK (pERK1/2) in the PVN of rats during stress. The results of these experiments give specific support as to the anatomical location of where both tonic and phasic CORT act at to regulate stress-stimulated HPA axis activity and further illustrate that the responsiveness of the HPA axis to stress is modulated by tonic CORT activity.

Dephosphorylation of pERK within in peripheral cell types has been shown to be dependent on glucocorticoid up-regulation of the *mkp-1* gene. It is unclear whether glucocorticoid induction of the *mkp-1* gene may serve as a regulatory mechanism for stress-stimulated increase in pERK within the neuroendocrine cell populations of the HPA axis. Chapter 3 shows a series of experiments that examined the ability of tonic and phasic CORT to modulate *mkp-1* mRNA levels within the intrinsic cellular elements of the HPA axis and within extrinsic medial prefrontal cortex brain regions that facilitate stress responses. The results suggested that stress-induced *mkp-1* mRNA expression likely plays a role in stress-dependent regulation of MAP-kinases in both extrinsic and intrinsic anatomical sites to the HPA axis.

Chapter 4 considers several basic experimental issues that surround the innovative *in vitro* and *in vivo* glucocorticoid studies that originally characterized temporal phasic glucocorticoid effects. As mentioned, many of those early studies were conducted under less than optimal experimental conditions while also employing an extremely wide range of stressors. Moreover, most of those studies relied on indirect measures of hormone levels that produced results that were inconsistent within and between studies. Also, over the last 30-40 years there has been limited replication and reexamination of some of these temporal glucocorticoid phenomena, particularly under similar experimental conditions (e.g. same stressor and glucocorticoid treatment). Most of the current and classic phasic glucocorticoid research has focused on isolating the effects of phasic glucocorticoids from the producing stressor through pretreatment paradigms, resulting in insufficient documentation and examination of negative feedback glucocorticoid effects that take place during stress. For these reasons the series of experiments in chapter 4 reexamine the temporal requirements and restrictions of intermediate and fast glucocorticoid negative feedback control over the HPA axis initial and ongoing hormone response to stress by exposing rats to phasic CORT before or after stress onset. The results from these experiments clearly illustrate that the HPA axis differentially responds to whether phasic CORT exposure is before or after stress onset. The following chapters will also provide further experience-dependent gene expression when relevant to locate possible spatial intrinsic and extrinsic HPA anatomical sites of phasic and tonic CORT negative feedback.

Chapter II

Tonic, but not phasic corticosterone constrains stress activated extracellular regulated-kinase 1/2 immunoreactivity within the hypothalamic paraventricular nucleus

Abstract

The negative feedback actions of corticosterone (CORT) depend on both phasic and tonic CORT secretion patterns to regulate hypothalamic pituitary adrenal (HPA) axis activity. How these two different CORT secretion pattens influence specific intracellular signal transduction pathway activity within the cellular elements of the HPA axis has not been determined. For example, it is unknown whether CORT has suppressive actions over signal transduction events within medial parvocellular paraventricular nucleus (PVN) corticotropin-releasing hormone (CRH) neurons and if these suppressive actions are responsible for alterations in PVN transcriptional processes and neurohormone secretion associated with stress. The extracellular-regulated kinase (ERK) is a stress activated intracellular signaling molecule that is potentially subject to glucocorticoid negative feedback regulation. We tested the ability of CORT to modulate levels of the active (phosphorylated) form of ERK (pERK1/2) in the PVN of rats. Acute psychological stress (restraint) produced a rapid increase in the number of PVN pERK1/2 immunopositive cells within CRH neurons. Absence of tonic CORT via adrenalectomy (ADX) produced no change in basal pERK1/2 cell counts, but augmented the increased pERK1/2 cell counts elicited by acute restraint. Treatment of ADX rats with CORT in the drinking water normalized this enhanced pERK1/2 response to stress. In contrast, treatment of ADX rats with a phasic increase in CORT 1 hour prior to restraint had no effect on pERK1/2 cell counts, despite substantially suppressing stress-induced PVN *crh* gene expression and ACTH secretion. This tonic CORT inhibition of stress-induced activation of ERK1/2 may involve both alteration of the activity of stress-dependent neural inputs to PVN CRH neurons and alteration within those neurons of stress-dependent intracellular signaling mechanisms associated with ERK activation.

Introduction

Glucocorticoids potently suppress stress-induced hypothalamic-pituitary-adrenal (HPA) axis activity (negative feedback) as measured by ACTH secretion [49]. Both phasic and tonic glucocorticoid secretion patterns contribute to the regulation of basal and stimulated HPA-axis activity [44,45]. Tonic glucocorticoid actions (sometimes referred to as permissive actions) generally refer to effects from the long-term presence (hours to days) of basal circulating glucocorticoids. Tonic glucocorticoid actions produce a somewhat constant modulation over physiological function, such as HPA axis hormone secretion [44].

In contrast, phasic glucocorticoid actions (sometimes referred to as suppressive and stimulating actions) can be attributed to the effects that stem from a rapid and transient increase in glucocorticoids elicited by an acute exposure to stress. Phasic glucocorticoid actions produce additional dynamic modulation of physiological function including HPA axis activity [44]. Tonic glucocorticoid negative feedback effects have been studied by long-term removal of endogenous glucocorticoids (adrenalectomy) combined with various replacement regimens of exogenous glucocorticoids [56-58]. Phasic glucocorticoid negative feedback effects have been studied by pretreating subjects with an exogenous dose of glucocorticoids prior to HPA axis stimulation [52,70,72].

The underlying cellular and molecular mechanisms by which tonic and phasic glucocorticoids act at specific anatomical sites to regulate HPA axis activity remains largely undetermined. Glucocorticoid negative feedback within the hypothalamic element of the HPA axis suppresses both the coupling of cellular excitation with neurohormone secretion and gene induction [68,134]. Nevertheless, it is largely unknown whether tonic or phasic glucocorticoid

activity inhibits specific stress-induced intracellular signal transduction events within CRH neurons.

Extracellular signaling-regulated kinase (ERK) is one member of a family of mitogen-activated protein kinases (MAPK), and it is a functionally versatile intracellular signaling molecule (1). For example, ERK activity is responsive to an array of intercellular stimuli (e.g. classic neurotransmitters, growth factors, cytokines and hormones), and as a result the active (phosphorylated) forms of the two isoforms of ERK (phospho-p44/phospho-p42 or pERK1/2) participate in regulation of a variety of cellular processes (e.g. cell proliferation, cell differentiation, cell death, as well as neuronal plasticity and neuroexcitability). Interestingly, pERK1/2 may be an important intracellular mediator of stress-induced HPA axis activity. For example, mice challenged with 30 minutes of restraint displayed an increase in PVN pERK1/2 cell counts [100], and others have found that intravenous infusion of rats with a physiological stressor (2-deoxy-D-glucose) produced an increase in pERK1/2 immunoreactivity that was present in PVN CRH neurons [107,135]. In some instances pERK1/2 can selectively activate specific transcription factors, such as Elk-1 [136]. Also pERK1/2 can indirectly activate the transcription factor cAMP response element binding protein (CREB) through phosphorylation of other signaling intermediates such as ribosomal-s6-kinase [102]. CREB is a primary transcription factor responsible for induction of PVN *crh* gene expression [103], therefore pERK1/2 may contribute to stress-induced PVN *crh* gene expression. pERK1/2 also plays a role in maintaining enhancement of neuronal excitability [137], consequently pERK1/2 may be a mediator of stress-induced alterations in CRH neuronal excitability and subsequent neurohormone secretion. These examples of pERK1/2 cellular function overlap with cellular processes believed to be altered by

glucocorticoid negative feedback, and therefore make pERK1/2 a candidate target for glucocorticoid regulation within CRH neurons.

In this study we characterized both basal and stimulated PVN pERK1/2 immunoreactivity in response to tonic and phasic glucocorticoid manipulations. We first, determined if pERK1/2 levels can be rapidly increased in rat PVN CRH neurons by an acute exposure to a psychological stressor (restraint). We then examined whether tonic changes in corticosterone (CORT) exposure may influence PVN pERK1/2 levels by removing endogenous CORT via adrenalectomy (ADX) and providing some of those rats with exogenous CORT replacement in their drinking water. This particular CORT water replacement regimen normalizes the augmented HPA axis response that ADX rats display when challenged with acute stress [57,59]. We also examined whether restraint-stimulated PVN pERK1/2 levels were subject to modulation by a 1 hour phasic CORT pretreatment. We have previously shown that this particular phasic CORT pretreatment is sufficient to inhibit stress-induced ACTH secretion and PVN *crh* hnRNA expression [70]. Phasic glucocorticoid negative feedback is evident when exogenous glucocorticoids are administered either within a few minutes (fast negative feedback) or in a moderately short interval of time (~1 hour or more; short-term delayed negative feedback) prior to the onset of stress [70,72]. Short-term delayed glucocorticoid negative feedback effects differ from fast negative feedback effects in that their inhibitory actions depend on alterations in *de novo* protein synthesis [67,138]. Short-term delayed glucocorticoid negative feedback may be especially important for the regulation of the HPA axis in the context of recurring acute episodes of stress or chronic stress, and it is the focus of this study.

The phosphorylated form of ERK1/2 may be used as an experience dependent marker of recent neuronal PVN activation [135] in a similar fashion that immediate early genes are conventionally used [139]. Additionally, pERK1/2 may serve as a stress-induced intermediate signaling molecule for both *crh* gene expression and hormone release in PVN CRH neurons. Consequently, measuring changes in pERK1/2 cell counts may provide specific information about the actions of CORT (tonic and phasic) within the PVN by localizing those effects to events that modulate ERK activation.

Methods

Subjects

Young adult (250-320g) male Sprague-Dawley rats (Harlan Labs, Indianapolis, IA) were housed two per-cage (polycarbonate tubs, 47 cm x 23 cm x 20 cm) and were maintained on a 12 hour light/dark cycle with rat chow and tap water available ad libitum unless otherwise indicated. All test day manipulations were performed during the first half of the rat's light period when basal HPA axis activity is at its diurnal trough. Rats were given two weeks to acclimate to the facility before exposure to experimental procedures. All experiments were conducted in accordance with ethical procedures and were approved by the University of Colorado Institutional Animal Care and Use Committee.

Surgery

In experiments 2-4, rats received either bilateral adrenalectomy (ADX) or were sham-ADX. During surgery all rats were anesthetized with halothane. Bilateral incisions were made through the skin and peritoneal wall in close proximity to each kidney. Sham-ADX rats underwent the same surgical procedure as ADX rats except adrenal glands were not removed. All ADX and sham-ADX rats had a post-surgical recovery period of 4 days. ADX rats were given 0.9% saline water ad libitum unless indicated otherwise.

Restraint Stress

Stress challenge consisted of placing rats in clear plexiglas tubes (23.5 cm in length and 7 cm in diameter; with multiple air holes). The size of the tube restricted lateral, forward and backward movement but did not interfere with breathing. Restraint is widely accepted as a

psychological stressor within the stress neurobiology field. This stressor categorization has not only a conceptual basis, but is also supported by neurocircuit activity studies [140].

Experimental procedures

Experiment 1a and b: time-course for stress-induced PVN pERK1/2 cell counts, and colocalization of stress-induced pERK1/2 in CRH neurons. **1a:** The time-course experiment examined 5 treatment groups of adrenal intact rats (N = 34). Four groups of rats (n = 6) were placed in restraint for either 5, 10, 20 or 30 minutes. Immediately after restraint these rats were killed by guillotine decapitation. The 5th group of rats (n = 10) were used as a no stress control comparison group, and were left undisturbed in their home cages within a closed room separate from the restrained rats. **1b**: The colocalization experiment examined 2 treatment groups of adrenal intact rats (N = 12). Stress challenged rats (n = 6) were restrained for 15 minutes and then decapitated. Non-stressed rats (n = 6) were left undisturbed in their home cages in a room separate from restrained rats and killed at the same time of day as stress challenged rats.

Experiment 2: effects of long term absence of endogenous CORT (ADX) on basal and stress-induced ACTH secretion and PVN pERK1/2 cell counts. This experiment examined 4 separate treatment groups (2 x 2 between subjects factorial design; n = 4; N = 16 rats) with treatment factors of tonic CORT condition (sham-ADX or ADX) and stress challenge (no stress or 15 minute restraint). On the test day rats were either restrained for 15 minutes or left undisturbed in their home cage prior to decapitation.

Experiment 3: effects of tonic CORT treatment of ADX rats on basal and stress-induced ACTH secretion and PVN pERK1/2 cell counts. This experiment examined 6 separate treatment groups (3x2 between subjects factorial design; n = 6; N = 36 rats) with

treatment factors of tonic-CORT condition (sham-ADX , ADX, or ADX + CORT) and stress challenge (no stress or 15 minute restraint). After surgery, ADX + CORT rats were given ad libitum a drinking solution of 0.9% saline that contained CORT and 0.1% ethanol. The CORT drinking solution was made by dissolving CORT (Steraloids Inc. Newport, RI, USA) in absolute ethanol (25 mg/ml) and then diluting that solution 1:1000 into 0.9 % saline tap-water, to give a final CORT concentration of 25 μg/ml and ethanol concentration of 0.1%. ADX rats were given ad libitum a drinking solution containing 0.9% saline and 0.1% ethanol. Sham-ADX rats were given ad libitum a drinking solution of tap-water containing 0.1% ethanol. On the test day rats were either restrained for 15 minutes or left undisturbed in their home cage prior to decapitation.

Experiment 4: Effects of a phasic 1 hour CORT pretreatment of ADX rats on basal and stress-induced ACTH secretion, PVN pERK1/2 cell counts and PVN *crh* gene expression. This experiment examined 4 treatment groups (2 x 2 between groups factorial design; n = 6; N = 24 rats) with treatment factors of stress challenge (no stress or 15 minute restraint) and 1 hour CORT pretreatment (CORT or vehicle). All rats were ADX and were given 0.9% saline ad libitum. On the test day, rats were pretreated 1 hour before stress challenge with CORT (2.5 mg/kg, i.p.) or vehicle (10% ethanol, 30% propylene glycol and 60% sterile saline, i.p.). One hour after drug pretreatment stressed rats were restrained for 15 minute and then were decapitated. The remaining rats were returned to their home cage after injection and left undisturbed until decapitation at a post injection time matched to that of the stress challenged rats.

Brain tissue processing

Due to the high level of PVN pERK1/2 immunoreactivity present in brains of rats that had been deeply anesthetized and perfused with buffer followed by 4% paraformaldehyde at the time of death (our unpublished observations), we instead used a guillotine to decapitate unanesthetized rats and adopted a whole brain postfixation method suitable for immunohistochemistry [107,135]. In brief, brains were placed in 0.1 M sodium acetate buffered 4%-paraformaldehyde (PFA; pH 6.5) and stored over night (12-15 hours; 4 ° C). Brains were transferred to a 4% PFA solution buffered with sodium borate (pH 9.5; 4 ° C). After 4-5 days, brains were transferred to cryoprotectant (0.1 M sodium acetate/20% glycerol, 4 ° C). After two days in cryoprotectant, brains were flash frozen in -30 ° C isopentane chilled with dry ice and stored at -80 ° C. Coronal brain sections (30 μm thick) were cut on Leica Microsystems cryostat (model 1850, Bannockburn, IL, USA) through the extent of the PVN (approximately 1.80 mm caudal from bregma), and stored at 4 ° C in cryoprotectant.

Immunohistochemistry

pERK1/2 immunoperoxidase histochemistry: tissue sections were immunostained for pERK1/2 using a standard immunoperoxidase procedure [141]. In experiments 2 and 3, pERK1/2 was detected with a monoclonal primary antibody (rabbit anti-phospho p44/p42 MAPK, 1:500 dilution; catalog #4376 , Cell Signaling Technology, Danvers, MA). In experiments 1 and 4, pERK1/2 was detected with a polyclonal primary antibody (rabbit anti-phospho p44/p42 MAPK, 1:4000 dilution; #9101 Cell Signaling Technology). The same secondary antibody was used for all immunoperoxidase assays (goat anti-Rabbit IgG, 1:500; # BA1000, Vector Labs, Southfield, MI). After incubation with ABC complex reagents (1:500;

Vector Labs) a dark purple precipitate was generated using 0.2 mg/ml diaminobenzidine (Sigma; # D5905, St. Louis, MO) and 6 mg/ml nickel ammonium sulfate (Fisher Scientific; #N48-500, Pittsburgh, PA) as substrates for the peroxidase mediated reaction. Sections were then rinsed and mounted in 0.1M Tris buffer (pH 7.4) onto 10% gelatin coated slides, dried, delipidized, and coverslipped. We chose to use two separate anti-pERK1/2 antibodies in order to validate that the specific pattern of pERK1/2 immunoreactivity that we observed in the PVN would generalize across two separate antibody preparations. Both anti-pERK1/2 antibodies yielded similar results in terms of the relative number of pERK1/2 immunoreactive cells under basal and stress conditions, however the monoclonal rabbit antibody (#4376, Cell Signaling Technology) tended to produce higher background immunostaining.

pERK1/2 and CRH co-localization immunofluorescence histochemistry: PVN pERK1/2 was detected with rabbit primary antibody (anti-phospho p44/p42 MAPK, 1:4000 dilution; #9101 Cell Signaling Technology) and secondary antibody (Alexa-594 anti-rabbit IgG, 1:500 dilution; # A-11021 Invitrogen, Carlsbad, California). PVN CRH peptide was detected with guinea pig primary antibody (anti-CRH monoclonal antibody, 1:20000 dilution; # T-5007 Peninsula Laboratories, member of the Bachem group, Torrance, California) and secondary antibody (Alexa-488 anti-guinea pig IgG, 1:500 dilution; #A-11073 Invitrogen, Carlsbad, California). Floating tissue sections were incubated over night (4 °C) in a 0.01 M phosphate buffered saline (PBS; pH 7.4) solution containing both primary antibodies, 0.3% triton-X and 1.5% normal goat serum (Vector Labs; # PCN-5000). Sections were then rinsed and incubated for 2 hours in a 0.01 M PBS solution containing both secondary antibodies, 0.3% triton-X and 1.5% normal goat serum. Sections were then rinsed and mounted onto gelatin coated slides, with

addition of a few drops of the nuclear 4',6-diamidino-2-phenylindole fluorescent stain. As an immunostaining control procedure, some sections were treated the same as described above except that either one or both primary antibodies were omitted from the overnight incubation solution. For those control sections, no distinct cellular immunostaining was evident when applying the appropriate flourescent viewing conditions for the absent antibody.

Quantification

pERK1/2 immunoperoxidase cell counts: bright field photomicrographs (200X; Zeiss microscope, Axioimager M1) of peroxidase immunostained PVN sections were captured using Axio-Vision imaging software (version 4.6, Zeiss). At least 4 separate PVN hemispheric images per brain were captured and scored by individuals that were blind to treatment group assignments throughout this process. The target region of interest (ROI) was the medial parvocellular PVN region as determined by matching digitized rat hypothalamic structures to rat brain atlas images [142]. Immunopositive cells within the PVN were manually counted using NIH-ImageJ (freeware, http://rsbweb.nih.gov/ij/) touch-count feature which allowed the investigator to mark each cell within the ROI that was judged to be immunopositive. A threshold grey level for immunopositive cells was chosen that was approximately 50% of the maximum grey level cell signal over background.

pERK1/2 immunofluorescence cell counts: 2-color channel flourescent (mercury bulb illumination) photomicrographs (200X; Zeiss microscope: Axioimager M1) of PVN sections were captured using Axio-Vision imaging software as noted above. At least 4 separate PVN hemispheric images per brain were captured for co-localization assignments. For assessment of co-localization of pERK1/2 immunoreactivity with CRH immunoreactive neurons, a software

application (Adobe Photoshop-CS3, San Jose, CA) was used to view and tag immunopositive cells within a red channel image layer that displayed fluorescencent labeled pERK1/2 cells and a green channel image layer that displayed fluorescencent labeled CRH cells. Each image layer was separately marked with red-dots over pERK1/2 immunopositive cells or green-dots over CRH immunopositive cells. The two marked layers were then merged, and the number of cells containing both red and green marks was determined. The region of interest for analysis was restricted to an outline of the CRH immunopositive cell region within the PVN. Cell counts were performed by an individual that was blind to the treatment group assignment for each image.

ACTH radioimmunoassay and CORT ELISA

Trunk blood was collected into EDTA-containing vacutainer tubes (Becton-Dickinson; Franklin Lakes, New Jersey, USA), placed on wet ice and then centrifuged for 15 minute (4 °C). Plasma was then aliquoted into microfuge tubes and snap-frozen on dry ice. The blood processing procedure was completed within 45 minute after decapitation. ACTH (pg/ml) was determined in duplicate (100 µl plasma) by competitive radioimmunoassay protocol as described previously [59]. Radio-labeled ^{125}I ACTH-Tracer was obtained from DiaSorin (Cat # 20515, Stillwater MN) and primary ACTH anti-serum Rb 7 (diluted to a final concentration of 1:30,000) was provided courtesy of Dr. William Engeland, University of Minnesota. The detection limit for this assay was 15 pg/ml; the intra-assay coefficient of variability was 6% and inter-assay coefficient of variability was 6%.

Plasma CORT measurement was conducted using 20µl of plasma with an enzyme immunoassay kit (Assay Design, Ann Arbor, MI, USA) according to manufacturer's instructions.

Sensitivity for the CORT assay was 27 pg/mL. The intra-assay and inter-assay coefficients of variability for the corticosterone assay were approximately 4% and 6%, respectively.

CRH hnRNA in situ hybridization and quantification (experiment 4)

Coronal PVN brain sections (post fixed and processed as described above) were floated in 0.5 M PBS and mounted onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Tissue was then further fixed in 4% PFA for 30 minute at room temperature and then processed as previously published (2). Generation of ^{35}S-UTP labeled cRNA probes for *crh* hnRNA used a cDNA template corresponding to a 696 basepair nucleotide intronic sequence (genomic location between 104765-10476512; plasmid containing cDNA provided courtesy of Dr. R. T. Thompson, University of Michigan). *crh* hnRNA probes were subsequently linearized from pGEM-3 vectors (Promega, Madison, WI) with the restriction endonuclease HindIII and transcribed using T7 RNA polymerase. The identity of *crh* cloned DNA was verified by DNA sequencing (University of Colorado Molecular, Cellular and Developmental Biology sequencing facility). After the hybridization assay procedure, slides were exposed to x-ray film (Kodak Biomax MR film) for 20 days.

Semiquantitative analyses were performed on digitized images from x-ray films using the linear range of the gray values obtained from an acquisition system (Northern Lights lightbox, model B 95, Ontario, Canada; CCD camera, model XC-77, Sony, Tokyo, Japan; image capture with National Institutes of Health scion Image v1.59 software) as previously described [143]. Signal pixels of the region of interest were defined as those with a gray value of 3.5 standard deviations above the mean gray value of background (lateral hypothalamus or corpus callosum). The PVN region was determined by matching digitized rat hypothalamic structures to

rat brain atlas diagrams (3). The product of the number of pixels and the average pixel values above the set background were then computed, providing an integrated densimetric measure of arbitrary units (integrated gray level). Quantification and analysis of images were performed by individuals that were blind to treatment condition assignments.

Statistical Analysis

Statistical analyses were conducted using the SPSS statistical program 10.5 (Chicago, IL, USA) for Macintosh operating system. One way analysis of variance (ANOVA) (experiment 1a), two way ANOVA (experiments 2-4), or Student's t-test (experiment 1b) were used to determine main effects and interactions for all treatment factors. In cases where there was an overall significant F-test, pairwise comparisons of interest were assessed by Fisher's least significant difference test (FLSD); alpha = 0.05. Data presented in graphs represent group means ± standard error of the mean.

Results

Experiment 1a: restraint rapidly increased ACTH and CORT secretion, as well as PVN pERK1/2 cell counts.

Restraint significantly increased ACTH ($F_{4,31} = 9.7$, $P < 0.05$) and CORT ($F_{4,31} = 46.3$, $P < 0.05$) secretion, and restraint increased the number of cells within the PVN that were immunoreactive for pERK1/2 ($F_{4,22} = 4.6$, $P < 0.05$)(figure 2.1). Posthoc tests indicate that hormone secretion and pERK1/2 immunoreactivity were increased within 5 minute after restraint onset. Both CORT levels and pERK cell counts remained elevated throughout the duration of 30 minute restraint. Mean ACTH levels had started to decline at the 30 minute time-point, however the 30 min levels were not significantly different from the peak levels present at the 10 minute time-point.

Experiment 1b: approximately 50% of restraint-stimulated pERK1/2 immunoreactive cells were colocalized with PVN CRH immunoreactive cells.

To assess the extent to which restraint-stimulated increases in pERK1/2 cell counts occurred within PVN CRH neurons, we used a double-label CRH and pERK1/2 immunofluorescence histochemistry procedure. Restraint (15 min) increased both single labeled PVN pERK1/2 immunoreactive cell counts ($t_{(11)} = 3.3$, $P < 0.05$) and doubled labeled PVN pERK1/2 and CRH immunoreactive cell counts ($t_{(11)} = 3.4$, $P < 0.05$) (figure 2.2). Approximately 50% of stress-stimulated pERK1/2 immunoreactivity was colocalized within PVN CRH neurons. There was no effect of restraint on the total number of CRH neuron counts (data not shown).

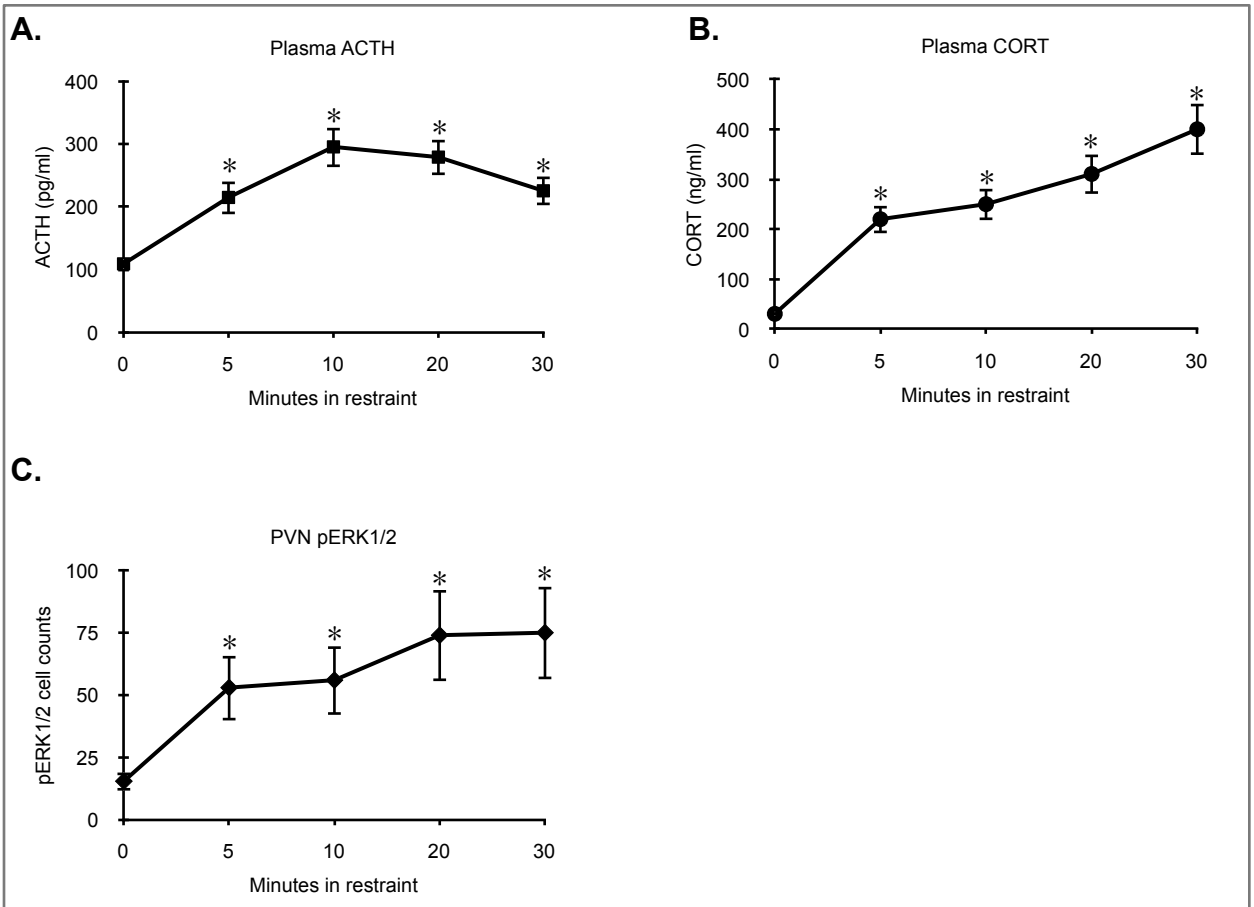

Figure 2.1 Stress-induced HPA hormone and pERK time course. (A-C) Restraint significantly increased ACTH and CORT secretion, and increased PVN pERK1/2 cell counts in adrenal intact rats. Rats not challenged with restraint displayed low basal ACTH, CORT and PVN pERK1/2 cell counts. Five minute of restraint rapidly increased ACTH, CORT and pERK1/2 cell counts. The increased ACTH and CORT hormone levels and pERK1/2 cell counts remained elevated over the course of a 30 minute restraint challenge. *, represents a difference compared to unstressed rats (time 0); $p < 0.05$, FLSD.

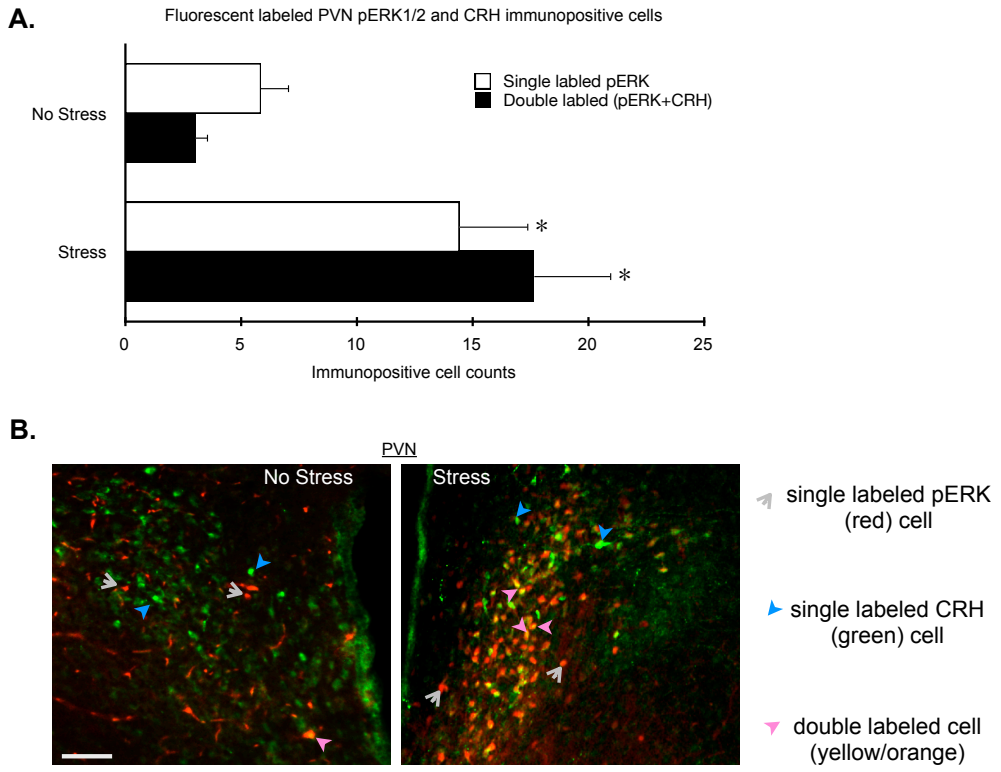

Figure 2.2 Colocalization of basal and stress-stimulated pERK1/2 cell counts in PVN CRH neurons. (A) Both the number of single labeled (pERK1/2 alone) and double labeled (pERK1/2 and CRH) PVN pERK1/2 immunopositive cells were increased in response to 15 minute of restraint. Approximately 50% of stress-stimulated immunofluorescence pERK1/2 was colocalized within PVN CRH neurons. *, represents a significant stress effect compared to non-stressed group ($p < 0.05$, FLSD). (B) Displays a representative merged immunofluorescence image of a single PVN hemisphere section from either a non-stressed (right) rat or stressed (left) rat. Single labeled pERK1/2 immunoreactive cells are labeled with red immunofluorescence (gray arrows), single labeled CRH immunoreactive cells are labeled with green immunofluorescence (blue arrow heads), and pERK1/2+CRH doubled immunoreactive cells are evident by yellow/orange immunoflourescence (pink arrow heads). Scale bar = 100 μm.

Experiment 2: long-term absence of endogenous CORT increased basal and stress-stimulated ACTH secretion, and stress-stimulated pERK1/2 cell counts within the PVN.

As expected, ACTH secretion was increased by 15 min of restraint challenge (stress challenge: $F_{1,12} = 15.8$, $P < 0.05$) (figure 2.3a). In addition, basal and stress-stimulated ACTH secretion were substantially increased in ADX rats compared to sham-ADX rats (tonic CORT condition: $F_{1,12} = 34.01$ $P < 0.05$) (figure 2.3a). PVN pERK1/2 immunoreactivity was increased after 15 min of restraint stress compared to non-restrained rats regardless of surgical condition (stress challenge: $F_{1,10} = 25.8$, $P < 0.05$) (figure 2.3b). There was also an overall effect of ADX on PVN pERK1/2 cell counts (tonic-CORT condition: $F_{1,10} = 13.3$, $P < 0.05$) (figure 2.3b). Although, statistical analysis only supported a trend for a significant stress challenge by tonic CORT condition interaction ($p = .09$), posthoc tests did indicate that there was a greater effect of stress on PVN pERK1/2 cell counts in restrained ADX rats compared to sham-ADX rats. Non-stressed PVN pERK1/2 immunoreactive cell numbers were comparably low in both sham-ADX and ADX rats (figure 2.3b).

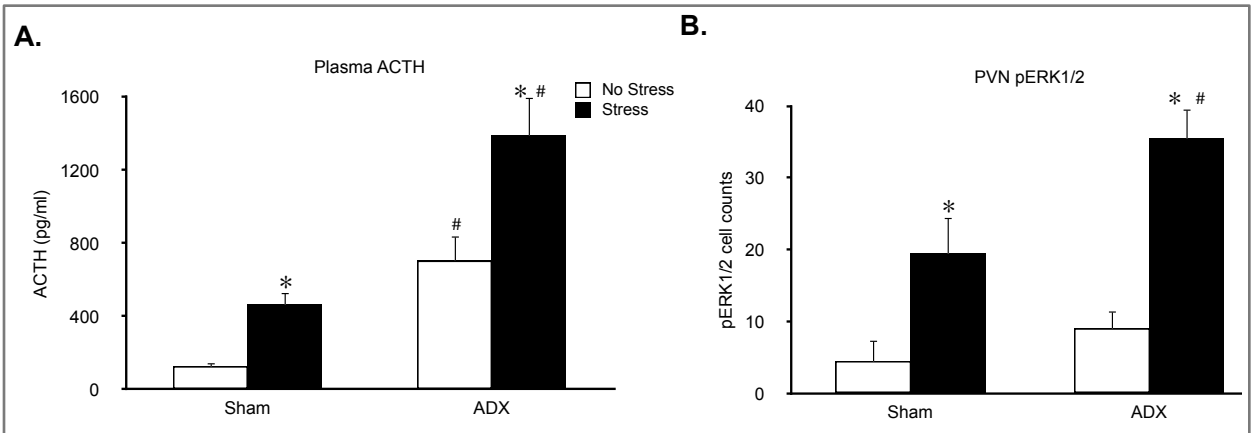

Figure 2.3 ADX effects on ACTH and pERK. ADX resulted in a marked increase in both basal and stress-stimulated ACTH secretion, as well as stress-stimulated PVN pERK1/2 cell counts. (A) ACTH secretion was increased by 15 minute restraint. Both basal and stress-stimulated ACTH secretion were substantially increased in ADX rats. (B) PVN pERK1/2 cell counts were also increased by restraint. ADX rats displayed a greater increase in PVN pERK1/2 cell counts compared to sham-ADX rats. *, represents a significant stress effect compared to the no stress group within the same surgical condition ($p < 0.05$, FLSD); #, represents a significant tonic CORT condition (ADX) effect compared to the sham group within same stress condition ($p < 0.05$, FLSD).

Experiment 3: CORT replacement (via drinking water) of ADX rats normalized basal and stress-stimulated ACTH secretion and stress-stimulated PVN pERK1/2 cell counts.

As was the case in experiment 2, 15 min of restraint increased ACTH secretion regardless of tonic CORT condition (stress challenge: $F_{1,30} = 45.12$, $P < 0.05$). ADX led to significantly greater basal and stress-stimulated ACTH secretion (tonic CORT condition: $F_{2,30} = 28.01$, $P < 0.05$) (figure 2.4a). Treatment of ADX rats with a tonic CORT drinking water regimen was sufficient to normalize both basal and stress-stimulated ACTH secretion (stress challenge by tonic CORT condition interaction: $F_{2,30} = 9.52$ $P < 0.05$, followed by FLSD *post hoc* tests; Figure 4a). Also, consistent with experiment 2, PVN pERK1/2 immunoreactive cell counts were increased after 15 min of restraint stress (stress challenge: $F_{1,28} = 86.1$, $P < 0.05$), and this increase was greater in ADX rats (tonic CORT condition: $F_{2,28} = 5.2$ $P < 0.05$) (figure 2.4 b,c). Interestingly, treatment of ADX rats with CORT drinking water normalized the magnitude of stress-stimulated PVN pERK1/2 cell counts (stress challenge by tonic CORT condition interaction: $F_{2,28} = 11.3$, $P < 0.05$, followed by FLSD *post hoc* tests; Figure 2.4b,c). Our CORT water replacement regimen resulted in low, but detectable basal levels of circulating plasma CORT in non-stressed ADX rats ($M = 40.4$ ng/ml, $SEM = 1.4$) and ADX Stressed rats ($M = 20.4$ ng/ml, $SEM = 0.5$). These CORT levels were comparable to basal plasma CORT levels of non-stressed sham-ADX rats ($M = 50.0$ ng/ml, $SEM = 0.4$), whereas stressed sham-ADX rats had an expected large increase in CORT levels ($M = 320.0$ ng/ml, $SEM = 4.1$). ADX rats that were not given CORT in their drinking water had plasma CORT levels below the detection limits of the assay.

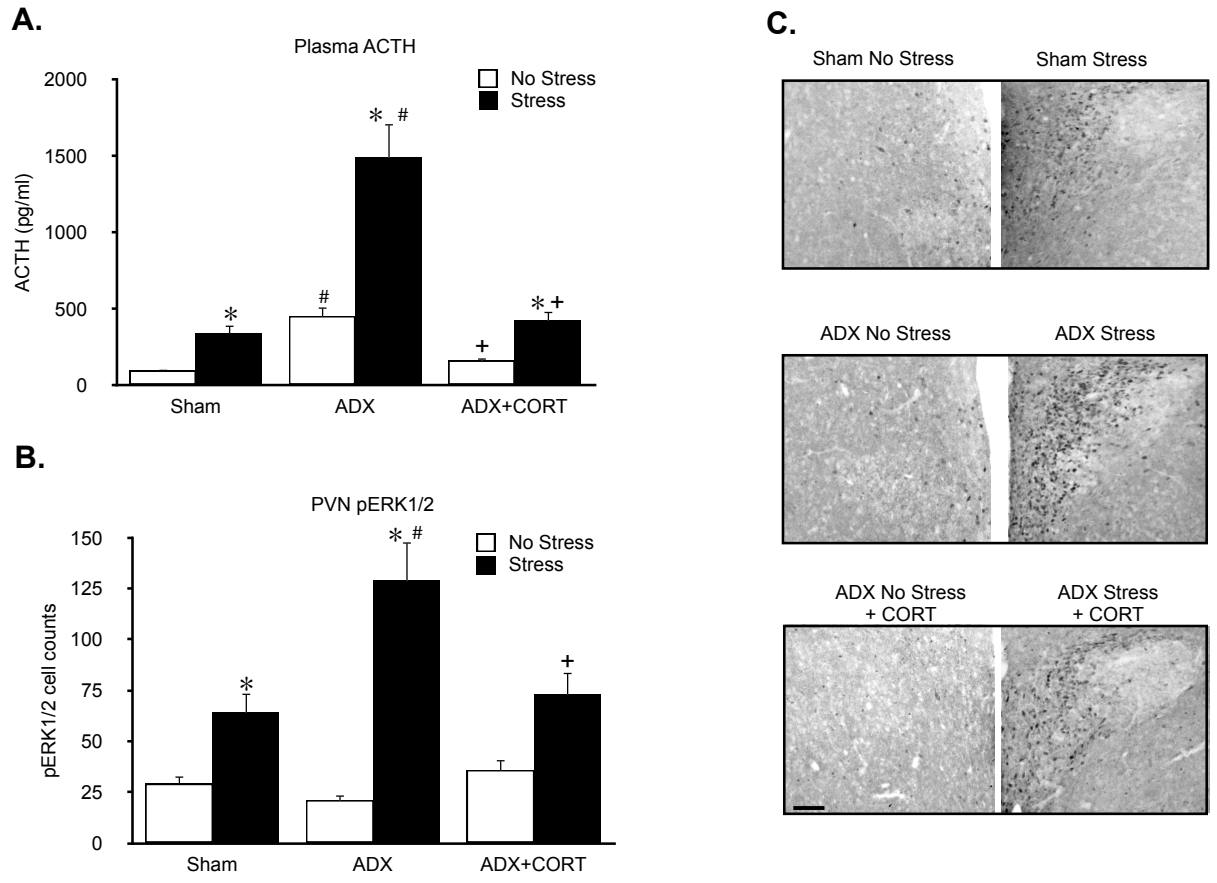

Figure 2.4 Effects of tonic CORT replacement in drinking water. Tonic CORT replacement of ADX rats normalized basal and stress-induced ACTH secretion, and normalized stress-induced PVN pERK1/2 cell counts. (A-B) Restraint (15 minute) increased ACTH secretion and PVN pERK1/2 cell counts, and ADX augmented these stress-stimulated effects. Tonic CORT water replacement of ADX rats normalized basal and stress-stimulated ACTH secretion to levels comparable to those displayed by sham-ADX rats. Tonic CORT water replacement also normalized the increase in stress-stimulated PVN pERK1/2 cell counts. *, represents a significant stress effect compared to no stress group within the same tonic CORT condition ($p < 0.05$, FLSD); #, represents a significant tonic CORT condition effect compared to sham rats within the same stress condition ($p < 0.05$, FLSD); +, represents a significant CORT replacement (ADX+CORT water) effect compared to non-replaced ADX rats within the same stress condition ($p < 0.05$, FLSD). (C) Shows representative photomicrographs from each treatment group for pERK1/2 immunoperoxidase histochemistry in one hemisphere of the PVN (3rd ventricle on the right side of no stress condition images and on the left side of stress condition images). Scale bar = 100 μm.

Experiment 4: a phasic increase in CORT 1 hour prior to restraint challenge suppressed stress-stimulated ACTH secretion and stress-induced PVN crh gene expression, but not stress-stimulated PVN pERK1/2 cell counts.

Restraint (15 min) significantly increased ACTH secretion ($F_{1,20} = 7.0$, $P < 0.05$), PVN pERK1/2 immunoreactive cell number ($F_{1,20} = 23.4$, $P < 0.05$) and PVN *crh* hnRNA levels ($F_{1,20} = 17.4$, $P < 0.05$) (figure 2.5a-d). CORT pretreatment (1 hour) significantly inhibited basal and stress-stimulated ACTH secretion (phasic CORT treatment: $F_{1,20} = 40.1$, $P < 0.05$, and stress by phasic CORT treatment interaction: $F_{1,20} = 11.4$, $P < 0.05$) and it inhibited stress-induced *crh* hnRNA expression (phasic CORT treatment: $F_{1,20} = 25.4$, $P < 0.05$, and stress by phasic CORT treatment interaction: $F_{1,20} = 17.5$, $P < 0.05$) (figure 2.5 a-c). In contrast, CORT pretreatment had no effect on stress-stimulated PVN pERK1/2 immunoreactive cell numbers (figure 2.5d).

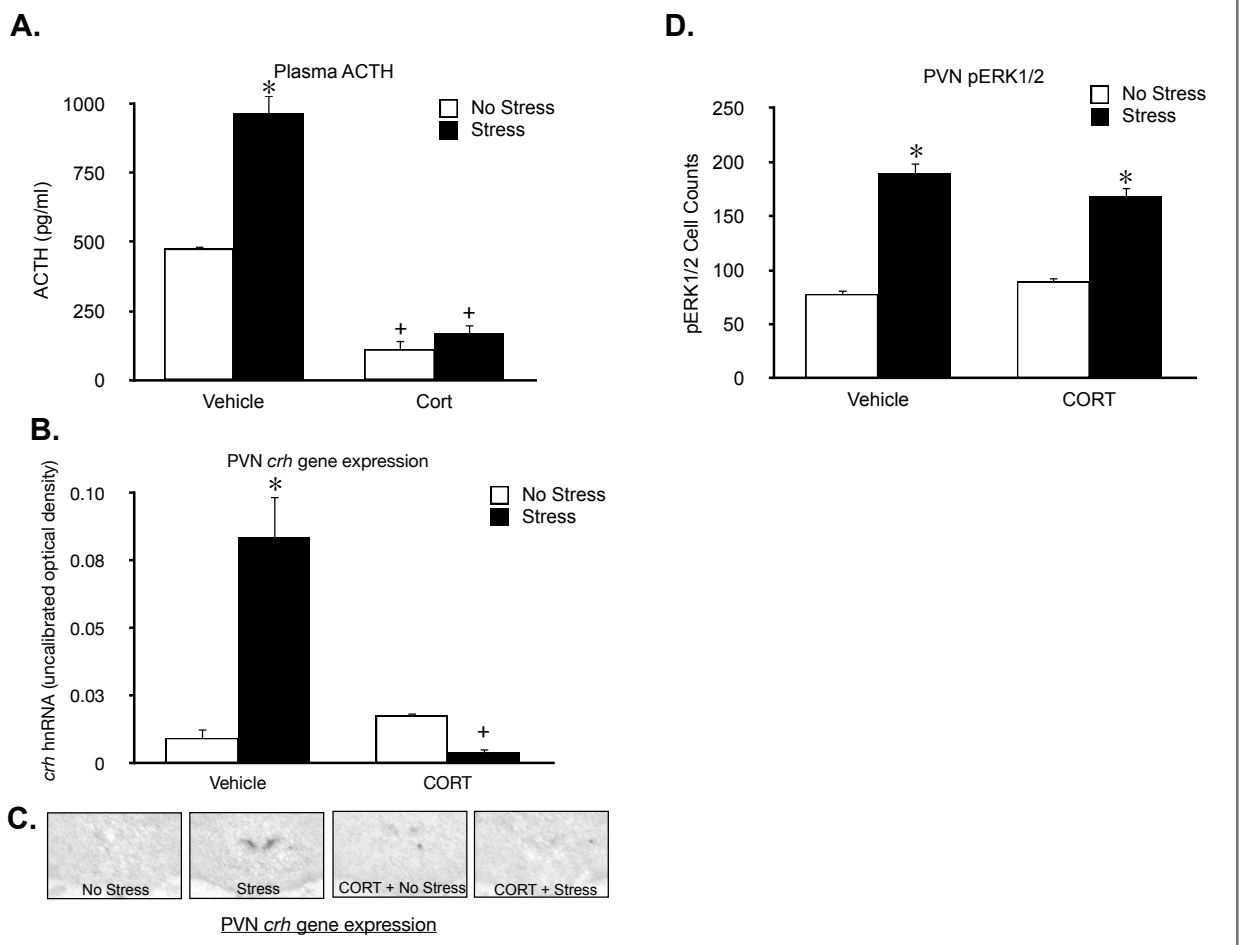

Figure 2.5 Effects of 1 hour phasic CORT pretreatment on PVN pERK *crh* and ACTH. Phasic increase in CORT 1 hour prior to restraint challenge suppressed stress-stimulated ACTH secretion and PVN *crh* gene expression, but not stress-stimulated PVN pERK1/2 cell counts in ADX rats. (A) Basal and restraint-stimulated ACTH secretion was suppressed 1 hour after phasic CORT treatment. (B-C) Stress-induced PVN *crh* gene expression was suppressed by this CORT pretreatment. (C) Shows representative autoradiograms for in situ hybridization of PVN *crh* hnRNA expression. Images are centered over the PVN containing ventral-medial portion of coronal brain sections. (D) Phasic CORT pretreatment had no effect on stress-stimulated PVN pERK1/2 cell counts. *, represents a significant stress effect compared to non-stressed rats within the same phasic CORT condition ($p < 0.05$, FLSD); +, represents a significant phasic CORT effect compared to vehicle treated rats within the same stress condition ($p < 0.05$, FLSD).

Discussion

From these experiments we determined that CORT constrains in a tonic, but not phasic manner the extent to which the intracellular signal transduction protein ERK1/2 is activated by an acute psychological stress challenge in the rat PVN. Thus, the long-term absence of endogenous CORT (via ADX) augmented restraint-stimulated PVN pERK1/2 immunoreactivity, and treatment of ADX rats with CORT in the drinking water was sufficient to normalize pERK1/2 immunoreactive levels. As previously reported [67], a short-term phasic CORT treatment 1 hour prior to restraint substantially suppressed basal and restraint-stimulated ACTH plasma levels, and suppressed stress-induced PVN *crh* hnRNA levels. However, we found that this short-term phasic CORT pretreatment did not suppress the increase in PVN pERK1/2 immunoreactivity produced by restraint. This study also served to further characterize the dynamic response of a MAPK pathway in the PVN of rats to stress. We found that exposure of rats to an acute psychological stressor (restraint) resulted in a rapid (within 5 minutes) increase in the number of PVN pERK1/2 immunopositive cells, and that this increase was maintained over a 30 minute period of restraint. Approximately half of restraint-induced pERK1/2 immunopostive cells within the medial parvocellular portion of the PVN were colocalized within CRH neurons.

This specific MAPK pathway may play an important role in the coupling of CRH neuron excitation with both gene induction as well as neurohormone secretion. A number of genes are rapidly induced in the PVN with acute stress [59,144,145]. Khan et al. [135] provide evidence for stress-stimulated activation of ERK1/2 in CRH neurons to depend on noradrenergic alpha-1 receptors located in the PVN. Increased norepinephrine in the PVN is associated with *crh* and *c-fos* gene induction [135,146,147]. Moreover, ERK1/2 activity is upstream of activation of several transcription factors (e.g. CREB, AP-1, ELK and NGFI-B) that are instrumental in the activity-

dependent expression of most, if not all, of the genes that have been found to be rapidly induced in the PVN by acute stress [104-106]. One research group has recently demonstrated that inhibition of ERK1/2 phosphorylation reduces the firing rate of CRH neurons in response to norepinephrine, suggesting that pERK1/2 also plays a functional role in the modulation of neurohormone release from these neurons [47]. Similarly, pERK1/2 may also function as a dynamic intermediate signaling molecule for gene expression and hormone release within corticotrophs. For instance, CRH receptor stimulation of corticotrophs recruits the MAPK pathway for the modulation of *pomc* gene transcription, and pERK1/2 is thought to participate in the mediation of stimulated ACTH secretion [92].

Given the accumulating evidence for an important role of this MAPK pathway in HPA axis function, the focus of this study was on characterization of the regulatory influence of glucocorticoids on basal and stimulated pERK1/2 levels within the hypothalamic element of the HPA axis. By comparing the regulatory effects of glucocorticoids on PVN pERK1/2 levels with other activity-dependent HPA axis related measures, we can also make some inferences about which aspects of this neuroendocrine system are affected by changes in tonic and phasic glucocorticoids.

We first consider the implications of our data for understanding tonic glucocorticoid regulation of basal HPA axis activity. Although ADX produces a large increase in basal ACTH levels, the absence of tonic CORT activity did not change basal PVN pERK1/2 cell counts. We have previously found that the basal expression of several experience dependent genes (*c-fos*, *zif268* and *ngfi-b genes)* within the PVN also remain unaltered by the loss of tonic CORT activity [58,59]. In contrast, the basal expression of the neurohormone encoding genes, *crh* and *avp*

upregulate in the PVN with ADX [59,148]. There is evidence for both the *crh* and *avp* genes to be directly regulated in a negative fashion by glucocorticoids, and consequently their basal expression may be tonically constrained by endogenous glucocorticoids [97,149]. There is no evidence for a direct glucocorticoid repressive effect on the expression of *c-fos*, *zif268* and *ngfi-b* genes within the PVN. Thus, the absence of an increase in the basal expression of these genes after ADX may reflect an absence of net excitatory drive to CRH neurons. The parallel absence of increased basal pERK1/2 levels is consistent with this interpretation. Consequently, the large increase in basal ACTH in ADX rats may be due to removal of tonic CORT inhibition that occurs directly at the level of neurohormone production and basal secretion independent of changes in basal CRH neuron firing rate. Alternatively, it may reflect a direct inhibitory effect at the level of the anterior pituitary, as is supported by some recent studies [150,151].

Whereas the tonic presence of CORT may not alter basal CRH neuron activity, tonic CORT clearly constrains the stress reactivity of CRH neurons. In this study we establish that the absence of tonic CORT activity in ADX rats challenged with restraint showed an exaggerated increase in PVN pERK1/2 cell counts and ACTH secretion. We have previously demonstrated that ADX rats exposed to restraint have an exaggerated increase in the expression of five genes within the PVN that are rapidly induced by acute stress (*c-fos*, *crh*, *avp*, *zif268*, and *ngfi-b*) [58,59]. Stress-stimulated Fos protein levels in the PVN are also increased in ADX rats F[60]. Together, these studies indicate that the loss of systemic tonic CORT regulation leads to an amplified stress-stimulated response of intracellular PVN activity (i.e. increased signal transduction, gene transcription and protein translation). Whether the augmented stress-induced HPA axis activity that results from ADX is a consequence of increased extrinsic drive to PVN

neurons or an increase in responsivity of those neurons to stimulation remains to be determined. The absence of tonic CORT activity does not alter stress-induced *c-fos* mRNA or protein expression in a number of brain regions outside of the PVN including the hippocampus [60,152,153]. Interestingly, the loss of tonic CORT increases the extent to which noradrenergic inputs in the PVN suppress GABAergic transmission [154]. This decreased GABAergic activity could result in an unrestricted stress-induced excitatory drive to PVN CRH neurons of ADX rats.

HPA axis hormone secretion is substantially regulated by tonic CORT activity [44,45]. For example, both basal and stress-stimulated ACTH plasma levels are increased by the removal of endogenous glucocorticoid activity via adrenalectomy [56,57,59]. Interestingly, treatment of ADX rats with a constant low dose of CORT normalizes basal but not stress-stimulated ACTH secretion patterns [56]. In contrast, the augmented stress levels of ACTH that result from the long-term absence of CORT activity can be normalized by treatment of rats with CORT containing drinking water [57,58]. This replacement regimen closely mimics endogenous circadian CORT secretion patterns [57]. Consistent with these previous studies, we found that providing ADX rats free access to CORT containing drinking water was sufficient to not only normalize the exaggerated ACTH plasma secretion patterns, but also the increased stress-stimulated PVN pERK1/2 cell counts. This shared profile for tonic CORT regulation of stress-stimulated ACTH secretion and PVN pERK activation may reflect a common cellular mechanism exerted by CORT at multiple levels of the HPA axis. Alternatively, tonic CORT may regulate stress reactivity at sites central to or within the PVN, which is then expressed throughout the downstream HPA axis anatomical elements.

In addition, to the tonic regulatory effects of CORT on the HPA axis, there is a temporally dynamic inhibition of the axis in response to a phasic increase in CORT. These inhibitory effects are evident within minutes (fast feedback) or after a delay, such as those present after the 1 hour pretreatment interval used in this study [49]. In previous studies we found that 1 hour systemic CORT pretreatment inhibited not only stress-stimulated ACTH secretion, but also stress-induced *crh* gene expression in the PVN and stress-induced *pomc* gene expression in the anterior pituitary [69,70]. We also found [67] that the inhibition of stress-stimulated ACTH secretion by 1 hour CORT pretreatment depended on *de novo* protein synthesis which is consistent with the notion that the underlying suppressive mechanism of delayed glucocorticoid negative feedback is distinct from that present during glucocorticoid fast feedback [45]. In this study we found that the suppressive effects of 1 hour CORT pretreatment on stress-induced PVN *crh* gene transcription and ACTH secretion was not associated with a suppressive effect on PVN pERK1/2 activity. One hour CORT pretreatment also does not inhibit stress-induced PVN *c-fos* gene expression [67,70]. The disassociation between the ability of phasic CORT to inhibit stimulated *crh* hnRNA expression 1 hour after treatment, but not stimulated PVN pERK1/2 activity or *c-fos* mRNA expression, suggests that this CORT treatment does not alter at least some of the stress-induced neural input to PVN neurons or the corresponding stress-induced intracellular signaling activity of those neurons.

It is likely that the suppressive actions of 1 hour CORT pretreatment on stimulated ACTH secretion is a result of CORT negative feedback actions that directly alter PVN and corticotroph hormone secretion. Studies (*in vivo and in vitro)* support a direct inhibitory effect of glucocorticoids on corticotroph intracellular processes necessary for corticotroph ACTH

hormone release [79,138,150]. Two recent studies also provide evidence for glucocorticoids to act directly at the level of the PVN to inhibit stress-stimulated neurohormone secretion. Others have found that microinfusion of dexamethasone in the PVN produces a rapid inhibition of stress-stimulated ACTH secretion [72]. We have recently found that microinfusion of CORT into the PVN 1 hour before stress challenge also inhibits ACTH secretion [68]. Although, the mechanisms underlying this direct phasic glucocorticoid inhibitory action at CRH neurons remains to be established, they appear to not depend on altered MAPK pathway activity.

There are a couple of caveats to note when considering the absence of an effect of phasic CORT manipulation on stress-stimulated pERK1/2 activity that we observed in this study. First, we examined pERK1/2 activity at one particular time point (1 hour after CORT treatment), but cellular and molecular effects of CORT can vastly change with timing [46,67], therefore, stimulated PVN pERK1/2 activity may be more responsive to phasic CORT activity within other negative-feedback time-intervals (e.g. more rapid or delayed time frames). Second, the ultradian pattern of endogenous CORT secretion may have differential functional effects on CRH neuron function that are not evident with our acute and long-term pharmacological CORT manipulations [50,155]. Nonetheless, our findings further illustrate the differential tonic and phasic effects of CORT on the cellular and systems-level function of the HPA axis. The ERK1/2 MAPK pathway appears to be a key intracellular signal transduction pathway for the coupling of CRH neuron excitation with gene induction and neurohormone secretion. Modulation of PVN ERK1/2 activation may be a molecular process by which glucocorticoids tonically regulate stress reactivity of the HPA axis.

Chapter III

Corticosterone constrains stress-dependent upregulation of mitogen-activated kinase phosphatase-1 (*mkp-1*) mRNA expression in the hypothalamic paraventricular nucleus and anterior pituitary, but not stress-induced upregulation of *mkp-1* gene expression in the medial-prefrontal cortex.

Abstract

Stress-induced activation of the hypothalamic paraventricular nucleus (PVN) corticotropin-releasing hormone (CRH) peptide synthesis and release is dependent on the intracellular activation of the extracellular-regulated kinase (ERK). We have recently demonstrated that the endogenous glucocorticoid of rat corticosterone (CORT) constrains stress-stimulated upregulation in the activated form of phospho-ERK (pERK) in the medial-PVN. Inactivation (dephosphorylation) of pERK within in peripheral cell types has been shown to be dependent on glucocorticoid upregulation of the MAP kinase phosphatase (*mkp-1*) gene, this glucocorticoid dependent increase of the *mkp-1* gene may serve as a regulatory mechanism for stress-stimulated increase in pERK within the neuroendocrine cell populations of the PVN. There is little documentation of *mkp-1* gene expression in forebrain regions (prefrontal and PVN) and endocrine cell populations (anterior pituitary) that are responsible for the HPA axis hormone release during an acute psychological stress challenge. Using young adult male Sprague-Dawley rats, we tested whether CORT could induce upregulation of *mkp-1* mRNA expression within intrinsic (PVN and anterior pituitary) cell populations to the HPA axis and extrinsic neuronal cell populations of the medial prefrontal cortex (prelimbic or PrL) and infralimbic or IL subregions), during basal and acute stress challenge (restraint). Results indicated that PVN *mkp-1* mRNA was significantly increased in response to 15 minute restraint and continued to increase with the duration of stress (30 min). We also found that *mkp-1* mRNA expression was upregulated within the PrL and IL cortex. Stress-dependent increase in *mkp-1* mRNA within the PrL, IL, PVN, and anterior pituitary was independent of an acute increase in CORT activity. However, the long-term absence of endogenous CORT (4-day adrenalectomy) augmented stress-induced *mkp-1* mRNA expression within the PVN and anterior pituitary, but not in the medial-PFC. These results

suggest that stress-induced *mkp-1* mRNA expression plays a role in stress-dependent regulation of MAP-kinases in both extrinsic and intrinsic anatomical sites to the HPA axis, and CORT constrains stress-induced *mkp-1* mRNA gene expression within the intrinsic cell populations of the HPA axis.

Introduction

The adrenal glucocorticoid response to stress requires activation of the hypothalamic-pituitary-adrenal (HPA) axis [44,45]. More specific, the hypothalamic paraventricular nucleus (PVN) corticotrophin-releasing (CRH) neurons secrete CRH peptide in the hypophyseal portal system; this portal vasculature system links PVN CRH neurons to the anterior pituitary. CRH stimulates anterior pituitary corticotrophs to release adrenocorticotrophin releasing hormone (ACTH). Secreted ACTH travels in the systemic circulation of the body, subsequently causing the production and secretion of the endogenous adrenal glucocorticoid of rats corticosterone (CORT). Stress-stimulated release of CORT depends on the hypothalamic PVN CRH neurons to coordinate excitatory and inhibitory inputs from the perlimbic (PrL) and infralimbic (IL) subregions of the medial prefrontal cortex (PFC), as well as other limbic forebrain regions (amygdala and hippocampus) [156,157]. These CRH neuroendocrine neurons utilize intracellular signaling proteins to translate stress induced excitatory input from the medial PFC and other forebrain regions into appropriate intracellular responses (i.e. CRH release) [46,47]. In particular, stress-stimulated PVN CRH neuron activity is tightly coupled with an increase in the phosphorylation (activation) of the extracellular-regulated kinase (pERK1/2) signaling protein [47,107,135,158]. Recently, pERK1/2 has been shown to be responsible for modulating the firing rate of PVN CRH neurons, which altered downstream CRH synthesis and release [47]. We have demonstrated that an acute exposure to psychological stress (restraint) increased the phosphorylated form of ERK1/2 (as measured by cell counts) in PVN CRH neurons, and this increase in pERK1/2 was constrained by tonic glucocorticoid activity (chapter 2).

ERKs are one of many singling proteins within the mitogen-activated protein kinase (MAP-kinases) pathway. MAP-kinases are inactivated by an intracellular signaling system

involving MAP kinase phosphatase (MKP-1) family of dual specificity phosphatases (Camps et al., 2000). MKP-1 phosphatases inactive pERKs by dephosphorylating the tyrosine and threonine residues [159]. Inactivation of pERK dependent inflammatory processes in peripheral cell types require glucocorticoid induced transcriptional upregulation of the *mkp-1* gene [160,161]. In mast cells glucocorticoids are necessary for the suppressive control over pERK1/2 induced pro-inflammatory processes by directly increasing the upregulation of *mkp-1* gene expression at the promoter region and reducing proteasomal degradation of MKP-1 proteins [87]. Similarly, glucocorticoids inhibit MAP-kinase activation by upregulating *mkp-1* mRNA in fibroblast and osteoblast cells [88,162]. Glucocorticoids also mediate anti-inflammatory effects in immune cells by directly enhancing the upregulation of *mkp-1* gene expression [110] Also, the tumor suppressor p53 protein prevents MAP-kinase mediated cellular proliferation by upregulating *mkp-1* gene expression [111].

Glucocorticoids may also inactive pERK through the transcriptional upregulation of the *mkp-1* gene within neuronal and endocrine cell populations that are associated and/or required for stress-stimulated hormone secretion (e.g. medial-PFC, PVN and pituitary). There is limited characterization profiling *mkp-1* gene expression within the medial-PFC, PVN or anterior pituitary after exposure to an acute psychological stress challenge coupled with glucocorticoid manipulations (tonic and phasic). Therefore, we tested how tonic and phasic glucocorticoid activity influenced *mkp-1* mRNA expression within the medial-PFC, PVN and anterior pituitary. It is important to note that both tonic and phasic adrenal glucocorticoid activity differentially regulate the PVN and anterior pituitary responses to stress [67,158]. Tonic activity is characterized by a circadian variation in basal circulating levels of glucocorticoids, which

constrain the HPA hormonal response to acute stress [44,59,158]. In contrast, phasic actions are attributed to the rapid and transient increase in glucocorticoids evoked by stress, which produce additional potent inhibitory action over HPA axis response to stress [44,67,68].

Preliminary results demonstrated that PVN *mkp-1* mRNA expression was significantly upregulated after exposure to an acute psychological stress challenge. However, it was unclear if this increase in *mkp-1* mRNA expression was the result of stress or the phasic increase in glucocorticoids that accompany the acute stress challenge, and how tonic glucocorticoids influence *mkp-1* gene expression. In a follow up experiment, we examined if medial-PFC (PrL and IL cortex), PVN and anterior pituitary *mkp-1* gene induction was dependent on stress or glucocorticoid activity by removing endogenous glucocorticoids (adrenalectomy) from select groups of rats and then exposing some of those rats to restraint. To further examine whether the induction of *mkp-1* mRNA in the medial-PFC and PVN had any dependency on the phasic increase in glucocorticoids, we injected an acute dose of CORT 1 or 3 hours before decapitating rats. The timing between 1 and 3 hours is sufficient to allow for glucocorticoids to significant increase *mkp-1* gene expression in mast cells [87].

Methods

Animals

Young adult male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IA) weighing between 280 and 305 g at time of experimentation were housed two per-cage. The colony room lights were regulated on a 12 hour light/dark cycle and rats were given rat chow (Purina Rat Chow, Ralston Purina, St. Louis, MO, USA) and tap water ad libitum. Rats were allowed at least a 2 week acclimation to the colony room before any initiation of experimental procedures. All experiments were performed during the first half of the rats' inactive period, when basal CORT secretion is at its circadian trough. Handling and testing of all rats conformed to ethical procedures approved by the University of Colorado Institutional Animal Care and Use Committee.

Surgery

Rats were adrenalectomized bilaterally (ADX) or were sham-ADX. After being anesthetized with halothane, rats received bilateral incisions made through the skin and peritoneal wall next to each kidney. Sham-ADX rats experienced the same procedure as ADX rats, but adrenal glands were not removed. Moreover, all ADX and sham-ADX rats were given 3 days to recover from surgery before the experimental test day. ADX rats were given 0.9% saline drinking water ad libitum.

Restraint Stress

Stress challenge (except experiment 3) consisted of placing rats in clear plexiglas tubes (23.5 cm in length and 7 cm in diameter; with multiple air holes). The size of the tube restricted lateral, forward and backward movement but did not interfere with breathing. Restraint is widely

accepted as a psychological stressor within the stress neurobiology field, which has not only a conceptual basis, but is also supported by neurocircuit activity studies [140].

Experimental Procedures

Preliminary experiment 1: effect of stress over the expression of PVN *mkp-1* mRNA. Using 11 rats, we examined PVN *mkp-1* mRNA levels after rats were exposed to two different durations of stress and third group of control rats that were not subjected to stress. Two groups of rats (n = 4) were exposed to restraint stress for either 15 or 30 minutes. Immediately after restraint, these rats were killed by guillotine decapitation. A third group of control rats (n = 3) were used for a non-stress comparison, and were not disturbed until decapitation. Immediately after decapitation, all brains were immediately removed and flashed frozen in isopentane (between -30 and -40 C), after which brains were stored in a -80 C freezer for later analysis.

Experiment 2: effect of long term absence of endogenous glucocorticoid (ADX) and acute stress challenge (restraint) on ACTH secretion, PVN, anterior pituitary and PFC (PrL and IL) *mkp-1* mRNA expression. On testing day, both ADX and adrenal intact rats were either subjected to 30 minutes of restraint or left in their respective homecage for a non-stress control comparison group (2x2 between subjects factorial design; n=6; N=24). Immediately after decapitation, brains were immediately removed and flash frozen in isopentane (between -30 and -40 C), after which brains were stored in a -80 C freezer for later analysis. In addition, trunk blood was collected into EDTA coated tubes (vacutainer, Becton-Dickinson), whereby, all samples were centrifuged for 15 min 4 C temperature in order to separate plasma from the red blood cells. Plasma samples were then aliquoted into microfuge tubes and were immediately snap frozen on dry ice.

<u>Experiment 3</u>: effect of a phasic 1 or 3 hour corticosterone (CORT) pretreatment on medial-PFC and PVN *mkp-1* mRNA expression of non-stressed adrenal intact rats. This experiment was comprised of 4 separate treatment groups (2 x 2 factorial between-subjects design; n=6; N=24 rats). Treatment factors consisted of a drug pretreatment group (CORT or vehicle) and Drug pretreatment time point (1 or 3 hour). On test day, rats were injected with an acute dose of CORT (2.5 mg/kg, i.p.) or vehicle i.p. 1 or 3 hours before decapitation. This exogenous CORT treatment procedure produces plasma CORT levels in rats that closely match the endogenous CORT levels and time course associated with a moderate intensity stressor, such as restraint (Pace et al., 2009), while also not producing added stress to the rats (i.e. intraperitoneal injections do not increase CORT secretion) (Osterlund and Spencer, 2011). After each injection, rats were returned to their home cage and home room. Paired cage mates rats received either a vehicle or CORT drug treatment, all cage mates were placed into the same injection time condition to minimize any disturbance that may be caused by removing a rat from its cage mate at injection or sacrifice time. This procedural measure ensured that blood and tissue samples were collected under the same conditions. Trunk blood collection and brain extraction were rapidly performed after decapitation in an area adjacent from the home room (note pituitary were not taken). Test day procedures occurred during lights on between 0800 and 1300 hour or the inactive period of rats, and time of day was counterbalanced across treatment groups. Rats were habituated to both i.p. injections by poking rats with the blunt end of a 1 ml syringe (no needle attached) for 2 min over a 3-day period before testing. CORT was purchased from Steraloids Inc. (Newport, RI, USA) and dissolved in vehicle (10% ethanol, 30% propylene glycol and 60% sterile saline)

ACTH radioimmunoassay

Trunk blood was collected into ethylenediaminetetraacetic acid-containing vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ, USA), placed on wet ice and then centrifuged for 15 min (4 °C). Plasma was then aliquoted into microfuge tubes and snap-frozen on dry ice. The blood processing procedure was completed within 45 min after decapitation. ACTH (pg /ml) was determined in duplicate (100 ul of plasma) by competitive radioimmunoassay protocol as described previously [67]. Radiolabeled 125-I ACTH-Tracer was obtained from DiaSorin (catalogue #20515; DiaSorin, Stillwater, MN, USA) and primary ACTH anti-serum Rb 7 (diluted to a final concentration of 1:30 000) was provided courtesy of Dr W. Engeland (University of Minnesota, twin cities campus, department of Neuroscience). The detection limit for this assay was 15 pg/ml; the intra-assay coefficient of variability was 4%.

in situ Hydridization assay (ISH)

Coronal PVN brain sections (post fixed and processed as described above) were floated in 0.5 M PBS and mounted onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Tissue was then further fixed in 4% PFA for 30 minute at room temperature and then processed as previously published [163]. Generation of 35S-UTP labeled cRNA probes for *mkp-1* mRNA used a cDNA template corresponding to a 466 nucleotide mature sequence (for exact genomic location enter the blast identification code AF357203 and NM053769 at the National Center for Biotechnology Information-NCBI blast website http://blast.ncbi.nlm.nih.gov) plasmid containing cDNA was generated within the Spencer lab. *mkp*-1 mRNA probes were subsequently linearized from pGEM-3 vectors (Promega, Madison, WI) with the restriction endonuclease HindIII and transcribed using T7 RNA polymerase. The identity of *mkp-1* cloned DNA was verified by DNA

sequencing (University of Colorado Molecular, Cellular and Developmental Biology sequencing facility). After the hybridization assay procedure, slides were exposed to x-ray film (Kodak Biomax MR film) for 14 days. Semiquantitative analyses were performed on digitized images from x-ray films using the linear range of the gray values obtained from an acquisition system (Northern Lights lightbox, model B 95, Ontario, Canada; CCD camera, model XC-77, Sony, Tokyo, Japan; image capture with National Institutes of Health scion Image v1.59 software) as previously described [143]. Regions of interests (PVN, anterior pituitary, prelimbic cortex, and infralimbic cortex) were determined by matching digitized rat hypothalamic structures to rat brain atlas diagrams [142]. Quantification and analysis of images were performed by individuals that were blind to treatment condition assignments. For the PVN, measurements for four sections were taken for both hemispheres of the brain for all rats (4-8 measurements for each rat brain). Measurements were also taken for four to six sections of the anterior pituitary. For the medial-PFC prelimbic and infralimbic subregions, measurements were taken from four to six tissue sections were used from both hemispheres (4-12 measurements fir each rat brain). Optical densities for each region of interest were measured using the program ImageJ provided by National Institutes of Health (version 1.42q, http://rsb.info.nih.gov/in). Graphs showing relative differences between groups were created by measuring the average uncalibrated optical density for each rat and then averaging the those densities of each rat to give an overall mean value for that treatment effect.

Statistical Analysis

A one-way (preliminary experiment 1) or two-way (experiment 2 and 3) factorial analysis of variance was calculated for dependent measures using the Statistical Package for the Social

Sciences (Chicago, IL, USA) software program for the Macintosh. When warranted significant F-test results were followed with a Fishers Least Significant Difference (FLSD) post hoc test in order to assess significant differences between pairs of groups. An alpha-level of $P \leq 0.05$ was used to determine statistical significance.

Results

Preliminary Experiment 1: PVN mkp-1 mRNA significantly increased after a 15 and 30 minute restraint challenge.

Restraint significantly increased PVN *mkp-1* mRNA ($F_{(3,11)} = 4.2$, $P < 0.05$) (figure 3.1) Post-hoc tests indicate that restraint of 15 minutes produced a robust increase in *mkp-1* mRNA expression when compared to non-stressed rats, additionally restraint of 30 minutes also produced a robust increase in *mkp-1* levels when compared to both non-stressed rats and rats that were restrained for 15 minutes. It is noteworthy to point out that the PVN expressed almost undetectable levels of MKP-1 mRNA during a non-stressed state.

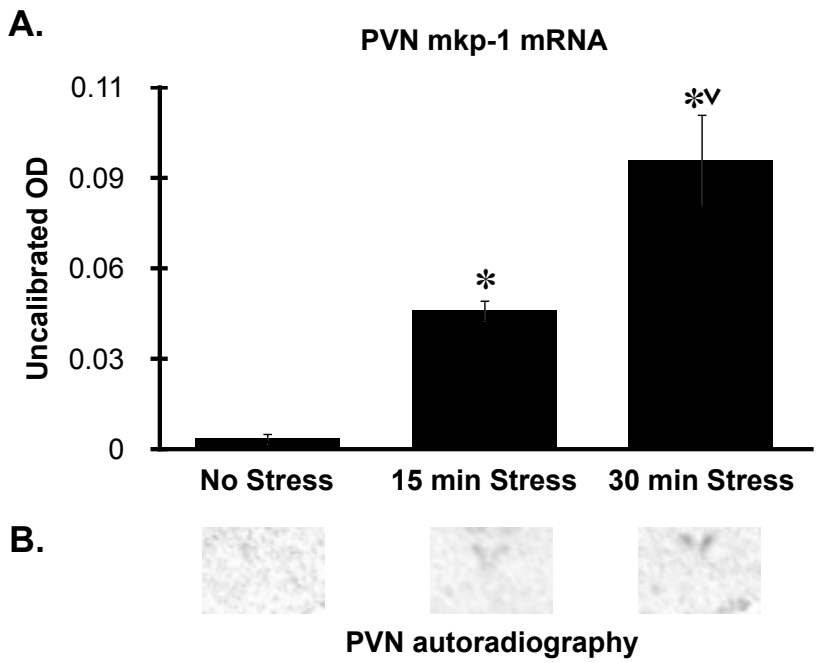

Figure 3.1 Stress induced PVN *mkp-1* mRNA expression. PVN *mkp-1* mRNA was significantly increased after an acute restraint challenge of 15 minutes when compared to non-stressed rats. Rats that were in restraint for 30 minutes displayed a significantly increases in *mkp-1* mRNA levels compared to both basal and 15 minute restraint groups. No *mkp-1* mRNA expression was detected during basal conditions. *, represents a significant stress effect compared to the no stress group, and V, represents a significant stress effect compared to 15 minute stress group, $p < 0.05$, FLSD.

Experiment 2: long-term absence of endogenous glucocorticoids (ADX) augmented stress-stimulated ACTH secretion, as well as mkp-1 mRNA expression in the PVN and anterior pituitary, but not medial-PFC (PrL and IL cortex).

We surgically (ADX) removed endogenous glucocorticoids (phasic and tonic) from select rats to assess whether glucocorticoids regulate *mkp-1* mRNA expression within the PVN and anterior pituitary, as well as the PrL and IL surgeons of the medial-PFC during both basal and stress-stimulated states. As expected, restraint challenged rats displayed an increase in ACTH secretion (stress effect: $F_{(1,19)} = 78.3$, $p<0.05$), and the absence of glucocorticoids resulted in a substantial increase in basal and stress-stimulated ACTH secretion (ADX effect: $F_{(1,19)} = 33.8$, $p<0.05$) (figure 3.2).

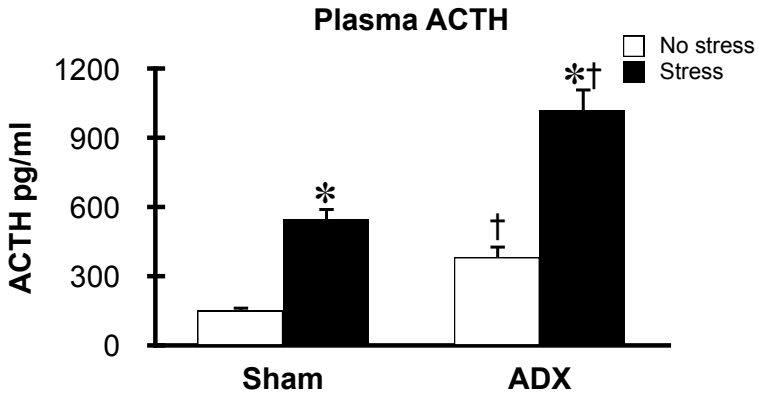

Figure 3.2 ADX induced augmentation in basal and stress-induced ACTH. ACTH secretion was increased by 30 minute restraint. ADX resulted in an augmented increase in both basal and stress-stimulated ACTH secretion. Both basal and stress-stimulated ACTH secretion were substantially increased in ADX rats. *, represents a significant stress effect compared to the no stress group within the same surgical condition ($p < 0.05$, FLSD); †, represents a significant tonic CORT condition (ADX) effect compared to the sham group within same stress condition ($p < 0.05$, FLSD). Below panel are representative autoradiograms for in situ hybridization of PVN (left) and PFC mkp-1 mRNA expression, arrows indicate region of interest.

Under non-stress conditions PVN and anterior pituitary *mkp-1* gene levels were next to undetectable (figure 3.3 a,b). Rats challenged with 30 minutes of restraint displayed a significant increase in *mkp-1* mRNA expression within both PVN (stress effect: $F_{(1,19)} = 37.4$, $p<0.05$) and anterior pituitary ($F_{(1,19)} = 29.8$, $p<0.05$), figure 3a and b. ADX augmented stress-stimulated *mkp-1* gene expression within the PVN (stress x ADX interaction $F_{(1,19)} = 11.0$, $p<0.05$) and anterior pituitary (stress x ADX $F_{(1,19)} = 5.1$, $p<0.05$), but had no effect on basal levels of *mkp-1* mRNA activity (figure 3.3 a,b).

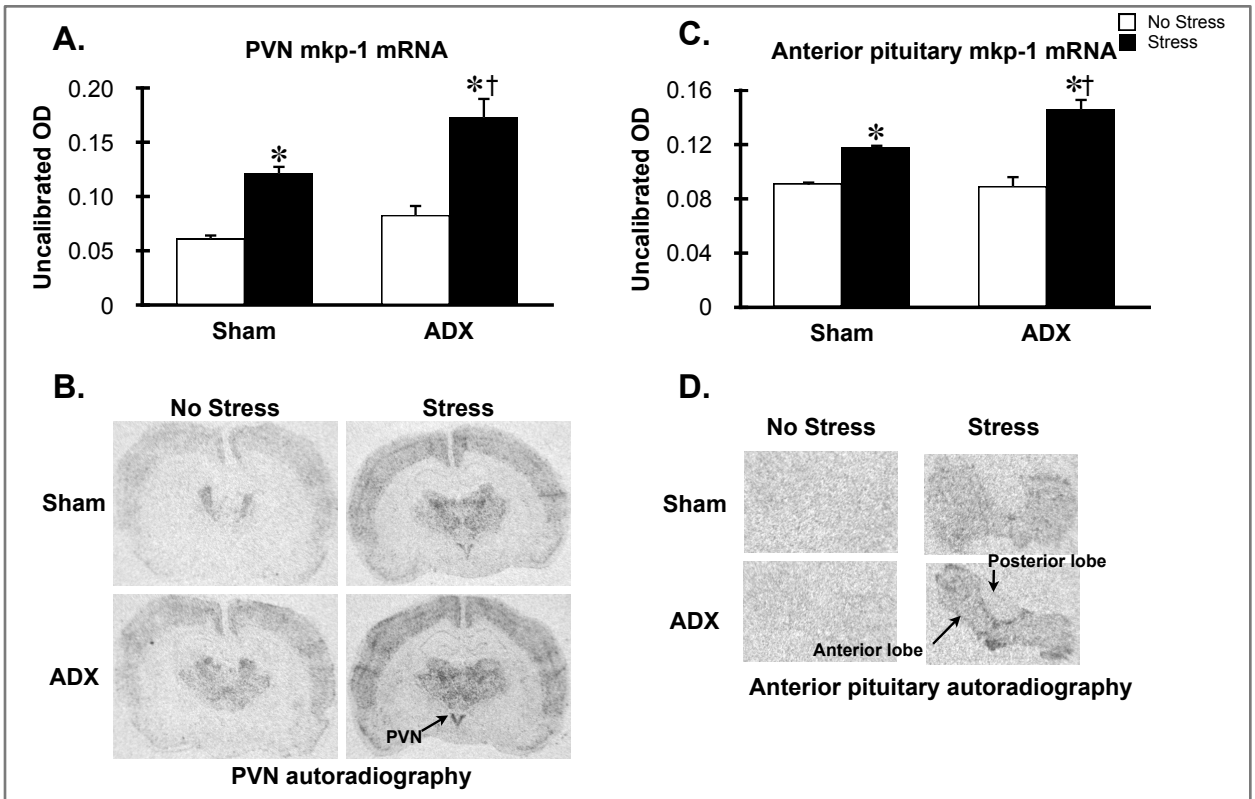

Figure 3.3 Effect of ADX on PVN and anterior pituitary *mkp-1* mRNA expression. Restraint (30 minutes) significantly increased both PVN (A) and anterior pituitary (B) mkp-1 mRNA. ADX resulted in a marked increase in stress-induced mkp-1 levels in both the PVN and anterior pituitary, though ADX did not increase basal PVN nor anterior pituitary mkp-1 expression. *, represents a significant stress effect compared to the no stress group within the same surgical condition ($p < 0.05$, FLSD); #, represents a significant tonic CORT condition (ADX) effect compared to the sham group †, within same stress condition ($p < 0.05$, FLSD). Below panel A and B are representative autoradiograms for in situ hybridization of PVN and anterior pituitary mkp-1 mRNA expression, arrows indicate region of interest.

Non-stressed rats displayed constitutive basal *mkp-1* gene expression within both PrL and IL subregions of the medial-PFC. An acute stress challenge (30 minutes of restraint) significantly increased *mkp-1* mRNA in the PrL cortex (stress effect: $F_{(1,19)} = 14.7$, $p<0.05$) and the IL cortex ($F_{(1,19)} = 42.8$, $p<0.05$) (figure 3.4a,b). We found that ADX did not result in a significant alteration in basal or stimulated PrL cortex *mkp-1* gene expression. However, the loss of glucocorticoids activity did increase basal IL cortex *mkp-1* gene expression, but did not alter the stress-induced increase in *mkp-1* levels $F_{(1,19)} =7.9$, $p<0.05$, although there appears to be a similar trend to PVN *mkp-1* mRNA expression.

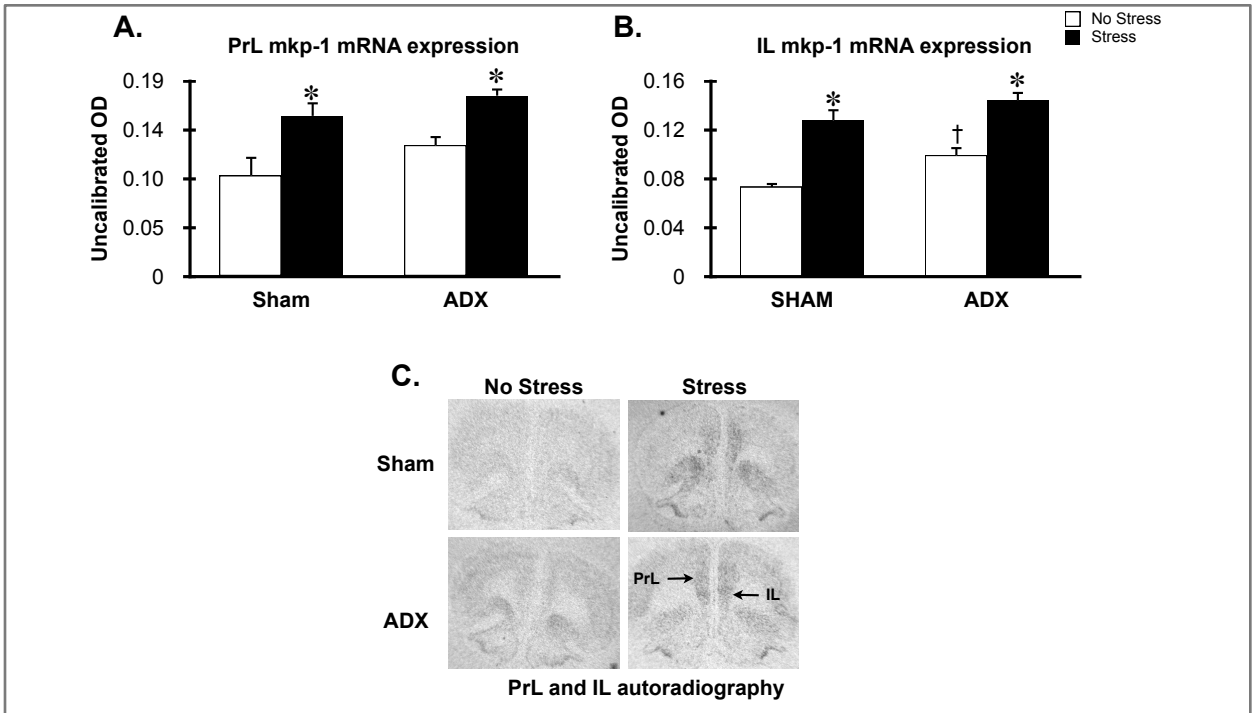

Figure 3.4 Effect of ADX on PrL and IL PFC mkp-1 mRNA expression. Both the prelimbic (PRL, panel A) and infralimbic (IL, panel B) subregions of the PFC displayed constitutive mkp-1 gene expression, and 30 minutes of restraint significantly increased basal PrL and IL mkp-1 mRNA levels. The loss of glucocorticoid activity via ADX resulted in selective augmentation in IL basal mkp-1 mRNA levels (B), but not PrL mkp-1 levels (A). ADX did not alter stress-induced mkp-1 mRNA in the PrL (A) cortex or the IL (B) cortex. *, represents a significant stress effect compared to the no stress group within the same surgical condition ($p < 0.05$, FLSD); †, represents a significant tonic CORT condition (ADX) effect compared to the sham group within same stress condition ($p < 0.05$, FLSD). Below panel A and B are representative autoradiograms for in situ hybridization of PrL and IL cortex mkp-1 mRNA expression, arrows indicate region of interest.

Experiment 3: a phasic increase in CORT did not upregulate PVN or medial-PFC mkp-1 mRNA levels 1 or 3 hours after treatment.

To assess the extent to which a phasic rise CORT would be sufficient to upregulate *mkp-1* gene transcription in either the PVN or medial-PFC we pretreated adrenal intact rats with vehicle or CORT 1 or 3 hours before decapitation. As mentioned both injection time points do not produce added stress to the rats resulting in stimulated adrenal CORT secretion [67]. We found that an acute CORT pretreatment 1 hour or 3 hour did not upregulate *mkp-1* gene expression within the PVN nor enhance the constitutive *mkp-1* expression in the medial-PFC, figure 5. Similar to non-stressed conditions in experiment 1 and 2 we found that there is almost undetectable levels of *mkp-1* mRNA within the PVN. Also, *mkp-1* mRNA expression within the medial-PFC was transiently reactive to the handling of the rat as the 1 hour pretreatment enhanced *mkp-1* mRNA expression compared to the 3 hour pretreatment group. This transient increase in *mkp-1* levels was not dependent on CORT treatment, but rather injection time point as both drug treatments in the 1 hour group displayed similar increases in gene expression, (injection time point: $F_{1,10} = 2.4$, $P < 0.05$).

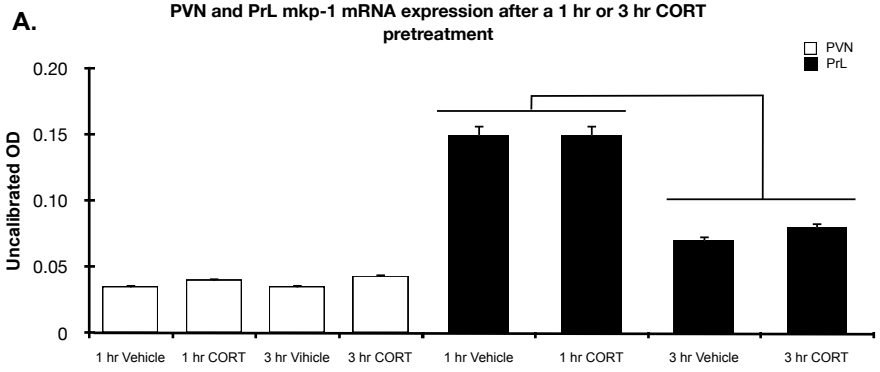

Figure 3.5 Effect of Phasic CORT on PVN and medial PFC *mkp-1* mRNA expression. Phasic CORT treatment 1 or 3 hours after administration did not increase prefrontal (PFC) nor PVN *mkp-1* levels. Although, the PFC displayed strong basal *mkp-1* expression, but not the PVN. The PFC *mkp-1* basal expression was slightly increased in response to 1 hour drug injection, $p < 0.05$, FLSD.

Discussion

We found that *mkp-1* mRNA transcription was increased in an experience-dependent fashion within the intrinsic cell populations of the HPA axis (PVN and anterior pituitary) and extrinsic medial-PFC (PrL and IL cortex). Exposure to psychological stress (restraint) produced a significant upregulation of *mkp-1* mRNA expression in the PVN, anterior pituitary and medial-PFC (PrL and IL). During a non-stimulated state both PVN and anterior pituitary displayed undetectable levels of *mkp-1* mRNA, however, the medial-PFC displayed constitutive basal *mkp-1* mRNA expression. We also found that the long-term absence of endogenous glucocorticoids (via ADX) augmented stress-induced *mkp-1* gene expression within the PVN and anterior pituitary, but not in the PrL or IL. In addition, we found that a phasic increase in glucocorticoids did not upregulate PVN or medial-PFC *mkp-1* gene levels. Taken together these results suggest that stress-induced *mkp-1* expression plays a role in activity-dependent regulation of MAP-kinase activity in both extrinsic and intrinsic anatomical sites to the HPA axis, and tonic glucocorticoid activity regulates PVN and anterior pituitary *mkp-1* transcription in a suppressive fashion, but not medial-PFC *mkp-1* gene expression.

MAP-kinases are essential intracellular signal transduction proteins that couple extracellular excitation to an array of different cellular functions (e.g. axonal branching, cellular proliferation, cellular apoptosis, and cellular differentiation). MAP-kinases also perpetuate proinflammatory processes in a number of different cells such as, fibroblast cells, mast cells and glial cells [87,161,164]. MKP-1 proteins are important and necessary intracellular regulators of MAP-kinases. Interestingly, glucocorticoids provide anti-inflammatory effects in those cells by suppressing MAP-kinase signaling activity through the transcriptional upregulation of the *mkp-1* gene at the promoter level, while in some cases also reducing proteasomal degradation of

functional MKP-1 proteins [87]. MAP-kinases are also important intracellular mediators of stress-induced excitatory processes in PVN CRH neuroendocrine neurons, as MAP-kinases couple extracellular stress signals to intracellular neurohormone transcriptional and translational processes, as well as neurohormone release [47,107,135]. It has not previously been determined whether glucocorticoids regulate stress-induced MAP-kinase activity within the PVN, anterior pituitary or medial-PFC by transcriptionally upregulating the *mkp-1* gene. We provide evidence in this study that exposure to acute psychological stress results in the transcriptional upregulation of the *mkp-1* gene within PVN, anterior pituitary and medial-PFC and that glucocorticoids constrain stress induced PVN and anterior pituitary *mkp-1* mRNA levels.

Our preliminary experiment showed that PVN *mkp-1* gene expression was rapidly induced within 15 minutes of an acute stress challenge and is significantly increased as the duration of stress continued (30 minutes after stress onset), this is characteristically similar to the expression profile of other PVN immediate early genes (e.g. *c-fos*, *zif268* and *ngfi-b*) [58,59]. Duman et al [28,165]. MKP-1 is also increased in an experience-dependent fashion, albeit this increase was under extreme experimental conditions (e.g. electric shock treatment and chronic unpredictable stress). We show in this study that the exposure to a moderate acute psychological stressor (restraint) [93,166] not only significantly increased *mkp-1* levels in the PVN, but also in the medial-PFC (PrL and PVN). This increase in PVN and medial-PFC *mkp-1* mRNA levels was not dependent on a phasic increase in glucocorticoids or on the prior presence of tonic glucocorticoids, as neither the exogenous glucocorticoid pretreatment (1 hour or 3 hour) or the removal of endogenous glucocorticoids resulted in an upregulation *mkp-1* gene expression in both forebrain regions measured. Despite the potent inhibitory actions that phasic glucocorticoids

provide over stress-induced PVN *crh* gene transcription and corticotroph ACTH secretion, these suppressive actions are not associated with an inhibitory effect over stimulated PVN MAP-kinase activity [158]. Taken together these findings demonstrate that neither phasic nor tonic glucocorticoid activity regulate stress dependent activation of PVN MAP-kinases by increasing *mkp-1* gene transcription. Rather, stress-induced upregulation of *mkp-1* mRNA transcription indicates that the *mkp-1* gene serves as an activity-dependent intracellular regulatory mechanism for MAP-kinases within the PVN and medial-PFC.

In this study, the long-term absence of glucocorticoid resulted in an augmentation in stress-induced *mkp-1* gene expression in the PVN and anterior pituitary, but not in the medial-PFC (PrL and IL cortex). Consistent with previous studies (Osterlund et al., 2011, Pace et al., 2009), we also found that the absence of tonic glucocorticoids also produced a large increase in basal and stimulated ACTH levels. However, in this study ADX did not alter basal *mkp-1* gene expression within the PVN or anterior pituitary but did produce an augmentation in stress-induced *mkp-1* gene expression within the neuroendocrine and endocrine cell populations of the HPA axis. These results suggest that stress-stimulated *mkp-1* expression within the PVN and pituitary are constrained by glucocorticoids. In support of this, the stress-dependent induction of immediate early genes *c-fos*, *zif268* and *ngfi-b* within the PVN all remain unaltered during non-stimulated conditions, regardless of the absence of endogenous glucocorticoids, but are significantly augmented in ADX rats exposed to stress [58,59,153]. These immediate early genes are not known to contain a glucocorticoid response element, suggesting that glucocorticoids indirectly modulate these genes during stress. These results indicate that stress-induced hypothalamic PVN gene expression are predominately constrained by glucocorticoid activity,

and the absence of this particular glucocorticoid-dependent regulatory influence results in a significant increase in stimulated PVN gene activity.

In contrast to the ADX induced augmentation of PVN gene expression, the extrinsic medial-PFC neural circuits that participate in the regulatory control of HPA system during stress exposure [156,157,167] are not altered by the long-term absence of glucocorticoids. For example, stress (30 minute restraint) significantly increased the immediate early genes *arc* and *c-fos* mRNA levels within the medial-PFC, however, these experience-dependent genes remained unaffected in ADX rats exposed to same stress conditions [168]. In addition, we did not find an augmentation in the stress-induced upregulation of the *mkp-1* gene within the PrL or IL of ADX rats. This suggests that the stress-dependent activation of the medial-PFC is not generally regulated by tonic glucocorticoids. Our finding also indicate that stress-induced activation of the medial-PFC MAP-kinase pathway is regulated by the stress-dependent increase in *mkp-1* gene expression and not by glucocorticoids [169]. Further, this supports that the observed ADX induced augmentation in stress-stimulated HPA axis activity (as measured by PVN gene induction and ACTH release) is due to a lack of glucocorticoid constraint over the hypothalamic responsiveness to stress, rather than an ADX induced augmentation in the extrinsic medial-PFC neural inputs that are responsible for appropriate activation of the HPA system in response to stress.

Whether glucocorticoids influence transcriptional processes is highly dependent on differences in cellular function and location. For example, glucocorticoids provide suppressive transcriptional regulation of the *crh* gene in the PVN CRH neuroendocrine neurons [170], but in other forebrain regions such as the amygdala and bed nucleus of the stria terminalis

glucocorticoids either upregulate *crh* gene expression or have no effect [171]. In this regard glucocorticoid regulation of the *mkp-1* gene is similar, as the *mkp-1* expression varies widely. Depending on cell type and anatomical location, *mkp-1* gene expression is either upregulated by glucocorticoids [87,88,160], or is induced by a number of different stimulating factors [172]. As already mentioned, glucocorticoids provide anti-inflammatory effects in peripheral cells like fibroblast and mast cells by suppressing MAP-kinase signaling activity through transcriptional upregulation of the *mkp-1* gene. Similarly, glucocorticoids can suppress hippocampal inflammation processes through MKP-1 dependent upregulation in rat microglia cells [173]. Alternatively, cannabinoids may also protect neurons from inflammatory damage by transcriptionally increasing *mkp-1* mRNA expression in cultured microglia cells [174]. Brain-derived neurotrophic factor (BDNF) has also been shown to upregulate hippocampal *mkp-1* mRNA during neuronal development [2,175]. Also, during the induction phases of long-term potentiation, hippocampal *mkp-1* mRNA is also rapidly upregulated [106]. We show in this manuscript that forebrain (medial-PFC and PVN) and anterior pituitary *mkp-1* gene expression is upregulated in an experience-dependent fashion (i.e. via stress) and is tonic glucocorticoids constrain this expression rather than increase its transcription.

Initially, we did not expect that the medial-PFC or PVN *mkp-1* expression would be unresponsive (not upregulated) to phasic glucocorticoid treatment. Rather, we predicted that this phasic increase in glucocorticoids would produce a robust increase in neuronal and endocrine *mkp-1* levels. Clearly, under acute stress conditions both tonic and phasic glucocorticoids do not mediated the upregulation of medial-PFC, PVN and anterior pituitary *mkp-1* expression, and thereby do not serve as a mechanism for direct glucocorticoid negative feedback regulation of

medial-PFC, PVN or anterior pituitary MAP-kinase activity. Instead, the upregulation of *mkp-1* in the medial-PFC, PVN and pituitary serves as an activity-dependent intracellular regulatory mechanism for the modulation of stress activated MAP-kinases. MAP-kinase have been proposed to upregulate *mkp-1* expression [87,176], and can stabilize functional MKP-1 proteins from rapid degradation [87]. Whether, MAP-kinase activation is sufficient to mediate stress-induced upregulation of medial-PFC, PVN or anterior pituitary *mkp-1* gene expression in an experience-dependent fashion remains to be determined. These findings indicate that the interaction between MAP-kinases and *mkp-1* are dynamic and require further comprehensive examination, but herein we further demonstrate that glucocorticoid upregulation or down-regulation of *mkp-1* gene expression is dependent on cellular function and location.

Chapter IV

The hypothalamic-pituitary-adrenal axis response to an acute psychological stress challenge is determined by the timing of corticosterone exposure relative to the onset of stress.

Abstract

A phasic increase in corticosterone (CORT) secretion suppresses stress-stimulated hypothalamic-pituitary-adrenal (HPA) axis hormone release through mechanistically distinct temporal domains that are referred to as fast or intermediate CORT negative feedback (e.g. protein synthesis independent or dependent, respectively). Studies in the 1960s and 1970s proposed that the suppressive actions of fast and intermediate negative feedback were separated by an intervening time-period in which glucocorticoid exposure before (between 10 to 45 min) failed to produce a functional negative feedback effect over stimulated HPA hormone secretion. It was also proposed that the induction of fast negative feedback depended on the rate at which glucocorticoid blood levels increased. However, most of those studies relied on indirect measures of hormone levels and in vivo studies were typically conducted under non-physiological conditions. There has been a marked lack in examining whether some of those temporal phasic glucocorticoid phenomena occur under the same stressor and same glucocorticoid treatment. Also, most of those studies focused on using pretreatment paradigms that separated the effects of glucocorticoids from the stress. Thus there is little documentation of whether glucocorticoids produce a measurable negative feedback effect during stress. Using jugular catheterized young adult male Sprague-Dawley rats, we tested the ability of phasic CORT (0.3mg/kg) treatment prior to stress onset (1 hour, 15 minute, or 30 second) or 5 minutes post-stress onset to suppress the initial and ongoing adrenocorticotropic hormone (ACTH) response to restraint. Also, we measured stress-induced prolactin secretion, paraventricular nucleus (PVN) and anterior pituitary *c-fos* mRNA expression, and corticotroph *pomc* hnRNA expression. Although, prolactin is not secreted via the HPA axis, it is responsive to stress and provides useful information about spatial CORT negative feedback effects. We found that the

preexposure to CORT an hour to seconds prior to stress prevented the ACTH initial and ongoing response to that stress challenge. However, CORT exposure during stress did not provide a further suppressive effect over ACTH. In follow up experiment we adrenalectomized (ADX) rat to remove their endogenous adrenal CORT response and selectively treated these rats with phasic CORT or vehicle 5 minute after stress onset. We found that ADX treated with CORT during stress resulted in a delayed (timed) shutoff over the ACTH ongoing response to stress, but not the initial response. We also found that none of our suppressive phasic CORT effects over ACTH secretion were coupled to an additional suppressive effect over stress-induced PVN and anterior pituitary *c-fos* mRNA expression, nor stress-stimulated prolactin secretion. However, our suppressive phasic CORT effects were all accompanied by an inhibition over *pomc* hnRNA. Overall these results suggested that phasic CORT activity before the onset of stress almost completely prevented subsequent ACTH response to stress. Stress evokes a transient resistance over the initial HPA hormone response to phasic CORT treatment during stress. However this CORT treatment reverently produced a delayed (timed) suppressive effect over the continued ACTH response. The experience-dependent gene expression and prolactin results all suggest that exposure to CORT before or during stress produces suppressive effects at the level of the HPA axis and do not regulate the stress induced stimulatory drive to the PVN.

Introduction

The stress-dependent release of glucocorticoids begins with the most integrative component of the hypothalamic-pituitary-adrenal axis (HPA axis) axis, the corticotropin-releasing-hormone (CRH) neurons located in the medial hypothalamic region of the paraventricular nucleus (PVN). These neuroendocrine neurons secrete CRH through stress-dependent mechanisms that lead to release of CRH peptide into the hypophyseal portal system located in the median eminence. CRH activates endocrine corticotroph cells to release adrenocorticotropic hormone (ACTH) into systemic circulation. Stress leads to a somewhat sustained secretion of ACTH, which has an extremely high clearance rate (~3-4 minute half-life) [48]. Stress-stimulated ACTH secretion subsequently leads to the production and secretion of glucocorticoid hormones, (cortisol in humans and corticosterone in rats). This Stress-dependent phasic corticosterone (CORT) secretion pattern is phasic because it produces a rapid and transient increase in CORT, which also results in a potent negative feedback effect on the HPA axis [44,67,68].

A large portion of classic and current glucocorticoid negative feedback research has focused on isolating the effects of phasic glucocorticoids from stress effects on HPA axis activity by pretreating animals with an acute exogenous dose of glucocorticoids prior to HPA axis stimulation [45,49,69-72,79,85,177-179]. This particular glucocorticoid manipulation has generated a considerable body of evidence which suggests that the regulatory actions of phasic glucocorticoids is manifest through mechanistically discrete temporal patterns of onset and duration [45,46,49,67,69]. This classic model of phasic glucocorticoid negative feedback function was proposed originally by Dallman and Yates [45,49,52,54,55]. They provided

evidence indicating that negative feedback actions of glucocorticoids fundamentally operate in distinct timeframes that were referred to as intermediate and fast negative feedback.

Both *in vitro* and *in vivo* evidence indicate that intermediate glucocorticoid negative feedback effects depend on genomic (transcriptional and translational) processes [67,81,86,96,180,181]. Intermediate feedback has a delayed induction phase of ~1 hour, and has suppressive actions over stimulated HPA axis activity that persist for hours [3,67]. In contrast, fast glucocorticoid negative feedback has a rapid induction phase that is dependent on non-genomic cellular alterations (protein-protein interactions), which produce an immediate suppressive action over stimulated HPA axis activity [72,75-78]. Dallman and Yates believed that fast feedback only lasted for several minutes (<10) because the suppressive effect only persisted as long as there was an increase in the rate of glucocorticoid secretion, and the suppressive effect was lost once glucocorticoid levels peaked and were no longer rapidly increasing (i.e. fast negative feedback is rate-sensitive) [45,182]. Other studies have suggested that fast glucocorticoid actions are dependent on the rate in which glucocorticoid increases, in contrast, intermediate glucocorticoid actions are dependent on the magnitude of glucocorticoid concentration [50,74,183-186]. The classic stress literature has also provided some evidence that fast and intermediate negative feedback actions are also separated by a 45 minute window during which the prior exposure to phasic glucocorticoids fails to produce a suppressive effect over stimulated HPA hormone secretion [38,49,91,187]. These early studies referred to this timeframe as a glucocorticoid negative feedback "silent period." The existence of a silent period was reinforced by the fact that intermediate glucocorticoid effects take time to develop and second because fast glucocorticoid effects are rate-sensitive (i.e. persisted as long as there was an rapid

increase in glucocorticoid levels), therefore the time between the two could be refer to as a silent period [186].

Several fundamental problems exist with these early *in vitro* and *in vivo* glucocorticoid negative feedback studies. For instance, many of those studies were not carried out under optimal experimental conditions. Animals were sometimes anesthetized while a wide range of physiological and psychological HPA activators were used (e.g. histamine, ether, laparotomy, noise-stress, immobilization, shock, hypovolemia, hypothermia, hypoglycemia, hypoxia, leg-break). ADX completely removes all endogenous CORT activity, it is often difficult to determine whether any lack in HPA axis suppression observed under this surgical condition is dependent on disruption of tonic or phasic CORT without also selectively and appropriately reestablishing tonic or phasic CORT activity[44]. Furthermore, some of the studies that documented the temporal restrictions and requirements of intermediate and fast negative feedback were conceptually based on experiments that relied on experimental tools and techniques that typically produced unreliable and inconsistent results (e.g. crude hypothalamic explants, unreliable, inadequate and indirect CORT, ACTH and CRH measurements etc.)[45,49,52].

In addition to these early experimental drawbacks, other persistent and fundamental problem exists. First, there has been a marked lack in examining whether intermediate and fast temporal phasic glucocorticoid effects occur under the same experimental conditions (i.e. same stressor and glucocorticoid treatment). Second, most of the current and classic phasic glucocorticoid negative research has focused on isolating the effects of glucocorticoids from stress through pretreatment paradigms, which unintentionally, has resulted in a lack of experimental support documenting whether phasic glucocorticoids actually produce a

measurable fast negative feedback effect during stress. Third, no single experiment has accurately provided a real-time hormone profile of the HPA axis initial and ongoing response to an acute psychological stress challenge coupled with the multiple temporal phasic glucocorticoid manipulations. Lastly, and perhaps most pertinent, many studies examining temporal glucocorticoid effects have not adequately attempted to validate the physiological relevance of their glucocorticoid treatment, nor have they provided evidence of validation that their experimental design did not inadvertently induce HPA activity. There has been limited replication and reexamination of some of these temporal glucocorticoid negative feedback phenomena, which warrants the need for further study.

Our objective for these series of experiments was to reexamine the temporal requirements and restrictions (silent period and rate dependency) of intermediate and fast glucocorticoid negative feedback on the HPA axis hormone response to stress using the same validated experimental conditions (surgical procedure, psychological stress challenge and glucocorticoid treatment i.e. CORT). Since ACTH secretion is known to have a dynamic and continued reaction to stress [48,59,93], we also aimed to document the initial and ongoing stress-stimulated secretion profile of ACTH secretion response to intermediate and fast phasic CORT conditions. In addition, we wanted to determine whether fast negative feedback was present during stress, thus we examined the initial and ongoing HPA axis hormone response to a phasic CORT treatment given after stress onset. Importantly, we have adapted a surgical procedure that allows us to implant an indwelling jugular catheter into rats which permits us to repeatedly measure HPA axis activity while allowing us to intravenously infuse CORT at multiple pre- or post- stress time points without directly handling the animals. This technique provided a detailed and tight

temporal profile of the basal, initial and ongoing response of HPA axis hormone response to stress. This initial experiment demonstrated that our surgical and experimental procedure did not result in unwanted stimulation to HPA axis, therefore demonstrating its usefulness to further examine how intermediate and fast phasic CORT effects the HPA axis initial and ongoing hormone response to stress. These series of experiments examined temporal phasic CORT effects on ACTH secretion, as well as stress-dependent PVN and anterior pituitary *c-fos* gene expression and lactotroph prolactin secretion. Measuring changes in stress-dependent PVN and anterior pituitary *c-fos* mRNA in coordination with stress-stimulated prolactin release has provided useful information that has allowed for us to target intercellular and intracellular suppressive effects of phasic CORT actions [67-70].

Methods

Subjects

Young adult (290–320 g) male Sprague–Dawley rats (Harlan Labs, Indianapolis, IA, USA) were housed two per cage (polycarbonate tubs, 47 · 23 · 20 cm) and were maintained under a 12 : 12 h light ⁄ dark cycle with rat chow and tap water available ad libitum, unless otherwise indicated. All test day manipulations were performed during the first half of the rat's light period when basal HPA axis activity is at its diurnal trough. Rats were given 2 weeks to acclimate to the facility before exposure to surgical and experimental procedures. All experiments were conducted in accordance with ethical procedures approved by the University of Colorado Institutional Animal Care and Use Committee.

Surgical Procedures

Surgical jugular catheterization of rats (all experiments) was adapted from Graham et al 2009 and Burvin et al 1998. Catheters were made from flexible silastic tubing (0.094 cm o.d x 0.043 cm i.d., Green Rubber, Woburn, MA) and were previously treated with tridodecylmethyl ammonium chloride heparin (Polysciences Inc., Warrington, PA). Subcutaneous placement of chronic indwelling jugular vein catheters were surgically positioned by advancing the silastic tip into the right atrium [188], while then securing the catheter with Mersilene surgical mesh (General Medical, New Haven, CT) to the jugular vein. The other end of the catheter was passed subcutaneously to exit the animals' dorsal surface immediately behind the shoulder blades through a 22 gauge cannula (Plastics One, Roanoke, VA, #C313G-5UP/SPC 38172 22GA 5-UP) imbedded in dental cement on a Marlex surgical mesh (Bard Inc., Cranston, RI) [188,189]. All rats were anaesthetized with halothane during surgery, given 0.33ml of Baytril (antibiotic), and

0.1ml of Buprenorphine (pain killer, 0.1 mg/kg). Following surgery, all rats were housed one per cage, catheters were flushed daily (except test-day) with 0.5cc saline-heparinized-gentamicin (0.9%-20unit-5unit) solution to help maintain catheter patency and minimize infection. Rats were allowed no less than a 4-day recovery period in their home cages before experimental procedures began.

For the adrenalectomy (ADX) procedure (experiment 1 and 4), rats received an additional bilateral ADX immediately after surgical placement of jugular catheter with continuing halothane anesthetization. Bilateral incisions were made through the skin and peritoneal wall in close proximity to each kidney. All jugular catheterized and ADX rats had a post-surgical recovery period of 5 days. After surgery, these ADX rats were given a drinking water solution of 0.9% saline that contained CORT and 0.1% ethanol ad libitum. The CORT drinking solution was made by dissolving CORT (Steraloids Inc. Newport, RI, USA) in absolute ethanol (25 mg⁄ml) and then diluting that solution 1:1000 into 0.9% saline tap water to give a final CORT concentration of 25 ug⁄ml and an ethanol concentration of 0.1%. These ADX rats were switched to (ad libitum) a drinking solution containing only 0.9% saline and 0.1% ethanol 12 hours prior to testing.

Manual blood sampling apparatus

The sampling apparatus included a cannula connector with spring-cover (product # C313CS) and inner spring polyethylene tube (product # C313CT/SPC, i.d. 0.58 x o.d. 1.27 Plastics-One, Roanoke, Va.). The spring cover was attached to a cage-mounted swivel, whereby a 3 ml syringe was used to manually sample or infuse drug (vehicle or CORT). 24 hours post surgery, rats were acclimated to the sampling apparatus by fastening them to the sampling-spring line for no less than 2 hours daily for 2-3 days prior to testing, test day included.

Blood sample collection procedures

Experiment 1: rats were placed into restraint tubes for 2 hours. Subsequently all rats' tails were nicked by removing the distal 0.5 mm of the tail using a razor blade. ~200-300ul of blood was collected into EDTA-coated 1.5 ml microfuge tubes by milking blood from the lateral tail vein while the rats remained in the restraint tube, as described in Vahl et al 2005. Blood samples were immediately centrifuged for two minutes on a bench-top centrifuge. After centrifugation 20ul of plasma was aliquoted in duplicate samples and was immediately snap-frozen on dry ice. Samples were obtained within roughly 1–2 min per rat per sample. Serial samples were obtained by careful removal of the scab from the tip of the tail and repeating the same tail-milking method.

Experiment 2, 3 and 4: repeated blood samples were taken from indwelling jugular catheters by using a 3 ml sterile syringe (sterile syringes were used for each serial samples), roughly collecting 300-500ul of blood. Each sample was dispensed into EDTA-coated 1.5 ml microfuge tubes and immediately centrifuged for two minutes on a bench-top centrifuge. Rats received a 200uL sterile saline (0.9%) infusion after each repeated blood sample. After centrifugation 100ul of plasma were aliquoted in duplicate and were immediately snap-frozen on dry ice. All rats were sacrificed by guillotine decapitation following the 30-minute restraint challenge or comparable home-cage condition (experiment-2) to collect trunk blood. Trunk blood was collected into EDTA coated vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ, USA), placed on wet ice and centrifuged for 10 min (4 °C). Plasma was then aliquoted into microfuge tubes and snap-frozen on dry ice for subsequent hormone plasma level analysis. The blood processing procedure was completed within 45 min after decapitation. Note, rats did not receive

heparin at least 20 hours prior to testing, this was done to prevent heparin contamination of ACTH blood samples.

Restraint Stress Challenge

All experiments used a stress challenge that consisted of placing rats in clear plexiglas tubes (length 23.5 cm, diameter 7 cm; with multiple air holes). The size of the tube restricted lateral, forward, and backward movement but did not interfere with breathing. Restraint is widely accepted as a psychological stressor [140].

Experimental Procedures

Experiment 1 (Validation of CORT drug treatment): the purpose of this experiment was to examine the initial concentration, duration of elevated concentration, and systemic clearance of plasma CORT produced by an acute intravenous CORT (0.31mg/kg) treatment of ADX rats exposed to a 2 hour restraint challenge. To accurately examine the time-course of this exogenous phasic CORT treatment we surgically fitted all rats with a indwelling jugular catheters (N = 10) and ADX all rats to prevent endogenous CORT contamination. On test day (after 4 day recovery) we infused CORT (0.31mg/kg) via jugular catheter and immediately placed rats into restraint for 2 hours. We repeatedly sampled blood through a tail nick at 2, 5, 10, 15, 30, 45, 60, 90, and 120 minutes following restraint/CORT treatment. Immediately after the restraint challenge rats were killed by guillotine decapitation.

Experiment 2 (Validation of experimental procedure): This experiment served as a validation measurement that our testing procedure and repeated blood sampling methods did not significantly alter or stimulate the HPA axis hormone response of non-restrained rats as well as the effect of a 30-minute restraint stress challenge on corticotroph ACTH secretion, lactotroph

prolactin secretion and adrenal cortex CORT secretion (N = 20, n = 10). All rats were adrenal intact and surgically fitted with an indwelling jugular catheter. On test day, rats in the stress challenge group were placed in restraint tubes for 30 minutes and repeated blood samples were collected 5, 15, and 30 minutes post-stress onset. A single baseline sample was taken 1 minute prior to the onset of restraint (30 seconds before drug treatment). Trunk blood collection and brain extraction were rapidly performed after decapitation in an area adjacent from the home room. Only CORT and ACTH plasma samples were collected and measured serially.

Experiment 3 (reexamination of temporal phasic CORT effects): the purpose of this experiment was to reexamine the temporal actions and restrictions of CORT negative feedback effects over the HPA axis initial and ongoing response to an acute psychological stress challenge. Adrenal intact rats (N = 72, divided into three separate cohorts, n = 24 rats per cohort) were surgically fitted with an indwelling jugular catheter. Rats were infused with either CORT (0.3 mg/kg) or vehicle (10% ethanol mixed with 30% propylene glycol and 60% sterile saline) either 1 hour, 15 minutes, or 30 seconds prior to stress-onset (30 minute restraint) or 5 minutes following stress onset. Drug infusion was followed by 0.3 cc saline injection to ensure that drug cleared the sampling and catheter line. Blood samples were taken 5, 15, and 30 minutes post-stress onset. A single baseline sample was taken 1 minute prior to the onset of restraint (30 seconds before drug treatment) (figure 4.1) for experimental design. Only ACTH levels were measured serially; trunk blood, brains and pituitaries were also extracted and collected at the 30 minute kill time-point (rapidly performed after decapitation in an area adjacent from the home room).

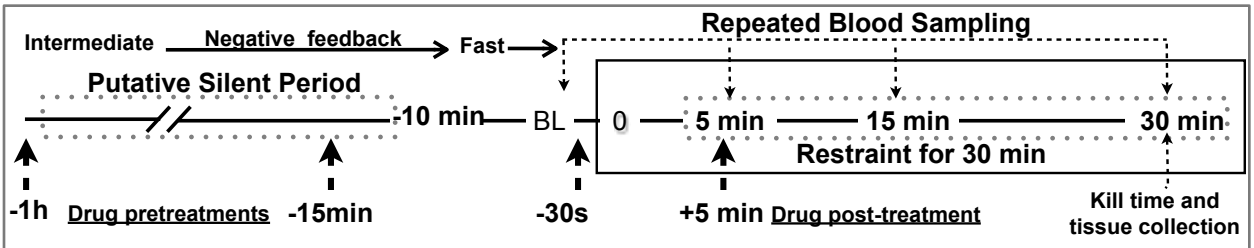

Figure 4.1 Test day timeline. Rats were pretreated with vehicle or CORT either 1 hour, 15 minute, 30 before 30 minute restraint challenge, another group of rats received a vehicle or CORT treatment 5 min post-stress onset. Repeated blood samples were taken at baseline (30 seconds prior to restraint challenge) and 5, 15, and 30 minute. Gray dotted box indicates putative silent period. Trunk blood, brains and pituitaries were taken immediately after rats were killed at 30 minute after stress onset. BL; indicates baseline sample.

Experiment 4 (examination of fast CORT negative feedback during stress): the purpose of this experiment was to examine whether an exogenous CORT treatment in ADX rats would produce an inhibitory effect over stimulated HPA axis activity during an ongoing stress challenge. All ADX rats (N = 24, n = 6) were surgically fitted with an indwelling jugular catheter and given a 5-day post surgery recovery period before testing. Rats were infused with either CORT (0.3 mg/kg) or vehicle (10% ethanol mixed with 30% propylene glycol and 60% sterile saline) 30 seconds prior to stress onset or 5 minutes post stress onset. Drug infusion was followed by 0.3 cc saline infusion to insure the drug cleared the sampling and catheter line. All rats were restrained for 30 minutes, and repeated blood samples began 5, 15, and 30 minutes post-stress onset. A single baseline sample was taken 1 minute prior to the onset of restraint (30 seconds before drug treatment); refer to figure 1 for timeline. Only ACTH levels were measured serially; trunk blood, brains and pituitaries were also extracted and collected at the 30 minute kill time-point (rapidly performed after decapitation in an area adjacent from the home room). ADX rats were placed on a tonic CORT water replacement regimen, which we have shown in recent

studies that this CORT regimen is a sufficient method of reinstating tonic CORT activity [58,59,158]. Note, rats were placed on a CORT water regimen to normalize the augmentation in HPA axis activity that accompanies the long-term absence of tonic CORT activity [57-59,67] and taken off this regimen 12 hours prior to testing.

ACTH radioimmunoassay

ACTH (pg/ml) was determined in duplicate (100ul plasma) by competitive radioimmunoassay protocol. The RIA procedure was adapted from a previously established protocol [190]. For experiments 2 and 3, radiolabeled ^{125}I ACTH was obtained from DiaSorin (Minneapolis, MN). Primary ACTH anti-serum Rb 7 (diluted to a final concentration of 1:30,000) was obtained from Dr. William Engeland (University of Minnesota) [67]. Experiment-3 we used ^{125}I ACTH tracer from PerkinElmer Inc (Santa Clara, CA), diluted in 1ml of 0.05M PBS (with EDTA, and BSA pH 7.4). Primary ACTH anti-serum Rb 7 (Dr. England) was diluted to a final concentration of 1:15,000.

CORT and Prolactin enzyme linked immunosorbant assay immunoassay

Plasma CORT levels: were assayed via an enzyme immunoassay kit (Assay Design, Ann Arbor, MI, USA) in accordance with the manufacturer's protocol. Intra-assay coefficient of variance was 7.2%. **Plasma prolactin:** levels were determined via an enzyme immunoassay kit (#589701; Cayman Chemical, Ann Arbor, MI, USA) in accordance with the manufacturer's protocol [68]. The sensitivity for this assay was approximately 2.0ng⁄ml, and the intra-assay coefficient of variance was 7.2%.

In situ hybridization

Brains and pituitaries were rapidly extracted (experiment 3 and 4) after decapitation, frozen in chilled isopentane (at temperatures held between -30°C and 40°C), and stored at -80°C. Coronal brain sections (12um thick) were collected through the extent of the PVN ~1.80 mm posterior to bregma [142]. Horizontal pituitary sections (12 µm thick) were collected from the middle portion of the tissue. All tissues were sectioned on a cryostat (Leica Microsystems, model 1850, Bannockburn, IL, USA). Both brain and pituitary sections were thaw mounted onto Superfrost Plus slides (Fisher Scientific) and stored at -80°C. For in situ hybridization, tissue was postfixed in buffer solution containing 4% paraformaldehyde for 1 hour at room temperature and then processed as published previously [163]. ^{35}S-labelled antisense riboprobes were generated and in situ hybridization performed as described previously [67]. Probes were generated using plasmids containing a portion of the cDNA sequences encoding for *c-fos* mRNA (courtesy of Dr T. Curran, St Jude Children's Research Hospital, Memphis TN, USA), *pomc* hnRNA (courtesy of Dr Stanley Watson, University of Michigan, Ann Arbor, MI, USA) [67]. X-ray film (Kodak Biomax MR film) was exposed to slides for 14 days (*c-fos* mRNA) and 48 hours (*pomc* hnRNA). Densitometry was performed on film using IMAGEJ software (NIH, Bethesda, MD, USA).

Image analysis and quantification

Semiquantitative analyses were performed on digitized images from x-ray films using the linear range of the gray values obtained from an acquisition system (Northern Lights lightbox, model B 95, Ontario, Canada; CCD camera, model XC-77, Sony, Tokyo, Japan; image capture with National Institutes of Health scion Image v1.59 software) as previously described [143]. Regions of interests (PVN and anterior pituitary) were determined by matching digitized rat hypothalamic structures to rat brain atlas diagrams [142]. Quantification and analysis of images

were performed by individuals that were blind to treatment condition assignments. For the PVN, measurements for four sections were taken for both hemispheres of the brain for all rats (4-8 measurements for each rat brain). Measurements were also taken for four to six sections of the anterior pituitary. Optical densities for each region of interest were measured using the program ImageJ provided by National Institutes of Health (version 1.42q, http://rsb.info.nih.gov/in). Graphs showing relative differences between groups were created by measuring the average uncalibrated optical density for each rat and then averaging the densities of each rat to give an overall mean value for that treatment effect.

Statistical analysis

Statistical analysis was conducted using the IBM Predictive Analytics Software (PASW, version 21.0, Chicago, IL) for Macintosh operating system. For repeated plasma samples a one-way (experiment 1-4), two-way (experiment 2) and three-way (experiment 3 and 4) repeated measures analysis of variance (ANOVA) statistical test were used to determine main effects and interactions for all treatment conditions, followed by a Bonferroni corrected post hoc test ($p<0.05$) and a separate independent t-test (experiment 2-4) to determine simple-main effects for between treatment conditions. Statistical analysis of trunk blood (experiment 2 only) was done by conducting an independent t-test. Two-way univariate-ANOVA (experiment 3 and 4) was used to examine changes in both trunk blood hormone levels and gene expression for all treatment conditions and in cases where there was an overall significant F-test, pairwise comparisons of interest were assessed by Fisher's least significant difference test (FLSD). Significance was determined by $p<0.05$. All graphs depict group means ± standard error of the means.

Results

Experiment 1 (validation of CORT drug treatment)

This experiment examined the initial concentration, sustained concentration levels, and systemic clearance of an acute intravenous CORT (0.31mg/kg) treatment of ADX rats exposed to a 2 hour restraint challenge. We collected blood from rats that were restrained because we needed to measure our exogenous CORT treatment time course under the same stress condition that was used for all experiments and second it provided a convenient method for collecting repeated blood from the tail.

Plasma CORT from repeated tail-bleed sampling (figure 4.2)

Repeated blood samples were taken at (2, 5, 10, 15, 30, 45, 60, 90 and 120 minutes) following stress onset/CORT treatment. One-way repeated measures ANOVA indicated an overall significant mean difference in plasma CORT levels ($F_{(8,63)} = 45.9$, $p<0.05$). Bonferroni pairwise comparison indicated that plasma CORT levels at 2, 5, 10, 15 minutes post treatment contained significantly ($p<0.05$) higher concentration of plasma CORT, compared to all other repeated plasma samples (i.e. 30, 45, 60, 90, 120 minute time-points). Bonferroni analysis also indicated that 30 minutes after stress onset/treatment the concentration of CORT had markedly declined (~20ug/100ml) and continued to clear from circulation 45 and 60 minutes after treatment, reaching near complete clearance by the 90 minute sampling time-point. These results demonstrated that an acute intravenous CORT treatment (0.31mg/kg i.v.) produced an immediate peak in plasma CORT concentration of ~40-45 ug/100ml within 2 minutes after treatment; these peak CORT plasma levels are similar to levels of CORT that adrenal intact rats display when exposed to the same stress challenge [59]. Additionally, these peak CORT levels were maintained

for 15 minutes (5, 10 and 15 minute repeated CORT samples), declined by 30 minutes, and cleared the circulation within 1-2 hours after treatment.

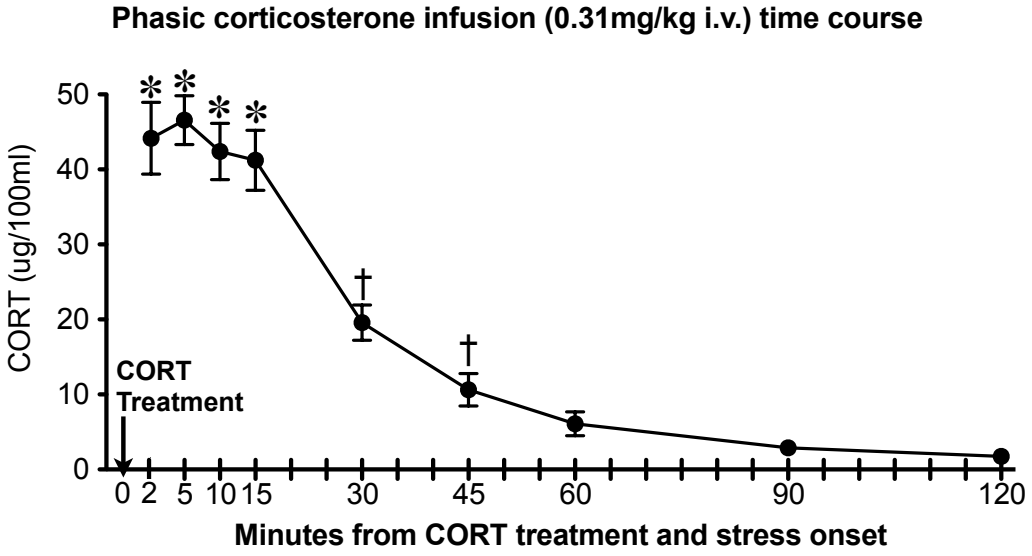

Figure 4.2 Exogenous CORT time course (experiment 1). Physiological relevance of exogenous acute CORT treatment. Exogenous CORT (0.31mg/kg i.v.) treatment (time-point zero) significantly increased CORT. Bonferroni pairwise comparison ($p<0.05$) indicated that an acute intravenous CORT treatment (0.31mg/kg i.v.) produced a significant increase in CORT within 2 minute, represented by *, peak CORT plasma levels were maintained for 15 minutes (5, 10 and 15 minute time-points) and CORT plasma levels significantly declined by the 30 and 45 minute time-point, represented by †. All rats were adrenal adrenalectomized. BL; indicates baseline sample.

Experiment 2 (validation of experimental procedure)

This experiment characterized whether our experimental procedures and conditions added unwanted activation of the HPA axis hormone response. Additionally, we examined the initial and ongoing HPA axis ACTH and CORT response of rats to an acute 30 minute restraint challenge.

Plasma ACTH and CORT from repeated intravenous sampling (figure 4.3a,b)

Repeated blood samples were taken at baseline (30 seconds prior to restraint challenge) and 5, 15, and 30 minutes following stress onset (figure 4.1 and 4.3). One-way repeated measures ANOVA indicated overall mean differences in both stress-stimulated ACTH levels ($F_{3,12} = 16.8$, $p<0.05$) and CORT levels ($F_{3,12} = 29.1$, $p<0.05$) of vehicle treated rats. Bonferroni pairwise comparison indicated a significant ($p<0.05$) stress effect (increase in hormone) at the 5, 15 and 30 minute time points compared to baseline samples for both ACTH and CORT plasma levels, (figure 4.3a,b). One-way repeated measures ANOVA did not support any difference in ACTH or CORT plasma levels of home-cage control rats, (figure 4.3b). Two-way mixed model repeated measures ANOVA indicated an overall effect of restraint on ACTH secretion (ACTH time condition: $F_{(1,8)} = 16.07$, $p < 0.05$) and CORT secretion ($F_{(1,7)} = 40.7$, $p < 0.05$), an overall significant mean difference in plasma ACTH (ACTH time condition: $F_{(3,24)} = 15.23$, $p < 0.05$) and CORT (CORT time condition: $F_{(3,21)} = 23.7$, $p < 0.05$), and an interaction between stress and change in ACTH levels ($F_{(3,24)} = 16.8$, $p < 0.05$) and an interaction between stress and change in CORT levels ($F_{(3,21)} = 15.3$, $p < 0.05$) (figure 4.3a). Independent t-test indicated that plasma ACTH levels were significantly increased in restrained rats compared to non-stressed rats at the 5 minute ($t_8 = 4.5$, $p<0.05$), at the 15 minute ($t_{10} = 5.2$, $p<0.05$) and at the 30 minute ($t_{10} = 4.1$,

$p<0.05$) time-points, as well as plasma CORT levels at the 5 minute ($t_9 = 6.1$, $p<0.001$), at the 15 minute ($t_9 = 5.5$, $p<0.001$) and at the 30 minute ($t_9 = 6.1$, $p<0.001$) time-points. No significant difference between baseline samples for ACTH and CORT of non-stress rats compared to restrained rats was found. Taken together, these results clearly show that stress rapidly increases ACTH and CORT secretion, while experimental procedures did not cause any additional activation of the HPA axis as indicated by no stress comparison group (figure 4.3a,b).

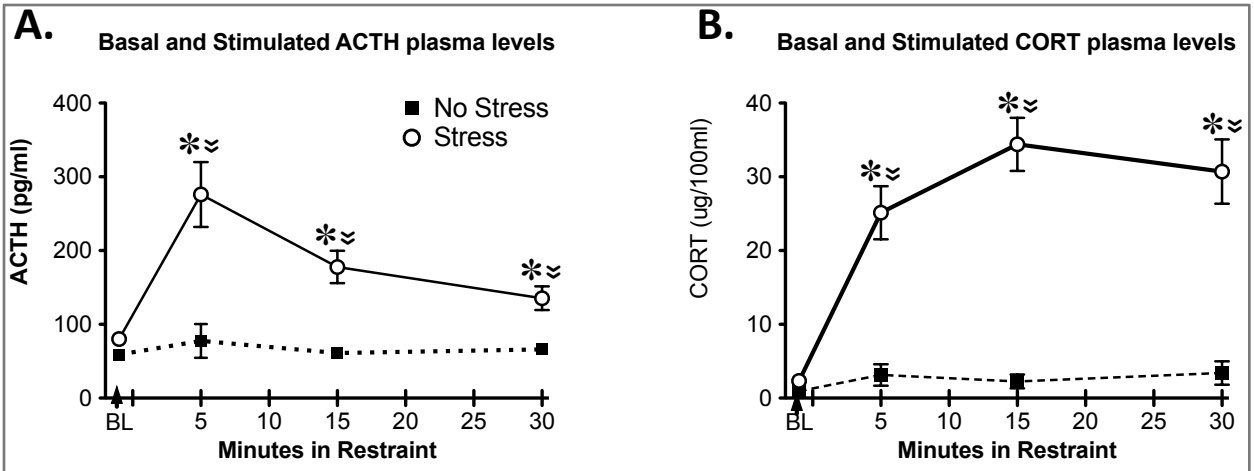

Figure 4.3 Validation of experimental procedures (experiment 2). Validation that our repeated blood sampling procedure. ACTH (A) and CORT (B) secretion were rapidly increased by restraint 5 minutes after stress-onset and continued to stay elevated 15 and 30 minutes after stress onset. *, represents significant stress effect when compared to non-stress comparison groups ($p<0.05$); ⩔, represents significant stress effect within treatment group compared to baseline sample (Bonferroni, $p<0.05$). BL; indicates baseline sample.

Trunk plasma ACTH and prolactin levels at the 30 minute post-stress onset (figure 4.4a,b)

Trunk plasma blood samples were collected following a 30-minute period of restraint stress or no stress control, used to establish control levels of ACTH and prolactin secretion. There was a significant difference in restraint-stimulated ACTH ($t_{10} = 4.0$, $p<0.05$) and prolactin ($t_{10} = 4.3$, $p<0.05$) plasma levels 30 minutes after stress onset when compared to no stress group (figure 4.4a,b). These results show that prolactin secretion is increased in response to restraint challenge.

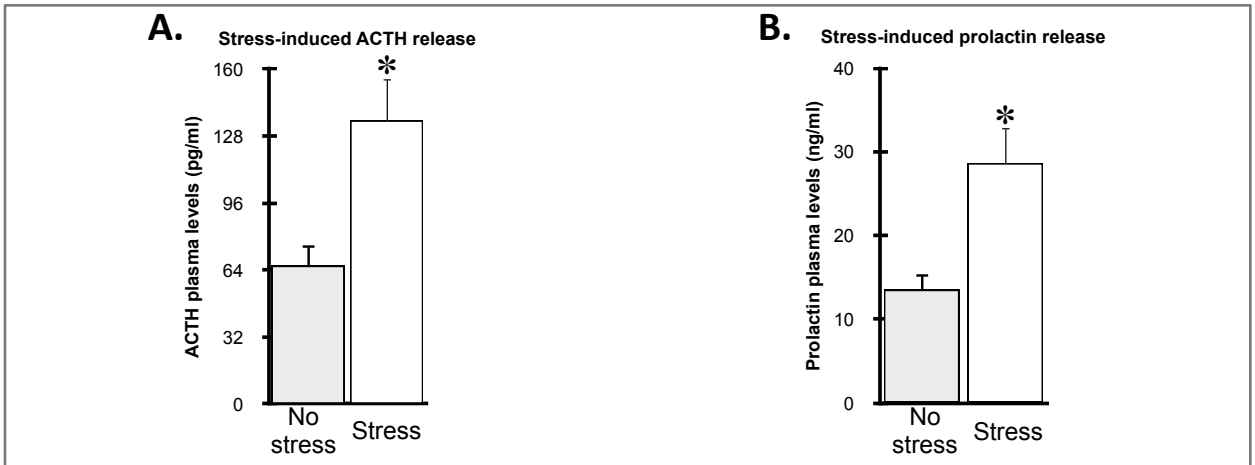

Figure 4.4 Stress induced ACTH and prolactin (experiment 2). Stress-evoked prolactin release was not inhibited by pre- or post- CORT treatments. Stress increased ACTH (A) and prolactin (B) secretion when compared to no stress groups ($p<0.05$), as represented by *. Both prolactin and ACTH trunk blood samples were collected immediate after 30 minutes of restraint.

Experiment 3 (reexamination of temporal phasic CORT effects):

This experiment examined the initial and ongoing stress-stimulated HPA axis response to the effects of a systemic infusion of phasic CORT administered 1 hour, 15 minutes, or 30 seconds prior to or 5 minutes following stress onset.

Plasma ACTH from repeated intravenous sampling (figure 4.5a-d)

Repeated blood samples were taken at baseline (30 seconds prior to restraint challenge) and 5, 15, and 30 minutes following stress onset (figure 4.1). One-way repeated measures ANOVA indicated overall mean differences in stress-stimulated ACTH levels of vehicle treated rats with the 1 hour ($F_{3,18} = 8.4$, $p<0.05$), 15 minute ($F_{3,24} = 20.9$, $p<0.05$) and 30 second ($F_{3,10} = 7.7$, $p<0.05$) pretreatments. Bonferroni pairwise comparison indicated a significant ($p < 0.05$) increase in ACTH in response to restraint of all vehicle treated rats at the 5, 15 and 30 minute repeated sampling time points when compared to appropriate baseline sample. Furthermore, one-way repeated measures ANOVA analysis indicated overall mean differences in stress-stimulated ACTH levels of CORT treated rats with the 1 hour ($F_{3,24} = 8.4$, $p<0.05$), 15 minute ($F_{3,24} = 20.9$, $p<0.05$) and 30 second ($F_{3,10} = 7.7$, $p<0.05$) CORT pretreatment time-points (figure 4.5a-d); however, Bonferroni pairwise comparison indicated that only the post stress-onset 5 minute sampling time point for the 1 hour, 15 minute and 30 second CORT pretreatment groups were significantly different from all other repeated sampling time points ($p<0.05$), which suggests a small and transient increase in HPA ACTH response to stress regardless of CORT treatment (figure 4.5a,b,c). One-way repeated measures ANOVA indicated an increase in ACTH for rats that received a 5 minute post-stress onset vehicle ($F_{3,18} = 12.1$, $p<0.05$) and CORT ($F_{3,15} = 8.4$, $p<0.05$) treatment (figure 4.5d). Bonferroni analysis indicated that increased ACTH levels

occurred at the 5, 15 and 30-minute time points compared to baseline sample, suggesting that restraint rapidly and significantly increased ACTH secretion regardless of drug treatment. Three-way repeated measures ANOVA indicated an overall effect of drug (CORT) treatment ($F_{(1,50)}$ = 26.5, $p<0.001$), significant change in repeated ACTH levels (time condition: $F_{(3,150)}$ = 35.8, $p<0.001$), and a drug by time condition interaction ($F_{(3,150)}$ = 26.5, $p < 0.05$). Independent t-test indicated that stress-stimulated ACTH levels were significantly suppressed by 1 hour CORT treatment (5 minute: t_{16} = 5.7, $p<0.001$, 15 minute: t_{16} = 4.1, $p<0.001$, 30 minute $t_{1,16}$ = 2.4, $p<0.05$), by 15 minute CORT treatment (5 minute: t_{15} = 4.0, $p<0.001$, 15 minute t_{14} = 4.4, $p<0.001$, 30 minute t_{14} = 4.1, $p<0.001$) and by the 30 second CORT treatment (15 minute: t_{14} = 2.6, $p<0.05$ and 30 minute: t_{13} = 2.6, $p<0.05$) (figure 4.5a,b,c). There was no statistical support for a significant suppressive effect of CORT at the 5 minute post-stress treatment time-point (figure 4.5d). Overall, these results show that phasic CORT pretreatment (1 hour, 15 minute and 30 second) significantly inhibited stress-stimulated HPA axis ACTH secretion, but not the post-stress CORT treatment.

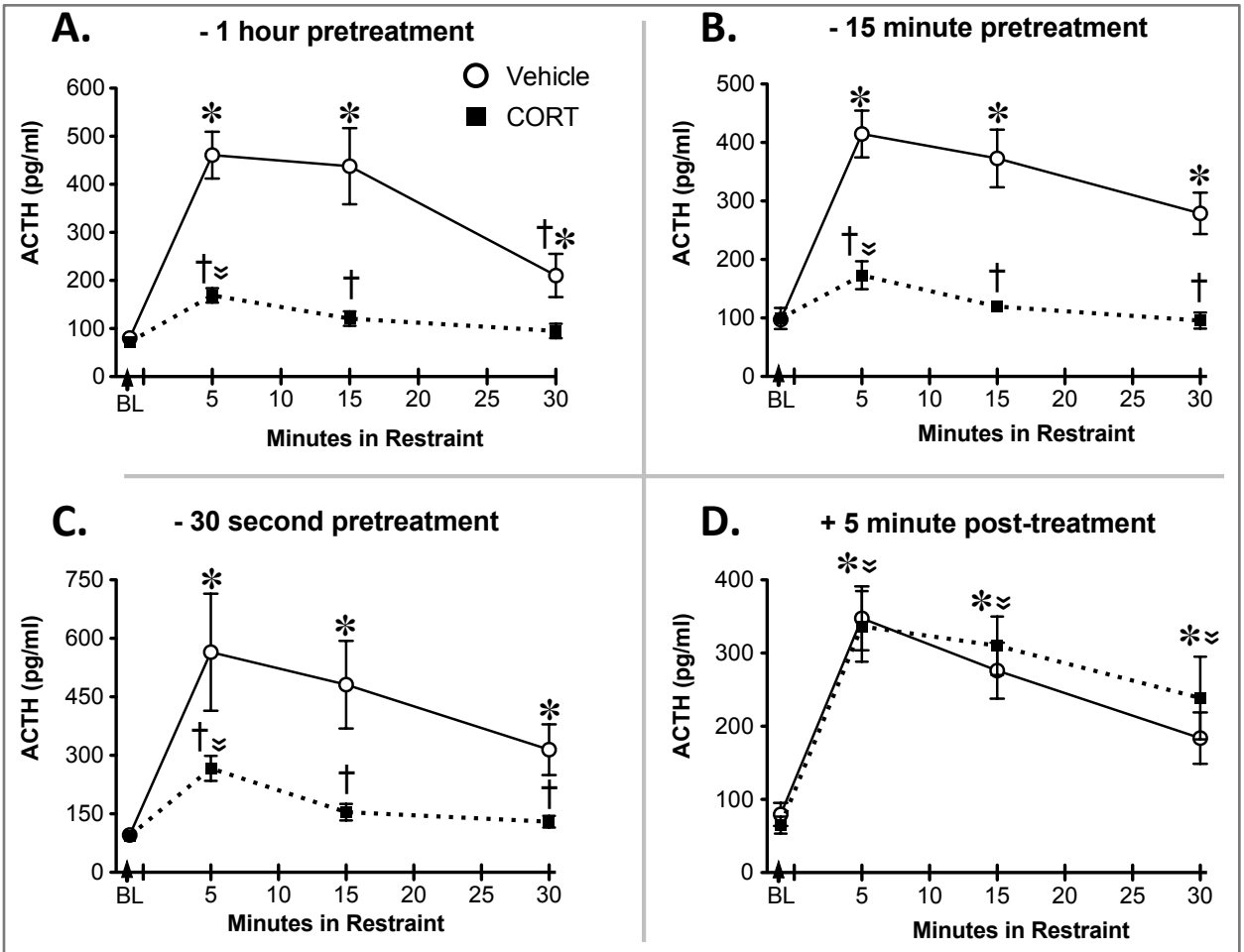

Figure 4.5 Reexamination of temporal phasic CORT effects (experiment 3). Phasic CORT treatment before, but not after stress onset blocked the stress stimulated ACTH. 1 hour (A), 15 minute (B), and 30 second (C) CORT pretreatment significantly inhibited restraint induced ACTH secretion at the 5, 15 and 30 minute repeated samples. Statistical analysis also indicated that the 1 hour (A), 15 minute (B), and 30 second (C) vehicle treated rats displayed a significant increase in ACTH at the 5, 15 and 30 minute repeated samples. The 1 hour (A), 15 minute (B), and 30 second (C) CORT treated rats displayed a slight and transient increase in ACTH at the 5 minute time-point. Both the 5 minute (D) post-stress onset vehicle and CORT treated rats displayed significant increase in ACTH levels at the 5, 15 and 30 minute repeated samples. †, significant CORT effect when compared to same vehicle treated group ($p<0.05$); *, represents significant stress effect within same vehicle condition compered to baseline (Bonferroni, $p<0.05$); ⩒, represents significant stress effect within same CORT condition compered to baseline (Bonferroni, $p<0.05$). BL; indicates baseline sample.

Trunk plasma ACTH and Prolactin levels at the 30 minute post-stress onset time-point (figure 4.6)

Plasma ACTH and prolactin levels were measured after a 30 minute restraint period to determine if there was an effect of CORT treatment on ACTH and prolactin secretion. Two-way ANOVA indicated that a phasic CORT pretreatment 1 hour ($F_{1,15} = 4.187$, $p<0.05$), 15 minute ($F_{1,15} = 6.803$, 0.05), and 30 second ($F_{1,12} = 7.067$, $p<0.05$) inhibited restraint-stimulated ACTH secretion (figure 4.6a). There was no statistical support for a mean difference in ACTH levels of CORT treated rats compared to vehicle treated rats in the 5 minute post-treatment group. Two-way ANOVA analysis determined that there was no main effect of CORT on prolactin levels at 1 hour, 15 minute, and 30 second pretreatment groups or in the 5 minute post-treatment group, (figure 4.6b).

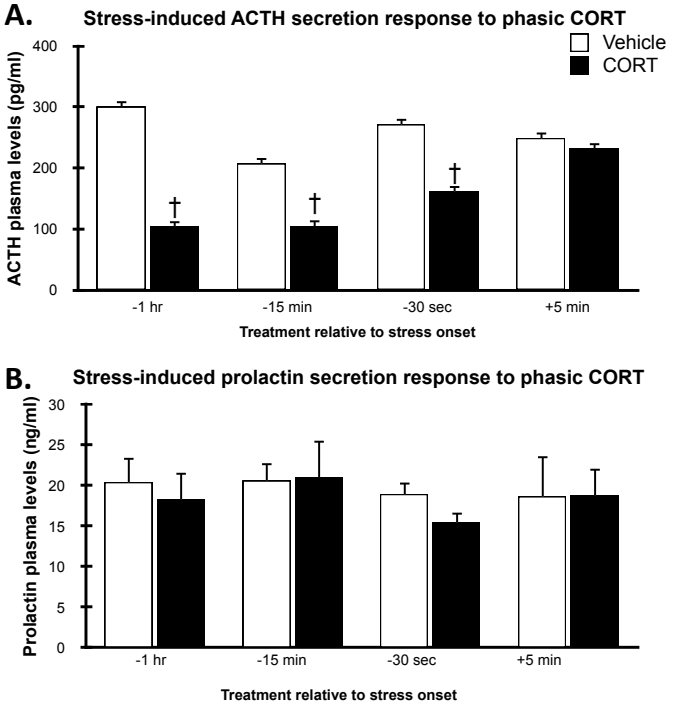

Figure 4.6 Effect CORT pretreatment on ACTH and prolactin secretion (experiment 3). Phasic CORT treatment did not block the stress stimulated prolactin secretion. 1 hour, 15 minute, and 30 second CORT pretreatment significantly inhibited restraint stimulated ACTH (A) secretion, but not prolactin secretion (B). †, represents a significant CORT effect when compared to vehicle treatment time condition ($p<0.05$, FLSD). Both prolactin and ACTH trunk blood samples were collected immediate after 30 minutes of restraint.

PVN c-fos mRNA levels and anterior pituitary c-fos mRNA and pomc hnRNA levels (figure 4.7 and 4.8)

CORT treatment before and after stress onset did not alter stress induced PVN *c-fos* mRNA levels nor anterior pituitary *c-fos* mRNA as there was no two-way ANOVA statistical support for a CORT effect on *c-fos* mRNA expression (figure 4.7a,b). However, two-way ANOVA indicated a main effect of CORT ($F_{1,68} = 12.3$, $p < 0.05$) and FLSD pair wise analysis indicated that 1 hour, 15 minute, and 30 second CORT pretreatment suppressed stress-induced levels of *pomc* hnRNA, but not post 5 minute CORT treatment (figure 4.8a,c). These results suggest that CORT did not modulate stress-induced excitatory drive to the PVN and anterior pituitary.

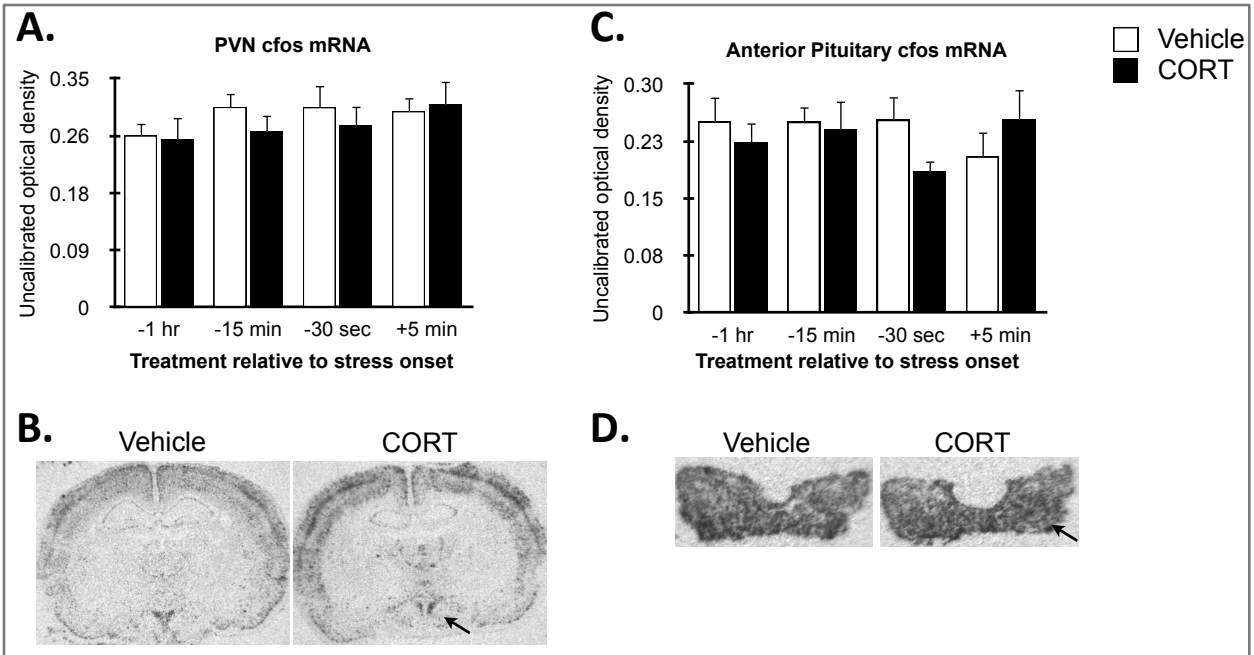

Figure 4.7 Effect CORT pretreatment on PVN and anterior pituitary *c-fos mRNA* expression (experiment 3). Phasic CORT treatment did not block the stress-induced PVN or anterior pituitary *c-fos* levels. CORT pretreatment 1 hour, 15 minute, 30 second or 5 minute post CORT treatment did not inhibit restraint-induced PVN (A,B) or anterior pituitary (C,D) *c-fos* mRNA experssion*. Panel (B) PVN and panel (C) anterior pituitary are relevant representative autoradiograms for in situ hybridization for *c-fos* mRNA expression. Tissue was collected immediately after restraint. Black arrow indicates PVN (B) and anterior pituitary (C).

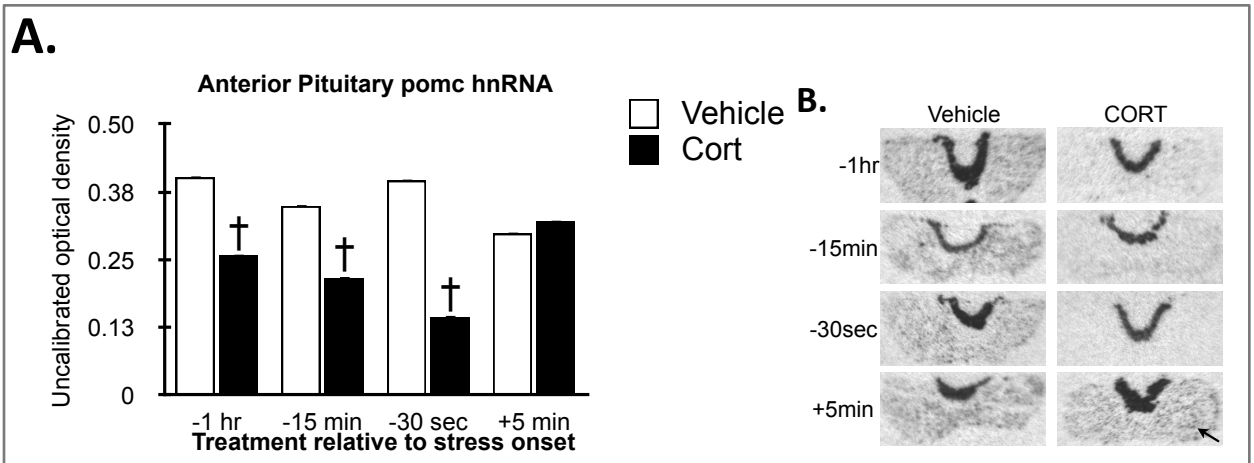

Figure 4.8 Effect CORT pretreatment on corticotroph pomc hnRNA expression (experiment 3). Phasic CORT pretreatment blocked stress-induced corticotroph pomc hnRNA. 1 hour, 15 minute, and 30 second CORT pretreatment significantly inhibited restraint induced pomc hnRNA expression (A,B) secretion. The 5 minute post CORT treatment did not inhibit pomc hnRNA. †, represents a significant CORT effect when compared to vehicle treatment time condition ($p<0.05$, FLSD). Panel (B) relevant representative autoradiograms for in situ hybridization for anterior pituitary pomc hnRNA expression. Tissue collected 30 minutes after stress onset. Black arrow indicates anterior pituitary.

Experiment 4 (examination of fast CORT negative feedback during stress)

Experiment 3 suggested that there is an absence of a fast CORT negative feedback effect during stress. Alternatively, this also suggested that stress-stimulated increase in endogenous phasic CORT during stress provides maximum constraint over the ongoing ACTH response to stress (i.e. essentially producing a ceiling level negative feedback effect), thereby excluding further suppressive action from an exogenous CORT treatment given during stress.

The purpose of experiment 4, was to examine whether stress-stimulated phasic increase in CORT provides a measurable negative feedback effect during stress, therefore, we eliminated the physiological ability of rats to produce a phasic CORT response by removing their adrenal

glands (ADX). We additionally, treated some those ADX rats with an exogenous phasic CORT treatment (30 second pretreatment or a post-stress 5 minute) to reestablish the possible loss in suppressive action over the ACTH response. We measured the initial and ongoing stress-stimulated ACTH response of ADX rats response to these phasic CORT treatment through serial ACTH plasma samples. In addition, we measured stress-dependent PVN and anterior pituitary gene levels 30 minutes after stress onset.

Plasma ACTH from repeated intravenous sampling (figure 4.9a,b)

Repeated blood samples were taken at baseline (30seconds prior to restraint challenge) and 5, 15, and 30 minutes following stress onset. One-way repeated measures ANOVA indicated overall mean differences in stress-stimulated ACTH levels of rats treated with vehicle 30 second pre-stress ($F_{3,12}$ = 53.6 $p<0.05$) and 5 minute post-stress ($F_{3,12}$ = 26.3 $p<0.05$) (figure 4.9a,b). Bonferroni analysis indicated a significant ($p < 0.05$) increase in ACTH of vehicle treated rats at the 5, 15, and 30 minute time points compared to appropriate baseline sample for both the 30 second and 5 minute vehicle treatment groups. Interestingly, one-way repeated measures ANOVA also indicated overall mean differences in stress-stimulated ACTH levels of CORT treated rats at the 30 second pretreatment time-point ($F_{3,12}$ = 11.1, $p<0.05$) and at the 5 minute post-stress onset time-point ($F_{3,12}$ = 52.4, $p<0.05$) (figure 4.9a,b). Bonferroni pairwise analysis indicated that 30 second CORT pretreated rats displayed significantly different plasma ACTH levels at the 5 minute and 15 minute sampling time points ($p<0.05$) when compered to the baseline sample, while the 30 minute repeated sample was not significantly different from baseline. Bonferroni pairwise comparison also indicated that a post stress onset 5 minute CORT treatment of ADX rats displayed significantly different plasma ACTH levels at the 5 minute and

15 minute sampling time points, but not the 30 minute when compared to baseline ($p<0.05$). Three-way repeated measures ANOVA indicated an overall drug (CORT) effect ($F_{1,15} = 4.5$, $p<0.05$), significant change in repeated ACTH levels (time condition: $F_{3,40} = 34.4$, $p<0.001$) and an interaction between drug and ACTH time condition ($F_{3,40} = 16.5$, $p<0.001$). Further independent t-tests indicated that a 30 second pretreatment with CORT provided a suppressive alteration in restraint-stimulated increase in ACTH secretion of ADX rats at the 5 minute ($t_7 = 2.4$, $p<0.05$), 15 minute ($t_8 = 2.3$, $p<0.05$), and 30 minute ($t_{1,10} = 5.8$, $p<0.001$) post stress onset repeated blood sampling time-points (figure 4.9a). Further, t-test analysis indicated that in the 5 minute CORT post-treatment group, there was a delayed suppressive effect over stress induced ACTH secretion at the 30 minute time point ($t_{10} = 5.1$, $p<0.001$) (figure 4.9b). Overall, these results suggest that ADX rats pretreated with CORT 30 seconds before stress onset have a significant ACTH response to restraint at all post-stress onset repeated samples, but that the CORT pretreatment still provides a suppressive alteration in that response when compered to vehicle treated rats (i.e. returning stimulated ACTH levels back to non-stimulated base-line levels). Additionally, ADX rats treated with CORT 5 minutes post-stress onset also displayed a significant ACTH response to acute stress, but this particular CORT post-treatment provided a delayed suppressive alteration in that response that was only observed 30 minutes after stress onset. Overall, these results show that CORT produces a significantly different suppressive pattern in stress-stimulated ACTH release which depends on the timing of CORT treatment relative to stress onset.

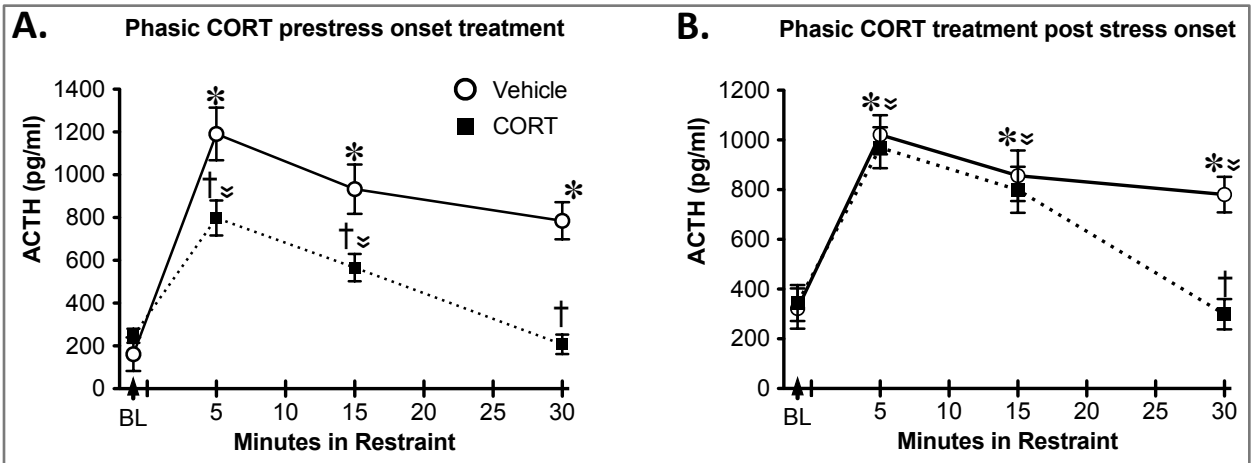

Figure 4.9 Examination of fast CORT negative feedback during stress (experiment 4). CORT treatment during ongoing stress of ADX rats produced a delayed suppressive effect over stimulated ACTH secretion. 30 second (A) CORT pretreatment inhibited restraint induced ACTH secretion at the 5, 15 and 30 minute repeated sample time-points, however, the 5 minute (B) post CORT treatment only suppressed restraint stimulated ACTH release at the 30 minute time-point. The 30 second (A) and post 5 minute (B) vehicle treated rats displayed a significant increase in ACTH at the 5, 15 and 30 minute repeated samples. Groups that received a CORT treatment at 30 seconds before (A) or 5 minutes after (B) stress onset displayed a significant increase in ACTH plasma levels at the 5 minute and 15 minute time-points, but not at the 30 minute time-point. †, significant CORT effect when compared to same vehicle treated group ($p<0.05$); *, represents significant stress effect within same vehicle condition compered* to baseline (Bonferroni, $p<0.05$); ≪, represents significant stress effect within same CORT condition compered* to baseline (Bonferroni, $p<0.05$). BL; indicates baseline sample.

PVN c-fos mRNA levels and anterior pituitary c-fos mRNA and POMC hnRNA levels (figure 4.10 and 4.11)

ANOVA statistical tests indicted that phasic CORT treatment 30 seconds before stress onset and a 5 minute post stress onset treatment did not suppress PVN *c-fos* mRNA levels anterior pituitary *c-fos* mRNA levels (CORT effect: $F_{1,14} = 8.7$, $p<0.05$ (figure 4.10c,d). However, both CORT treatments did suppress corticotrophs *pomc* hnRNA levels (CORT effect: $F_{1,20} = 85.4$, $p<0.05$ (figure 4.11a,b) compared to vehicle.

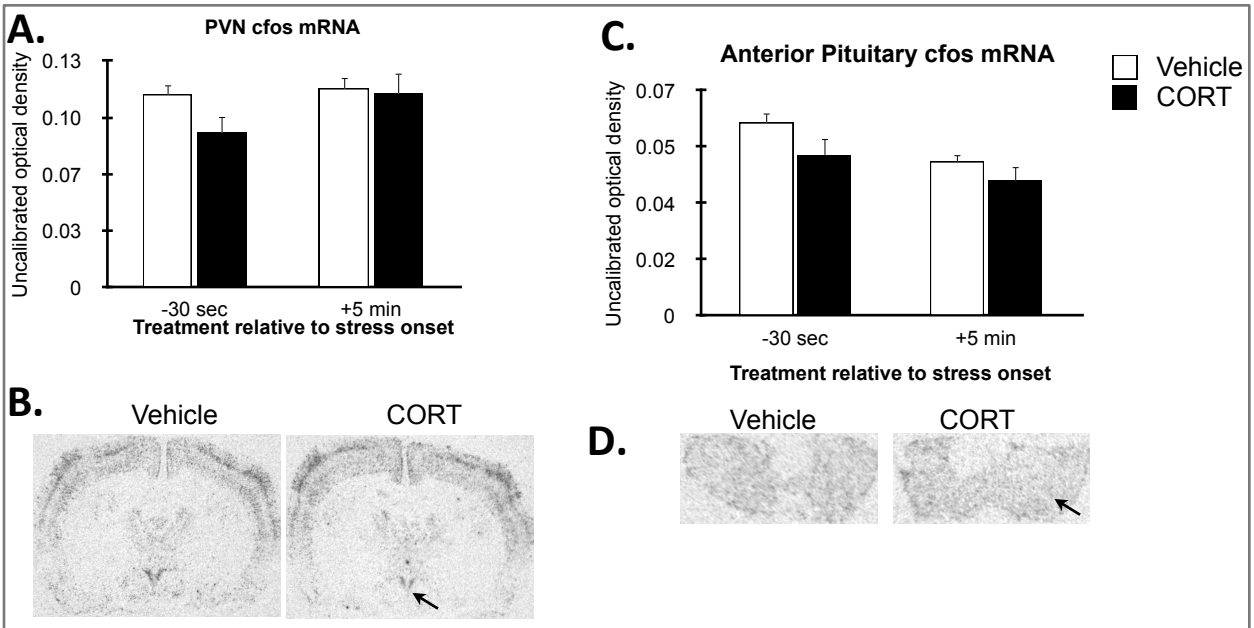

Figure 4.10 Phasic CORT post-treatment effect on PVN and anterior pituitary *c-fos* mRNA (experiment 4). (experiment 4). Neither phasic CORT treatment before or during stress produced an inhibitory effect over PVN to anterior pituitary cfos gene expression. 30 second CORT pretreatment and a 5 minute CORT post-treatment did inhibited restraint-induced PVN (A,B) and anterior pituitary (C,D) cfos mRNA expression. †, represents a significant CORT effect when compared to vehicle treatment time condition ($p<0.05$, FLSD). Panel (B) PVN and panel (C) anterior pituitary show relevant representative autoradiograms for in situ hybridization

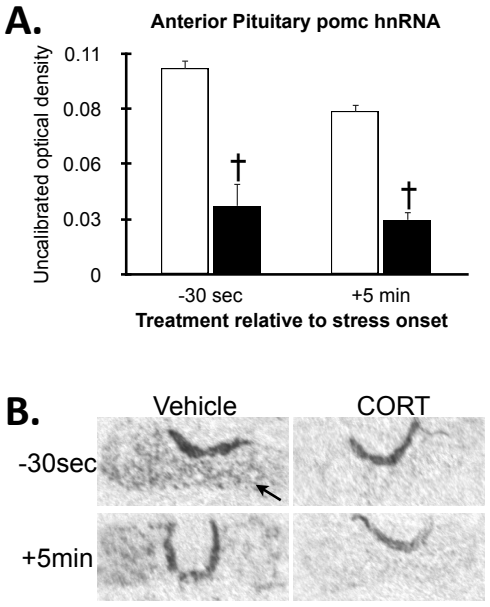

Figure 4.11 Phasic CORT post-treatment effect on corticotrophs pomc hnRNA (experiment 4). Neither phasic CORT treatment before or during stress produced an inhibitory effect over PVN to anterior pituitary cfos gene expression. 30 second CORT pretreatment and a 5 minute CORT post-treatment did inhibited restraint-induced PVN (A,B) and anterior pituitary (C,D) cfos mRNA expression. †, represents a significant CORT effect when compared to vehicle treatment time condition ($p<0.05$, FLSD). Panel (B) PVN and panel (C) anterior pituitary show relevant representative autoradiograms for in situ hybridization for cfos mRNA expression. Tissue collected 30 minutes after stress onset. Black arrow indicates PVN (B) or anterior pituitary (C).

Discussion

We demonstrate in this series of experiments that the timing of phasic CORT exposure relative to stress onset (pre- or post-stress restraint initiation) produced a significantly different pattern of HPA hormone suppression. The preexposure to phasic CORT (seconds, minutes or hours) produced a significant suppressive effect over the initial ACTH response to that stress. Regardless of when CORT pretreatment occurred, rats were able to show a slight and transient ACTH response to stress at the 5 minute time-point only. Whether this slight and transient ACTH response translates into a subsequent adrenal CORT response remains to be determined. However, all phasic CORT pretreatments significantly prevented the HPA axis ongoing (the remaining 15 minutes) response to stress. Exposure to phasic CORT minutes after stress onset produced no additional suppressive action over the initial (first 15 minutes) HPA axis ACTH response to stress, but eventually produced an inhibitory effect over the ongoing ACTH response to that stress challenge. This observation indicates that stress evokes an HPA resistance to the presence of phasic CORT after stress onset. Essentially, phasic CORT regulation over HPA axis hormone release is dependent on the state of the HPA axis (basal or stimulated) relative to the timing (pre or post) of phasic CORT treatment. We also found that regardless of our phasic CORT inhibition of stress-stimulated ACTH secretion these effects were not coupled with a similar inhibitory effect over stress-induced PVN and anterior pituitary *c-fos* mRNA expression as well as stress-stimulated prolactin secretion. However, we did find that *pomc* hnRNA expression was suppressed by both the preexposure to phasic CORT and exposure to CORT during stress in experiment 4.

Intermediated and fast CORT negative feedback

In these experiments we demonstrate that phasic CORT pretreatment (seconds, minutes and up to an hour) blocked subsequent HPA axis initial and ongoing ACTH response to that stressor. The literature provides some evidence that fast CORT negative feedback and intermediate (1-3 hours) CORT negative feedback are separable not only by time but also by mechanism. We have shown in a recent *in vivo* study that the underlying mechanisms of intermediate CORT negative feedback control of HPA hormone response to restraint depend on CORT-induced *de novo* protein synthesis [67]. We additionally demonstrated that the temporal window for intermediate genomic dependent negative feedback actions of phasic CORT extend up to 3 hours [67,69]. Certain *in vitro* studies support a protein synthesis-dependent functional glucocorticoid inhibitory effects on corticotroph function within a similar timeframe (Dayanithi & Antoni 1989, Woods et al. 1992, Clark & Kemppainen 1994, Tierney et al. 2003).

While intermediate CORT negative feedback function is dependent on genomic activity, which takes considerable time to develop [3,46,67,79], fast CORT negative feedback relies on non-genomic actions within the cell. Fast feedback occurs too quickly to depend on glucocorticoid dependent transcriptional and translational processes [3,79]. Recent studies suggest that fast glucocorticoid negative feedback regulates HPA axis reactivity to stress through a glucocorticoid receptor-dependent activation of a retrograde cannabinoid system that results in suppressive modulation of stress-induced excitatory synaptic input to the PVN CRH neurons [72,75,76]. Classic glucocorticoid literature indicates that fast glucocorticoid negative feedback is transient, lasting less than 10 minutes [52,182]. Here we demonstrate that fast feedback produced a significantly robust inhibitory action over the ACTH response to stress. Additionally, we also demonstrate that the temporal window for the induction of fast feedback by a pre-stress

phasic CORT increase is longer than previously believed, extending up to 15 minutes and can be induced within seconds rather than an induction phase of minutes. However, the underlying mechanisms responsible for this extended phasic CORT action is unclear. This relatively short temporal window of 15 minutes likely rules out CORT induced genomic actions and suggests that phasic CORT likely alters the HPA axis response through intracellular CORT mediated interactions.

The idea that fast negative feedback is short-lived stems from studies that have suggested that fast negative feedback is lost when phasic glucocorticoid levels plateau and begin to decline and could only measure a negative feedback effect an hour or more later when glucocorticoid induced genomic actions begin to take effect [64]. Both *in vivo* and *in vitro* studies extending from the early 1960's suggested that animals exposed to rapidly increasing concentration of exogenous glucocorticoids (rate-sensitivity) within minutes before stress onset significantly inhibited the HPA axis response to stress [64]. Work from the Jones laboratory in the 1970s showed that fast feedback at the level of hypothalamus displayed glucocorticoid rate-sensitivity [91,191,192]. Others have also shown that fast glucocorticoid inhibition of ACTH release was only seen when CORT levels increased rapidly as opposed to when the magnitude of CORT concentrations leveled or began to clear (falling phase), suggesting that this feedback is rate-sensitive [73,74]. The Lightman group has recently shown that pulsatile (ultradian) CORT activity had a "phase-dependent" inhibitory effect that occurred during the rising phase of plasma CORT but not present during the falling phase, this effect was also observed during stress [50]. However, whether the observed phase dependent effect in this study was truly rate-sensitive could not be discerned since multiple pulses of CORT were used. In contrast to some studies we

found that the induction of fast negative feedback is not dependent on the rate of CORT increase. Here we show that CORT exposure within 30 seconds to 15 minutes before the onset of stress produced a potent inhibitory action over the HPA axis ACTH initial and ongoing response to restraint. This inhibitory action clearly was not rate-sensitive, as this particular temporal window of negative feedback effect occurred when our exogenous CORT treatment (15 min pretreatment) had circulating levels that were already at a maximum concentration and had continued to plateau.

The glucocorticoid negative feedback silent period

Initial studies of fast and intermediate glucocorticoid negative feedback actions showed those effects to be separated by a glucocorticoid feedback inactive period (silent period), which is described as a 15-45 minute temporal window whereby prior stress onset of glucocorticoid administration fails to produce a functional negative feedback effect over stimulated HPA hormone secretion [45,52]. The possible existence of a silent period has been reinforced from other studies that have suggested fast negative feedback has a rate-sensitive requirement that produces a rapid induction phase and a transient duration phase that lasts less than 10 minutes, while intermediate glucocorticoid genomic actions take multiple hours to develop. We found that if CORT exposure preceded an acute stress challenge by seconds, minutes or 1 hour, we then observed a potent inhibitory action over the HPA axis ACTH response to that stressor (restraint). This indicated that the suppressive actions of CORT were not restricted to an interval of time whereby CORT fails to produce a functional negative feedback effect over HPA axis activity, however, this was not necessarily true if CORT exposure was present after stress onset. The primary reason for this negative feedback discrepancy is that most of the evidence that indicates

the existence of a glucocorticoid silent period was fundamentally based on observations derived from experimental assays and techniques that produced unreliable and inconsistent results (e.g. insensitive, inadequate and/or indirect CORT, ACTH and CRH assays etc.) [182,193].

Isolating the negative feedback effects of CORT

Most of the phasic glucocorticoid negative feedback literature has focused on separating glucocorticoid effects from stress effects through pretreatment experimental designs (exposure to CORT before stress). The problem herein is that a phasic increase in endogenous CORT is inevitably stress-dependent, and so we do not have an adequate understanding of how glucocorticoid pretreatments apply to a normal stress situation. We do have some understanding of how a phasic increase in CORT produced by stress influences the HPA axis response to a second stressor. Exposure to back-to-back stressors change the subsequent responsiveness of neurological, physiological and endocrine systems, in particular the HPA system. For instance, prior exposure to inescapable tailshock (a potent stressor) primed or sensitized the HPA axis hormone response to a subsequent pedestal stress challenge [194]. In contrast, prior exposure to pedestal stress decreased the HPA axis hormone response to a subsequent restraint stress challenge, but did not completely abolish the HPA hormone response to restraint [63]. The problem is that we really do not know whether glucocorticoid negative feedback effects from exogenous glucocorticoid pretreatment paradigms can be attributed to negative feedback actions that may take place during an acute stressor situation or a back-back stressor situation without superimposing or examining whether these glucocorticoid effects take place during these unique stressor situations. What is more important, is that no one particular study has accurately examined what negative feedback effects are produced by a phasic rise in CORT that

accompanies a typical stress response. The lack in documentation of phasic CORT effects during stress is significant because stress leads to a rapid increase and sustained secretion of ACTH (as opposed to a single bolus release of ACTH) [48], which implies the necessity for immediate negative feedback control over the HPA axis ongoing ACTH response to stress.

Negative feedback effects of CORT during stress

One of the key findings of this study is that phasic CORT exposure after stress onset did not produce an observable negative feedback effect in adrenal intact rats challenged with restraint. This suggests that a restrictive temporal period may occur after stress onset wherein CORT may be unable to provide a suppressive regulation over the HPA axis initial and ongoing response to that stress. Elizabeth Young demonstrated that cortisol treatment of rats given 2 minutes after stress onset provided some inhibitory effect over rat ACTH hormone secretion 30 minutes after treatment [195]. Interestingly, rats in Young's study did exhibit a significant ACTH and CORT response to restraint when compared to appropriate baseline samples indicating that restraint still evoked a significant HPA axis response despite cortisol treatment. It is also noteworthy to point out that cortisol is not primarily produced by rats and has a greater ability to penetrate the blood-brain-barrier compared to CORT. Also, the cortisol treatment in Young's study was given at a time-point were CORT levels are documented to still be relatively low. Typically stress evoked increase in CORT begins to rise by 3 minutes albeit these CORT levels are still low [64]. To date, the glucocorticoid negative feedback literature lacks studies that have accurately documented the negative feedback actions of CORT that typically accompany an acute stressor situation.

In this series of studies we used a 5 minute post-stress onset time point to treat rats due to evidence which demonstrates that stimulated (stress or ACTH treatment) increase in endogenous phasic CORT begins to significantly rise by 3 minutes and peaks between 15-20 minutes after stimulation [182,196]. In addition, serial blood profiling from our validation experiment indicated that endogenous CORT was significantly increased from basal levels within 5 minutes and peaked within 15 minutes after stress on set. Therefore, we would expect endogenous fast glucocorticoid actions to begin to manifest between 5 and 15 minutes post stress onset. Interestingly, we found that CORT administered to adrenal intact rats 5 minutes post-stress onset was unable to produce a further suppressive effect over the HPA axis response to restraint. This result suggests that stress evoked an HPA resistance to the suppressive action of CORT. Alternatively, this also suggested that stress-stimulated increase in endogenous phasic CORT during stress provides maximum constraint over the ongoing ACTH response to stress, producing a ceiling level negative feedback effect, thereby excluding further suppressive action from an exogenous CORT treatment given during stress. The question still remains, does endogenous phasic CORT produce a measurable negative feedback effect over the HPA axis initial and ongoing response to stress?

To test whether there is an absence of a fast negative feedback during stress or a negative feedback ceiling-level effect produced by endogenous phasic CORT, we eliminated the physiological ability of rats to produce a phasic CORT response by removing their adrenal glands (ADX). We then selectively reinstated phasic CORT to rats challenged with restraint with either post-stress 5 minute CORT or vehicle treatment. We found that an exogenous reinstatement of phasic CORT activity 5 minutes post-stress onset (during stress) provided a

delayed (timed) suppressive effect over the ongoing HPA hormone response to restraint, but not the initial ACTH response. As a comparison group we administered a 30 second CORT or vehicle pretreatment to another group of ADX rats, which provided a suppressive effect over the initial and ongoing ACTH response. These observations indicate that the timing of phasic CORT exposure relative to that of stress onset resulted in a significantly different pattern of HPA hormone suppression. Consequently, phasic CORT regulation over HPA axis hormone release is dependent on the state of the HPA axis (basal or stimulated) relative to the timing of phasic CORT administration. We found that phasic CORT treatment during an ongoing stress challenge produced a delayed suppressive effect over stimulated ACTH secretion of ADX rats. In contrast, exposure of CORT seconds, minutes or hours before the same stress challenge almost completely prevented the HPA ACTH response to stress.

Our observed delayed feedback effect of CORT during stress suggests that the HPA axis initial resistance may be an adaptive characteristic that allows for an appropriate HPA hormone response to acute stress. However, eventually phasic CORT leads to the shut off of the HPA axis ongoing hormone response to acute stress. The results from these experiments also indicated that the prior presence of CORT (analogous to that produced by a previous stressor) within a temporal proximity window of seconds to hours of additional stress challenge will likely have an almost complete inhibitory effect over the initial HPA hormone response and complete suppression of the ongoing response to that stressor.

It is also important to point out that tonic CORT replacement in the drinking water did provide a partial normalization over the basal levels of ACTH (baseline plasma levels were within the expected range typically produced by this CORT replacement regimen) [59]. We have

shown that without tonic presence of CORT (via ADX), the HPA hormone response to stress is significantly amplified [58,59]. These studies from our lab have also shown that tonic CORT provides an important normalization over the initial (first 15 minutes of restraint) and ongoing (30 to 60 minutes) HPA hormone response to stress [59]. This effect clearly demonstrates that tonic CORT constrains the magnitude of HPA axis hormone response to stress. Although tonic CORT replacement normalizes the HPA stress response, it does not provider a timed shutoff over the HPA hormone response. This emphasizes the importance for phasic CORT control and shut-off over the continued HPA hormone response to stress that we demonstrate here.

Spatial effects of phasic CORT

Temporal glucocorticoid negative feedback actions depend on glucocorticoid-sensitive cellular elements within the HPA axis [45,67,68]. As a result, the temporal suppressive actions of phasic CORT activity are associated with distinct HPA axis hormone changes that may parallel intracellular alterations in PVN and pituitary experience-dependent gene expression such as *c-fos*, *crh*, and *pomc* [67-70,93,134,139,158]. We have repeatedly demonstrated that PVN and anterior pituitary *c-fos* mRNA expression is almost undetectable during unstimulated states but is significantly increased in response to restraint challenge [58,59,67,69,70]. As mentioned in the general introduction (chapter 1), we have also demonstrated that both systemic or local PVN microinfusion of CORT 1 hour before stress challenge produce a negligible inhibitory effect on stress-induced PVN *c-fos* gene expression [67,68]. However, this treatment strongly inhibits stress-induced PVN *crh* hnRNA and corticotroph *pomc* hnRNA expression and ACTH secretion. As also mentioned in chapter 1, the stress-induced prolactin that released from lactotrophs in the anterior pituitary is independent of HPA axis activity and is unaffected by a 1 hour CORT

microinfusion to the PVN [68]. Taken together, these results demonstrate that 1 hour intermediate CORT pretreatment inhibition of ACTH is not associated with an inhibitory effect over the stress-induced excitatory drive to the PVN as indicated by its lack of suppressive effect over PVN *c-fos* gene expression and prolactin secretion.

We found in this study that the suppressive effects of phasic CORT produced by exposure 1 hour, 15 minutes, and 30 seconds before or during stress was not coupled with an inhibitory effect over stress-induced PVN and anterior pituitary *c-fos* mRNA expression or stress-stimulated prolactin secretion. However, we did find that 1 hour, 15 minute, and 30 second CORT pretreatment did inhibit both stress-induced anterior pituitary corticotroph *pomc* hnRNA expression and ACTH secretion. As previously mentioned PVN CORT micro-infusion 1 hour before stress onset produced the same suppressive pattern (decrease in ACTH release and *pomc* hnRNA, but not *c-fos* mRNA or prolactin secretion) [68]. Taken together, this consistent temporal pattern of glucocorticoid inhibition supports the notion that suppressive intermediate phasic CORT activity acts locally at the PVN to suppress stress-stimulated excitatory release of CRH, without altering stress-associated stimulatory inputs to the PVN. The Shipston group has shown *in vitro* evidence that intermediate temporal glucocorticoid activity induces a protein that interferes with CRH-stimulated corticotrophs ACTH secretion [86,96], indicating that intermediate glucocorticoids act at the level of anterior pituitary to suppress stimulated ACTH release. It is likely that the suppressive effect over the *pomc* gene expression that we show in this series of experiments is also a result of direct glucocorticoid negative feedback within corticotrophs [67,69]. Evidence also supports that fast negative feedback is at least in part mediated by glucocorticoid-induced cannabinoid activity that subsequently reduces presynaptic

glutamate stimulation of PVN CRH neurons [72,75,76,95]. Also, fast glucocorticoid suppression of stimulated HPA axis hormone activity has been shown to depend on direct independent actions within the anterior pituitary [79]. It is therefore likely that the fast negative feedback that we show here immediately before and during stress onset is also dependent on phasic CORT actions at the level of the PVN and pituitary.

We found that none of our CORT treatments suppressed overall stress-induced *c-fos* mRNA in the anterior pituitary. At first glance this suggests that our phasic CORT treatments did not inhibit PVN CRH release and suppression of ACTH must be attributed to a direct CORT effect at the level of the anterior pituitary, rather than the PVN. However, corticotrophs constitute an incredibly small proportion of the endocrine cells in the anterior pituitary [197]. In contrast, lactotrophs occupy a larger portion of the anterior pituitary and are stress-reactive [68,112,198]. Visual comparison of the anterior pituitary autoradiograms characteristically show that stress-induced *c-fos* mRNA has a dense and dispersed expression pattern throughout the anterior pituitary, unlike the limited and punctate expression of stress-induced *pomc* hnRNA [67-69,158]. Stress-stimulated prolactin levels were not altered by our systemic CORT treatment or by intra-PVN CORT microinfusion [68]. Consequently, any putative CORT suppression of corticotroph *c-fos* mRNA expression would have been masked by the lack of a suppressive CORT effect over *c-fos* expression in lactotrophs. There is evidence for glucocorticoid (Dex) suppression of stress-induced prolactin in the rat, this suppressive effect requires *de novo* protein synthesis [199]. This study suggests that glucocorticoids likely produce a timed-dependent delay in suppression over stress-induced activation of lactotrophs. Taken together, these observations support the

possibility that stress-induced *c-fos* expression in the anterior pituitary is primarily attributable to lactotrophs, respectively.

Conclusion

Overall, these results demonstrate that initial and ongoing response of ACTH to stress is not simply arrested by the presence of phasic CORT. The series of experiments presented here demonstrate that the HPA axis initial and ongoing response to an acute psychological stress challenge is determined by the timing of phasic CORT exposure relative to the onset of stress. Phasic CORT present during stress is required to maintain the time-course shutoff of the HPA axis response to an acute stressor. However stress can transiently evoke HPA axis resistance to the suppressive effects produced by CORT exposure during stress. Alternatively, preexposure to phasic CORT hours to seconds before stress onset essentially prevents the HPA axis response restraint. Furthermore, none of our CORT treatments suggested that exposure to phasic CORT before or during stress alter or dampen the excitatory stress inputs to the HPA axis. This suggests that phasic CORT produces suppressive effects directly at the anatomical level of the PVN and anterior pituitary regardless of pre-stress onset or post-stress onset CORT treatment.

Chapter V

General Discussion

Textbook glucocorticoid negative feedback control of HPA axis activity is often grossly over simplified as a homeostatic negative feedback system that is analogous to a home thermostat. The thermostat simply reacts (on or off) to changes in temperature only after they occur. Similarly, the HPA axis control of glucocorticoids is often thought of as a simple reactive system, whereby the HPA axis merely responds to stress by releasing glucocorticoids and in turn glucocorticoids shut-off the HPA axis (negative feedback). However, the interplay between the HPA axis, stress, and glucocorticoids are quite complex. Stress comes in a seemingly endless variety of forms, and not all stressors produce equivalent HPA hormone responses. Also, the HPA axis is often employed to anticipate impending stress. In some cases of physiologic stress (e.g. hypovolemia, hypoxia, infection, injury, poison, toxins, etc.) the HPA axis is in a sense reactive; physical stressors activate specific sensory receptors and associated neural pathways that work to produce immediate homeostatic changes that are accompanied by activation of the HPA axis [200-203]. We are often unaware of these physiological changes. By contrast, psychological stressors (e.g. major life events, traumatic threats, disease, anxiety, depression, etc.) do not necessarily produce changes in sensory systems as physiologic stressors do, rather they activate stress responsive forebrain neural circuits (PFC, amygdala, hippocampus, limbic system, etc.), as well as similar hormonal systems (e.g. sympathoadrenal medullary system and the adrenocortical system) [140]. Psychological stressors are perceived threats that require higher order neural activity that lead to activation of the HPA axis to help an organism prepare to meet, avoid, anticipate and/or adapt to imminent adverse challenges. Also, unlike the thermostat analogy, the HPA axis does not simply respond to all stressors equally (i.e. it is not just on or off). For instance, both PVN gene induction and HPA axis hormone release is significantly

correlated with the intensity of the stress challenge [93]. Furthermore, not all stressors and associated glucocorticoid responses equally impact the HPA axis, as the prior exposure to an intense stressor can in some cases sensitize the HPA hormone response to another stressor. Alternatively, pre-exposure to a mild stress challenge can decrease the HPA hormone response to another moderate stress challenge [166,194]. The PVN CRH neuroendocrine neurons must respond to different stressors by initiating and producing appropriate release of CRH peptide which consequently generates the necessary glucocorticoid response that is needed to deal with the threat at hand.

Chapter 2-4 further illustrate that functional negative feedback control of stress-stimulated HPA axis hormone activity mechanistically depends on three distinct glucocorticoid actions—first, the physiological secretion pattern (tonic and phasic), second, temporal actions (intermediate and fast), and third, the intercellular (systems levels changes) and intracellular (molecular changes) spatial actions. The "moment to moment" status of these three glucocorticoid actions (secretion profile, spatial and temporal actions) establish how the HPA axis will initially respond to stress and continue to respond to stress (chapter 2-4). In other words, the HPA axis is more than a simple 'on' and 'off' switch, as the thermostat analogy would suggest. The system must integrate and be sensitive to changes in glucocorticoid status, physiological demands, and homeostatic challenges. The ambiguity that still surrounds how these three glucocorticoid actions control normal HPA axis activity has likely precipitated the inability to completely understand how glucocorticoids contribute to wide spectrum of physical and mental disorders.

Part I

Tonic CORT stabilizes the HPA response (initial, continued and recovery) to acute stress

From the serial blood sampling profile in chapter 4 we can conclude that the HPA hormone response to stress (30 minute restraint challenge) can be separated into two phases, an initial phase and a continued (ongoing) phase. The initial stress phase refers to the evoked rapid increase and sustained HPA hormone response to stress, which corresponds roughly to the first 15 minutes of restraint. The continued stress phase refers to the sustained hormone response to restraint (roughly the last 15 minutes of restraint). There is a third phase that is also important to mention—a post-stress recovery phase, which refers to when HPA hormone activity has completely returned to basal levels. Previous studies from our lab indicate that the stress recovery period can occur toward the end or after cessation of a stress challenge [59,93,166,204]. These three phases of HPA hormone activity will be used throughout this discussion to help characterize and identify the presence or absence of specific tonic and temporal phasic CORT negative feedback actions.

Chapters 2 and 3 illustrate that diurnal tonic CORT expression is required for maintaining normal HPA hormone responsiveness to an acute psychological stress challenge. Upon removal of this tonic circulating presence of CORT (via ADX) the HPA axis initial and continued hormone response to stress is significantly amplified or augmented (figure 5.1 A) [59]. Additionally, the long-term absence of tonic CORT also significantly disrupts post-stress recovery of the HPA system (figure, 5.1 A) [59]. Here (chapter 2) we show that reinstatement with tonic CORT normalizes HPA axis augmentation produced by ADX (figure 5.1 B). Previous studies from the Spencer lab have also shown that tonic CORT provides an important recovery

(albeit a partial recovery) over the initial, ongoing and recovery phases of the HPA axis response to restraint (figure 5.1B) [59]. Taken together, these results clearly demonstrate that tonic CORT constrains the magnitude of HPA axis hormone response (initial, continued and recovery) to stress. Although, tonic CORT replacement normalizes the HPA hormone activity, it does not shut-down stress-stimulated HPA hormone responses. Experiments presented in chapter 4 show that phasic CORT given before or during restraint challenge either prevents or produces a timed shut-off over the HPA hormone response to stress; as opposed to the constraining effect tonic CORT has on the system. Whether phasic CORT prevents HPA axis hormone reactivity to stress or produces a timed shut-off over the system depends on when exposure to CORT occurs (i.e. before or during stress).

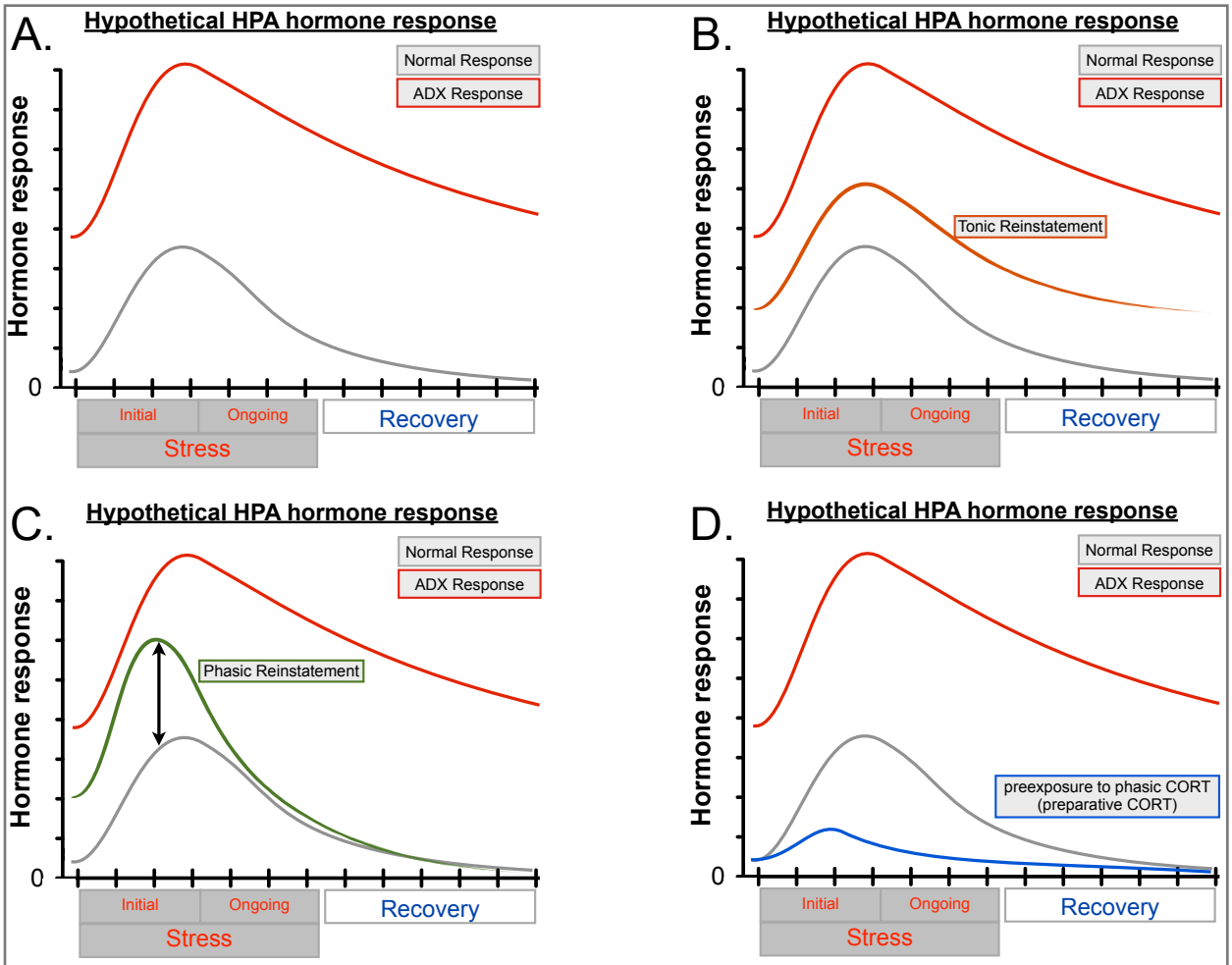

Figure 5.1 Hypothetical HPA hormone response to stress.
A-D, gray-line depicts normal physiological secretion profile of HPA hormone response to stress. A, ADX augments HPA hormone initial, ongoing and recovery response to stress (adapted form Pace et al 2009). B, tonic CORT reinstatement normalizes ADX induced HPA hormone augmentation (constrains HPA axis). C, phasic CORT reinstatement suppresses the ongoing HPA hormone responses, but no the initial, arrow indicates HPA resistance to phasic CORT. D, preparative CORT effects produced by phasic CORT pretreatment significantly suppresses the initial HPA hormone response to stress and prevents the ongoing hormone response.

Phasic CORT exposure during stress produces a timed shut-off over the continued HPA response to stress

We assume that the phasic increase in CORT that accompanies an acute stressor produces immediate (fast) suppressive action over the HPA axis [44]. However, there is little evidence documenting this effect despite the vast existing glucocorticoid negative feedback literature. Furthermore, not many studies have accurately produced conclusive documentation which illustrates that stress induced phasic increases in CORT provide negative feedback control over the HPA axis continued response to stress. In chapter 4, we clearly show that reinstatement of phasic CORT 5 minutes post-stress onset produced a timed shut-off over the HPA continued hormone response to stress (figure 5.1 C). This negative feedback effect manifested roughly 25 minutes after treatment. The initial HPA hormone reactivity was completely unaffected by this post-stress onset phasic CORT treatment (figure 5.1 C as indicated by the arrow), which suggest stress-evokes HPA resistance to CORT. Whether phasic CORT is necessary for the shut-off or simply regulates the time-course of HPA axis recovery to stress is somewhat unclear. There is some evidence which demonstrates adrenalectomized rats HPA hormone response to stress does recover over time, albeit the period last much longer than is necessary (figure 5.1 A) [59]. Furthermore, tonic replacement of ADX rats does not adequately normalize stress-stimulated HPA hormone recovery, the time-course is still longer than expected and basal hormone levels are still augmented compered to adrenal intact rats [59] (figure 5.1 B). Taken together, these results all suggest that phasic CORT modulates the shutoff time-course of the HPA hormone response to stress, effectively also reducing the post-stress HPA axis recovery period (figure 5.1C).

The observed CORT-induced negative feedback pattern during stress does not necessarily fit the glucocorticoid negative feedback model that was originally proposed by Dallman and Yates in the 1960, nor does it fit text-book negative feedback whereby glucocorticoids simply turn-off stress-induced HPA hormone secretion. Rather, we find that phasic CORT negative feedback during stress is restricted from producing any suppressive action over the initially hormone response to restraint (figure 5.1 C), which suggests that stress engages intercellular and/ or intracellular mechanisms that temporally ensures that the HPA axis can respond to an acute stress episode. If there truly is a stress evoked biological mechanism in place that momentarily prevents CORT from producing negative feedback it is unknown. Alternatively, this initial lack in control over the ACTH response to stress is perhaps due to the partial normalization of the HPA axis as a result of our tonic CORT replacement regimen. In either case, this HPA axis negative feedback resistance is short-lived, as phasic CORT eventually provided a timed shut-off over the HPA system (figure 5.1 C).

Preparative phasic CORT essentially prevents the HPA axis response to subsequent acute stress

A decade ago Sapolsky differentiated some negative feedback actions based on whether glucocorticoids alter the physiological HPA response to a subsequent stressor as “preparative” actions [44]. Sapolsky also stated that classifying some glucocorticoid actions as preparative is new and poorly understood, however, a decade later we have a clearer understanding of some of the temporal and mechanistic requirements of preparative CORT actions. First, purely preparative CORT actions exist and are quite complex. Our lab has previously shown that the acute pre-exposure (1 hour) to a pedestal stressor reduced the HPA reaction to a second

heterotypic stressor (restraint); moreover, the same pre-exposure to pedestal stress significantly reduced the habituated HPA hormone response restraint [63]. In this case we see that the preparative effects of CORT (produced by the pedestal stress) provided an added (and likely adaptive) suppressive influence over the reactiveness of the HPA axis to the second stressor, restraint.

In chapter 4, we effectively separated preparative phasic CORT effects from the producing stressor by pretreating animals with exogenous CORT. We demonstrated that pre-exposure to phasic CORT within seconds (fast) of and up to an hour (intermediate) before stress onset significantly suppressed the immediate HPA hormone response to stress and completely prevented the continued hormone response to stress (Figure 5.1 D). As mentioned in chapter 4, the initial studies that characterized intermediate (~1 to several hours) and fast (less than 10 minutes) glucocorticoid negative feedback actions suggest that these temporal negative feedback effects are separated by an inactive period (silent period). This silent period is roughly a 15-45 minute temporal window between fast and intermediate negative feedback wherein glucocorticoid administration fails to produce a negative feedback effect over subsequent stress-induced HPA axis activity [45,52]. In contrast to this literature, we found that a preexposure with CORT seconds, minutes, and an hour before stress produced a potent inhibitory effect over subsequent HPA hormone reactivity to stress, indicating that preparative suppressive CORT actions are not temporally restricted.

Our results in chapter 4 also indicate that preparative CORT negative feedback effects can be separated into two different temporal categories, intermediate and fast negative feedback. I have shown recent *in vivo* evidence which indicates that the underlying mechanisms of

intermediate negative feedback effects produced by a preexposure of CORT depend on CORT-induced *de novo* protein synthesis (genomic effects) [67]. This suggests that intermediate preparative CORT effects demonstrated by Pace et al (2001) likely also depend on CORT-induced genomic actions. I have also demonstrated that intermediate preparative suppressive CORT actions extend up to 3 hours before a subsequent stress challenge and these actions continue to be dependent on CORT-induced *de novo* protein synthesis [67]. However, the putative CORT-induced protein(s) responsible for these negative feedback actions have yet to be completely identified. However, evidence herein and from other publications continue to suggest that putative CORT-induced protein(s) likely influence stress-stimulated excitatory-exocytosis coupling mechanism that lead to HPA peptide release [80-85]. On the other hand, there is also *in vivo* support that indicates preparative fast negative feedback effects regulate HPA axis reactivity to stress through a GR-dependent activation of a retrograde cannabinoid system [72]. Therefore, it is likely that any preparative suppressive CORT effects within close temporal proximity (seconds to minutes) to stress also depend on similar non-genomic actions. In Part II, I will show some support that fast-negative feedback actions may also take place in PVN CRH nerve terminals.

Part II

Spatial suppressive actions of tonic and phasic CORT

Measuring changes in ACTH plasma levels provides information about the current state of the HPA axis. For instance, exposure to stress increases ACTH release indicating that the HPA system is in an active state. This ACTH release proportionally increases as stress intensifies further indicating that the HPA system is in a heightened state of activation [166]. Additionally, if the application of CORT suppresses ACTH release, then we know the HPA axis is in a general state of CORT-induced suppression. However, measuring only systemic ACTH levels does not provide information about the anatomical and cellular location of where suppressive CORT effects takes place. Both stress-stimulated HPA axis activity and the suppressive actions of CORT correspond to distinct HPA cellular alterations involving changes in gene expression and signaling protein activity (chapters 2-3). As mentioned chapter 1, we have additionally demonstrated that phasic CORT suppression produces time-dependent changes in gene expression that can further help identify possible spatial CORT effects [67,69,70]. In chapter 2-4, we systematically compared functional HPA hormone output to changes in experience-dependent gene expression as well as signal transduction protein immunoreactivity within hypothalamic PVN neuroendocrine neurons and anterior pituitary cells, to identify possible anatomical locations of CORT negative feedback.

In chapter 2, we used experience-dependent intracellular signaling pathway analysis (changes in pERK1/2 immunoreactivity as measured by cell counts) as a marker of recent neuronal HPA activation and to spatially define the anatomical location of tonic CORT effects. As mentioned in chapter 2, ERK participates in stress-induced PVN activity by translating

excitatory inputs into increased CRH production and release [47,107,135]. We found that restraint produced a rapid increase in the number of PVN pERK1/2 immunopositive cells within CRH neurons. This increase in pERK1/2 was significantly augmented in ADX rats and normalized by tonic CORT replacement (via drinking water). Our lab has also demonstrated that ADX augments stress-induced PVN experience-dependent *crh*, and *c-fos* gene expression [58-60]. Interestingly, the absence of tonic CORT activity does not alter stress-induced *c-fos* or *arc* gene expression in medial-PFC and hippocampus, two brain regions that provide excitatory and inhibitory control over the PVN [152,153,168]. Taken together, these results indicate that the ADX-dependent augmentation of stress-induced HPA axis activity is not a consequence of an increase in stimulatory drive to the PVN. Rather, ADX-dependent amplification of stress-induced response of intracellular PVN signal transduction and gene transcription is produced by the absence of tonic CORT control of sites central to or within the PVN CRH neurons (figure 5.2 blue arrows) [154,158]. This disruption of tonic CORT regulation over these PVN locations is likely reflected throughout the HPA axis (i.e. augmentation in all hormones).

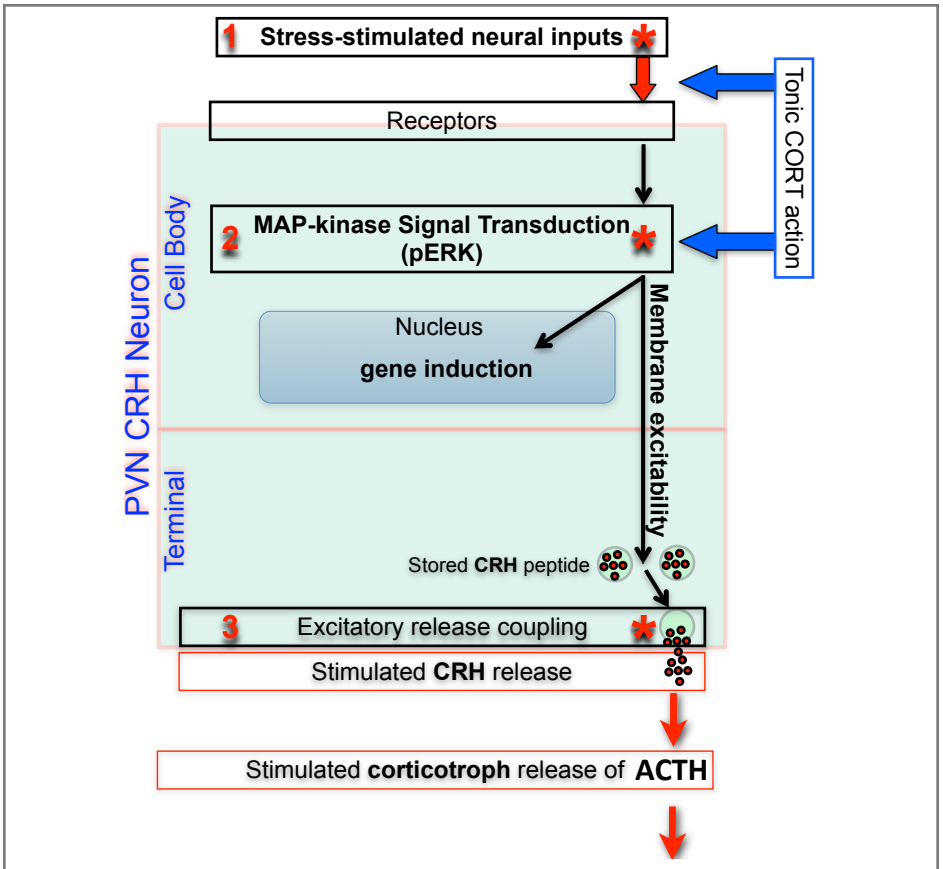

Figure 5.2 Hypothetical spatial actions of tonic and phasic CORT. *, depicts stress-stimulated activation of stress responsive extrinsic neural inputs to the PVN receptors. *, shows that PVN CRH intracellular processes that lead to gene induction and CRH releases are dependent on MAP-kinase signal transductionactivity. Red numbers (1-3) indicate extracellular or intracellular location for direct CORT negative feedback actions to occur. Blue arrows indicate possible spatial actions of tonic CORT activity. Figure was adapted from Watts et al. 2005.

Chapters 2 and 4 provide evidence that suggests that intermediate preparative phasic CORT treatment (1 hour) directly suppresses the excitatory intracellular coupling mechanisms responsible for PVN CRH release or similar suppressive action at the level of the anterior pituitary. First, results in chapter 2 demonstrated that stress induced pERK1/2 immunoreactivity was not affected by an intermediate phasic CORT treatment. This indicates that the suppressive intermediate preparative CORT effect over stress-induced ACTH release was independent of altering stress-induced PVN intracellular MAP-kinase signal transduction responses that are necessary for stress-induced gene induction and CRH release (figure 5.2, compartment 2). Furthermore, in chapter 4, we found that stress-induced PVN and anterior pituitary *c-fos* mRNA expression as well as or prolactin secretion were not suppressed by an intermediate phasic CORT treatment. Together these results (chapter 2 and 4) support the notion that extra-HPA axis stress-induced excitatory drive to the PVN remains unaltered by intermediate preparative CORT activity. We have provided recent evidence (noted in chapter 1) that an intermediate micro-infusion with CORT into the PVN inhibits corticotroph ACTH release but not PVN *c-fos* gene induction [67,68]. Collectively, these observations provide support that intermediate phasic CORT does not alter stress-induced excitatory drive to the PVN, stress-induced transynaptic inputs of the PVN, and/or stress-induced intracellular responses that reflect *c-fos* gene induction (figure 5.2, compartment 1 and 2). Rather, intermediate phasic CORT can provide independent suppressive alteration of the intracellular mechanisms associated with PVN CRH release (figure 5.2, compartment 3).

We can also extend our knowledge about the spatial location of fast preparative CORT negative feedback. Results in chapter 4 illustrate that fast phasic CORT pretreatment did not

suppress restraint-induced PVN and anterior pituitary *c-fos* gene expression or prolactin secretion. These findings suggest that fast CORT inhibition of ACTH secretion may be associated with CORT-induced alteration PVN CRH release (figure 5.2, compartment 2 or 3). This negative feedback notion is somewhat inconsistent with recent evidence showing that glucocorticoid-induced fast feedback inhibition of the HPA axis is in part mediated by a reduction in stress-induced transynaptic drive to the PVN [72]. However, this inhibitory effect was not associated with alteration in stress-induced c-Fos protein expression. These cumulative results do suggest that fast glucocorticoid inhibition of ACTH secretion is not associated with alteration of stress-induced PVN CRH intracellular signal transduction activity responsible for induction of the *c-fos* gene (figure 5.2, compartment 2).

As previously mentioned, it is possible that fast CORT negative feedback may also independently alter stress-induced excitation-exocytosis coupling mechanisms associated with PVN CRH release (figure 5.2, compartment 3). I have characterized some basic immunoreactivity that shows significant colocalization of GRs within PVN CRH nerve terminals (external zone of the median eminence) (figure 5.3). This colocalization supports the possibility that CORT activation of GRs within the CRH terminals may also facilitate suppressive modulation of CRH release, by directly inhibiting the intercellular protein cascades that are responsible for excitatory CRH peptide release (figure 5.2 and 5.3).

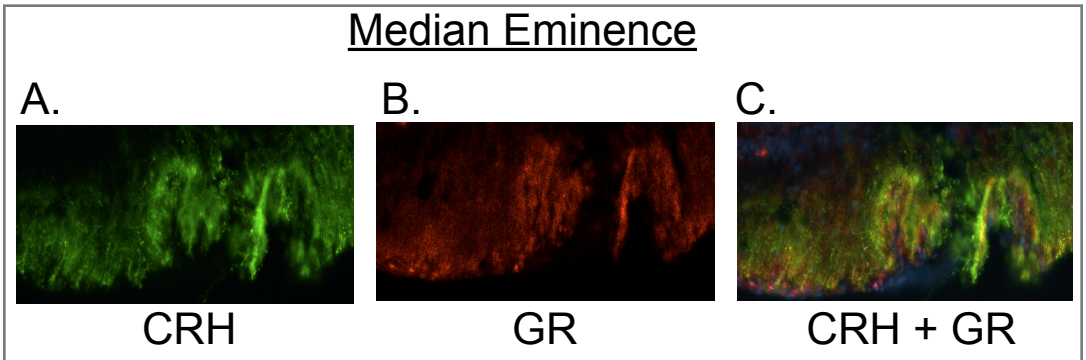

Figure 5.3 Median Eminence CRH and GR colocalization. Figure shows a significant colocalization of GRs within CRH terminals (median eminence). Immunoreactivity: (A) CRH green, (B) GRs red and (C) CRH and GR colocalization.

Hypothetical stress-dependent induction of medial-PFC and PVN *mkp-1* mRNA

As mentioned previously, regulation of stress-stimulated HPA axis activity depends on multiple glucocorticoid actions that vary with different secretion patterns (tonic and phasic) and anatomical site of action (intrinsic or extrinsic to the HPA axis). We also know that hypothalamic PVN CRH neuron function is regulated through the activation (phosphorylation) of ERK. Interestingly, inactivation of pERK within immune cells is dependent on CORT induced MAP kinase phosphatase (*mkp-1*) gene and protein expression. In chapter 3, we proposed that CORT induced *mkp-1* expression in peripheral tissues could be a model for ERK regulation in the PVN and medial-PFC (which are both important sites for HPA hormone control). We found that stress but not CORT upregulated *mkp-1* gene expression in the medial-PFC, PVN, and anterior pituitary. Additionally, the long-term absence of endogenous CORT augmented stress-induced *mkp-1* gene expression within the PVN and pituitary. Phasic CORT did not upregulate medial-PFC or PVN *mkp-1* gene expression. These results indicate that tonic CORT constrains stress-induced *mkp-1* mRNA expression, rather than up-regulating its expression, which further indicates that CORT does not regulate pERK1/2 activity via upregulation of the *mkp-1* gene. Rather, these results suggest that stress-induced *mkp-1* expression is an important activity-dependent intracellular regulator of stress-induced pERK activity in both extrinsic and intrinsic anatomical sites to the HPA axis. Furthermore, these results suggest that stress either directly (through unknown intracellular signaling activity) leads to the upregulation of medial-PFC and PVN *mkp-1* mRNA (figure 5.3) or that stress upregulates *mkp-1* mRNA transcription via activation of the MAPK pathway, some evidence suggests that pERK leads to the induction of phosphatase activity (figure 5.3) [205].

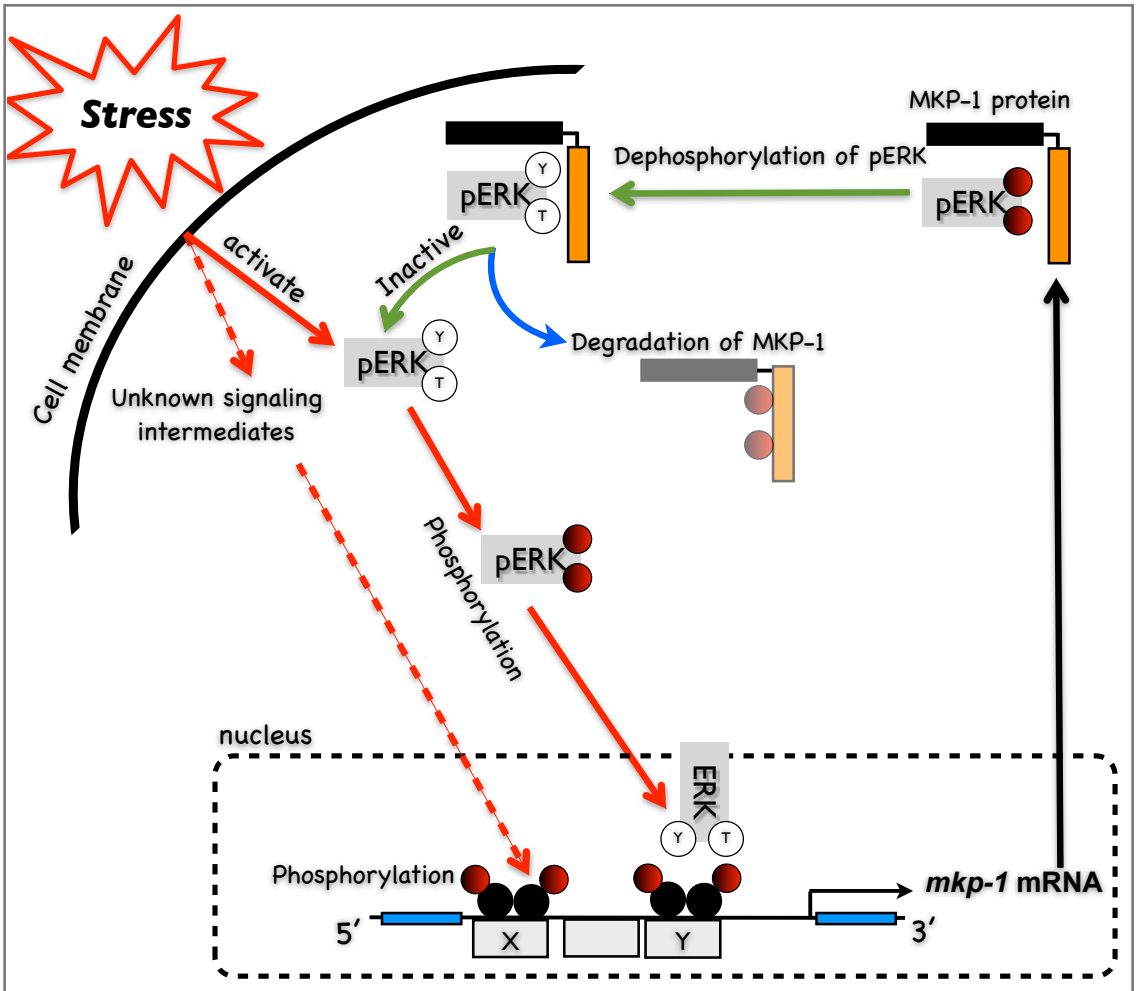

Figure 5.3 Hypothetical stress-deponent induction of medial-PFC and PVN *mkp-1* mRNA.

It is possible that the event of stress directly through ***unknown*** intracellular signaling activity leads to the upregulation of *mkp-1* mRNA or that stress upregulates mkp-1 mRNA via activation of the MAPK pathway. The MAPK signaling pathway has been proposed to induce its own negative feedback by regulating phosphatase activity. Figure was adapted form Li et al, 2001.

Summary

The series of experiments presented in chapters 2-4 demonstrate that proper stress reactive HPA axis functionality is established by the interaction between distinct secretion patterns, spatial effects, and temporal aspects through which glucocorticoids operate. In light of the results found in these studies, we can provide an updated working model of CORT negative feedback: The HPA axis initial and ongoing response to an acute psychological stress challenge is determined by the timing of phasic CORT exposure relative to the onset of stress. First, preparative suppressive effects produced by preexposure of phasic CORT hours to seconds before stress onset prevent the HPA axis response to stress. Second, phasic CORT exposure during stress results in the timed shut-off the HPA axis ongoing response to stress, however, the initial response is transiently resistant to phasic CORT. Third, the overall magnitude of HPA axis responsiveness to stress is modulated by the presence and state of tonic CORT activity. Fourth, these phasic and tonic CORT effects have distinct spatial locations within the HPA axis where their activity is reflected (figure 5.2). These negative feedback effects are likely not limited to a single anatomical site with the HPA axis, but studies herein strongly suggest that CORT influences PVN CRH release.

It is not a mystery that many of the adverse effects of stress on health are precipitated and exacerbated by impaired glucocorticoid regulation of the HPA axis [206,207]. However, the underlying mechanisms that instigate these stress related pathological conditions are not completely understood. A major contributor to this problem is the lack of a thorough understanding of the basic characteristics that define normal HPA axis activity and glucocorticoid negative feedback. Regardless of the reasons for this lack of HPA knowledge, the studies presented here advance some of our basic understanding of both the spatial and temporal

level aspects of CORT negative feedback control of the HPA axis response to stress, some of which may be applied to the clinical setting.

By temporally monitoring the HPA axis hormone reactivity to stress and various CORT treatments we identified some interesting and novel characteristics about the interplay between stress, the HPA axis and CORT. First, stress can transiently evoke HPA axis resistance to the suppressive effects produced by the exposure of phasic CORT during stress. This knowledge may be directly applicable to the clinical setting. For instance, there is evidence indicating that depressed patients display decreased sensitivity to fast glucocorticoid negative feedback [26]. This decreased sensitivity may be related to an exacerbation in overall HPA axis resistance to the suppressive effect of glucocorticoids. Second, we also demonstrated that phasic CORT treatment after stress onset eventually stops the HPA axis continued hormone response to stress (i.e. timed shutoff). Therefore, it is also possible that patients with decreased sensitivity to fast negative feedback have an irregularity in the underlying mechanisms that mediate this particular timed glucocorticoid shutoff effect. Both of these possibilities convey the necessity for further examination in identifying the systems, cellular, and/or molecular mechanisms responsible for both stress evoked HPA resistance to glucocorticoids as well as mechanisms that mediate normal suppressive phasic glucocorticoid timed shutoff of the HPA system.

The last and perhaps most intriguing future direction is to further examine the necessary innate and/or environmental conditions that may render an individual unable to effectively mount sufficient preparative glucocorticoid actions. Successful preparative glucocorticoid responses are crucial for tempering the effects of back-to-back stressors as well as rationing physiological resources if homeostatic threats continue as they often can or do [44]. It is reasonable to assume

that some stress related health issues directly correspond to the inability of some individuals to organize and initiate coordinated physiological and behavioral preparative responses to deal with multiple stressors that occur within close temporal proximity. As mentioned preparative CORT effects not only provide an adaptive suppressive effect over a subsequent acute novel stress challenge, preparative CORT actions also increase habituated responses to repeated stressors [63].

Although, the work presented in this thesis certainly broadens or knowledge of glucocorticoid regulatory control over stimulated HPA axis activity, it only represents an immensely small portion (albeit not insignificant) of the innovative research that has occurred and of the research that needs to continue. The original and ongoing reason I find stress interesting, is unlike many clinical conditions, everyone has and will continue to personally experience stress on a regular basis.

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