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Chronic Estrogen-Induced Alterations in Adrenocorticotropin and Corticosterone Secretion: Relationship to Mineralocorticoid and Glucocorticoid Receptor Regulation in the Female Rat Anterior Pituitary Gland and Brain

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CHRONIC ESTROGEN-INDUCED ALTERATIONS IN ADRENOCORTICOTROPIN AND CORTICOSTERONE SECRETION: RELATIONSHIP TO MINERALOCORTICOID AND GLUCOCORTICOID RECEPTOR REGULATION IN THE FEMALE RAT ANTERIOR PITUITARY GLAND AND BRAIN

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To Pattie and my parents

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VITA

The author, Loyd Hutchins Burgess, was on November 14, 1956 in Chicago, Illinois to Herbert and Dorothy Burgess.

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A post-doctoral position has been arranged with Dr. David Sibley at the NIH, where he will investigate structure-function relationships among Dopamine receptor subtypes.

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LIST OF ABBREVIATIONS:

ACTH	Adrenocorticotropic Hormone
ADX	Adrenalectomy
AHP	Afterhypepolarization
ALDO	Aldosterone
AP	Anterior Pituitary Gland
AVP	Arginine Vasopressin
bp	Basepairs
BW	Body weight
C	Centigrade
cAMP	Cyclic 5', 3' adenosine monophosphate
CA	Cornu amonis
CAT	Chloramphenicol Acetyltransferase
CBG	Corticosterone Binding Globulin
cDNA	Complementary Deoxyribonucleic Acid
cm	centimeter
CORT	Corticosterone
CRF	Corticotropin Releasing Factor(s)
CRE	cAMP responsive element
CRH	Corticotropin Releasing Hormone
CSF	Cerebrospinal Fluid
DEPC	Diethylpyrocarbonate
DEX	Dexamethasone
DST	Dexamethasone Suppression Test
E	Estrogen
ER	Estrogen Receptor
ERE	Estrogen Response Element
g	gram
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Element
HIPP	Hippocampus
HPA	Hypothalamic-Pituitary-Adrenal
HPOA	Hypothalamic-Pre-optic Area
HRU	Hormone Response Unit
hsp	Heat shock protein
in	inch
ir	immuno-reactivity

Kb	kilobase
Kd	Dissociation constant
KDa	kiloDalton
kg	kilogram
M	molar
mA	milli-ampere
MBH	Medial basal Hypothalamus
mg	milligram
min	Minute
MR	Mineralocorticoid Receptor
mRNA	messenger Ribonucleic Acid
N	Amino
NE	Norepinephrine
NF1	Nuclear Factor 1
nM	nanomolar
NT	Nuclear Translocation
OVX	Ovariectomy
pg	picogram
PMS	Premenstrual Syndrome
POA	Preoptic Area
POMC	Pro-opiomelanocortin
PVN	Paraventricular Nucleus
RIA	Radioimmune Assay
S	Svedberg Unit
SDS	Sodium dodecyl sulfate
sec	Second
SON	Supraoptic Nucleus
SP1	Serum protein 1
sc	Subcutaneous
UV	ultraviolet
V	volts
VIP	Vasoactive Intestinal Peptide
5-HT	5-hydroxytryptamine
11*B*-HSD	11*B*-Hydroxysteroid Dehydrogenase

CHAPTER I

Introduction

Activation of the hypothalamic-pituitary-adrenal (HPA) axis is a characteristic physiological response to a stressor. Stress, in turn, can be thought of as the body's response to a stimulus or challenge (stressor) that disturbs or interferes with the normal physiological equilibrium or homeostasis of the organism. The HPA axis is one of the primary effector systems which functions to minimize the deviations from the homeostatic state, as well as return the organism to equilibrium following disturbances. Both physical and psychological stressors result in the secretion of factors, including corticotropin releasing hormone (CRH), arginine vasopressin (AVP), and oxytocin (OT), into the hypophysial portal circulation (Plotsky, et al., 1985a). These factors then travel to and stimulate the corticotroph cell in the anterior pituitary gland, causing their release of adrenocorticotropin (ACTH). In turn, circulating ACTH will increase the release of corticosterone (CORT) from the adrenal gland.

Previous studies have demonstrated that the estrogen (E) status of the female rat may affect its endocrine response to stress. It has been shown that a sex difference exists, in both circulating CORT and in the CORT response to stress, with females having higher levels than males (Kitay, 1961; Crithlow, et al., 1963). Furthermore, a relationship between the day of the estrous cycle and plasma CORT

levels has also been shown, with basal and stress responsive CORT levels being the highest on proestrus when E levels are the highest (Phillips, et al., 1978; Raps, et al., 1971; Buckingham, et al., 1978; Viau, et al., 1991; Pollard, et al., 1975).

The effects of high CORT levels are largely beneficial, protecting against other components of the body's own stress response (such as inflammation and immune reactions), mobilizing energy stores, maintaining osmotic balance, effecting neurochemistry, and influencing behaviors. However, these adaptive responses can become detrimental, resulting in immunosuppression, steroid diabetes, hypertension, and neuronal loss, if CORT secretion remains unchecked (Munck, et al., 1984; Sapolsky, et al., 1986).

At present, the factors which underlie the effect of E on CORT secretion have not been examined. Several possibilities exist, because the magnitude and duration of the CORT response is under complex regulation. E may be affecting a variety of neuronal components, such as neurotransmitters, peptides, and other hormones, which are involved in controlling the excitation of the HPA axis. In addition, the feedback regulation by CORT on the HPA axis may also be influenced by E.

Negative feedback regulation of the HPA axis is mediated predominately through the binding of CORT to intracellular CORT receptors (King and Mainwaring, 1974; McEwen, et al., 1986). These receptors for CORT have been previously classified into Type I and Type II receptors (Funder, et al., 1973; Reul and DeKloet, 1985), and have been shown to be the products of the mineralocorticoid receptor (MR) gene and the glucocorticoid receptor (GR) gene, respectively (Arriza, et al.,

1987; Miesfeld, et al., 1986). For clarity, in this dissertation I will employ the designations of MR and GR for the Type I and Type II receptors. Both receptor types have been implicated in negative feedback regulation by CORT (Dallman, et al., 1989; Ratka, et al., 1989), and both are found in varying amounts at the three major sites involved in negative feedback. **The working hypothesis of this project is that the altered hormonal response to stress seen in the presence of E is due to an effect of E on CORT receptor regulation.**

The specific aims of the studies performed for this dissertation are:

> 1) To characterize the effect of E on the CORT and ACTH response to stress in the female rat, and determine if E-induced alterations in CORT negative feedback inhibition contribute to these altered hormonal responses.
>
> 2) To explore one possible mechanism by which E alters the response of the HPA axis by analyzing the effect of E on CORT receptor regulation and function.
>
> 3) To further investigate the influence of E on CORT receptor function by examining the effect of E on regulation of CORT receptor mRNAs.

The initial experiments conducted for this dissertation characterized the ACTH and CORT responses to stress in female rats in the absence of circulating E or the presence of chronic E replacement. In E treated animals following stress, an elevated and prolonged secretory response implied an effect of E on CORT negative feedback. This pattern of secretion was similar to that described in the aged male rat, where hyperactivation of the HPA axis was associated with deficits in hippocampal CORT receptors (Sapolsky, 1986). In addition, a similar pattern of stress induced CORT

secretion was shown following the administration of synthetic glucocorticoid receptor antagonists (Ratka, 1989). In both of those studies, the altered pattern of hormone secretion was attributed to an attenuation of the negative feedback signal. Consequently, I examined the possibility that alterations in CORT negative feedback were contributing to the observed hormonal changes in the presence of E.

Previous studies have shown that CORT receptors are autoregulated; CORT and DEX treatment decreases receptor number, while adrenalectomy (ADX) increases it (Sapolsky and McEwen, 1985; Reul, et al., 1987). The down-regulation of these receptors is a functional consequence of MR and GR activation, and can be used to assess functional changes in CORT receptors. In the next experiment, we exploited the property of autologous receptor regulation to determine if the E status of the animal affects CORT receptor regulation.

The final study in this dissertation investigated the mechanism by which E might affect CORT receptor function. In this study, I examined the effect of E on autologous regulation of CORT receptor mRNAs. The examination of receptor mRNA levels has the advantages of requiring no surgical manipulations, such as adrenalectomy which is necessary for the measurement of receptors by binding assay, as well as providing insight into the cellular level at which regulation may be occurring.

Our studies demonstrate that E treatment can impair GR-mediated negative feedback. Furthermore, they show this may result from a functional impairment of the GR; E treatment interferes with autologous regulation of both the GR and its mRNA.

CHAPTER II

Review of Related Literature

Historical Perspectives

The importance of the adrenal glands was first documented by Addison in 1849, in a paper describing the disease (which now bears his name) of hypofunction of the adrenal cortex (Halpin, 1986). It was not until 1927 that Smith demonstrated that adrenocortical atrophy resulted from pituitary gland removal, suggesting hormonal control of the adrenal cortex. Subsequent studies used pituitary extracts to prevent this atrophy (Evans, 1933; Collip, et al., 1933), leading eventually to the isolation and characterization of adrenocorticotropin (ACTH) (Li and Dixons, 1956).

As early as 1948, Harris proposed a portal vessel-chemotransmitter hypothesis, whereby the portal circulation represented the functional link between the central nervous and endocrine systems. The existence of a corticotropin-releasing factor of hypothalamic origin was then demonstrated by Saffran and Schally, and Guillemin and Rosenberg in 1955. Due to the problems associated with bioassays, as well as the multiplicity of factors affecting ACTH release, it was not until 1981 that unequivocal evidence for the existence of corticotropin releasing hormone (CRH) was presented (Vale, et al., 1981; Speiss, et al., 1981).

Controversy has existed concerning the role played by glucocorticoids in

mediating the body's response to environmental perturbations. In 1936, Hans Selye described the "general adaptation syndrome" as the sum of all nonspecific, systemic reactions of the body which develop following long continuous exposure to stressors. Among these responses is activation of the pituitary-adrenocortical axis and the subsequent increase in circulating steroid hormones. Two types of adrenal steroids, one affecting salt and water metabolism and the other carbohydrate metabolism, were called mineralo-corticoids and gluco-corticoids, respectively (Selye, 1946). During the 1930's and 1940's, glucocorticoids were thought to enhance the body's normal defense mechanisms, conferring "increased resistance to stress" (Sayers, 1950). Selye's monumental review (Selye, 1946) not only classified stress responses in terms of the general adaptation syndrome, but went one large controversial step further by presenting his concept of "diseases of adaptation." Diseases such as diffuse collagen disease, allergy, and rheumatic diseases were suggested to be the result of excessive adaptive responses to stress, or the general adaptive syndrome gone awry. Selye's "pendulum hypothesis," proposing that "excess mineralocorticoid predisposes the body for inflammation, while excess glucocorticoid augments the danger of infection," attempted to provide a unified concept to the field of adrenocortical physiology (Selye, 1950).

The discovery of glucocorticoid's anti-inflammatory effects in the late 1940's by Kendall and others, not only led to an outright dismissal of the concept of diseases of adaptation, but also weakened the idea that glucocorticoids protect against stress (Kendall, 1971; Gaunt, 1974). The physiologists of that time were at a loss to

explain how glucocorticoids could suppress, rather than enhance, the normal defense mechanism of inflammation (Sayers, 1950). The easiest solution was to segregate the anti-inflammatory effects as pharmacological rather than physiological (Gaunt, 1974).

In the 1960's, studies began to address the question of the glucocorticoid's mechanism of action by examining the effects of steroids added at physiological concentrations directly to isolated tissues and cells (Munck and Brinck-Johnsen, 1967). With the discovery of the glucocorticoid receptors, a common cellular and molecular mechanism for the actions of glucocorticoids was proposed (Munck and Brinck-Johnsen, 1968; Schaumburg and Bojesen, 1968; Baxter, 1976; Munck and Leung, 1977). This common mechanism allows the physiologist to accept that the effects of glucocorticoids on the single cell may in fact, reflect the effects that circulating glucocorticoids would have on similar cells in the whole organism.

The Hypothalamic-Pituitary-Adrenal Axis

Overview

Corticosterone (CORT) secretion from the adrenal gland is under control of the brain via the hypothalamic-pituitary-adrenal (HPA) axis. This neuroendocrine axis represents a cascade of neural and humoral reactions driven by both the circadian pacemaker and by the environment (figure 1). Plasma CORT levels, therefore, display pronounced circadian fluctuations, ranging from peak levels of circulating CORT at the onset of the animal's active period, to minimal amounts of CORT at the end of this period (Dallman, et al., 1987). Superimposed on this diurnal change in CORT, are sudden dramatic increases in plasma CORT in response to environmental

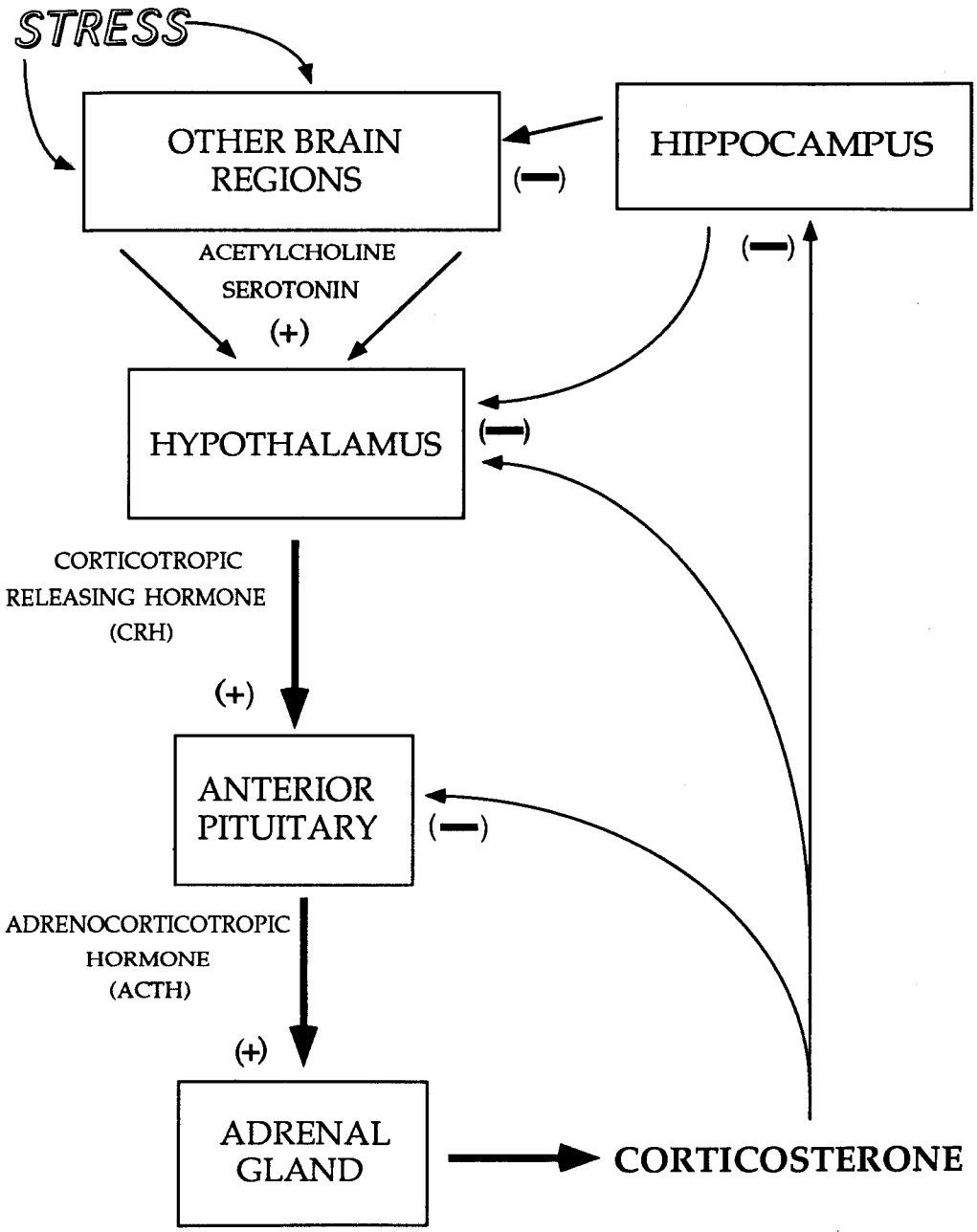


FIGURE 1. Hypothalic-pituitary-adrenal axis. (+), stimulatory signals (-), inhibitory signals

perturbations. In fact, the response to these stressors itself displays analogous diurnal fluctuations, with the response being greatest in the AM (Bradbury, et al., 1991b).

In response to changing environmental conditions, the HPA axis responds quickly and in a complex manner involving multiple regulatory factors of hypothalamic, neurohypophysial, and peripheral origins (Plotsky, et al., 1985a). These factors include CRH (Vale, et al., 1981), vasopressin (AVP) (Gilles, et al., 1982), epinephrine (Giguere and Labrie, 1983), and possibly oxytocin (OT) (Antoni, et al., 1983a). The presence of these factors in hypophyseal portal plasma of the rat has been shown (Recht, et al., 1981; Gibbs and Vale, 1982; Johnston, et al., 1983; Gibbs, 1984; Plotsky and Vale, 1984; Plotsky, et al., 1985a). Furthermore, the presence of receptors for CRH (Wynn, et al., 1983), AVP (Van Leeuwen and Wolters, 1983), and epinephrine (Petrovic, et al., 1983) has been demonstrated in anterior pituitary tissue. These factors then act on the anterior pituitary corticotroph, enhancing the synthesis and release of proopiomelanocortin (POMC) gene products (figure 2). One of these, ACTH, acts in turn on the adrenal cortex to cause a rapid (within 10 minutes) rise in plasma CORT levels following the stimulus. To complete this closed regulatory loop, circulating CORT regulates the HPA axis by feedback inhibition at several different sites (figure 1).

Anatomy of the HPA axis

Both physical and emotional stressors result in signals traveling via multiple neural pathways to the hypothalamus (Swanson and Sawchenko, 1980; Swanson, 1987; Plotsky, et al., 1989), where they cause the release of CRH from the

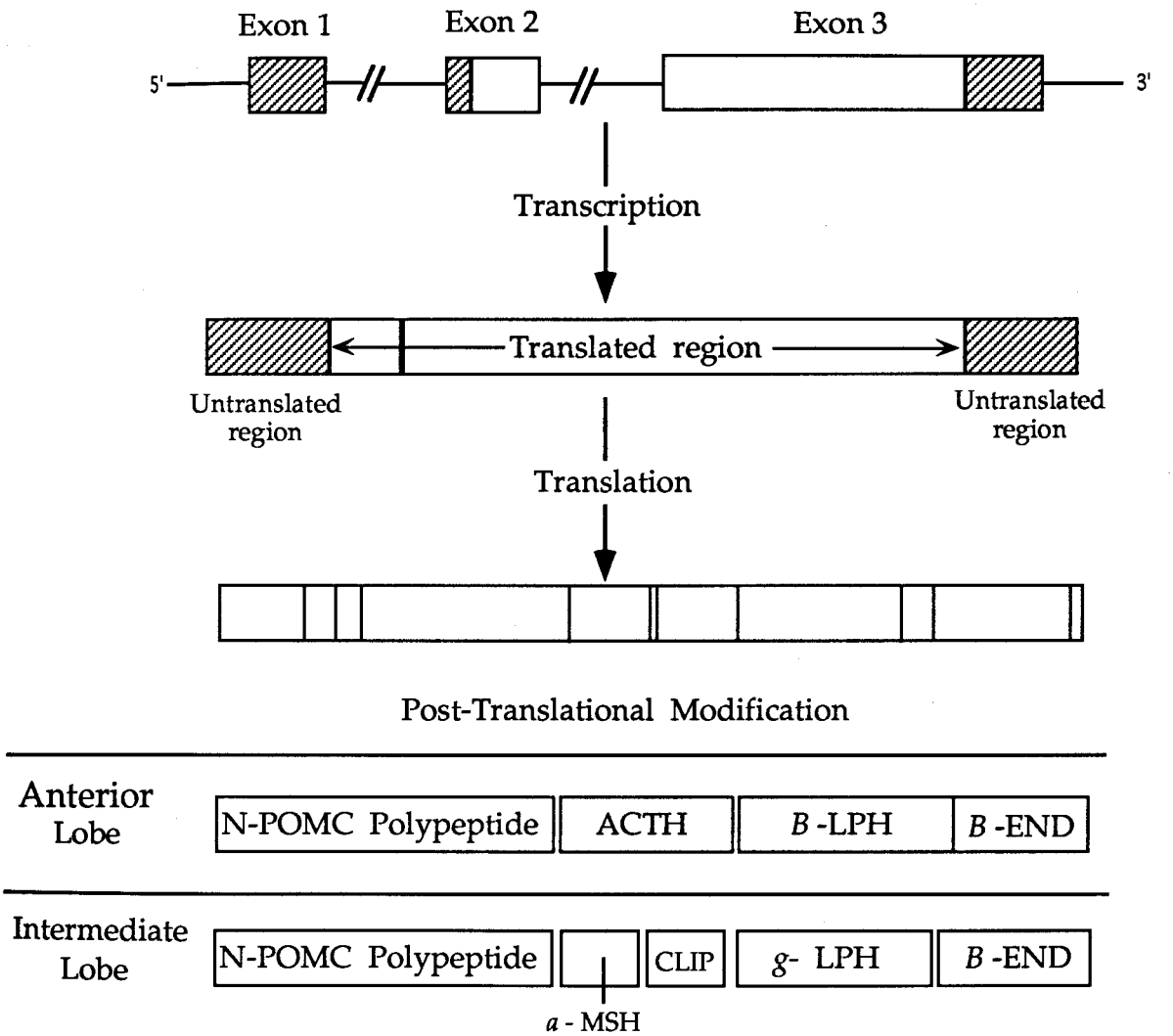


FIGURE 2. Proopiomelanocorticon (POMC) gene expression. Differential posttranslational processing in the two lobes of the pituitary gland are shown. Adrenocorticotropin (ACTH), Melanocyte-stimulating hormone (MSH), Lipotropic hormone (LPH), Endorphin (END)

neurosecretory cells of the paraventricular nuclei (PVN) (Bloom, et al., 1982; Merchenthaler, et al., 1983; Swanson, et al., 1983; Plotsky and Vale, 1984). Anatomical and physiological evidence demonstrates that collaterals from the somatosensory and viscerosensory pathways enter the hypothalamus via multisynaptic neural pathways (Palkovits, 1987; Swanson, 1987). Signals from special sensory stimuli may also reach the PVN via collaterals of their pathways, as evidenced by the abolishment of the ACTH response to such sensory stimuli following posterior hypothalamic deafferentation (Feldman, 1985). Emotional stimuli may generate limbic signals which reach the PVN through direct (septal) or indirect projections. Electrical stimulation of limbic areas such as the hippocampus, septum, and amygdala, was shown to cause elevations of plasma CORT which could be abolished by anterior hypothalamic deafferentation (Feldman, et al., 1982).

Upon reaching the neurosecretory cells of the PVN, these signals are transformed into a multifactorial signal (Plotsky, et al., 1985a), which includes CRH - the primary regulator of ACTH secretion (Rivier and Plotsky, 1986). The axons of CRH-containing cells project to the median eminence, and terminate around the primary capillaries of the hypothalamo-pituitary portal circulation (Antoni, et al., 1983b; Swanson, et al., 1983; Merchenthaler, et al., 1983; Leranth, et al., 1983). CRH, along with other factors such as arginine vasopressin (AVP) and epinephrine, are secreted from the nerve terminals in a stimulus specific fashion into the portal circulation (Gibbs, 1985; Antoni,1986; Rivier and Plotsky, 1986). These capillaries travel down the infundibular stalk to reach the adenohypophysis. CRH (and other

factors) then diffuse from the capillaries in the pituitary and can bind to corticotrophs expressing CRH-receptors (or other appropriate receptors).

Corticotropin Releasing Hormone

Following the isolation and characterization of CRH by Vale and colleagues (Vale, et al., 1981; Speiss, et al., 1981), studies have demonstrated that CRH stimulates the release of ACTH both in vivo (Rivier, et al., 1982a; Donald, et al., 1983) and in vitro (Vale, et al., 1983b). Furthermore, the specificity of CRH in this release has been shown by using *a*-helical CRH as an antagonist (Rivier, et al., 1984), and by immunoneutralization by polyclonal (Rivier, et al., 1982b; Nakane, et al., 1985) and more recently monoclonal (van Oers, et al., 1989) anti-CRH antibodies. When injected intracerebroventricularly, CRH stimulates the secretion of ACTH (Ono, et al., 1985a) and cortisol, the primary endogenous glucocorticoid in rhesus monkeys and man (Rock, et al., 1984). Thus, CRH is the major physiological regulator of HPA activity. Additional studies have shown that the exogenous-CRH-induced release of ACTH is blocked by CRH antisera, as is the ACTH response to several different stressors, including ether, restraint, cold, formalin, and leg fracture (Rivier and Vale, 1982b; Linton, et al., 1985; Ono, et al., 1985b; Nakane, et al., 1985; van Oers, et al., 1989).

Numerous studies have reported changes in corticotropin-releasing factor(s) (CRF) bioactivity and CRF-like immunoreactivity in the hypothalamus following various stressors (Hiroshige, et al., 1971; Sato, et al., 1975; Buckingham, 1979; Moldow and Fischman, 1982; Chappell, et al., 1986; Moldow, et al., 1987; Haas and

George, 1988a; Murakami, et al., 1989). These changes in CRH content may reflect alterations in secretion, storage, synthesis, or degradation. Finally, direct measurements of CRH in hypophysial portal blood by Plotsky and Vale (1984) have shown elevations in CRH following hemodynamic stress.

More recently, measurements of CRH mRNA has allowed a more direct method to monitor changes in CRH expression. Increases in hypothalamic CRH mRNA, following insulin-induced hypoglycemia, were shown by Northern blot analysis (Suda, et al.,1988). Studies employing in situ hybridization histochemistry have also demonstrated increases in the levels of CRH mRNA, specifically in PVN parvocellular neurons, in response to a variety of stressors. These included hypertonic saline injection (Lightman and Young, 1987, 1988, 1989), streptococcal cell-wall-induced arthritis (Sternberg, et al., 1989), intermittent footshock (Imaki, et al., 1991), chronic electroconvulsive shock (Herman, et al., 1989b), restraint and swim stress (Harbuz and Lightman, 1989).

Adrenocorticotropic Hormone

CRH acts primarily on the corticotroph cells of the anterior pituitary gland (Grossman, et al., 1982; Wehrenberg, et al., 1984; Rivier and Plotsky, 1986) to increase the release of ACTH, as well as other POMC derived peptides, eg. *B*-endorphin and *B*-lipotropin (figure 2) (Vale, et al., 1983a; 1983b; Gibbs, et al., 1983). In addition, CRH spurs the synthesis of the precursor POMC (Reisine, et al., 1985) and elevates POMC mRNA levels in vivo (Bruhn, et al., 1984b; Dallman, et al., 1985).

It is clear, however, that CRH is not the sole activator of POMC expression in the pituitary. Studies have shown that some of the neuropeptides found co-localized with CRH in the parvocellular subdivision of the PVN, such as vasopressin, angiotensin II, cholecystokinin, and enkephalin (Kiss, et al., 1984; Sawchenko, et al., 1984; Swanson et al., 1986), could potentiate CRH action at the level of the pituitary (Antoni, 1986; Rivier and Plotsky, 1986).

It is well documented that ACTH is the principal stimulator of glucocorticoid secretion from the adrenal gland (Sayers, 1950; Ingle, 1952; Keller-Wood and Dallman, 1984; Baxter, 1986). ACTH acts on the cells of the zona fasciculata and the zona glomerulosa to cause the synthesis and secretion of CORT and aldosterone (Baxter, 1986).

Corticosterone

CORT can affect cell metabolism and growth in almost every cell of the body. The effects of circulatory CORT are largely beneficial, protecting against the body's own stress response (anti-inflammatory), mobilizing energy stores (glucogenesis), and maintaining osmotic balance (Axelrod and Reisine, 1984; Munck, et al., 1984). In addition to these peripheral effects, CORT has profound influences on many of the brain's neurotransmitter systems. CORT induces changes in neurotransmitter receptor levels, second messenger pathway regulation, and regulation of certain synthetic enzymes, which are listed in Table I. These may be some of the biochemical mechanisms by which CORT influences certain aspects of adaptation, mood, attention, and learning (McEwen, et al., 1986).

TABLE I

Glucocorticoid Effects on the Brain's Neurotransmitter Systems

Neurotransmitter System	*Treatment*	*Reference*
Deceases cortical glutamate & aspartate	ADX Reversed with CORT	Rindi and Ventura, 1961 Sutherland et al., 1964
Increases tryptophan hydroxylase activity	acute CORT acute DEX	Yanai and Sze, 1983 Singh et al., 1990
Increases hippocampal 5-HT_1 receptor density	acute ADX (1 hr) chronic ADX (6 days) Both reversed with CORT	Martire et al., 1989 Biegon et al., 1985
Increases hypothalamic tyrosine hydroxylase	ADX (2 days) Reversed with CORT	Shen and Ganong, 1976
Decreases hypothalamic dopamine *B*-hydroxylase	ADX (2 days) Reversed with CORT	Shen and Ganong, 1976
Decreases a_2-noradrenergic receptor density in PVN	ADX Reversed with CORT	Jhanwar-Uniyal and Leibowitz, 1986
Increase dopamine turnover Increase dopamine release	acute CORT acute CORT	Versteeg et al., 1983 Imperato et al., 1989
Decrease hippocampal NE- and VIP-stimulated cAMP production	acute CORT acute DEX	Harrelson and McEwen, 1987
Decreases hippocampal VIP levels	ADX Reversed with CORT/DEX	Rotsztejn et al., 1980

Abbreviations:

5-HT, 5-hydroxytryptamine

NE, norepinephrine

VIP, vasoactive intestinal polypeptide

Some or all of the aforementioned effects may be involved in CORT's mediation of the stress response. Almost any perturbation in homeostasis will result in CORT secretion. It has been suggested that CORT may alter cell metabolism simply in a permissive manner, to increase readily available energy resources during stress (Ingle, 1954). Alternatively, it has been proposed that CORT secretion occurs to prevent an overreaction to stress (Munck, et al., 1984). Disturbances in homeostasis result in the stimulation of primary defense mechanisms, such as inflammation following tissue damage or enhanced activity of the immune system in response to infection. In order to prevent these responses from becoming detrimental, these primary defense reactions must be terminated soon after the threat to homeostasis has disappeared. Munck (1984) suggested that during stress CORT exerts an inhibitory influence on the synthesis and release of several intracellular mediators of these primary defense reactions.

Importantly, while CORT plays a positive, protective role in controlling the responses to stress, chronically elevated CORT may act deleteriously. Unchecked CORT secretion has been shown to lead to steroid diabetes, immunosuppression, hypertension, osteoporosis, neuronal loss, and disturbances in mood and mental performance (Selye and Tuchweber, 1976; Munck, et al., 1984; Sapolsky, et al., 1985; McEwen, et al., 1986). Interestingly, these symptoms are characteristic of the aging process as well, and it has been proposed that high CORT levels may be related to brain aging and age-related neuropathology (Landfield, et al., 1978; 1981; Sapolsky, et al., 1986).

Sex Differences in HPA Function and the Role of Estrogen

A significant sex difference in both basal and stress-responsive circulating CORT levels has been reported, with females having higher levels than males (Kitay, 1961; Crithlow, et al., 1963; Le Mevel, et al., 1979). Consistent with the hypothesis that these sex differences are a result of circulating gonadal hormones, studies have shown that basal CORT levels are reduced following ovariectomy (OVX), and increased following estrogen (E) treatment (Kitay, 1963; Ramaley, 1976; Phillips and Poolsanguan, 1978; Lesniewska, et al., 1990). In further support of an E effect on CORT secretion, several studies have also shown a correlation between the day of the estrous cycle and plasma CORT levels: CORT levels are greatest on proestrus when E levels are highest (Raps, et al., 1971; Buckingham, et al., 1978; Phillips and Poolsanguan, 1978; Viau and Meaney, 1991). Similarly, intact females show a greater CORT response to stress on proestrus than if stressed on diestrus or estrus (Raps, et al., 1971; Pollard, et al., 1975; Phillips and Poolsanguan, 1978; Viau and Meaney, 1991). These cyclic changes in stress responses also appear to be related to circulating E levels, since following ovariectomy, female rats with E replacement had significantly higher post-stress CORT levels than controls (Phillips and Poolsanguan, 1978; Viau and Meaney, 1991; Burgess and Handa, 1992).

The factors which underlie the apparent effect of E on CORT secretion are unknown at present. Although Kitay, et al. (1965) demonstrated that E could increase adrenal production of CORT directly, several reports have demonstrated that the E-induced increases in CORT reflected increases in ACTH secretion, implicating a site

of action at the level of the pituitary or higher (Le Mevel, et al., 1978; Lesniewska, et al., 1990; Viau and Meaney, 1991; Burgess and Handa, 1992).

Previous studies have shown that E may directly affect levels of CRH immunoreactivity and mRNA. Chronic (3 weeks) E replacement in OVX female rats resulted in a significant decrease in hypothalamic CRH-ir content (Haas and George, 1988b), which is consistent with an increase in the release and/or a decrease in the synthesis of CRH. A subsequent study demonstrated that indeed a decrease in CRH synthesis occurred with chronic E treatment (Haas and George, 1989). CRH mRNA was elevated on the afternoon of proestrus, at the approximate time of the E-induced pre-ovulatory surge of LH (Bohler, et al., 1990). The subsequent decrease in CRH mRNA was even more dramatic and may be attributable to elevated CORT levels, resulting from afternoon increases (Watts and Swanson, 1989) combined with an effect of E. These data point to E acting at the level of the hypothalamic CRH neuron. Other studies, however, implicate actions at the level of the anterior pituitary; OVX resulted in a decreased sensitivity to hypothalamic extract-stimulated ACTH release, which was partially reversed by E (Coyne and Kitay, 1969).

Since the action of E is mediated through the estrogen receptor (ER), it is important to note that several studies have shown ER present not only in the hypothalamus, but also in the hippocampus of female rats (Pfaff and Keiner, 1973; Stumpf and Sar, 1978; Rainbow, et al., 1982; Cintra, et al., 1986). The use of autoradiographic (Pfaff and Keiner, 1973; Loy, et al., 1988), biochemical (Rainbow, et al., 1982; O'Keefe and Handa, 1990), and immunocytochemical (Cintra, et al.,

1986) methods to measure ER concentrations has demonstrated low levels of ER in the dentate gyrus, CA subfields, and subiculum, while higher levels were demonstrated recently by immunochemical means (Maggi, et al., 1989). In situ hybridization studies have shown the presence of levels of hippocampal ER mRNA either comparable to those found in the hypothalamus (Pellitier, et al., 1988), or lower levels comparable to those observed by autoradiography (Simerly, et al., 1990). While the reasons for the discrepancies in the levels of hippocampal ER are not clear, it is apparent that ER does exist in a functional state in the adult female rat hippocampus (O'Keefe and Handa, 1990). At present it is unknown whether ER are colocalized with MR and/or GR, or whether E exerts its influence transsynaptically.

Corticosterone Receptor Subtypes

The actions of CORT, both in the periphery and in the brain, are mediated through its binding to specific intracellular receptors (King and Mainwaring, 1974; McEwen, et al., 1986). These receptors are classified into Type I and Type II receptors (Funder, et al., 1973; Reul and DeKloet, 1985), which are discriminated by anatomical, biochemical, pharmacological, and most recently molecular characteristics (Reul and DeKloet, 1985; DeKloet, et al., 1986; Miesfeld, et al., 1986; Arriza, et al., 1987; Reul, et al., 1987b; Van Eekelen, et al., 1988). These characteristics are listed in Table II.

The Type I receptor, or mineralocorticoid receptor (MR), is a product of the MR gene (Arriza, et al., 1987). It is thought to be primarily involved in basal or tonic regulation of activities such as food seeking (Jhanwar-Uniyal, et al., 1986),

TABLE II

Characteristics of CORT Receptor Subtypes

Characteristic	*Type I*	*Type II*	*Reference*
	MR	GR	Arriza et al., '87 Miesfeld et al., 86
Affinity for corticosterone	High Kd 0.5nM	Lower Kd 2.5-5.0nM	Reul and DeKloet, '85
Affinity for aldosterone	High Kd 1.5-2.0nM	Low Kd >25nM	Krosowski and Funder, '83 Beaumont and Fanestil, '83
Affinity for dexamethasone	High Kd 0.8-2.6nM	High Kd 0.6-2.0nM	Brinton and McEwen, '88 Allen et al., '88
Affinity for RU 28362	Very low Kd >100nM	High Kd 1.2-2.0nM	Philibert and Mouguilewsky, '83
Neuronal localization	Restricted HIPP, septum	Widespread HIPP, septum, PVN, SON, medial amygdala	Reul and DeKloet, '85 Fuxe et al., '85 Van Eekelen et al., '87 Sarrieau et al., '88

Abbreviations:

MR, mineralocorticoid receptor
GR, glucocorticoid receptor
Kd, dissociation constant
HIPP, hippocampus
PVN, paraventricular nucleus
SON, supraoptic nucleus

exploration (Veldhuis and De Kloet, 1983), and sleep-related events (Micco, et al., 1980). A role for MR in mediating CORT regulation of HPA axis basal activity has also been shown (Dallman,et al., 1989; Ratka, et al., 1989). This receptor has a high affinity for CORT, the mineralocorticoid aldosterone, as well as the synthetic

glucocorticoid DEX, (Krosowski and Funder, 1983; Reul and DeKloet, 1985; Beaumont and Fanestil, 1983; Brinton and McEwen, 1988; Allen et al., 1988). The synthetic glucocorticoid RU 28362 binds to the MR with very low affinity, which allows its discrimination from the Type II receptor (Philibert and Mouguilewsky, 1983; Reul and DeKloet, 1985).

The Type I receptor has been localized predominately to the hippocampus and septum by both in vitro and in vivo binding studies, autoradiographic analyses, and most recently in situ hybridization (Reul and DeKloet, 1985; 1986; McEwen, et al., 1968; Van Eekelen, et al., 1988; Arriza, et al., 1988; Herman, et al., 1989a). The highest concentrations are in the dorsal subiculum, and CA1 field of the hippocampus, followed by the dentate gyrus, CA3 field, and lateral septum. Interestingly, low but detectable levels of MR (Reul and DeKloet, 1986) and MR mRNA (Swanson and Simmons, 1989) are found in the PVN.

The Type II receptor, or glucocorticoid receptor (GR), is a product of the GR gene (Miesfeld, et al., 1986). It is thought to function primarily in the regulation of the stress response, mediating the suppression by CORT of stress-activated processes (Reul, et al., 1987a). Recent studies have suggested that both MR and GR may act in mediating CORT negative feedback (Ratka, et al., 1989; Dallman et al., 1989). The GR, in contrast to the MR, has a higher affinity for the synthetic glucocorticoid RU 28362, a lower affinity for CORT, a similar affinity for DEX, and a very low affinity for aldosterone (Krosowski and Funder, 1983; Reul and DeKloet, 1985; Brinton and McEwen, 1988).

The anatomical distribution of the GR also differs from that of the MR, with the GR being found in neuronal populations throughout the central nervous system. The use of immunocytochemistry (Fuxe, et al., 1985; Van Eekelen, et al., 1987), in addition to binding and autoradiographic studies (Reul and DeKloet, 1985; 1986; Sarrieau, et al., 1988), has shown the highest concentration of the GR to be in the hippocampus, lateral septum, PVN, arcuate nucleus, supraoptic nucleus, and central amygdala. Within the hippocampus, the highest concentrations of the GR are in the CA1 and CA2 pyramidal cell fields, followed by the dentate gyrus, with lower levels found in the CA3 and CA4 fields (Van Eekelen, et al., 1987; Sarrieau, 1988). More recent reports, using in situ hybridization to examine the distribution of the MR and GR mRNAs, found the same pattern of distribution (Aronsson, et al., 1988; Van Eekelen, 1988; Herman, et al., 1989a; Sousa, et al., 1989). The probable coexistence of GR with MR in neurons in the CA1 and CA2 fields is not surprising, in light of the recent studies which suggest a role for both the GR and the MR in mediating CORT negative feedback (Ratka, et al., 1989; Dallman, et al., 1989). It has been postulated that the colocalization of MR and GR would expand the range of CORT concentrations that the neuron could respond to (Evans and Arriza, 1989).

Negative Feedback Regulation of the HPA axis

The magnitude and duration of CORT secretion following a stimulus is regulated by a variety of neuronal components, controlling the excitation of the HPA axis as well as negative feedback control (Antoni, 1986; Plotsky, et al., 1989; Keller-Wood and Dallman, 1984). The negative feedback actions of CORT have been

divided into 3 categories: fast or rate-sensitive feedback, intermediate or early delayed feedback, and slow or late delayed feedback (Keller-Wood and Dallman, 1984; Jones and Gillham, 1988). A summary of the their characteristics and sites of action are listed in Table III.

Fast feedback

The fast mode of feedback is dependent upon the rate of change in plasma CORT levels, with rapid inhibition of ACTH secretion occurring within seconds to minutes (Dallman and Yates, 1969). This rapid action of CORT happens during the time when CORT levels are rising, and appears to be limited to an effect on hormone release and not synthesis (Jones, et al., 1972; Abe and Crithlow, 1977). Both the rapidity of the response and the failure of cycloheximide pretreatment of perifused pituitaries or primary anterior pituitary cell cultures to affect this rapid inhibition, suggest that protein synthesis is not involved (Widmaier and Dallman, 1984; Abou-Samra, et al., 1986). Rather than the classical genomic mechanism of action, fast feedback may occur at the level of the cell membrane. Rapid membrane effects of glucocorticoids, such as changes in membrane potentials, have been demonstrated (Saphier and Feldman, 1988; Hua and Chen, 1989).

Sites for fast feedback

There is evidence for the occurrence of this rapid mode of feedback at sites above the hypothalamus, within the hypothalamus, and at the level of the anterior pituitary (Jones and Gillham, 1988). CORT has been shown to rapidly inhibit CRH

TABLE III

Negative Feedback Regulation of the HPA Axis at sites above the hypothlamus, the PVN, the hippocampus, and the AP.

Feedback Characteristics	*References*
Fast, seconds - minutes Rate sensitive Inhibits stimulus-induced CRH and ACTH secretion Protein synthesis not required	Jones and Gillham, '88 Sato et al., '75 Abe and Crithlow, '77 Abou-Samra et al., '86 DeKloet et al., '88
Intermediate, minutes - hours Inhibits stimulus-induced CRH and ACTH secretion, and CRH synthesis Protein synthesis required	Sato et al., '75 Suda et al., '84 Yokoe et al., '88 Plotsky et al., '84, '86 Abou-Samra et al., '86
Slow, > 12 hours Inhibits stimulus-induced CRH and ACTH secretion, CRH and ACTH synthesis, basal and stimulus-induced CRH and POMC gene expression	Roberts et al., '79 Gagner and Drouin, '87 Jingami et al.. '85 Beyer et al., 88 Harbuz and Lightman, '89 Herman et al., '89c, '90

Abbreviations:
CRH, corticotropin releasing hormone
ACTH, adrenocorticotropin
POMC, proopiomelanocortin
PVN, paraventricular nucleus
AP, anterior pituitary gland

release, as measured by bioactivity, both in vivo (Sato, et al., 1975) and from hypothalami incubated in vitro (Edwardson and Bennett, 1974; Buckingham and Hodges, 1977; Hillhouse and Jones, 1976). In addition, Abe and Crithlow (1977) have shown that some of the fast feedback events must be mediated within the hypothalamo-pituitary complex, since surgical isolation of the hypothalamus from the rest of the brain did not prevent fast feedback.

A recent study utilized a GR specific antagonist, RU 38486, to examine the role of the GR in mediating fast feedback inhibition of the CORT response to stress (DeKloet, et al., 1988). They demonstrated that bilateral intracerebral injections of RU 38486 into the PVN results in resistance to rapid feedback inhibition by CORT following stress. This suggests not only that a site for rapid feedback inhibition is located in or near the PVN, but also that the feedback is mediated via the GR.

In vitro studies have shown that CRH-stimulated ACTH release from primary pituitary cell cultures and perifused pituitaries are inhibited within 30 minutes of CORT or DEX treatment (Sayers and Portanova, 1974; Buckingham, 1979; Abou-Samra, et al., 1986; Widmaier and Dallman, 1984). While all these pituitary preparations demonstrate inhibition by CORT in the fast feedback time frame, none have been shown to have rate sensitivity. This suggests that the rate sensor may exist at a site above the pituitary. It therefore appears that CORT inhibits both CRH and ACTH secretion in the fast feedback mode.

Intermediate and Slow Feedback

The intermediate mode of feedback occurs minutes to hours after increases in

CORT, while the slow or late delayed mode results from continuous CORT exposure for 12 hours or more (Keller-Wood and Dallman, 1984; Jones and Gillham, 1988). While rapid feedback is sensitive to the rate of change in CORT levels, both early and late delayed feedback are dependent upon total CORT exposure. Thus delayed feedback represents an integral rather than level-sensitive control process. The peak level of CORT reached, the interval since exposure, and the total amount released (or administered) all contribute to the degree and duration of the inhibition achieved (Abe and Crithlow, 1980). In general, the longer the time and the greater the exposure to CORT, the greater the inhibition conferred on the system.

At the level of the anterior pituitary, apparently differing mechanisms of inhibitory actions serve to differentiate these two modes of feedback. The AtT20 cell line was derived from an anterior pituitary tumor, and displays corticotroph characteristics (Roberts et al., 1979). This cell line has greatly facilitated the studies of corticotroph function, since corticotrophs constitute only 3-5% of the cells in the anterior pituitary (Roberts et al., 1987). AtT20 cells, exposed to DEX for 5 - 25 hours, preferentially inhibit ACTH release, while both ACTH release and synthesis are inhibited after 2 or 3 days of exposure (Watanabe, et al., 1973). Further studies using AtT20 cells demonstrated that only stimulus-induced ACTH secretion is inhibited by glucocorticoids, and that this inhibition is blocked by cycloheximide treatment, suggesting a requirement for protein synthesis (Phillips and Tashjian, 1982). Thus for corticotroph cells at least, fast and intermediate feedback appear to inhibit stimulus-induced ACTH secretion only. Slow feedback, however, inhibits the

expression of POMC mRNA and ACTH synthesis, resulting in the inhibition of both basal and stimulated ACTH secretion (Roberts, et al., 1979).

Both fast and intermediate feedback probably occur under normal physiological conditions, ie. when increasing CORT secretion occurs in response to moderate or acute stress. Following chronic stress, however, both fast and intermediate feedback inhibition by CORT appear impaired (Young and Akil, 1985; Young, et al., 1990) Slow feedback probably occurs only under pathological conditions or after pharmacological treatment with glucocorticoids, ie. when plasma CORT levels are elevated for days.

Keller-Wood and Dallman (1984) have shown that in the time frame of intermediate feedback, stressors can be separated into two categories: 1) those not affected by short-term glucocorticoid treatment - or glucocorticoid resistant stressors; and 2) those stimuli that result in a suppressible response - glucocorticoid sensitive stressors. They exhibit differential responsiveness to glucocorticoids; CORT or DEX pretreatment of rats abolishes the normal increase in hypothalamic CRH bioactivity following certain glucocorticoid-sensitive stressors, while even large doses of DEX only partially reduce the pituitary-adrenocortical response to a glucocorticoid resistant stressor (Sato, et al., 1975). In steroid resistant stressors, fast feedback appears intact, while the intermediate feedback seems to have a much higher threshold. Following steroid sensitive stressors, intermediate feedback inhibition acts at or above the level of the hypothalamus to inhibit increases in CRH content, presumably by inhibiting CRH synthesis (figure 3) (Sato, et al., 1975). In response to a steroid

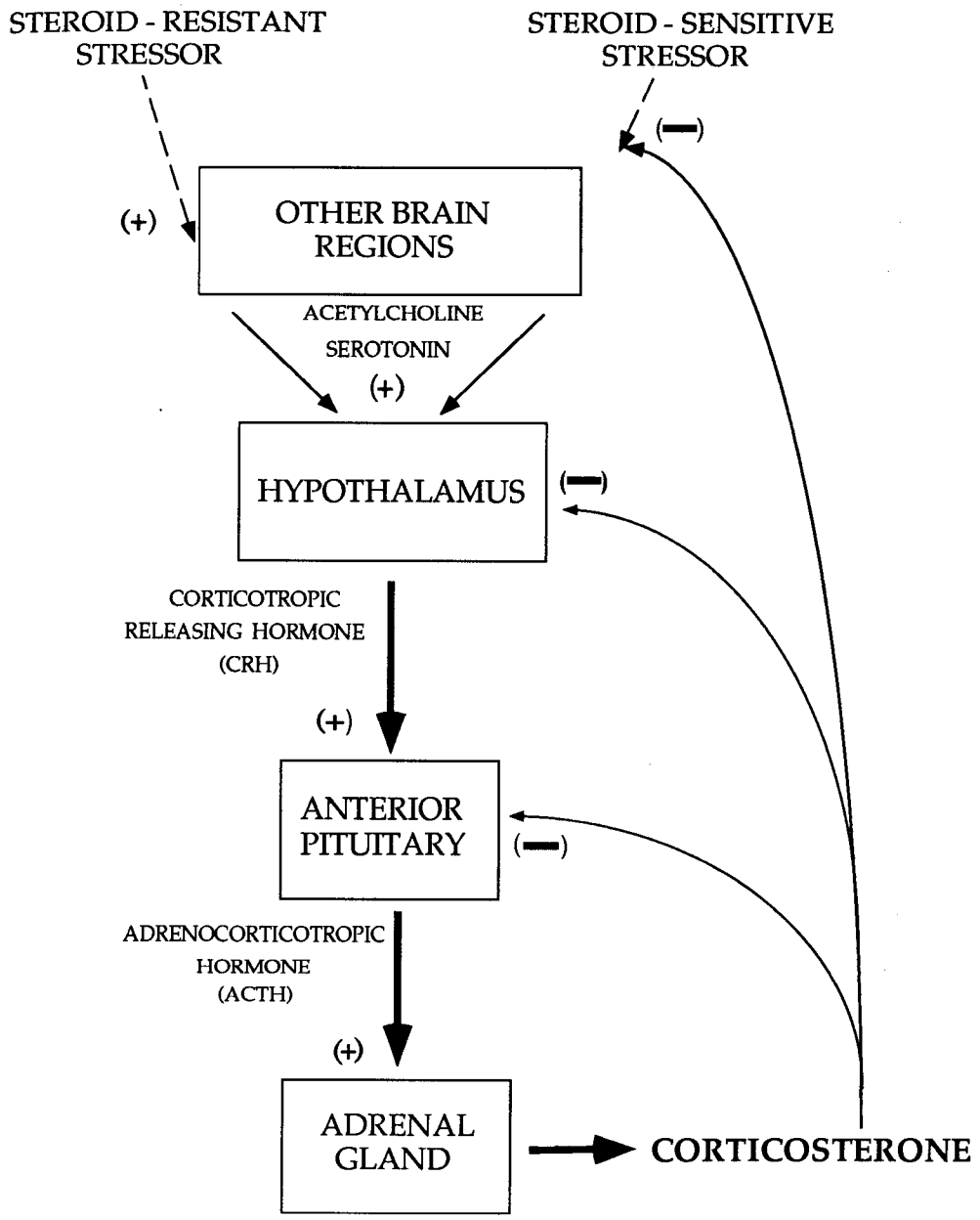


Figure 3. Hypothalamic-Pituitary-Adrenal Axis, Steroid-Resistant and Steroid-Sensitive Stressors.
(+), Stimulatory signals (−), Inhibitory signals

FIGURE 3. Steroid-resistant vs steroid-sensitive stressors of the hypothalamic-pituitary-adrenal axis. (+), stimulatory signals (-), inhibitory signals.

resistant stressor, intermediate feedback inhibition either fails to act on a different afferent pathway, or the signal from this different pathway overrides (nullifies) feedback inhibition acting at the CRH producing neuron (figure 3). In either case, synthesis. Thus, at the level of the hypothalamus or above, intermediate feedback inhibition affects both release and synthesis of CRH.

Sites of intermediate and slow feedback inhibition

Anterior pituitary sites. As early as 1956, the anterior pituitary was implicated as a feedback site; the injection of cortisol or DEX into the hypophyseal fossa results in decreased CORT responses to stress (Rose and Nelson, 1956). Numerous studies CRH content increases even with sustained ACTH release, suggesting increased CRH followed, which demonstrated that systemic administration of glucocorticoids results in a decrease in pituitary responsiveness to hypothalamic extracts (crude CRH preparations). This was shown in animals with intact hypothalami, hypothalamic lesions, median eminence lesions, or nembutal-morphine blocked stress-induced CRH release (Jones and Gillham, 1988). In vitro studies utilizing incubated whole pituitaries, perfused pituitaries, dispersed pituitary cells, or cultured pituitary cell lines have all demonstrated that glucocorticoids inhibit CRH-stimulated ACTH release (Keller-Wood and Dallman, 1984). While these early studies implicate a site of feedback at the pituitary, they are limited by the facts that only hypothalamic extracts were accessible as a source of CRH, and that only bioassays were available to assess changes in ACTH levels.

More recent work by Vale et al. (1983b) using purified CRH, has shown that 4 hours of DEX exposure results in maximal inhibition of CRH-induced ACTH secretion from cultured rat anterior pituitary cells. They also demonstrated this inhibition in vivo, using chlorpromazine-morphine-pentobarbital treated rats (Rivier, et al., 1982a). In this animal preparation, stress-induced CRH release is blocked. Four hours of DEX treatment prior to exogenous CRH administration completely abolishes the CRH-mediated rise in plasma ACTH, validating the pituitary as a feedback site. The dependence of intermediate feedback on protein synthesis was confirmed in a study where anterior pituitary cells coincubated with CORT and cyclohexamide demonstrate fast feedback inhibition, but not an intermediate inhibition (Abou-Samra, et al., 1986).

Another important feature of intermediate feedback at the pituitary level is that CORT does not block the potentiation of CRH-stimulated ACTH release by AVP, angiotensin-II, or norepinephrine (Oki, et al., 1991). Thus, AVP restores the CRH-stimulated ACTH response in 2 hour CORT treated anterior pituitary cells to the levels obtained with CRH alone in control cultures. Also, 20 hours of DEX pretreatment of anterior pituitary cells results in inhibition of CRH-stimulated ACTH secretion and cAMP accumulation, while the potentiation of both of these actions by AVP is unaffected (Bilezikjian, et al., 1987). The ability of cAMP-independent ACTH secretagogues to function in the presence of glucocorticoids, partially compensating for the inhibition of CRH, may be critical to the physiological stress response. It would allow continued ACTH secretion, via a glucocorticoid resistant

mechanism, in situations where it is appropriate despite already elevated plasma CORT levels.

While intermediate feedback at the level of the pituitary most likely inhibits secretion and synthesis of ACTH, slow feedback appears to alter POMC gene expression. Nakanishi, et al., (1977), using an in vitro translation system combined with immunoprecipitation of the translated product, first demonstrated that the levels of POMC mRNA increase following chronic adrenalectomy (ADX). Also, maximal decreases of POMC mRNA levels occur with 3 days of DEX treatment in chronic ADX animals. Subsequent studies utilizing solution hybridization methods (Schachter, et al., 1982; Birnberg, et al., 1983), and in situ hybridization histochemistry (Gee and Roberts, 1983; Fremeau, et al., 1986) demonstrated that adrenalectomy results in >10 fold increases in POMC mRNA levels in the anterior lobe. Although administration of DEX to chronic ADX animals results in a return of plasma ACTH levels to control values within 2 hours, a decrease in POMC mRNA levels is not seen until 8 hours, with a return to control values not seen until 5 days (Birnberg, et al., 1983).

Further evidence that slow feedback inhibition is mediated at the level of POMC gene expression is provided by studies of POMC gene transcription. Utilizing nuclear runoff transcription assays, POMC gene transcription has been shown to be elevated 20 fold within 1 hour of ADX, and maintained at this elevated rate for at least 4 hours after ADX (Birnberg, et al., 1983). Elevated transcription rates (2-4 fold) are still found 14 days following ADX (Eberwine and Roberts, 1984). DEX

administration at the time of ADX prevents the increase in transcription rate, while DEX administration to long-term ADX animals results in rapid inhibition of POMC gene transcription with maximal inhibition occurring within 30 min (Birnberg, et al., 1983; Affolter and Reisine, 1985). Administration of DEX to intact animals results in a 6-7 fold decrease in the basal transcription rate within 15 min (Eberwine and Roberts, 1984). The lag period of 6 to 8 hours between decreased POMC gene transcription rates and detectable reductions in POMC mRNA levels is consistent with a half-life of 18 to 24 hours, which is considered typical for a the stable class of eukaryotic mRNAs (Puckett, et al., 1975; Birnberg, et al., 1983). This rapid inhibition of both basal and stimulated POMC transcription suggests a direct action of glucocorticoids on transcription of the POMC gene.

Further support for a direct effect on the POMC gene in corticotrophs is provided by reports that utilized anterior pituitary primary cell cultures and the AtT20 pituitary tumor cell line (Gagner and Drouin, 1985, 1987; Eberwine, et al., 1987; Nakamura, et al., 1978; Roberts, et al., 1979). The transcription rate of the POMC gene is decreased within 10 min and is maximally reduced by 20 min following DEX administration (Eberwine, et al., 1987). Furthermore, the rapid (within 30 min) increase in the transcription rate following CRH administration and the rapid decrease following DEX treatment are both unaltered in the presence of cycloheximide (Gagner and Drouin, 1987). This suggests a direct effect of glucocorticoids, mediated by proteins already existing in the corticotroph, on POMC gene expression.

While in vitro studies using anterior pituitary cell cultures or cell lines

demonstrate direct effects of glucocorticoids on POMC transcription, mRNA levels, and ACTH secretion, the in vivo role of CORT feedback on the anterior pituitary POMC gene is not so clear. Although there appears to be a rapid effect of glucocorticoids on POMC transcription, there is a lag period (6-8 hrs) before this effect is manifested in reduced POMC mRNA levels and eventually reduced ACTH levels. This lag period is compatible with the appearance of delayed feedback, as defined by decreased plasma ACTH and/or CORT levels 12 or more hours after stress or glucocorticoid treatment.

While both CORT receptors are present in the anterior pituitary, in vivo uptake studies have demonstrated that DEX binds with several fold greater potency to those receptors than does CORT (DeKloet et al., 1975). Subsequent studies have revealed the presence of transcortin-like molecules, or corticosterone binding globulins (CBGs) in the pituitary which bind CORT but not DEX (Koch, et al., 1976; DeKloet and McEwen, 1976). These CBGs are present intracellularly and most likely compete with glucocorticoid receptors for the binding of CORT (DeKloet, et al., 1984). Thus, while the pituitary appears to be sensitive to CORT inhibition of ACTH secretion in vitro and in vivo, the presence of CBG in corticotrophs may make them less sensitive to the action of CORT than brain areas devoid of CBG, such as the hypothalamic-preoptic area and hippocampus (Koch, et al., 1979).

The normal response to ADX requires secretion of CRH and increased secretion of another ACTH secretagogue, probably vasopressin. ADX rats with anterolateral hypothalamic lesions exhibit no increase in pituitary POMC mRNA

levels, pituitary ACTH, or plasma ACTH levels, relative to sham-ADX lesioned rats, despite the removal of CORT feedback (Dallman, et al., 1985). Thus, in the absence of either exogenous or endogenous CRH, the corticotroph displays a complete lack of autonomy in response to ADX. Furthermore, exogenous CRH-driven corticotrophs from lesioned rats display the same (2.5 fold) increase in POMC mRNA in response to ADX as that seen in sham-lesion rats after ADX, while the rise in plasma ACTH is less in the CRH-driven ADX rats (2.6 fold vs 12 fold).

The lack of a corticotroph response in the absence of feedback (ADX) and drive (PVN lesion) suggests that at least for basal secretion feedback control is exerted at sites above the pituitary. Therefore, the anterior pituitary serves as a site for intermediate feedback inhibition of stimulus-induced ACTH secretion and synthesis, as well as for slow feedback inhibition of basal and stimulated POMC gene expression.

Hypothalamus. Several early studies demonstrated that injections or implants of CORT or DEX, when placed in the median eminence or anterior median hypothalamus, are capable of suppressing pituitary adrenal activity (Jones and Gillham, 1988). Delayed feedback inhibition by CORT or DEX has been shown to also affect the secretion of bioactive CRFs from the hypothalamic blocks maintained in vitro (Hillhouse and Jones, 1976; Jones et al., 1977; Holmes et al., 1985). While glucocorticoids must have acted within the hypothalamic tissue that was removed for in vitro incubation, the results must be considered in light of the perturbations of loss of afferent neural input, dramatically changed environment, and decreased viability of

the tissue.

More recently, DEX administration in the intermediate or slow feedback time domains (16 hours or 7 days) to intact animals has been demonstrated to significantly decrease immunoreactive CRH in the median eminence and plasma ACTH (Suda, et al., 1984; Yokoe, et al., 1988; Jessop, et al., 1990). Plotsky and Vale (1984) directly measured CRH levels in hypophysial-portal blood by RIA, and demonstrated that pretreatment with DEX prevents a hemorrhage-induced 2 fold increase in CRH secretion. In a subsequent study utilizing a pharmacological blockade of endogenous glucocorticoid synthesis, a 2 hour systemic infusion of CORT prevented a hypotension-induced rise in hypophyseal-portal CRH-ir (Plotsky, et al., 1986). These studies demonstrate a central site of glucocorticoid action somewhere proximal to the secretion of CRH, but do not delineate whether the site is in the PVN or pathways leading to it.

Removal of endogenous glucocorticoids by adrenalectomy leads to increases in immunoreactive levels of CRH in the parvocellular PVN neurons (Swanson et al., 1983; Merchenthaler, et al., 1983), and to increases in hypothalamic CRH gene expression, as measured by Northern blot analysis (Jingami, et al., 1985) and in situ hybridization histochemistry (Young et al., 1986a). Increases in vasopressin immunoreactivity, colocalized with CRH-ir in the parvocellular PVN (Tramu, et al., 1983; Kiss, et al., 1984; Sawchenko, et al., 1984) and median eminence (Whitnall, et al., 1985), have also been reported following ADX. Moreover, ADX-induced increases in vasopressin mRNA levels in CRH cells in the PVN have been observed,

with a shift in vasopressin mRNA containing cells into the medial, parvocellular region (Wolfson, et al., 1985; Davis, et al., 1986; Young, et al., 1986b). Systemic treatment with DEX at the time of ADX prevents both the increase in vasopressin mRNA and immunoreactivity, as well as their medial shift in the PVN (Davis, et al., 1986). Systemic CORT replacement, at the time of ADX, resulting in low circulating plasma CORT levels (3-8 ug/dl) is optimal for preventing the ADX-induced increase in PVN CRH mRNA levels, as measured by dot blot analysis or in situ hybridization (Beyer, et al., 1988; Swanson and Simmons, 1989). Similar levels of plasma CORT prevent the ADX-induced increase in plasma ACTH (Beyer, et al., 1988). These studies demonstrate that endogenous CORT clearly participates in basal regulation of the HPA axis by specifically influencing the gene expression of CRH and AVP in the PVN. Whether CORT is acting directly on the corticotroph neuron or on afferent connections to it is still unknown.

A role for magnocellular AVP in the stress response was demonstrated by Bruhn, et al. (1984a), who reported that AVP antagonists administered to PVN lesioned rats abolished their ACTH response to ether stress. A recent study examined chronically hyponatremic rats, which secrete virtually no magnocellular AVP and release significantly less ACTH compared to normonatremic rats, in response to ether stress (Dohanics, et al., 1991). The integrity of the parvocellular AVP neurons in the hyponatremic rats was shown by increased CRH-ir and AVP-ir following ADX as well as greatly increased plasma ACTH levels. These findings suggest that the magnocellular AVP neurons are important in mediating the physiological response to

stress.

Taken together, these studies point to the complexity of this system and the care one needs to take in drawing any general conclusions based on experiments utilizing such non-physiological manipulations as adrenalectomy. While ADX-induced increases in parvocellular AVP show DEX suppressibility, magnocellular AVP appears to be unaffected by DEX, although a recent study has shown GR to be present in these neurons (Kiss, et al., 1988). Plotsky, et al. (1985b) have shown that although increases in portal blood levels of AVP and not CRH are found in response to insulin-induced hypoglycemia, administration of antibodies to CRH abolishes the increase in ACTH secretion following this stressor. Their results further demonstrate the necessity for CRH, if only in a permissive role, in mediating the stress response.

Several studies have demonstrated direct effects of glucocorticoids on HPA activity at the level of the PVN. DEX filled capillaries, implanted with the tip near the PVN of ADX rats, suppressed the ADX-induced increases in parvocellular PVN CRH and AVP immunostaining and CRH mRNA, as well as plasma ACTH levels (Kovacs, et al., 1986, 1987, 1988; Sawchenko, 1987). These findings demonstrate a local effect of DEX directly on the PVN CRH neuron, or on afferents to those cells.

In all of these studies, similar implants of CORT instead of DEX, failed to achieve the same levels of suppression. While the authors attributed the difference to the effect of DEX being mediated by the GR, Type II DEX preferring receptor, I consider that the approximately 2 fold greater affinity of DEX versus CORT for the GR is insufficient to explain the results. It is unfortunate that the only data shown

were of controls, and DEX treated animals. Differences in the local CORT metabolism (see below for discussion of 11*B*-Hydroxysteroid dehydrogenase), or dispersal artifacts may be involved.

The above-mentioned studies clearly demonstrate that feedback inhibition by DEX of the non-physiological phenomenon of ADX is, in part, directly mediated at the PVN. Recently, feedback inhibition by DEX, of the PVN CRH mRNA response to acute hypertonic stress was examined using in situ hybridization histochemistry (Lightman and Young, 1989). DEX feedback, in the slow time domain, inhibited both basal and stress-stimulated PVN CRH mRNA levels. This occurred when DEX was administered via PVN implanted cannulae as well as systemically, providing evidence for direct inhibitory effects of DEX at the level of the PVN (Harbuz and Lightman, 1989).

An elegant study by Levin, et al. (1988) demonstrated a pivotal role of the hypothalamus in mediating the effects of CORT in the domain of slow feedback inhibition. This study combined experimental approaches utilizing hypothalamic lesions and ADX (Dallman, et al., 1985) with ones using ADX with various levels of constant CORT replacement (Akana, et al., 1985,1986). The results showed that ADX animals with lesioned medial basal hypothalami or PVNs failed to suppress CRH-driven ACTH secretion when replacement CORT levels were low (6 ug/dl). Only with stress-like replacement CORT levels (>30 ug/dl) were the plasma ACTH levels of the hypothalamic lesioned ADX animals lowered to the levels found in controls. Another finding was revealed by lesions which missed the PVN; those

animals had normal ACTH responses to ADX, which were only partially attenuated by low CORT levels (6 ug/dl). Thus, the PVN was shown to be necessary not only for the occurrence of the ACTH response to ADX, but also for suppression of that response by CORT feedback.

Overall, these results suggest that glucocorticoid feedback in the slow time domain clearly acts at the level of the PVN, to reduce basal, ADX-induced, and stress-stimulated CRH mRNA responses in the PVN. This in turn causes reduced levels of PVN CRH-ir and plasma ACTH.

Hippocampus. Studies examining the in vivo uptake of tracer doses of 3H-CORT given to ADX rats, provided the first demonstration of glucocorticoid receptors in the brain (McEwen, et al., 1968). The finding that the selective uptake of 3H-CORT was localized to the hippocampus, and not the expected areas such as the medial basal hypothalamus or pituitary, was the earliest study suggesting a role for the hippocampus in mediating negative feedback inhibition by CORT. More recent studies, using in vitro binding techniques, autoradiography, immunohistochemistry for GR, and in situ hybridization histochemistry, have demonstrated the presence of both hippocampal MR and GR (Reul and DeKloet, 1985, 1986; Fuxe, et al., 1985; van Eekelen, et al., 1987, 1988; Arriza, et al., 1988; Herman, et al., 1989a). Importantly, substantial amounts of MR were found only in the hippocampus and septum. Thus, any studies demonstrating a role for MR in mediating negative feedback inhibition also provide indirect evidence for feedback sites at the hippocampus or septum.

The preferential binding of 3H-CORT in the hippocampus (McEwen, et al., 1968), becomes somewhat more clearly understood in light of the two receptors' binding characteristics (Table II). The MR binds CORT with a greater affinity than the GR, while the GR binds the synthetic glucocorticoid DEX with equal or greater affinity compared to the MR (Krosowski and Funder, 1983; Reul and DeKloet, 1985; Reul, et al., 1987; Brinton and McEwen, 1988; Allen, et al., 1988).

In an effort to determine the CORT receptor subtype mediating basal feedback regulation, Dallman, et al. (1989) examined the potency of various plasma levels of either CORT or DEX on three variables in the ADX rat: plasma ACTH, thymus weight, and CBG production. Changes in the latter two have been shown to be mediated via the peripheral GR (Levin, et al., 1987). CORT was shown to be more potent than DEX in lowering ADX-induced ACTH levels, while DEX was 2 to 3 times more potent than CORT in decreasing thymus weight and CBG production (Dallman, et al., 1989). The agreement between the CORT and DEX concentrations needed to lower ACTH levels, and their reported Kd values for the MR, strongly suggest that the MR mediates slow feedback control of basal ACTH secretion. The fact that CORT remains more potent than DEX in the PM, argues that the MR mediates feedback at the diurnal peak as well. In contrast, GR mediation of feedback inhibition on the peripheral CORT targets is suggested by the greater potency of DEX than CORT in inhibiting plasma CBG and thymus weight.

Additional evidence was provided by studies using low levels of plasma CORT or MR antagonists to demonstrate MR mediation of basal feedback regulation by

CORT (Beyer, et al., 1988; Swanson and Simmons, 1989; Ratka, et al. 1989; Bradbury, et al., 1991). Due to the paucity of MR in the PVN, this would further strengthen arguments for an extrahypothalamic source of tonic inhibition on the CRH neuron.

Several studies have attempted to demonstrate direct effects of glucocorticoids on HPA activity at the level of the hippocampus, using CORT or DEX implants. CORT, but not DEX, implants into the dorsal hippocampus were reported to significantly attenuate, but not completely prevent, ADX-induced increases in plasma ACTH levels (Kovacs and Makara, 1988). This was in contrast to earlier studies which reported no significant changes in either CRH-ir, AVP-ir, or CRH mRNA in the PVN following hippocampal implants of either CORT or DEX (Kovacs, et al., 1986; Kovacs and Mezey, 1987). These conflicting results may be due to an insufficient amount of CORT in the implant to affect the entire hippocampus. A report by Bradbury and Dallman (1989) demonstrated that implantation of the GR antagonist RU 38486, in the dorsal hippocampus of ADX constant CORT replaced rats, resulted in dramatically elevated ACTH levels in the PM. This further suggests a role for hippocampal GR in mediating slow feedback inhibition by CORT.

Several early studies attempted to demonstrate a role for the hippocampus in mediating CORT negative feedback by removing the sites of CORT action, or by disrupting the transmission of the signal generated. Fornix transection, lesions in the hippocampus, or almost complete hippocampectomy result in flattened diurnal rhythms in plasma CORT, generally due to elevation of the nadir CORT levels and a

slight reduction in peak levels (Knigge, 1961; Nakadate and DeGroot, 1963; Moberg, et al., 1971; Fischette, et al., 1980). Wilson, et al. (1980) examined ACTH secretion, and reported a significant increase in PM levels in hippocampectomized animals compared to controls.

Stress responses, as measured by plasma CORT, have also been demonstrated to increase following dorsal (Feldman and Conforti, 1980) or complete hippocampectomy (Wilson, et al., 1980). Furthermore, DEX feedback inhibition of the ether response to stress, in the intermediate time domain, was shown to be significantly attenuated by dorsal fornix transection (Feldman and Conforti, 1976), dorsal (Feldman and Conforti, 1980), or complete hippocampectomy (Wilson, 1975; Magarinos, et al., 1987). These studies suggest that the hippocampus is involved in mediating basal and stress-responsive CORT feedback regulation of the HPA axis.

A recent study examined CRH and AVP mRNAs in the PVN, following either dorsal or complete hippocampectomy (Herman, et al., 1989c). Their results revealed increases, of a similar magnitude as seen following ADX (4 fold), in both CRH and AVP mRNA localized to the medial parvocellular neurons of the PVN. In addition, hippocampectomy resulted in increased plasma *B*-endorphin and CORT levels. These results suggest that the hippocampus serves as a site for basal inhibition of ACTH secretagogue synthesis in PVN neurons.

A subsequent study by Herman, et al. (1990) tested the hypothesis that elimination of neuronal afferents should leave PVN CRH and AVP, as well as HPA function mostly unaffected, if direct actions of CORT on the PVN were of primary

regulatory significance. To this end, they investigated the effects of anterior, posterior, or total deafferentations of the PVN, aimed at eliminating all indirect neuronal feedback, on levels of CRH and AVP mRNA in the PVN. Anterior and total deafferentations resulted in significant increases in both CRH and AVP mRNA, relative to controls, while posterior cuts had no significant effects. These data establish that the local effects of CORT within the PVN are incapable of maintaining normal expression of CRH mRNA in the absence of anterior neuronal input. This suggests that CORT regulation of PVN CRH neurons emanates in part from extrahypothalamic regions, such as the lateral septum, bed nucleus of the stria terminalis, or hippocampus, whose projections reach the PVN anteriorly.

Overall, these studies point to the hippocampus as a site mediating basal or tonic inhibition on CRH and AVP neurons in the PVN via fiber systems projecting through the forebrain. The fact that the increases seen following deafferentation were less than those seen after hippocampectomy, suggests that some of the inhibitory feedback signal may be transmitted multisynaptically through hippocampal projections to other areas, such as the amygdala.

To further investigate a hippocampal role in mediating feedback regulation of HPA axis activity, Sapolsky, et al. (1989) examined the effect of fornix transection on portal levels of CRH, and AVP, under basal conditions or following stress. Pharmacologically ADX rats with either low or high replacement plasma CORT levels were used, in order to ascertain the CORT receptor type involved. Transection of the fornix resulted in a loss of feedback inhibition of stress-induced CRH secretion, by

high CORT plasma levels in the intermediate time domain. Both basal and stress-induced AVP secretion were increased following fornix transection under conditions of low CORT feedback only. These results implicate the hippocampus as a site involved in GR mediated feedback inhibition of stress-induced CRH secretion, as well as MR mediated tonic inhibition of basal AVP secretion.

To further delineate the CORT receptor type mediating feedback inhibition on HPA axis activity, Sapolsky et al. (1990) measured CORT receptor occupancy both before and after hypotensive stress, and attempted to correlate that information with hypophysial portal blood levels of CRH and AVP . Combined occupancy of hypothalamic GR/MR and hippocampal GR correlated with initial portal levels of CRH; increased occupancy of those receptors was associated with decreased levels of CRH. CRH levels following stress, however, correlated with combined occupancy of hypothalamic GR/MR and hippocampal MR. Initial AVP levels correlated with occupancy of hippocampal GR, while no relationship was found between any receptor and stress levels of AVP. The lack of correlation between hippocampal GR or MR alone and basal or stress levels of CRH, respectively, suggests that multiple signals from different sites are involved in the feedback inhibition of CRH. Interestingly, the inhibitory curves for CRH and AVP differed, while 50% inhibition of initial CRH levels occurred with approximately 50% occupancy of receptors, 50% inhibition of initial AVP occurred with only about 12% occupancy of receptors. This tighter regulation of AVP fits with its role as a synergistic cofactor in regulating ACTH release.

Overall, these studies implicate the hippocampus as a site involved in basal feedback regulation as well as feedback inhibition of stress-induced increases in PVN CRH and AVP synthesis and secretion.

Summary of Negative Feedback Regulation of the HPA Axis

HPA axis activity, both basal and stress-induced, is subject to feedback inhibition by glucocorticoids. This feedback inhibition has been separated into 3 types, which differ in the time domains **within** which they act, the mechanisms **by** which they act, as well as the sites **at** which they act.

Fast, rate sensitive feedback acts within seconds to minutes, inhibiting hormone release (CRH, AVP, ACTH). It acts predominately at sites on afferent pathways to PVN CRH/AVP neurons, and/or the neurons themselves.

Intermediate, early delayed feedback is level sensitive and acts within minutes to hours, inhibiting release and synthesis of hormones (CRH, AVP, ACTH). It acts at sites on AP corticotrophs, afferent pathways to PVN CRH/AVP neurons, PVN CRH/AVP neurons, and/or hippocampal GR and/or MR containing neurons.

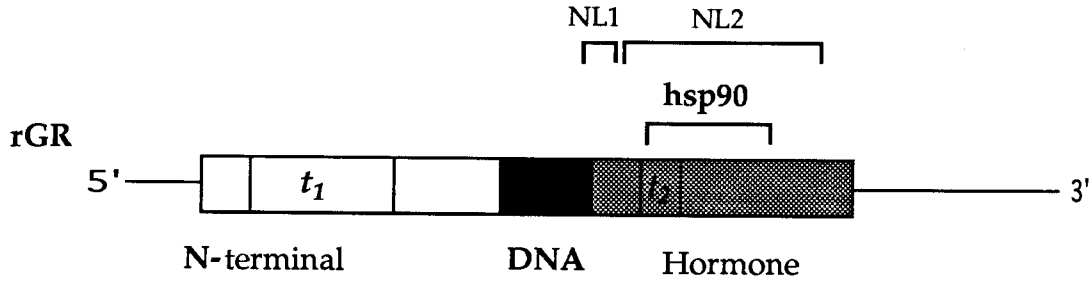
Slow, late delayed feedback is also level sensitive and acts within hours to days, inhibiting synthesis of hormones. It acts at sites on AP corticotrophs, afferent pathways to PVN CRH/AVP neurons, PVN CRH/AVP neurons, and/or hippocampal GR and/or MR containing neurons. It is probably involved only under pathophysiological or pharmacologically administered conditions.

Structure of the GR and the MR

The isolation and cloning of steroid receptor complementary DNAs has allowed for the identification of a superfamily of related genes, whose products are all hormone-responsive transcription factors (Evans, 1988; Carson-Jurica, et al., 1990). Analysis of the primary structure of MR (Arriza, et al., 1987; Patel, et al., 1989) and GR (Hollenberg, et al., 1985; Miesfeld, et al., 1986), and comparison with the primary structure of the receptors for thyroid hormones, estrogens, androgens, progesterones, vitamin D and retinoic acid, and the retroviral oncogene product v-erb A, has revealed a high degree of homology between these proteins. In addition, highly homologous proteins called orphan receptors have been discovered, whose ligands are still unknown. It is thought that this superfamily of receptors may constitute a group of descendants from a single ancestral gene (Evans, 1988; Carson-Jurica, et al., 1990).

Proteolytic digestion analysis of crude and purified GR first revealed the domain structure of the receptor protein (Gustafsson, et al., 1987). Utilization of the cDNAs for GR and MR, by expression of deletion mutations and chimeric proteins, has revealed discrete functions attributable to precise regions of the receptor proteins (Giguere, et al., 1986; Hollenberg, et al., 1987; Godowski, et al., 1987; Green and Chambon, 1987; Patel, et al., 1989).

The amino (N) terminal domain is highly variable between different types of steroid receptors, while being highly conserved between species (figure 4). The localization of the hormone independent trans-activational domains, *t*1 and enh2, in



hGR	83%	89%	90%
rMR	40%	76%	59%

FIGURE 4. Domain structure of the rat glucocorticoid receptor (rGR). The binding site for heat shock protein 90 (hsp90), as well as regions necessary for the transactivation (t_1 and t_2) and nuclear localization (NL1 and NL2) functions are designated. Below is a comparison of the amino acid homology for the N-terminal, DNA binding , and hormone binding domains between the rat GR and the human glucocorticoid receptor (hGR) and the rat mineralocorticoid receptor (rMR).

the human and rat, respectively, may account for this conservation (Hollenberg and Evans, 1988; Godowski, et al., 1988). The trans-activation domains function to alter the regulation of gene transcription, either positively or negatively. The majority of antibodies raised against GR, both polyclonal and monoclonal, are directed against the N-terminal region demonstrating its immunodominance (Gustafsson, et al., 1987).

The highest extent of homology both between species and between receptor types, is found in the DNA binding domain of the receptor proteins, suggesting a common mechanism of action and function (Evans, 1988). This domain is a cysteine-rich region responsible for the formation of two zinc-finger motifs, which are obtained by coordination of four cysteines to one zinc atom (Freedman, et al., 1988). The two zinc fingers allow for receptor binding distinction between the glucocorticoid responsive element (GRE) and other hormone responsive elements, thus conferring DNA binding specificity. Recently, a three-dimensional picture of the protein structure, obtained by structural nuclear magnetic resonance, revealed that the region contacting the DNA is an *a*-helical segment between the two fingers (Hard, et al., 1990).

The carboxy-terminal domain of the receptor molecule, or ligand binding domain, is also highly conserved between species, and considerably conserved between receptor types (figure 4). This may explain the ability of glucocorticoids to bind to both receptors with high, albeit different affinities. The steroid hormone is thought to bind in a hydrophobic pocket created in the 3-dimensional folded protein (Carlstedt-Duke, et al., 1988). Interestingly, the GR is functional without this

domain. Deletion of the hormone binding domain leads to a constitutive activator, with almost complete trans-activation capacity in the absence of hormone (Hollenberg, et al., 1987; Miesfeld, et al., 1987). It appears an intrinsic property of the hormone binding domain is to repress the trans-activation function in the absence of hormone (Hollenberg and Evans, 1988; Godowski, et al., 1988). Also contained within the hormone binding domain are regions necessary for the binding of heat shock protein 90 (hsp90) (Howard, et al., 1990), and for hormone-dependent nuclear translocation, NL2 (Picard and Yamamoto, 1987). The nuclear translocation functions of the NL1 and NL2 regions are thought to be involved in transporting the newly translated receptor protein from the cytoplasm into the nucleus (Picard and Yamamoto, 1987).

Another domain identified as being involved in hormone-independent nuclear translocation, NL1, maps to a 28 amino acid region adjacent to the DNA binding domain (Picard and Yamamoto, 1987). The region between the DNA binding and hormone binding domains is relatively hypervariable, and contains a segment which is very protease sensitive. It is thought that this segment represents a hinge region, and that hormone binding results in a conformational change in the protein at this region (Gustafsson, et al., 1987). A stretch of 6 and 8 proline residues found in this region in the human MR and rat MR, respectively, support a proposed hinge function for this region (Arriza, et al., 1987; Patel, et al., 1989).

GR/MR: Mechanism of Action

Based on several immunohistochemical studies, the intracellular localization of unoccupied GR, in contrast to other steroid receptors, was thought to be cytoplasmic

and not nuclear (Papamichail, et al., 1980; Govindan, 1980; Wikstrom, et al., 1987). Recent evidence suggests that diffusion of the receptor from the nucleus may be responsible for cytoplasmic immunoreactivity (Gasc, et al., 1989). Consistent with this is a study by Pekki, et al. (1992), using a freeze-drying and vapor fixation method to eliminate GR diffusion and redistribution, which reports that both occupied and unoccupied GR are found in the nucleus of uterine fibroblasts and cortical neurons.

Multiprotein docking complex

In the absence of hormone, the inactivated receptor (GR, MR, and other steroid hormone receptors) is present as a heteromeric complex that sediments at 9S and has a molecular weight of 310 kiloDaltons (kDa) (Holbrook, et al., 1983; Vedeckis, 1983). This complex consists of one GR molecule associated with two 90 Kda heat shock protein (hsp90) molecules (figure 5) (Sanchez, et al., 1985; Catelli, et al., 1985; Mendel and Orti, 1988). The hsp90 is a ubiquitous, abundant, and highly conserved protein, which is found in cells from primitive eukaryotes to humans. Although its expression in all these cells is increased in response to stress, suggesting a function essential to cell survival, this function is unknown at present (Schlesinger, 1986). Hsp90 may perform a chaperon function related to protein folding, a stabilization function protecting against degradation, and/or a role in protein transport (Pratt, 1990). While there is no evidence for a role in GR transport yet, it appears that the roles of the hsp90 in protein folding and stabilization may contribute to formation of the heteromeric complex (Pratt, 1990). This inactive 'docking' complex

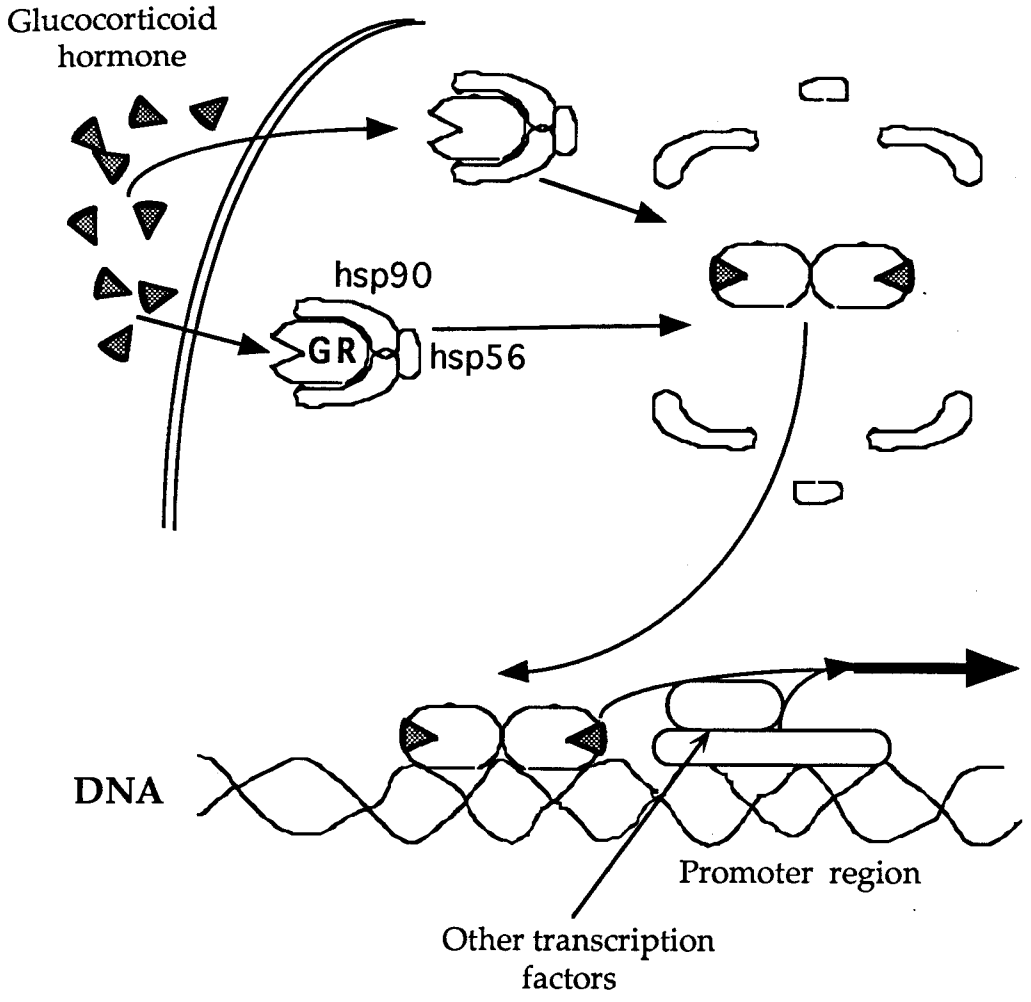


FIGURE 5. Interactions of glucocorticoid receptor (GR) with ligand and heat shock proteins (hsp90 and hsp56). Probable steps involved in GR-mediated signal transduction, resulting in transcriptional activation, are depicted.

is loosely associated with the nucleus, and following cell rupture in hypotonic buffer is recovered in the cytosolic fraction (Sanchez, et al., 1990). Other heat shock proteins, hsp70 and hsp56, are also present in the heteromeric complex (Sanchez, 1990), with hsp56 binding directly to hsp90 (Renoir, et al., 1990). Interestingly, a role for hsp70 in the passage of proteins across mitochondrial and endoplasmic reticular membranes has been shown (Rothman, 1989), suggesting that hsp70 may be involved in GR/MR translocation. The function of hsp56 is unknown at present.

The association of hsp90 with GR not only prevents DNA binding in the absence of hormone, but is actually required for a high affinity steroid binding conformation. Synthesis of GR without hsp90, or loss of hsp90 by heat inactivation, results in a GR with 10 fold lower affinity for hormone (Bresnick, et al., 1989; Nemoto, et al., 1990). Ligand binding of this GR causes receptor activation, but with a dramatically reduced efficiency (Picard, et al., 1990).

Hormone Binding

The initial step in the signal transduction pathway for gene activation involves the hormone binding to its high affinity site on the receptor molecule. The co-localization of the binding site for hsp90 and the region necessary for hormone-mediated receptor transformation, to the same region in the ligand binding domain, suggests that a structural feature exists which is responsible for transducing the free energy of steroid binding into derepression of receptor function (Pratt, 1990). Following hormone binding, hsp90 dissociates and the GR is converted to a DNA binding form (Pratt, 1987). This transformation of GR to the activated 4S, 90 kDa

form is thought to involve an irreversible conformational change that not only facilitates DNA binding, but also prevents reassociation with hsp90 (Bresnick, et al., 1989; Scherrer, et al., 1990). The activated form of the GR appears to be present as a homodimer (Wrange, et al., 1989), as has been shown for the ER (Kumar and Chambon, 1988).

DNA Binding

The activated forms of the GR and MR bind to specific DNA sequences in order to regulate gene transcription. The location and sequence of these glucocorticoid response elements (GRE) have been established using DNase I and methylation protection experiments, and deletion mutation studies (Gustafsson, et al., 1987; Beato, 1989). The consensus GRE sequence is a palindrome of 15 basepairs (bp) composed of two half palindromes of 6 bp separated by 3 nonconserved basepairs. Each half palindrome binds one receptor molecule of the activated GR dimer (Tsai, et al., 1988). The presence of glucocorticoids accelerates the kinetics of the activated receptor binding to the GRE, both the on-rate and off-rate, as well as the affinity of the receptor for the GRE (Schauer, et al., 1989; Becker et al., 1986). This suggests that the ligand bound receptor can scan the genome more rapidly in search for its site of action.

It is important to note that the MR also binds to the consensus GRE. Arriza et al. (1987) have shown that both aldosterone (ALDO) and DEX can induce human MR mediated activation of a plasmid construct containing GREs and a viral promoter linked to the reporter gene, chloramphenicol acetyltransferase (CAT). When the

human GR expression vector is cotransfected with the CAT reporter plasmid, DEX but not ALDO can induce CAT gene expression. Other promoters for glucocorticoid-responsive genes, tyrosine aminotransferase and tryptophan oxygenase, were also reported to be stimulated in vitro (Arriza, et al., 1988). In all these cases, however, the maximal activation of the promoters by the MR was only 5-15% of the levels reached with the GR. The exchange of the DNA binding regions of the MR and GR had no significant effect on the ability of either hybrid to regulate gene activity (Evans and Arriza, 1989). This provides further evidence that these receptors regulate a common gene network. Similar hybrids exchanging the N-terminal domains revealed that the GR N-terminal, which contains the *t*1 activation region, strongly activates transcription. The N-terminal domain of the MR, however, displays no such activity (Evans and Arriza, 1989).

The ability of both MR and GR, if they are coexpressed, to bind the same ligand, become activated, and regulate transcription of the same target genes provides for a hormonal system with complex and flexible physiological responses. Differing tissue-specific and developmental patterns of expression could expand the range of responses that could be affected. Furthermore, the differing affinities for CORT expands the dynamic range of hormone signal to which a cell can respond. Co-expression of the receptors in the same cell would also allow for synergistic or competitive interactions.

Trans-activation

Glucocorticoid binding by the GR is necessary not only for specific recognition

of GREs, but also for induction of the GR's trans-activation function (Webster, et al., 1988). The trans-activation domains mediate the receptors effects on gene transcription presumably by interacting with other regulatory components of the transcriptional machinery. One such regulatory component is the factor which binds to a consensus sequence, the TATA box, found within many promoter regions (Ptashne, 1988). A distance dependence has been observed between a GRE and the TATA box of the tk promoter, suggesting an interaction between the GR trans-activation domain and the TATA box factor (Ham, et al., 1988).

Examination of the promoter regions of several glucocorticoid-responsive genes revealed the presence of two or more GREs. Deletion analysis demonstrated that two GREs showed a synergistic effect, and that the increased levels of induction achieved were due to cooperative binding (Jantzen, et al., 1987). This cooperativity resulted in an increased binding affinity of the receptor to the GRE, as well as increased stability of the DNA-receptor complex formed (Schmid, et al., 1989).

Regulatory Synergism

Subsequent studies have determined that other sequences, in addition to the GREs, are necessary for certain genes to demonstrate glucocorticoid inducibility (Danesch, et al., 1987). Using various plasmid constructs transfected into various cell lines, interactions between GREs and several other transcription factor binding sites have recently been examined. Synergism between a GRE and another transcription factor binding site, a CACCC-box, was reported (Schule, et al., 1988). This synergism displayed a distance dependence of 10 bp between the binding sites,

corresponding to one complete turn of the double helix, suggesting a requirement for stereo-specific alignment and a protein-protein interaction between the two factors (Schule, et al., 1988). Furthermore, the CACCC box could be replaced by a CCAAT box, a nuclear factor 1 (NF1), or a serum protein 1 (SP1) binding site, thus demonstrating that the synergistic action was not restricted to the CACCC box binding protein (Strahle, et al., 1988).

The degree of synergism between different transcription factors has been shown to be strongly dependent on the cell line used in the study (Strahle, et al., 1988). This probably reflects the relative abundance of the various factors in these cell lines. This also offers a possible explanation for the variance seen in the level of glucocorticoid inducibility of natural genes in different cell lines, which does not always correlate with the amount of GR in the cell (Bocquel, et al., 1989).

Negative Regulation

A further complication in the case of glucocorticoid regulation of transcription, is that some physiological genes are negatively regulated by glucocorticoids. In the case of glucocorticoid repression of the human glycoprotein *a*-subunit gene, the inhibition was dependent on the overlap of a functional cAMP responsive element (CRE) and the GRE (figure 6) (Akerbloom, et al., 1988). The binding of GR caused a steric hindrance to the binding of a positive transcription factor to the CRE. Negative regulation by the GR due to the displacement of positive transcription factors has also been shown for the bovine prolactin gene (Sakai, et al., 1988), the rat *a*1-fetoprotein gene (Guertin, et al., 1988), and the rat POMC gene (Drouin, et al.,

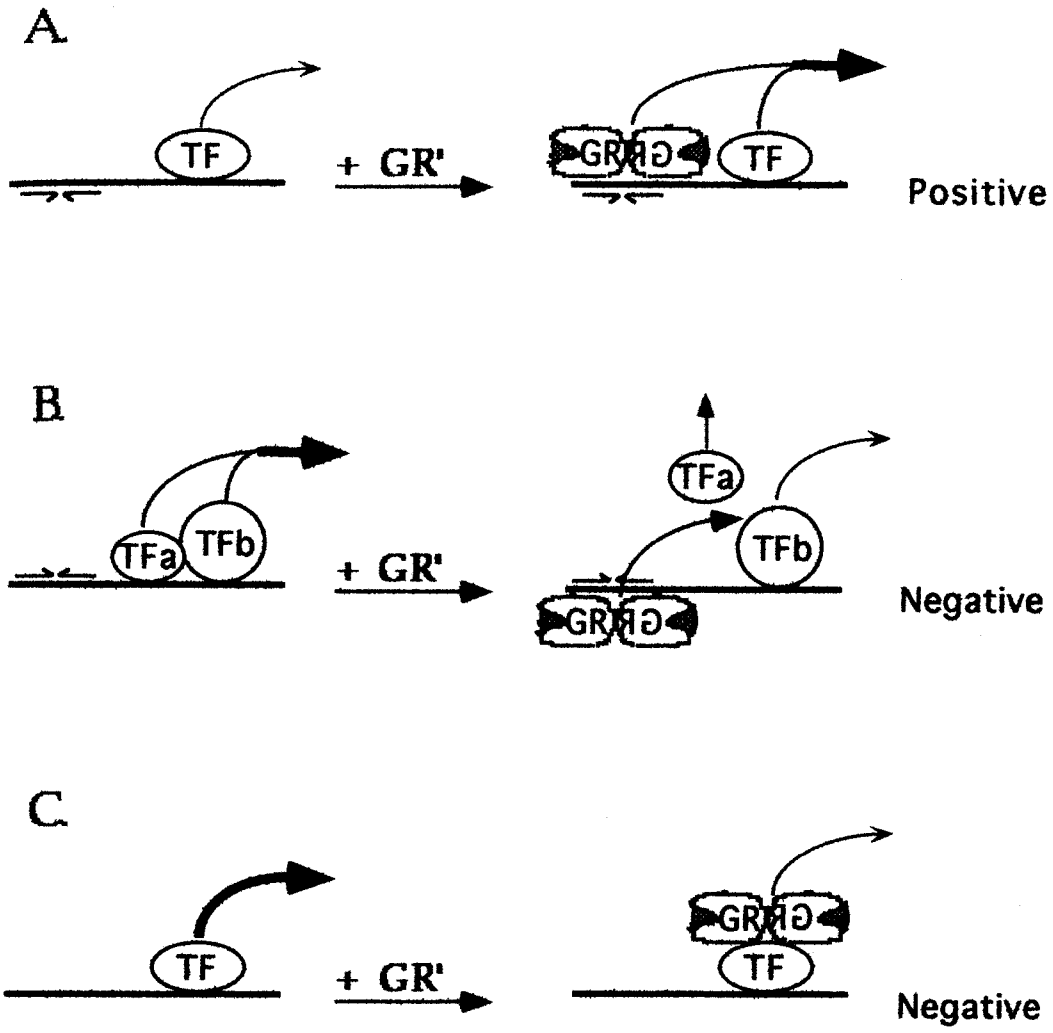


FIGURE 6. Schematic representation of positive and negative transcriptional effects of the activated glucocorticoid receptor (GR'). A. A synergistic interaction between GR' and a transcription factor (TF), resulting in positive transcriptional regulation. B. Displacement of a transcription factor by GR', resulting in negative regulation. C. Direct interaction between GR' and a transcriptional factor, resulting in negative regulation. Figure is modeled after Muller and Renkawitz, 1991.

1989). In these instances, the consensus sequence of the negative GRE differs from that of a positive GRE as well, possibly preventing positive regulation by GR binding (Sakai, et al., 1988; Beato, 1989).

Another mechanism of negative regulation does not involve DNA binding by GR; in fact no GR binding site is present. Instead, it involves the direct interaction between GR and the transcription factor AP-1 (Jonat, et al., 1990). AP-1 mediated induction of the collagenase gene is repressed by GR, even when the GR lacks the DNA binding domain. An immunoprecipitable AP-1/GR complex is formed which appears unable to trans-activate. Furthermore, GR mediated induction by DEX of a GRE-containing CAT-reporter gene construct was also reduced 10 fold in the presence of components of AP-1 (Jonat, et al., 1990).

A final example of negative regulation by yet a different mechanism can be illustrated using the proliferin gene. A fragment of the proliferin regulatory region was examined which exhibited GR and AP-1 binding. In addition this region conferred positive regulation by AP-1, and could confer both positive and negative regulation by glucocorticoids (Diamond, et al., 1990). The sequence differed from both positive and negative consensus GREs, and was termed a 'composite' GRE. The direction of regulation conferred by glucocorticoids was shown to depend on the intracellular composition and concentration of AP-1 complexes. Jun homodimers stimulated basal expression of proliferin, which DEX further enhanced. In contrast, Fos homodimers or Fos/Jun heterodimers strongly activated basal expression of proliferin, which DEX dramatically reduced. Thus a GR-Jun/Jun interaction results

in trans-activation at this composite GRE, while a GR-Fos/Jun interaction results in an inactive complex (Diamond, et al., 1990).

The emerging picture of glucocorticoid (hormonal) regulation of transcription is a complicated one, to say the least. A hormone response unit (HRU), comprised of both receptor and non-receptor binding sites, has been proposed as a functional model (Klein-Hitpass, et al., 1988). The strength of an HRU would then be determined by its DNA composition, as well as its environment (cell type). Its DNA composition comprises the number of hormone response elements combined with the number, type, and location of other transcription factor binding sites, while its environment includes the levels of GR and/or MR along with the levels of various transcription factors. The resulting interactions would then yield the observed pattern of induction and gene expression.

11*B*-Hydroxysteroid dehydrogenase (11*B*-HSD)

An entirely different mechanism of glucocorticoid regulation involves intracellular control of CORT levels by the microsomal enzyme 11*B*-HSD, which converts CORT to its inactive metabolite 11-dehydrocorticosterone (Monder and Shackleton, 1984). The specificity of the MR for ALDO, which is present in the plasma in 100-1000 fold lower concentrations than CORT, in mineralocorticoid target tissues such as the kidney, colon, and parotid gland is due to the presence of 11*B*-HSD in these tissues (Funder, et al., 1988; Edwards, et al., 1988). These studies reported that this enzyme displayed tissue specific expression, being localized only to peripheral mineralocorticoid target tissues, and was absent in the brain.

The elegant simplicity of this proposed mechanism was recently complicated by reports demonstrating that 11*B*-HSD activity is much more widespread than previously thought (Moisan, et al., 1990; Whorwood, et al., 1992). The presence of high levels of 11*B*-HSD bioactivity as well as high levels of 11*B*-HSD mRNA expression in various brain regions including the hippocampus, increases the complexity of regulation possible. In addition to regulation at the levels of GR and MR expression and function, tissues expressing 11*B*-HSD could regulate the amount of intracellular CORT available for receptor binding. Direct involvement in the regulation of CORT GR/MR interactions, or in HPA axis regulation by CORT remains to be shown.

Regulation of CORT Receptors

An important component of CORT receptor regulation is that both MR and GR are autoregulated. Decreased GR binding capacity following glucocorticoid exposure was first shown in AtT20 (mouse pituitary tumor) (Svec and Rudis, 1981) and HeLa S3 (human cervical carcinoma) (Cidlowski and Cidlowski, 1981) cell lines. MR in the rat kidney was also shown to be autoregulated: MR levels increase following ADX and are lowered by ALDO treatment (Claire, et al., 1981). Further evidence that decreases in GR binding reflected decreases in GR protein came from Western blot analyses, using GR antibodies. Twenty-four hours of glucocorticoid treatment decreases GR protein levels in a variety of cell lines and rat liver (Dong, et al., 1988; Hoeck, et al., 1989; Burnstein, et al., 1990).

Autoregulation has also been shown to occur in the brain and anterior

pituitary, with increases in CORT receptor levels following ADX (McEwen, et al., 1974; Koch, et al., 1978; Meaney and Aitken, 1985). Decreases in hippocampal CORT receptor concentrations occur in diabetic rats with chronically elevated plasma CORT levels (Tornello, et al., 1981), and in chronically stressed rats (Sapolsky, et al., 1984). Exogenous CORT administration, by pellet implantation or in the drinking water for 3 weeks (Tornello, et al., 1982), or injected for 4 days or 3 weeks (Sapolsky and McEwen, 1985), also reversibly decreases hippocampal CORT receptor levels. In contrast, DEX administration deceases CORT receptor levels in the pituitary, cortical amygdala, and the medial basal hypothalamus but not the hippocampus (Sapolsky and McEwen, 1985).

More recent studies, discriminating hippocampal CORT receptor subtypes, report that ADX results in an increase in GR after 1-2 weeks with no corresponding change in the levels of MR (Table IV) (Reul, et al., 1987a; 1987b). Furthermore, in the chronically (1 week) ADX animal, GR is subject to down-regulation by either CORT or DEX, but MR is not. In fact, an increase in hippocampal MR following DEX treatment was reported (Reul, et al., 1987a; Reul, et al., 1989). In contrast, constant ALDO or CORT replacement at the time of ADX was shown to significantly decrease hippocampal GR and MR concentrations relative to 3 day ADX values (Brinton and McEwen, 1988; Chao, et al., 1989). Down-regulation of murine hippocampal MR and GR by ALDO or DEX was also reported (Luttge, et al., 1989a; 1989b). These latter findings imply that coordinate regulation occurs, with the binding of ligand to either receptor influencing the regulation of both receptors.

The down-regulation of both hippocampal MR and GR by ALDO supports the hypothesis of coordinate regulation, since ALDO has only a very low affinity for GR. To further examine this hypothesis, Luttge et al. (1989a), using an MR specific antagonist, showed that the ALDO-induced down-regulation of GR, as well as MR, is dependent on ALDO/MR binding. Similarly, although DEX-induced down-regulation of MR is significantly attenuated by the MR antagonist, it is still significantly decreased relative to ADX controls (Luttge, et al., 1989b). This suggests that part of the down-regulatory effect of DEX was mediated by MR and part by GR. Unfortunately, no GR antagonist was administered with DEX and the MR antagonist to confirm this.

These studies support the hypothesis of coordinate regulation; the regulation of GR is controlled in part by MR mediated events, and MR regulation is mediated in part by GR. Thus, the binding of a ligand to either GR or MR may influence the regulation of both receptors.

A complicating factor in interpreting the abovementioned studies is that the endogenous ligand must be removed, usually by ADX, 18-24 hr before sacrifice. ADX allows the bound receptors to recycle out of the nucleus, so that they can bind radiolabeled ligand and be measured by a cytosolic in vitro binding assay. Examination of CORT receptor gene expression avoids these manipulations, which can themselves alter CORT receptor concentrations.

GR/MR mRNA Regulation

Recently, the availability of cDNA probes for MR and GR allows for the study

of CORT receptor gene regulation by analysis of their mRNAs (Miesfeld, et al., 1986; Arriza, et al., 1987). The examination of receptor mRNA avoids the surgical manipulations necessary for CORT receptor measurements using the binding assay, and also provides insight into the level at which regulation is occurring. Autologous regulation of CORT receptor mRNA has been shown by two recent studies, reporting that within 1 day, ADX causes an increased level of GR mRNA in the hippocampus (Reul, et al., 1989; Sheppard, et al., 1990). Furthermore, these increases are returned to intact values by DEX administration (Sheppard, et al., 1990). The anatomical specificity of this regulation has been demonstrated by examination of hippocampal subfields using in situ hybridization histochemistry. These studies revealed that chronic ADX (8 days) results in significantly elevated GR and MR mRNA specifically in the CA1-2 subfields (Herman, et al., 1989a). Interestingly, chronic CORT or DEX treatment, which decreases GR concentration (Reul, et al., 1987a), does not alter steady state levels of hippocampal GR mRNA (Chao, et al., 1989; Sheppard, et al., 1990) or MR mRNA (Chao, et al., 1989; Herman, et al., 1989a). This suggests that there may be changes in the efficiency of translation or posttranslational regulation (receptor protein turnover). A summary of the autologous regulation of CORT receptors is presented in Table IV.

TABLE IV

Autologous Regulation of CORT Receptors

Receptor Regulation	*Manipulation*	*Reference*
Increase HIPP GR Prevent above increase	ADX (7 days) CORT or DEX (7 days)	Reul et al., '87a,b
Increase HIPP MR	DEX (7 days)	Reul et al., '87a, '89
Decrease HIPP GR and MR	CORT or ALDO (3 days)	Brinton and McEwen, '88 Chao et al., '89
Decrease HIPP GR and MR	ALDO or DEX (1 day)	Luttge et al., '89a,b
Increase HIPP GR mRNA Prevent above increase	ADX (1 day) DEX (1 day)	Reul et al., '89 Sheppard et al., '90
Increase GR and MR mRNA in HIPP CA1-2 subfields	ADX (8 days)	Herman et al., '89a
No change in HIPP GR mRNA or MR mRNA	DEX (6 days) CORT (5-12 days)	Sheppard et al., '90 Chao et al., '89
Increase HIPP GR mRNA Decrease HIPP GR mRNA Increase AP GR mRNA	ADX (4 hours) ADX + DEX (4 hours) ADX + DEX (4 hours)	Sheppard et al., '90

Decrease GR transcription rates in rat liver and human lymphoma cells	DEX (1 day)	Dong et al., '88 Rosewicz et al., '88
Decrease GR mRNA half-life in AtT20 cells	DEX (1-5 hours)	Vedeckis et al., '89
Decrease GR protein half-life in GH_1 cells, HTC cells, and NIH 3T3 cells	DEX (1-2 days)	McIntyre and Samuels, '85 Dong et al., '88 Hoeck et al., '89

Abbreviations:

GR, glucocorticoid receptor

MR, mineralocorticoid receptor

ADX, adrenalectomy

CORT, corticosterone

DEX, dexamethasone

AtT20, corticotroph-like anterior pituitary cells

ALDO, aldosterone

HIPP, hippocampus

AP, anterior pituitary gland

GH_1, growth hormone producing anterior pituitary cells

HTC, hepatoma tissue culture

3T3, fibroblasts

Mechanism of Regulation

The mechanism by which glucocorticoids down-regulate their receptors has been a subject of extensive investigation in recent years. A shortened GR half-life is one mechanism supported by recent findings. A 2-fold reduction in the GR half-life occurs after glucocorticoid exposure, in both a rat pituitary cell line and hepatoma tissue culture cells, without a change in the GR's synthetic rate (McIntyre and Samuels, 1985; Dong, et al., 1988). These studies are consistent with pulse-chase

experiments in NIH 3T3 mouse fibroblasts, which also demonstrate an approximately two-fold reduction in GR half-life following DEX administration (Hoeck, et al., 1989). In contrast, Distelhorst and Howard (1989), also employing a pulse-chase technique, reported no difference in GR half-life in the presence or absence of DEX, in S49 mouse lymphoma cells. This discrepancy may be due to alternative mechanisms by which S49 mouse lymphoma cells regulate GR, as has been reported in the human CEM lymphoid cell line (Antakly, et al., 1989), or to methodological differences. Thus, a shortened GR half-life appears to be one mechanism by which down-regulation is achieved.

In every cell line demonstrating glucocorticoid-induced GR protein down-regulation, GR mRNA levels have also been shown to be significantly decreased, 50-95% (Okret, et al., 1986; Kalinyak, et al., 1987; Dong, et al., 1988; Rosewicz, et al., 1988; Hoeck, et al., 1989; Burnstein, et al., 1990). This suggests that regulation at the level of GR mRNA synthesis or stability is another mechanism involved in GR down-regulation. Using nuclear run-on transcription assays, the transcription rate of the GR gene was shown to be reduced by DEX in rat liver cells (Dong, et al., 1988) and human IM-9 lymphocytes (Rosewicz, et al., 1988). No effect of DEX on GR mRNA half-life was reported in hepatoma, pancreatic acinar (Dong, et al., 1988), or IM-9 cells (Rosewicz, et al., 1988). These studies, however, only examined GR mRNA half-life after maximal down-regulation (DEX pretreatment for 24 hours), and achievement of new steady-state levels of GR mRNA. Using actinomycin D to block transcription during glucocorticoid-induced down-regulation, Vedeckis et al. (1989)

demonstrated that GR mRNA half-life was reduced, from 3 h in the absence to 1 h in the presence of glucocorticoid, in AtT20 cells.

Further evidence for at least two independent mechanisms, one transcriptional and the other posttranslational, by which GR levels are regulated has been provided in a recent study by Okret et al., (1991). Using a glucocorticoid-resistant cell line which contains low levels of GR, but neither induces glucocorticoid regulated genes nor autoregulates GR mRNA levels, down-regulation of GR protein has been demonstrated to still occur.

A novel molecular mechanism, involving direct interaction of the ligand-receptor complex with GR gene coding regions and not traditional DNA transcriptional regulatory regions, may be one method by which glucocorticoids accomplish down-regulation of GR protein and mRNA levels. Evidence supporting this hypothesis was recently reported by two independent groups, utilizing cells transiently or stably transfected with human GR cDNAs. After demonstrating that the transfected cells were indeed producing intact and functional GR, DEX treatment (24 h) was shown to result in down-regulation of both GR protein and mRNA levels in the transfected cells. Expression of the hGR cDNA was driven by either the Rous sarcoma virus LTR promoter (Burnstein, et al., 1990; 1991) or the human metallothionein IIA promoter (Alksnis, et al., 1991), which were both shown to be nonresponsive to DEX. Neither expression vector could regulate the expression of a reporter gene, substituted in place of the hGR cDNA, in response to DEX. In addition, direct binding of 3H-DEX/GR complexes to a DNA fragment encoding the

entire coding region of hGR, but not the 3' untranslated region, was shown (Burnstein, et al., 1990). Therefore, it appears that the coding region of the GR cDNA, independent of transcriptional regulatory regions such as the promoter or enhancer regions, contains sequences that bind the ligand-receptor complex and mediate GR protein and mRNA down-regulation.

To further define the specific region of DNA involved, Burnstein et al. (1990), using deletion analysis, has demonstrated that a region between nucleotides 527 and 1526 contains at least some of the signals necessary for down-regulation of GR mRNA. Taking the deletion analysis one step further, Alksnis et al. (1991) has shown that DEX-induced down-regulation of receptor mRNA occurs in cells stably transfected with an expression vector, which encodes only the ligand binding domain and yields a 1.6 kilobase (Kb) mRNA. Whether these regulatory signals function by binding the activated receptor, thus inhibiting transcription, or by binding and altering the mRNA, thus acting posttranscriptionally, remains a question.

The autologous regulation of GR protein and mRNA levels appears to be mediated by two or more independent mechanisms of action. Posttranslational effects of glucocorticoids shorten GR protein half-life, while posttranscriptional effects decrease GR mRNA half-life. Decreases in GR mRNA transcription rates reflect effects of glucocorticoids on GR gene expression. While the decreases in transcription rates are probably mediated by traditional genomic ligand-bound-GR interactions, other novel means may be involved as well. The direct binding of the ligand-bound-GR to either novel regulatory regions on the DNA or to the GR mRNA

allows for complex regulatory interactions.

Clinical Implications

Two areas of research which have explored the interrelationships between HPA axis regulation and clinical illness are 1) depressive disorders and 2) premenstrual syndrome (PMS).

Extensive research has been conducted examining the relationship between the HPA axis and depressive disorders. Correlations have been shown between endogenous depression, a pathological mood state, and a reduced suppression of plasma cortisol levels following DEX injection (Carroll, et al., 1968; Rupprecht and Lesch, 1989). While the diagnostic specificity of the dexamethasone suppression test (DST) is less than was originally thought, the inability to suppress cortisol levels following DEX administration occurs significantly more frequently in depressed patients. Hypersecretion of cortisol during depression has been shown by several groups (Carroll, et al., 1976; Linkowski, et al., 1985; Gold, et al., 1986b; Christensen, et al., 1989; File, 1990). Recent studies have reported reduced suppression of ACTH and *B*-endorphin following DEX administration in depressed patients (Norman, et al., 1987; Meador-Woodruff, et al., 1987; Lesch, et al., 1988). Taken together with reports of increased CRH immunoreactivity in the CSF of depressed patients (Nemeroff, et al., 1984; Banki, et al., 1987; Gold, et al., 1988), this suggests a dysfunction of the HPA axis at or above the level of the hypothalamus. In addition to depression, anorexia nervosa is also associated with a hyperactive HPA axis and increased CRH levels in the CSF (Gold, et al., 1986a; Kaye, et al., 1987).

While the source of CRH in the CSF is most likely extrahypothalmic, its inverse relationship with CSF cortisol levels (Garrick, et al., 1987) suggests that its negative regulation by glucocorticoids would parallel hypothalamic CRH. Abnormal regulation of the HPA axis in depressed or anorexic patients, leading to hypercortisolemia or reduced suppression of cortisol in the DST, could involve several factors. These include 1) defective glucocorticoid-dependent negative feedback, possibly due to an abnormality of the glucocorticoid receptors, or 2) chronic CRH hypersecretion.

Premenstrual syndrome (PMS) is a late luteal phase anxiety disorder characterized by an increase in the onset of depressive syndromes and aberrant psychological behavior. Several studies have reported elevated cortisol levels in PMS patients versus controls, and suggested that a relationship may exist between PMS and endogenous depression (Haskett, et al., 1984; Watts, et al., 1985; Rubinow, et al., 1988). A recent study has reported transient increases in HPA axis activity in PMS patients (Rabin, et al., 1990). These patients exhibited an increased cortisol response to CRH administration during the luteal phase compared with normal women. Another recent study examined the relationship between symptoms and plasma hormone levels during two consecutive cycles in a group of women with PMS (Hammarback, et al., 1989). Their results indicate that high luteal phase plasma estradiol levels were correlated with higher premenstrual scores for adverse symptoms and lower scores for positive mood symptoms. The patients experienced more severe PMS in cycles with high luteal phase estradiol and progesterone concentrations.

Other studies have failed to uncover a clear relationship between changes in

estradiol across the menstrual cycle and PMS (Varma, 1984; Watts, et al., 1985; Rubinow, et al., 1988). In fact, estradiol administration, which inhibited ovulation, also alleviated the cyclical mood changes (Magos, et al., 1988). Thus, no clear cut correlation between estradiol levels and PMS exists. It has been suggested (Rabin, et al., 1990) that the severity of the possible HPA axis malfunction (hyperactivity) is less in patients with PMS than in patients with sustained hypersecretion of CRH or cortisol, where menstrual cycle dysfunction is commonly found (Villanueva, et al., 1986).

While a link between E and PMS has yet to be established, it appears that a relationship between transitory HPA axis hyperactivity and PMS exists. Thus, a common denominator of abnormal HPA axis function may exist in PMS, anorexia nervosa, and depression. While E may not be the primary etiologic agent, its ability to contribute to impaired HPA axis functioning strongly suggests a secondary supportive role. Support for a potential role for estrogen in the etiology of depressive disorders or mood swings can be inferred from the high incidence of clinical depression in females (Dohrenwend, et al., 1976; Weissman and Klerman, 1977).

Summary

Estrogen treatment in the female rat results in an enhanced hormonal response to stress, due to a hyperactivation of the HPA axis. The mechanisms by which E achieves this effect are unknown at present. Several possibilities have been implicated including alterations of CRH synthesis and secretion, increases in anterior pituitary responsiveness to CRH, and impairment of CORT negative feedback

inhibition. Examination of the ACTH and CORT patterns of secretion in response to stress revealed that plasma levels are elevated, and that the duration of the response is prolonged with E treatment. This altered pattern points to an impairment in the CORT negative feedback mechanism as a consequence of E treatment.

This feedback inhibition has been separated into 3 types, which differ in the time domains **within** which they act, the mechanisms **by** which they act, as well as the sites **at** which they act.

Fast, rate sensitive feedback acts within seconds to minutes, inhibiting hormone release (CRH, AVP, ACTH). It acts predominately at sites on afferent pathways to PVN CRH/AVP neurons, and/or the neurons themselves.

Intermediate, early delayed feedback is level sensitive and acts within minutes to hours, inhibiting release and synthesis of hormones (CRH, AVP, ACTH). It acts at sites on AP corticotrophs, afferent pathways to PVN CRH/AVP neurons, PVN CRH/AVP neurons, and/or hippocampal GR and/or MR containing neurons.

Slow, late delayed feedback is also level sensitive and acts within hours to days, inhibiting synthesis of hormones. It acts at sites on AP corticotrophs, afferent pathways to PVN CRH/AVP neurons, PVN CRH/AVP neurons, and/or hippocampal GR and/or MR containing neurons. It is probably involved only under pathophysiological or pharmacologically administered conditions.

The actions of CORT, including negative feedback inhibition, are mediated by two intracellular receptors, MR and GR. These receptors are products of the mineralocorticoid and glucocorticoid genes, respectively. Recent evidence suggests a

role for both receptors in mediating negative feedback. Receptor deficits, whether induced experimentally or naturally occurring, result in altered patterns of hormonal secretion similar to those observed with E treatment. **Consequently, our hypothesis is that the altered hormonal response to stress seen in the presence of E is due to an effect of E on CORT receptor regulation.**

The specific aims of the studies performed for this dissertation are:

1) To characterize the effect of E on the CORT and ACTH response to stress in the female rat, and determine if E-induced alterations in CORT negative feedback contribute to these altered hormonal responses.

2) To explore one possible mechanism by which E alters the response of the HPA axis by analyzing the effect of E on CORT receptor regulation and function.

3) To further investigate the influence of E on CORT receptor function by examining the effect of E on regulation of CORT receptor mRNAs.

CHAPTER III

Chronic Estrogen-induced Alterations in Adrenocorticotropin and Corticosterone Secretion, and Glucocorticoid Receptor-mediated Functions in Female Rats

Summary

The effect of estrogen (E) on the hypothalamic-pituitary-adrenal (HPA) axis was investigated in female Sprague Dawley rats. Animals were bilaterally ovariectomized (OVX) and a Silastic capsule (0.5 cm) containing 17*B* estradiol was subcutaneously implanted. Control animals received a blank capsule. Animals were sacrificed 21 days later. In E treated rats, we found significantly higher corticosterone (CORT) peak levels 20 min following a 5 sec footshock (1.0 mA) or exposure to ether vapors ($p < 0.05$) as compared to OVX controls. In addition, the recovery of the ACTH and CORT response to footshock stress was significantly prolonged ($p < 0.05$) in the presence of E. Furthermore, the ACTH and CORT secretory response to ether stress could be suppressed by exogenous RU 28362 (a specific glucocorticoid receptor agonist; 40 ug/100g BW for 4 days) in OVX controls ($p < 0.05$) but not in E treated animals. These data suggest that E can impair glucocorticoid receptor mediated delayed or slow negative feedback.

Consequently, we examined the influence of E on mineralocorticoid and glucocorticoid receptor concentrations using in vitro binding assays. E did not alter mineralocorticoid or glucocorticoid receptor concentrations in any of the brain regions

examined. The administration of RU 28362 (40 ug/100g BW for 4 days) to OVX'd control or E treated rats significantly downregulated hippocampal glucocorticoid receptor ($p<0.02$) in control rats only. In contrast, aldosterone administration (40 ug/100g BW for 4 days), significantly downregulated hippocampal glucocorticoid receptor ($p<0.0008$) in both control and E treated animals. Thus, E treatment results in a loss of the glucocorticoid receptor's ability to autoregulate, and suggests that E may cause a functional impairment of the glucocorticoid receptor even though receptor binding appears normal.

These findings suggest that the hyperactivation of the HPA axis following stress in E treated rats is in part due to impaired glucocorticoid receptor mediated slow negative feedback.

Introduction

Activation of the hypothalamic-pituitary-adrenal (HPA) axis is a characteristic physiological response to stress. Previous studies have demonstrated that the estrogen (E) status of the female rat may affect its endocrine response to stress. It has been shown that a sex difference exists, in both circulating corticosterone (CORT) and in the CORT response to stress, with females having higher levels than males (Kitay, 1961; Crithlow, et al., 1963). Additional studies have shown that ovariectomy (OVX) reduces basal CORT levels and that E replacement increases them (Kitay, 1963; Ramaley, 1976; Phillips and Poolsanguan, 1978). This is consistent with studies showing that basal (Raps, et al., 1971; Phillips and Poolsanguan, 1978; Buckingham, et al., 1978; Viau and Meaney, 1991) and stress responsive (Raps, et

al., 1971; Poolard, et al., 1975; Phillips and Poolsanguan, 1978; Viau and Meaney, 1991) CORT levels are highest on proestrus when E levels are the highest. Finally, it has been shown that female rats receiving E replacement following OVX have significantly higher post-stress CORT levels than controls (Phillips and Poolsanguan, 1978; Viau and Meaney, 1991).

At present, the factors which underlie the apparent effect of E on CORT secretion have not been examined. Several possibilities exist including E's effects on CRH synthesis or secretion, anterior pituitary gland (AP) sensitivity to CRH, or CORT negative feedback mechanisms. Previous studies have shown that E may directly effect levels of CRH immunoreactivity and mRNA (Haas and George, 1988; Swanson and Simmons, 1989; Bohler, et al., 1990). While chronic E treatment decreased hypothalamic CRH-ir (Haas and George, 1988), CRH mRNA was found to be elevated on the afternoon of proestrus, at the approximate time of the E-induced pre-ovulatory surge of LH (Bohler, et al., 1990). Using hypothalamic extracts to stimulate AP release of ACTH, Coyne and Kitay (1969) demonstrated decreased sensitivity of the AP following OVX, which was partially reversed by E. Studies in the aged rat showed that deficits in hippocampal CORT receptors are associated with the hyperactivation of the HPA axis found in response to stress (Sapolsky, et al., 1984; Salpolsky, 1986). This pattern is similar to the one we described for the E treated animal (Burgess and Handa, 1990).

Negative feedback regulation of the HPA axis, both fast and delayed, is mediated predominately through the binding of CORT to intracellular CORT receptors

found in the hippocampus, hypothalamus, and AP (Keller-Wood and Dallman, 1984; McEwen, et al., 1986; Jacobson and Salpolsky, 1991). Previous studies have classified receptors for CORT into Type I and Type II receptors (Funder, et al., 1973; Reul and De Kloet, 1985), which have been shown to be the products of the mineralocorticoid receptor (MR) gene and the glucocorticoid receptor (GR) gene, respectively (Arriza, et al., 1987; Miesfeld, et al., 1986). In the following studies, we will employ the designations of MR and GR for the Type I and the Type II receptors, respectively. Both receptor types have been implicated in negative feedback by CORT (Dallman, et al., 1989; Ratka, et al., 1989), and both are found in varying amounts at the three major sites involved in negative feedback.

In the following studies we 1) characterized the ACTH and CORT responses to stress in female rats in the absence of E (OVX) or with chronic E replacement; 2) examined the prospect that alterations in CORT delayed negative feedback are contributing to differing hormonal responses in the presence and absence of E; and 3) explored the possibility that the E status of the animal may influence CORT receptor regulation.

Materials and Methods

Animals

Adult female Sprague-Dawley rats (250-300g; Sasco, Omaha, NE) were housed in environmentally controlled quarters with a 12-h light/dark cycle (lights on at 0700 h), and food and water available ad libitum. Adrenalectomized rats were

maintained on 0.9% saline immediately following surgery.

Animals were bilaterally ovariectomized under ether anesthesia, followed either by subcutaneous (sc) implantation of a 0.5 cm Silastic capsule (0.078 in I.D. 0.125 in O.D., Dow Corning) filled with 17 *B*-estradiol (E) (Sigma Chemical Co., St.Louis, MO) or a sham-operation (control). Average serum estrogen levels achieved with this E capsule treatment are approximately 75 pg/ml (Handa and Rodriguez, 1991). Bilateral adrenalectomy was performed under ether anesthesia by making two dorsal flank incisions. Three days before blood sampling, Silastic catheters (0.045 in I.D. 0.065 in O.D., Dow Corning, Midland, MI) were inserted into the right atrium via the right jugular vein, exteriorized by passage under the skin to the back of the neck, and then connected to a vascular access port (Access Technologies, Skokie,IL) attached to the skin of the animal's back.

Experimental protocols

Exp 1a: Effect of E on the ACTH and CORT response to footshock stress: a repeated sampling paradigm

The timecourse of the ACTH and CORT response to stress was examined to determine if E treatment has a similar effect when performed in adulthood as that previously reported when OVX and E treatment was performed prepuberally. We used indwelling right atrial cannulae for repeated sampling from individual animals during the recovery period following footshock stress. These studies are important in determining if the observed effect of E is through a disruption of the negative

feedback actions of CORT. Female rats were bilaterally ovariectomized to reduce circulating E levels. One-half of the animals then received a sc implantation of a 0.5 cm Silastic capsule filled with 17 *B*-estradiol while control rats were OVX'd and sham treated. Animals were handled for three minutes each day for one week prior to testing. Eighteen days following treatment, Silastic tubing (0.045 in ID. 0.065 in OD., Dow Corning) was inserted into the right atrium via a nick in the right jugular vein, and externalized by connecting the tubing to a vascular access port (Access Technologies, Skokie, IL) which was secured to the skin of the animal's back. Stress responses were tested 3 days later (Day 21). On the day of testing, PE-50 tubing was connected to the ports 3 hrs prior to stress testing. The stressor used was 5 seconds of inescapable footshock (1.0 mA). Animals were placed into the shock chamber and returned to their home-cage immediately after the shock was administered. Blood samples (0.5 ml) were taken at 0, 5, 10, 15, 20, 30, 60, and 120 minutes following return to their home-cage. An equal volume of rat red blood cell preparation was replaced following each sampling to maintain hematocrit and osmotic balance (Coquelin and Bronson, 1981). Plasma was removed from samples and frozen at -70^0 C until assayed for ACTH and CORT by RIA.

Exp 1b: Effect of E on the CORT response to footshock stress: single point measurements

Data from experiment 1a. suggested that E treatment impaired negative feedback. To eliminate the effects of cannulation, we utilized animals sacrificed at

different timepoints to examine the pattern of the CORT response following footshock stress. OVX and E treatment were as described in experiment 1a. Animals were handled for three minutes each day for one week prior to testing. Twenty-one days after treatment, animals were removed from their home-cages, subjected to 5 seconds of inescapable footshock (1.0 mA) in a shock chamber, and immediately returned to their home-cages. Non-stressed controls were sacrificed immediately upon removal from their home-cage. Footshocked animals were sacrificed 20, 45, or 70 minutes following the stress. Animals were sacrificed by decapitation, trunk blood was collected, and plasma removed and frozen at -70^0 C. Plasma samples were assayed for CORT levels by RIA.

Exp 2: Effect of E on the CORT response to ether stress

This experiment was performed to determine if one minute of ether stress would prove a comparable stressor to the footshock used above. Ether stress would allow for collection of the 0 minute and 20 minute post-stress samples by tail vein bleeding from the same animal without the need for cannulation. Adult female rats were ovariectomized and E treated as described in experiment 1. Twenty-one days after treatment, rats were stressed by brief exposure to ether vapor. The ether stress protocol consisted of placing animals in a jar containing ether soaked gauze and removing them after 1 minute. The 0 min and 20 minute post-stress blood samples (0.5 ml) were subsequently collected from the tail veins. Plasma was removed and stored at -70^0 C until assayed for CORT by RIA.

Exp 3: Effect of E on DEX and RU 28362 mediated suppression of the neuroendocrine stress response

Our previous studies (Expts. 1, 2) suggest that an impairment of the negative feedback mechanism of CORT on the HPA axis is present following chronic E treatment. In this study we employed the synthetic glucocorticoid dexamethasone (DEX; 9*a*-Fluoro-16*a*-methylprednisolone, Sigma Chemical Co.,St. Louis, MO) or the specific GR agonist RU 28362 (11*B*, 17*B*-dihydroxy-6-methyl-17*a* (1-propynyl)-androsta-1,4,6-trione-3-one, Roussel-UCLAF, Romainville, France) to assess the responsiveness of the HPA axis to ether stress after chronic glucocorticoid suppression. Comparisons between the effects of DEX, which binds MR and GR (Krozowski and Funder, 1983; Allen, et al., 1988; Brinton and McEwen, 1988; Luttge, et al., 1989b), and RU 28362, which binds specifically to GR (Sarrieau, et al., 1988), could reveal the receptor type mediating these phenomena. OVX and E treatment were as described in experiment 1. Seventeen days after treatment, control and E treated animals were each divided into 3 groups: animals received daily sc injections of either DEX, RU 28362 (40 ug/100 g BW in oil, 0.2-0.3ml), or oil for 4 days. Twenty-four hrs after the last injection (Day 21), rats were stressed for 1 minute by exposure to ether vapor as described in experiment 2. Blood samples (0.5 ml) were collected from the tail vein at 0 min post-stress, and trunkblood was collected following decapitation at 20 min post-stress. Plasma was removed and stored at -70^0 C until assayed for ACTH and CORT levels by RIA.

Exp 4: Effect of E on MR and GR concentrations in the preoptic area (POA), medial basal hypothalamus (MBH), and hippocampus (HIPP)

Studies described in this paper have demonstrated that E elevates and prolongs the ACTH and CORT responses to stress in the female rat. This suggests an impairment of CORT negative feedback on the HPA axis, perhaps due to changes in specific populations of CORT receptors. Consequently, we measured MR and GR concentrations in various brain regions in control and E treated rats. E treatment of adult female rats was as described in experiment 1. Twenty days after surgery, rats were ADX'd bilaterally. This surgical manipulation is necessary to deplete endogenous ligand (CORT) and allow occupied receptors to recycle to the unoccupied form, since occupied CORT receptors do not readily exchange their ligand with radio-labeled ligand. Eighteen hrs after ADX, animals were sacrificed and the POA, MBH, and HIPP dissected for measurement of MR and GR using an in vitro binding assay.

Exp 5: Effect of E on the downregulation of MR and GR

In this experiment we examined the ability of ALDO, RU 28362, and DEX to downregulate MR and GR in the hippocampus in the presence or absence of E. E treatment of adult female rats was as described in experiment 1. Twenty-one days after surgery, animals were bilaterally ADX'd. Control and E replaced groups were subdivided into groups which received daily sc injections for 4 days of either ALDO, RU 28362, or DEX (40ug/100 g BW in oil, 0.2-0.3ml), or oil. Animals were sacrificed 24 hr after the last injection. MR and GR were measured in the hippocampus using an in vitro binding assay.

CORT receptor binding assays

MR and GR concentrations were determined using in vitro binding assays as previously described (Lorens, et al., 1990). Briefly, tissue is homogenized in TEGMD buffer (10 mM TRIS, 1.5mM EDTA, 10% glycerol, 25mM molybdate, 1mM dithiothreitol; pH 7.4) then centrifuged at 106,000 x g for 15 min. at 4^0 C. 100 ul aliquots of the supernatant cytosol are incubated with 5nM ^{3}H-Dexamethasone (DEX) or 5nM ^{3}H-CORT, in the presence of RU 28362 (to prevent binding to GR), for determinations of GR or MR respectively. For GR measurements, 1uM unlabeled RU 28362 (a GR specific agonist) is incubated in parallel tubes with ^{3}H-DEX to determine nonspecific binding. For MR measurements, 1uM unlabeled DEX is incubated in parallel tubes with ^{3}H-CORT plus 1 uM unlabeled RU28362 to assess nonspecific binding. DEX binds to both GR and MR, while RU28362 binds only to GR. Therefore, the binding of ^{3}H-DEX in the presence of RU28362 can be subtracted from the total ^{3}H-DEX binding to estimate binding to GR. DEX does not bind to corticosterone binding globulin (CBG). This allows the concentration of MR to be determined by subtracting ^{3}H-CORT binding in the presence of DEX and RU28362 from ^{3}H-CORT binding in the presence of RU28362 alone. Following incubations at 4^0 C for 16-20 h, samples were passed through Sephadex LH-20 columns to separate bound from free ligand. 600 ul of eluate was collected which contained bound radioactivity. Three ml of UltimaGold (Packard, Downers Grove,IL) were added to the eluate, and radioactivity was counted in a Packard 1900 LA liquid scintillation counter at 37% efficiency. All receptor data is expressed as

femtomoles bound per mg protein. The method of Lowry et al. (1951) was used to determine cytosolic protein concentrations.

Hormone assays

Blood samples were collected into tubes containing trasylol (1000 KIU) and EDTA. Plasma was removed and stored at -70^0 C until assayed for ACTH and CORT by RIA as previously described (Carnes, et al., 1987; Lorens, et al., 1990). ACTH plasma levels were determined using a double antibody RIA. The first antibody, directed against ACTH 7-18 (IgG, Inc. Nashville TN), was used according to manufacturer's instructions. Standard curves were made using 0.5 to 50 pg/tube of rat ACTH (Peninsula Inc. Belmont CA). The intra- and inter-assay variabilities were 6% and 10% respectively.

For CORT, binding proteins were heat denatured at 60^0 C for 1 h. Rabbit anti-CORT serum (Radioassay Systems Labs, Carson, CA) was used at a final dilution of 1:5,600 according to manufacturer's protocols. Standard curves were constructed from dilutions of CORT (4-Pregnen-11*B*,21-diol-3,20-dione, Steraloids, Wilton, NH). The intra- and interassay variabilities were 4.8% and 8.2%, respectively.

Plasma samples from DEX, RU 28362, and oil injected rats used in experiment 5 were analyzed for residual hormone by a competitive in vitro binding assay. Briefly, plasma was incubated with ^{3}H-DEX and a supernatant cytosol from liver, as a source of MR and GR, prepared as described above. Standard curves were constructed using dilutions of DEX and RU 28362, combined with the liver cytosol and ^{3}H-DEX. Unbound radioactivity was removed using dextran coated charcoal and

the bound radioactivity counted as described above. The sensitivity of this assay was approximately 100 pg/ml.

Statistical analysis

Temporal differences in the ACTH and CORT response to stress between control and E treated rats were detected by a two-way analysis of variance with repeated measures across time, followed by the Student-Newman-Keuls multiple comparison test. Differences in percent suppression in experiment 3. were analyzed by the non-parametric Kruskal-Wallis T test. All other data were analyzed by a two-way or three-way analysis of variance followed by the Student-Newman-Keuls multiple comparisons test (Zar, 1984).

Results

Exp 1a: Effect of E on the ACTH and CORT response to footshock stress: a repeated sampling paradigm

Plasma samples obtained by repeated sampling from individual animals during their recovery from footshock stress showed significantly elevated CORT and ACTH levels in E treated rats at 60 minutes ($p < 0.05$) versus controls (figure 7). Two way ANOVA with repeated measures across time, revealed a significant time by treatment interaction ($p < 0.007$).

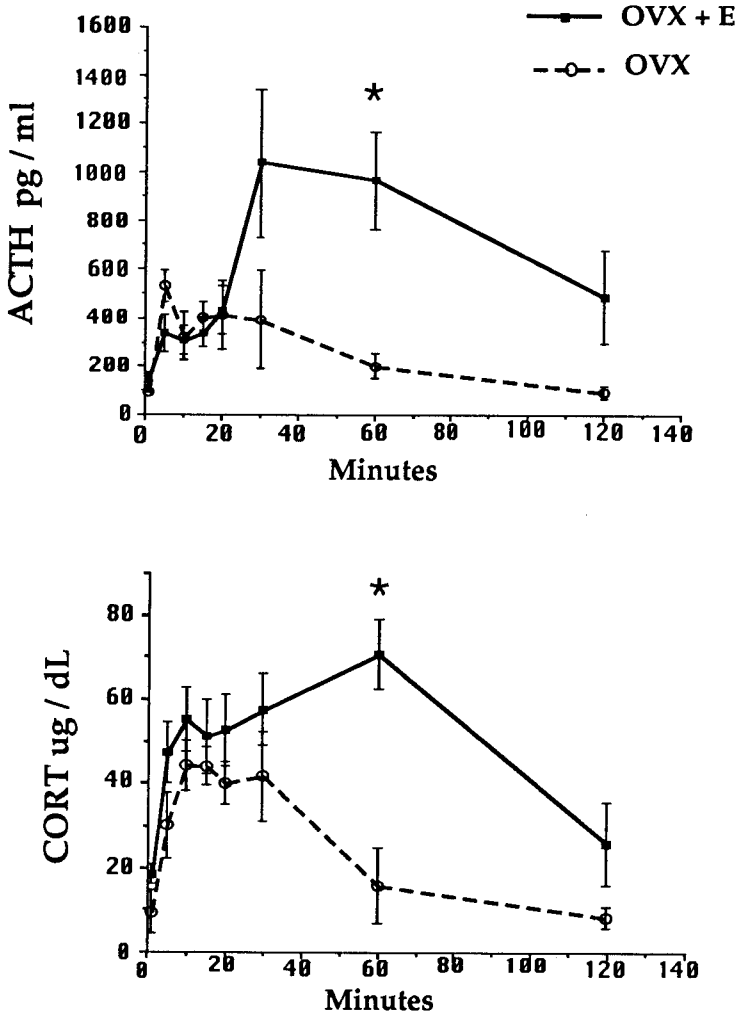


FIGURE 7. Time course of plasma CORT and ACTH levels following footshock stress to female rats 21 days after OVX or OVX with estrogen replacement (OVX + E). Serial blood samples were obtained by indwelling right atrial cannulae 0, 5, 10, 15, 20, 30, 60, and 120 min after 5 sec of footshock stress. Each *point* indicates the mean $\pm$ SEM of three to six determinations. Two-way ANOVA with one repeated measure across time indicated significant time, and time by treatment differences ($p < 0.007$). *, Significant increase ($p < 0.03$) from OVX control value. The 5-30 min CORT values in the OVX group and the 5-60 min CORT values in the OVX + E group were significantly elevated ($p < 0.05$) over the 0 min control values. The 5, 15, 20, and 30 min ACTH values in the OVX group, and the 30 and 60 min ACTH values in the OVX + E group were significantly elevated ($p < 0.05$) over the 0 min control values.

Exp 1b: Effect of E on the CORT response to footshock stress: single point measurements.

E treatment significantly elevated plasma CORT levels at 0, 20, 45, and 70 min post-stress ($p < 0.01$) as compared to controls (figure 8). Two-way ANOVA revealed a significant time by treatment interaction ($p < 0.005$).

Exp 2: Effect of E on the CORT response to ether stress.

To further examine the effect of E on the HPA axis, a second stress paradigm utilizing ether stress was performed. Twenty min following ether stress, CORT levels were significantly elevated ($p < 0.001$) in E treated and control groups. However, E treated animals had significantly greater plasma CORT levels at both 0 min and 20 min post-stress ($p < 0.05$) as compared to controls (figure 9).

Exp 3: Effect of E on DEX and RU 28362 mediated suppression of the neuroendocrine stress response.

DEX and RU 28362 treatment of OVX control rats significantly depressed plasma ACTH and CORT levels ($p < 0.01$) when sampled 0 min and 20 min after ether stress (figure 10). In the E treated rats, DEX treatment significantly decreased the plasma ACTH and CORT response ($p < 0.01$), however, there was no significant effect of RU 28362 at 0 min. RU 28362 was significantly less effective in suppressing the ACTH and CORT response at 20 min in E treated rats (69% & 78%,respectively) as compared to OVX controls (39% & 23%, respectively) ($p < 0.05$).

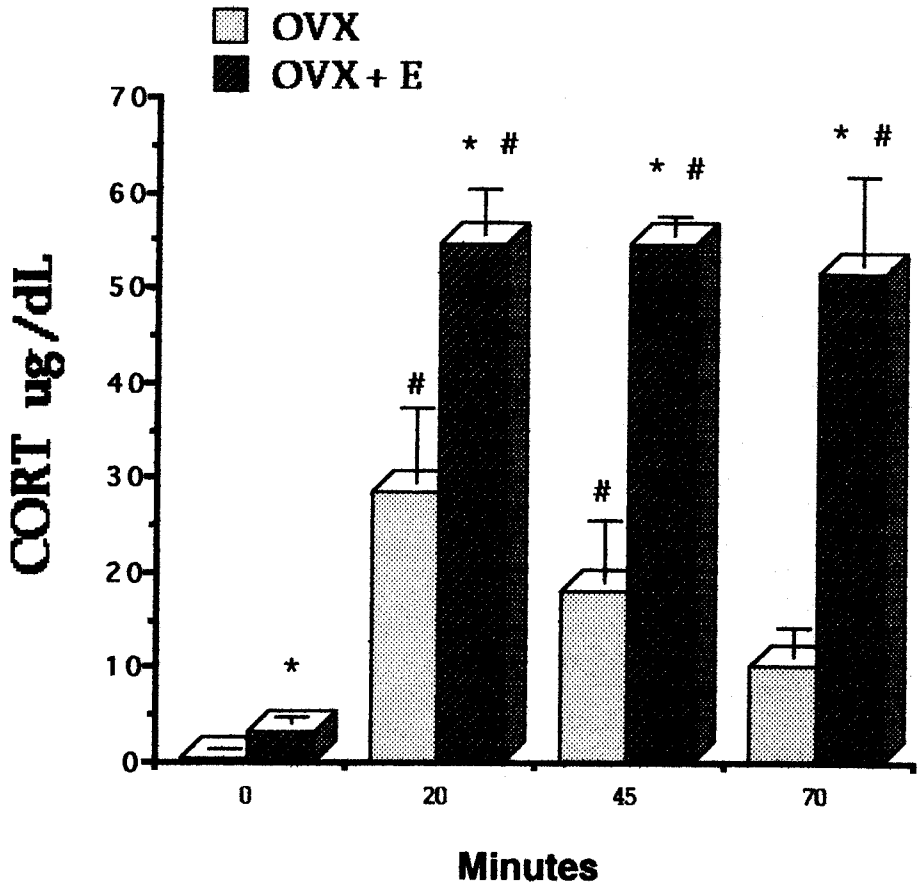


FIGURE 8. Plasma CORT levels 21 days after OVX or OVX and continuous treatment with E (OVX + E) are shown. Samples were obtained by decapitation at 0, 20, 45, and 70 min after 5 sec of footshock stress. Each *bar* indicates the mean $\pm$ SEM of five or six determinations. Two-way ANOVA indicated significant time, treatment, and time by treatment differences ($p < 0.005$). *, Significant increase ($p < 0.03$) from OVX value. #, Significant elevation ($p < 0.05$) over 0 min control values.

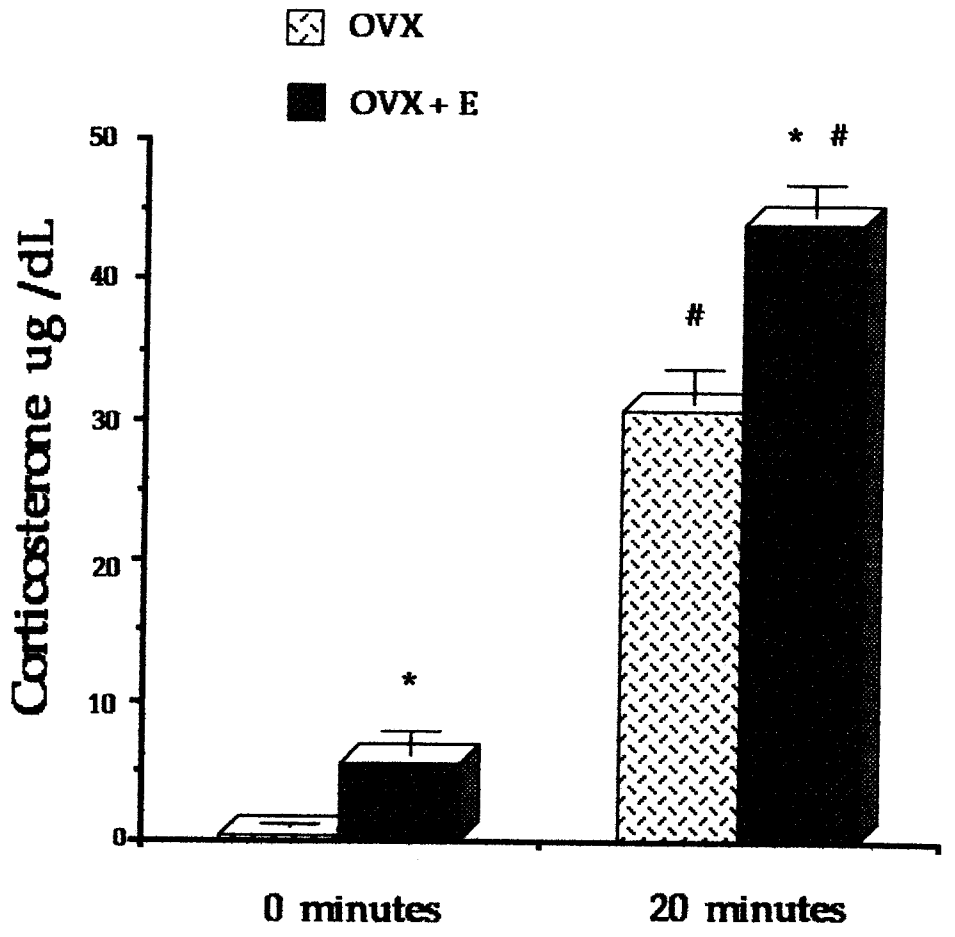


FIGURE 9. Plasma levels of CORT in female rats 21 days after OVX or OVX with an E capsule replacement (OVX + E). Samples were obtained at 0 and 20 min after 1-min exposure to ether vapors. Each *bar* indicates the mean $\pm$ SEM of five determinations. *, Significant increase ($p < 0.05$) from corresponding OVX value. #, Significant elevation ($p < 0.001$) over corresponding 0 min value.

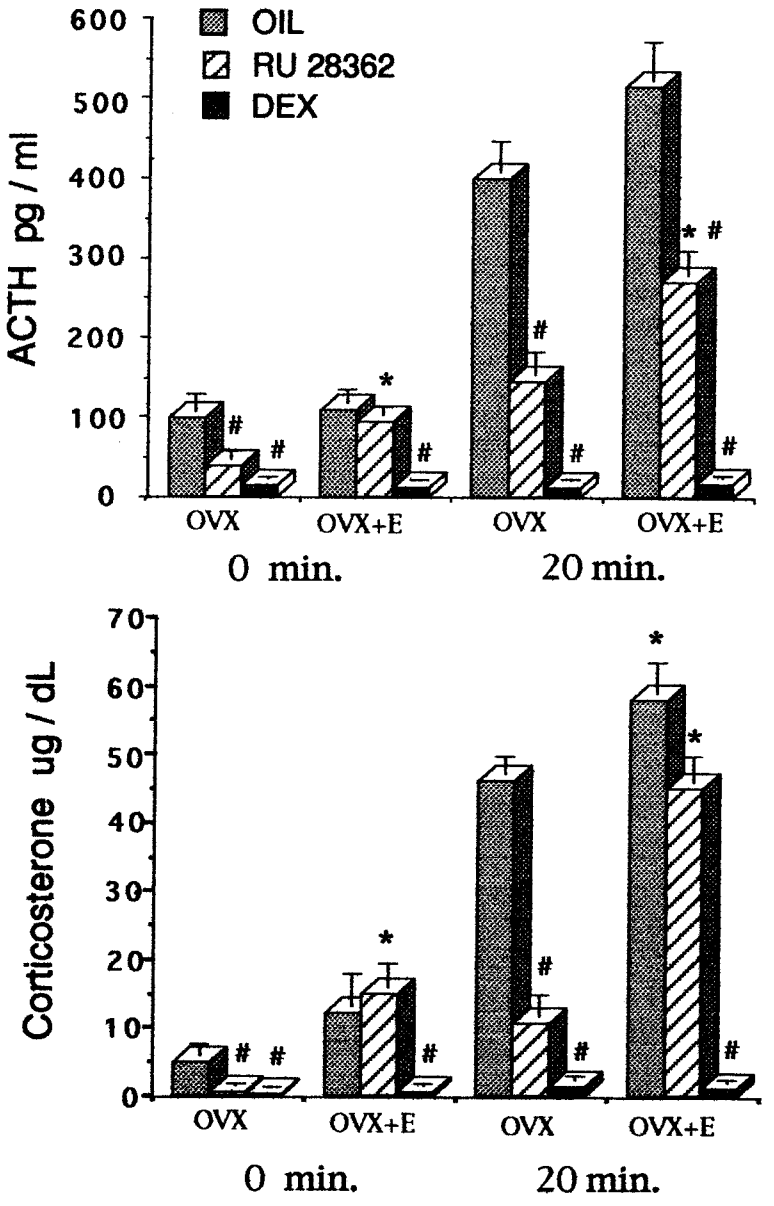


FIGURE 10. Basal and poststress plasma levels of ACTH and CORT after RU 28362 or DEX treatment. Animals were OVX for 17 days or OVX with E replacement (OVX + E). Animals were subsequently sc injected daily for 4 days with either RU 28362 or DEX (40 ug/100g BW in oil), or oil. Twenty-four hours after the last injection, plasma ACTH and CORT levels were measured 0 and 20 min after 1 min of ether stress. Each *bar* indicates the mean $\pm$ SEM of six determinations. Three-way ANOVA indicated significant treatment, injection, time, time by treatment, injection by treatment, and time by injection by treatment differences ($p < 0.03$). #, Significant decrease ($p < 0.01$) from oil control values. *, Significant difference ($p < 0.05$) from corresponding OVX timepoint.

Exp 4: Effect of E on MR and GR concentrations in the preoptic area (POA), medial basal hypothalamus (MBH), and hippocampus (HIPP).

E treatment had no effect on the concentrations of MR or GR in any of the brain regions examined (figure 11). Scatchard analysis of hippocampal saturation binding data revealed apparent dissociation constants (Kd) of 0.5nM and 0.6nM for MR and 1.3nM and 1.4nM for GR in OVX and OVX + E groups, respectively.

Exp 5: Effect of E on the downregulation of MR and GR.

In order to explore potential functional alterations in MR and GR, we examined the effect of E on the ability of ALDO, RU 28362, and DEX to downregulate MR and GR in the hippocampus.

Four days of either ALDO or DEX treatment significantly decreased the concentrations of MR and GR ($p < 0.05$) in the hippocampus in E treated and control groups (figure 12). Administration of the GR specific agonist, RU 28362, did not alter hippocampal MR levels in either group. RU 28362 treatment significantly decreased the concentration of hippocampal GR in the control group ($p < 0.05$), but not in the E treated animals. Scatchard analysis of saturation binding data for the hippocampus revealed no change in the KDs (0.6nM and 1.3nM, for MR and GR, respectively) in any of the treatment groups. Plasma samples, obtained at the time of sacrifice, from DEX and RU 28362 injected animals were assayed and found to contain no residual glucocorticoid receptor binding capacity.

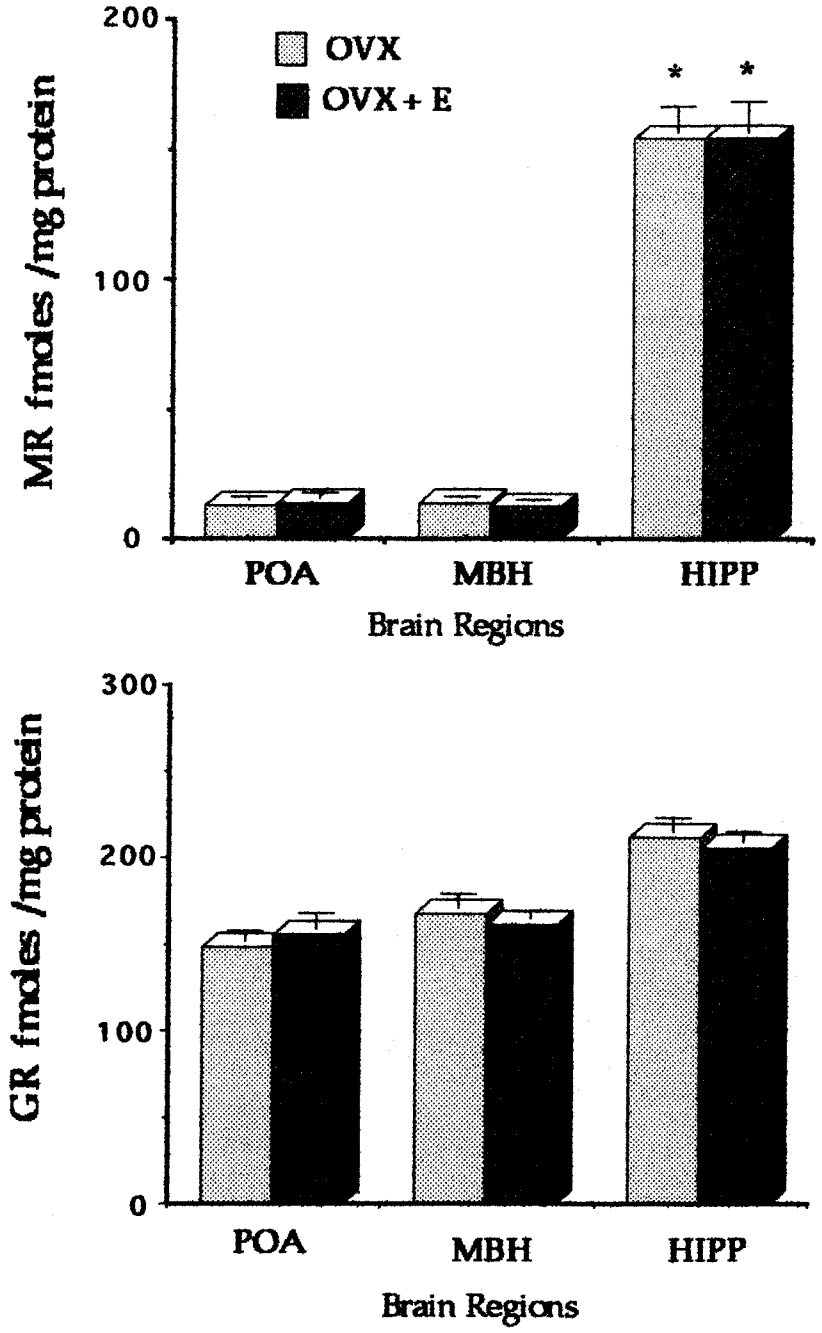


FIGURE 11. Concentrations of MR and GR in the pre-optic area (POA), medial basal hypothalamus (MBH), and hippocampus (HIPP) 21 days after OVX or OVX with E replacement (OVX + E). Each *bar* indicates the mean $\pm$ SEM of seven or eight determinations. *, Significant difference ($p < 0.001$) from other brain regions. No other significant differences in either receptor in any brain region examined were found.

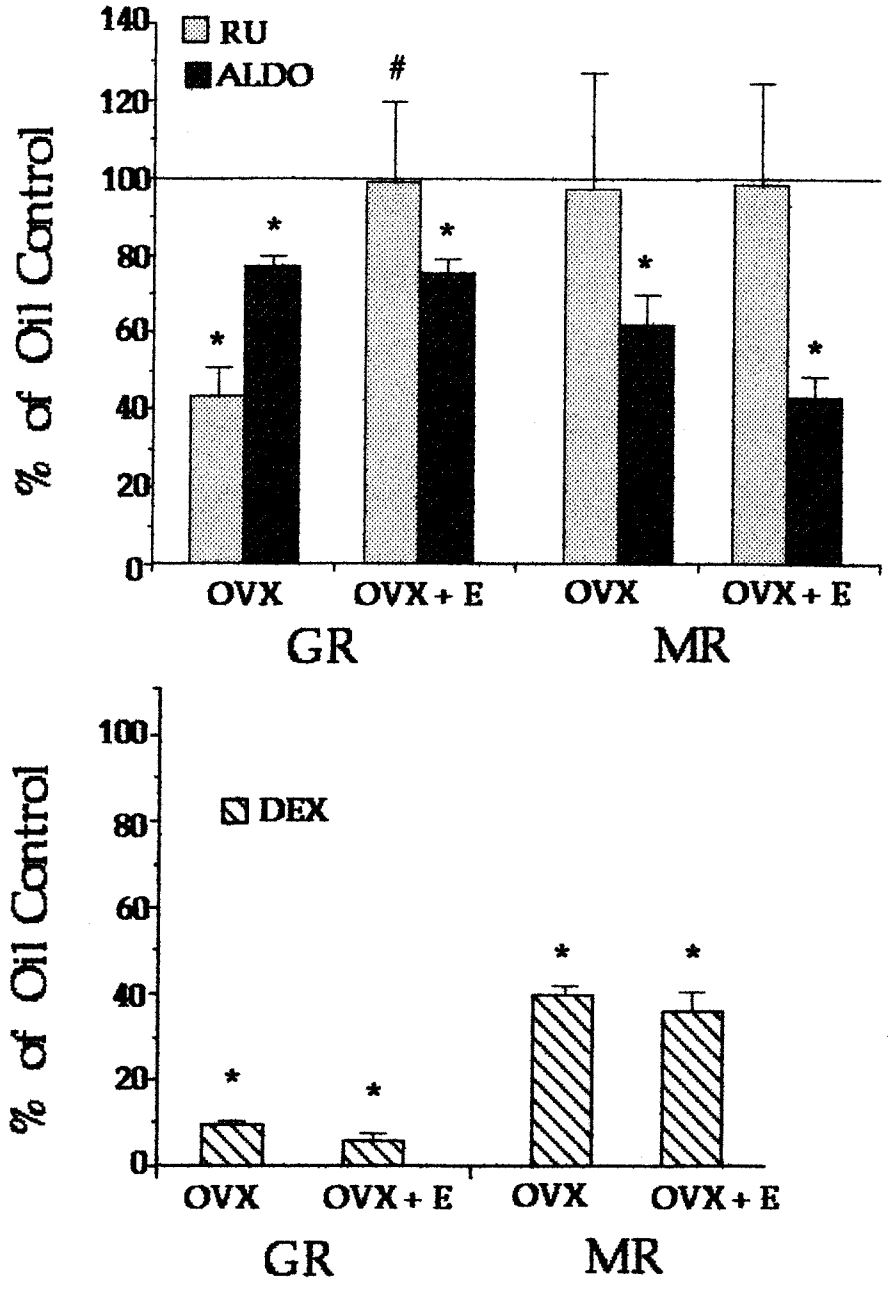


FIGURE 12. Concentrations of GR and MR in the hippocampus following ALDO or RU 28362 (A), or DEX (B) treatment. Animals were OVX for 17 days or OVX with E replacement (OVX + E). Animals were then adrenalectomized bilaterally and injected sc daily for 4 days with ALDO, RU 28362, or DEX (40 ug/100g BW in oil), or oil. Each *bar* indicates the mean $\pm$ SEM of six to ten determinations. *, Significant decrease ($p < 0.05$) from oil control. #, Significant difference ($p < 0.02$) from OVX value. OVX and OVX + E oil control values were 323 $\pm$ 25 and 348 $\pm$ 31 fmoles/mg protein, respectively for GR, and 210 $\pm$ 18 and 234 $\pm$ 21 fmoles/mg protein, respectively for MR.

Discussion

Previous studies have shown that female rats have higher basal plasma CORT levels as well as greater CORT responses to stress than males (Kitay, 1961; Crithlow, et al., 1963). These sex differences may be estrogen mediated, since OVX of prepuberal female rats results in reduced basal plasma CORT levels and E replacement reverses this change (Kitay, 1963). In addition, OVX'd rats with E replacement have significantly higher post-stress CORT levels than controls (Phillips and Poolsanguan, 1978; Viau and Meaney, 1991). Our initial experiments were designed to test for this effect of E in our model system, using a chronic OVX female with or without E replacement. Phillips and Poolsanguan (1978) have demonstrated that chronic OVX itself does not alter either basal or stress-induced CORT levels. Chronic OVX CORT levels were significantly reduced only from proestrus levels; they were quite similar CORT levels on all other days of the estrus cycle. Our data show that basal and post-stress CORT levels were higher in the presence of E, in agreement with these previous findings.

Since Kitay et al. (1965) have shown that E can increase adrenal production of CORT directly, it is possible that the effects of E are mediated at the level of the adrenal gland. Therefore, we subsequently measured plasma ACTH as well as CORT during the recovery period following stress. The elevated and prolonged ACTH response to stress in the presence of E points to an effect at the level of the pituitary gland or higher. The examination of the timecourse of recovery provides additional information to help in elucidating the mechanism by which E produces its effects.

These results clearly demonstrate an effect of E on ACTH secretion following two different types of physical stress. While an effect of E on the adrenal gland cannot be ruled out, our data demonstrate that there is a clear interaction of E at loci above the adrenal gland.

An impairment of CORT negative feedback could result in the pattern of hormonal secretion observed in the presence of E. Another possible explanation for the observed differences in ACTH and CORT secretion found in the presence of E is that E altered the clearance rate of the hormones. However, a recent study by Viau and Meaney (1991), demonstrating elevated stress levels of ACTH and CORT in acutely E treated animals compared to OVX controls, showed that clearance of ACTH and CORT was comparable in both groups.

In our hands, the basal levels of ACTH and CORT measured in cannulated animals were considerably higher than those found in animals sacrificed upon removal from their home cages. This apparent discrepancy can be explained by the studies of Fagin et al. (1983), who showed that even up to one week following cannulation, animals have elevated basal ACTH and CORT levels. However, our study demonstrating elevated post-stress CORT levels in E treated animals which were not cannulated, is consistent with our observations of elevated CORT and ACTH levels in E treated cannulated animals.

Importantly, not only the magnitude but the duration of the CORT response to stress was increased in E treated animals, resulting in an overall greater amount of circulating CORT during the 2 hours following stress. Higher basal levels of CORT

lead to greater occupancy of MR and GR, while higher stress levels lead to greater occupancy of GR (Reul, et al., 1987; Ratka, et al., 1989; Jacobson and Sapolsky, 1991). Correlations between CORT receptor occupancy and a variety of physiological endpoints such as stress-induced CRH and vasopressin release (Sapolsky, et al., 1990), circadian peak ACTH secretion (Bradbury, et al., 1991), and neuronal death (Sapolsky, et al., 1985) have been demonstrated. Thus the overall amount of CORT "seen" by the CORT receptors appears to be a very important factor when considering the physiological consequences of CORT secretion, in contrast to examining the relative change from baseline.

The pattern of the ACTH response to stress we obtained is very similar to the pattern of the CORT response to stress obtained by Ratka et al.(1989), who administered a GR antagonist prior to stress and subsequently examined CORT secretion. The anti-glucocorticoid delayed the shut-off of the stress response, presumably by antagonizing CORT negative feedback. The similarity of the CORT secretory patterns, in those studies and the present study, strongly suggests that in the presence of E there is an impairment of the GR mediated negative feedback.

We therefore assessed the responsiveness of the HPA axis following treatment with the synthetic glucocorticoids DEX or RU 28362. DEX and RU 28362 were administered to suppress the response of the HPA axis to stress. Since neither of these synthetic glucocorticoids are bound by CBGs, and DEX binds to both MR and GR, while the specificity of RU 28362 is for GR, this study also provides an insight into the receptor specificity of E's actions. Our results demonstrate that E interferes

with the ability of RU 28362, but not DEX, to suppress the ACTH and CORT response to stress. This implies that E is interfering with GR mediated slow negative feedback (Keller-Wood and Dallman, 1984), and demonstrates that MR also plays a role in negative feedback. DEX binding to MR may directly effect slow negative feedback, or alternatively, DEX binding to MR may prevent E's effects from being manifested on GR. Support for a role for MR in mediating negative feedback has been provided by Ratka et al. (1989), who showed that the pattern of the CORT response to stress was elevated and prolonged in the presence of an MR antagonist in a manner similar to that obtained following the administration of a GR antagonist. In addition, Dallman et al. (1989) showed that the IC_{50} values for inhibition of morning ACTH secretion by CORT and DEX are quite close to the reported Kd values for MR, implicating MR in the regulation of basal ACTH levels. A more recent report by Bradbury et al. (1991), using the MR antagonist spironolactone, has implicated both MR and GR involvement in the inhibition of circadian peak ACTH secretion. These studies are consistent with our data showing an impaired delayed negative feedback action of glucocorticoids following E treatment, perhaps as a consequence of interference with GR mediated signal transduction.

The differential effectiveness of RU 28362 and DEX in inhibiting CORT secretion, could also be explained by a differential availability of RU 28362 and DEX to the regions involved in negative feedback. However, autoradiographic studies using ^{3}H-RU 28362 and ^{3}H-DEX have shown that both effectively label GR in the hippocampus, HPOA, and other brain regions (Rhees, et al., 1975; Warenbourg,

1975; Sarrieau, et al., 1988). Both ^{3}H-ligands show uptake patterns similar to those obtained by immunohistochemistry using GR antibodies (Fuxe, et al., 1985) or in situ hybridization using GR specific probes (Aronsson, et al., 1988). Consequently, we conclude that both ligands have equal access to the brain regions involved in mediating negative feedback. Whether DEX acts more effectively than RU 28362 at the level of the anterior pituitary gland to inhibit stress induced CORT secretion, remains to be determined. The ability of DEX to bind to both MR and GR at saturating levels, while RU 28362 saturates only GR, strongly points to a selective effect of E on GR action.

The anterior pituitary gland, hypothalamus, and hippocampus are all involved in CORT negative feedback of the HPA axis (Wilson, et al., 1980; Kellerwood and Dallman, 1984; Plotsky and Vale, 1984). These three regions also have been shown to contain high levels of GR, and detectable to high levels of MR (Reul and De Kloet, 1985; 1986; Brinton and McEwen, 1988). The anterior pituitary gland contains transcortin, an intracellular corticosterone binding globulin which has a high affinity for CORT but a very low affinity for aldosterone or DEX (McEwen, et al., 1986). Its presence in the anterior pituitary gland complicates the distinction of MR from GR based on cytosolic binding assays. We have therefore limited our initial studies on E effects to brain regions which do not possess transcortin.

Negative feedback mechanisms involve CORT binding to MR and GR. Studies in the aged male rat (Sapolsky, et al., 1984; Sapolsky, 1986) point to decreases in receptor number as the mechanism responsible for the decreased negative

feedback, and the increased hormonal response to stress. We therefore examined MR and GR, to explore the possibility that E could be modulating CORT sensitivity by altering receptor concentrations.

Chronic E replacement (21 D) had no effect on MR or GR levels in any tissue examined, but does not rule out any acute changes in receptor levels that may have normalized over time. These data are in agreement with a recent study which reported no change in hippocampal levels of MR or GR after 15 days of E treatment (Ferrini, et al., 1990). In contrast, an earlier study had suggested that OVX resulted in an increase in hypothalamic CORT receptor levels, but receptor subtypes were not discriminated (Turner and Weaver, 1985). Our findings suggest that the steady state levels of CORT receptor proteins are unaffected by chronic E treatment. Alternatively, E treatment may induce a posttranslational change in GR, such as a shorter half-life, as has been shown following DEX treatment (Dong, et al., 1988). This could mask changes in CORT receptor levels, since GR levels have been shown to rise 24 hrs after ADX (Reul, et al., 1987). In either case, the functional efficacy of the CORT receptors is not addressed by binding studies. Rather, the in vitro binding assay employed to measure the levels of MR and GR relies only on the receptor's ability to bind ligand. While the levels of MR and GR appear unchanged, the capability of these receptors to regulate transcription remains undetermined.

Previous studies have shown that CORT receptors are autoregulated with receptor stimulation decreasing receptor number, and ADX increasing it (Sapolsky and McEwen, 1985; Reul, et al., 1987; Brinton and McEwen, 1988; Luttge, et al.,

1989b). The downregulation of these receptors was used in these studies to assess changes in CORT receptor function.

Prior studies have found downregulation of only GR by DEX in the rat (Reul, et al., 1987; Brinton and McEwen, 1988). In the mouse however, downregulation of both MR and GR by DEX treatment has been reported (Luttge, et al., 1989b). It is of note, therefore, that we found DEX to downregulate both GR and MR. Whether this is due to the use of female rats or differences in the binding assays remains to be determined.

Our findings, that E treatment interfered only with the ability of RU 28362 to downregulate GR while having no apparent effect on the ability of ALDO or DEX to downregulate GR, have two distinct implications. First, it is evident that E affects predominately GR mediated functions such as the suppression of the CORT response to stress and downregulation of GR. While RU 28362 accomplishes both these functions in the OVX rat, it is significantly less effective in the presence of E. In addition, DEX is slightly more effective than RU 28362 in both functional assays. This may be due to DEX's binding to both MR and GR. The binding of DEX to MR could account for its increased efficacy compared to RU 28362, as both have nearly identical reported Kd's for GR (Reul and De Kloet, 1985). The lack of an effect of E on ALDO-induced GR downregulation suggests that DEX bound MR would also be unaffected by E.

Our results suggest either: 1) the effects of DEX are being partially mediated through MR and that there may be interactions between DEX/MR and DEX/GR

mediated signals; 2) the binding of DEX to MR may somehow prevent the action of E on GR; or 3) the effect of E on GR is limited to the functioning of RU 28362 bound GR, while DEX bound GR can function unimpaired by E. Negative feedback and receptor downregulation have both been thought to be GR mediated (Reul and De Kloet, 1985; Reul, et al., 1987). However, more recent studies suggest a role for MR (Dallman, et al., 1989; Ratka, et al., 1989; Bradbury, et al., 1991), as well as the possibility of regulatory interactions between the receptor types (Brinton and McEwen, 1988; Luttge, et al., 1989a).

In summary, our findings have shown that E treatment elevates and prolongs the activation of the HPA axis following two different physical stressors. Although there were no observable changes in MR or GR concentrations in E treated rats, treatment with RU 28362, a GR specific agonist, failed to suppress the CORT response to stress and autoregulate GR in the presence of E. E treatment interfered with two functions mediated by GR: receptor downregulation and hormone suppression. These findings argue strongly for an effect of E on GR action and perhaps slow negative feedback as the mechanism by which E alters HPA function.

CHAPTER IV

Estrogen-induced Alterations in the Regulation of Mineralocorticoid and Glucocorticoid Receptor Messenger RNA Expression in the Female Rat Anterior Pituitary Gland and Brain

Summary

The influence of estrogen (E) on corticosterone (CORT) receptor mRNAs receptor function in neural tissue was investigated in female Sprague-Dawley rats. Animals were bilaterally ovariectomized (OVX), and a Silastic capsule (0.5 cm) containing 17*B*-estradiol was sc implanted. Control animals received a blank capsule. Animals were killed 1, 7, or 21 days later. In the anterior pituitary (AP), glucocorticoid receptor (GR) mRNA levels were significantly lower ($p<0.01$) in E-treated rats at all time points examined. Hippocampal GR mRNA levels were significantly decreased below OVX values ($p<0.01$) after 1 and 21 days of E treatment. In the hypothalamic-preoptic area (HPOA), GR mRNA levels were significantly lower ($p<0.01$) than OVX values only after 21 days of E treatment. Mineralocorticoid receptor mRNA levels were significantly lower after E treatment ($p<0.01$) at all time points and in all three tissues examined.

To determine if these effects of E on GR mRNA were a result of changes in GR autoregulation, we administered RU 28362 (40 ug/100 g BW for 4 days) or dexamethasone (DEX) (40 ug/100g BW for 4 days) to OVX control and OVX + E-

treated rats. DEX significantly down-regulated hippocampal GR mRNA ($p<0.05$) in both control and E-treated animals. In contrast, RU 28362 significantly down-regulated hippocampal GR mRNA ($p<0.05$) in control rats only. Similarly, in the HPOA, the administration of either DEX or RU 28362 reduced GR mRNA levels ($p<0.05$) in OVX control animals, but not in E-treated animals. Thus, E treatment results in a loss of the glucocorticoid receptor's ability to down-regulate its mRNA.

These findings demonstrate profoundly altered regulation of CORT receptor mRNAs in the presence of E, and provide further evidence that E may interfere with glucocorticoid receptor function.

Introduction

The estrogen (E) status of the female rat affects its endocrine response to stress. Previous studies have demonstrated that basal (Raps, et al., 1971; Phillips and Poolsanguan, 1978; Buckingham, et al., 1978; Viau and Meaney, 1991) and stress responsive (Raps, et al., 1971; Poolard, et al., 1975; Phillips and Poolsanguan, 1978; Viau and Meaney, 1991) corticosterone (CORT) levels are highest on proestrus when E levels are elevated. This is consistent with studies showing that ovariectomy (OVX) reduces basal and stress responsive CORT levels and that E treatment increases them (Kitay, 1963; Ramaley, 1976; Phillips and Poolsanguan, 1978; Viau and Meaney, 1991). Consistent with these studies, we have recently demonstrated an elevated and prolonged ACTH and CORT response to footshock stress following E treatment of ovariectomized rats (Burgess and Handa, 1992).

In this latter study, the pattern of the ACTH and CORT responses to stress in

the presence of E suggests an impairment of CORT negative feedback. The negative feedback regulation of the HPA axis is mediated predominately through the binding of CORT to intracellular receptors found in the hippocampus, hypothalamus, and AP (Kellerwood and Dallman, 1984; McEwen, et al., 1986; Jacobson and Salpolsky, 1991). Previous studies have classified receptors for CORT into Type I and Type II receptors (Funder, et al., 1973; Reul and De Kloet, 1985). These receptors have been shown to be the products of the mineralocorticoid receptor (MR) gene and the glucocorticoid receptor (GR) gene, respectively (Arriza, et al., 1987; Miesfeld, et al., 1986). Both receptor types have been implicated in negative feedback regulation by CORT (Dallman, et al., 1989; Ratka, et al., 1989), and both are found in varying amounts at the three major neural sites involved in feedback regulation. For clarity, in this paper we will employ the designations of MR and GR for the Type I and the Type II receptors, respectively.

Previously, we assessed the responsiveness of the HPA axis following GR specific suppression with RU 28362, or with the receptor nonspecific glucocorticoid, dexamethasone (DEX) (Burgess and Handa, 1992). Our results demonstrated that in the presence of E, the ability of RU 28362, but not DEX, to suppress the ACTH and CORT response to stress is impaired. Since RU 28362 binds specifically to GR (Sarrieau, et al., 1988) but DEX binds to both GR and MR (Krozowski and Funder, 1983; Allen, et al., 1988; Brinton and McEwen, 1988; Luttge, et al., 1989b), these data indirectly suggest that E interferes with GR mediated negative feedback.

In addition, we have found that E treatment interferes with the ability of

RU28362, but not aldosterone or DEX, to down-regulate GR. These data are consistent with the hypothesis that E impairs CORT negative feedback by altering GR function, but not the absolute concentrations of GR.

In the present study, we have chosen to further investigate the influence of E on CORT receptor function by exploring the effect of E on the regulation of CORT receptor mRNAs. The examination of receptor mRNA levels has the advantage over in vitro binding assays in that it requires no surgical manipulations such as adrenalectomy, which are necessary for binding assay analysis. In addition, the determination of receptor mRNA levels can provide insight into the cellular level at which regulation may be occurring. To this end, we have developed a sensitive solution-hybridization RNase protection assay to allow absolute quantitation of both MR and GR mRNAs in neural tissues.

Materials and Methods

Animals

Adult female Sprague-Dawley rats (250-300g; Sasco, Omaha, NE) were housed in environmentally controlled quarters with a 12-h light, 12-h dark cycle (lights on at 0700 h), and food and water available ad libitum.

Animals were bilaterally ovariectomized under ether anesthesia, followed either by subcutaneous (sc) implantation of a 0.5-cm Silastic capsule (id, 0.078 in.; od, 0.125 in.; Dow-Corning, Midland, MI) filled with crystalline 17 *B*-estradiol (E) (Sigma Chemical Co., St.Louis, MO) or a sham-operation (control). Animals were

sacrificed by decapitation 21 days after surgery and the commencement of hormone treatment.

RNA Isolation

Immediately following sacrifice by decapitation, pituitary glands and brains were removed and placed on ice. The anterior pituitary gland (AP), preoptic area (POA), medial basal hypothalamus (MBH), and entire hippocampal formation (HIPP) were dissected out and homogenized separately in 4M guanidinium isothiocyanate (Boehringer Mannheim, Indianapolis, IN) buffer containing 50mM sodium citrate (pH 7.0), 0.5% sarkosyl, and 0.1M *B*-mercaptoethanol. Total RNA was isolated, as previously described by Chirgwin, et al. (1979), by centrifugation through a 5.7 M CsCl cushion for 14-16 h at 147,000 x g at 15^0 C. The resulting pellets were resuspended in DEPC-treated H_2O, phenol/chloroform/isoamyl alcohol (24:24:1) extracted, and the aqueous phase stored under ethanol at -20^0C. Prior to assaying, samples were precipitated by the addition of NH_4OAc. RNA pellets were washed with 70% ethanol and then resuspended in DEPC-treated H_2O. The concentration and purity of the samples were analyzed by UV spectrophotometry at 260/280 nm.

Northern blot hybridization analysis

Total RNA (10-20 ug) was loaded onto 0.7% agarose formaldehyde gels and electrophoresed at 28 V for 18-20 hrs. RNA was then transferred onto nylon membranes (Nytran, Schleicher and Schull, Keene, NH) by capillary action. After transfer, the filters were dried for 2 hr under vacuum at 80^0C. Filters were

prehybridized at 60^0 C overnight in 50% formamide, 1M sodium phosphate, 1% SDS, 2X Denhart's, 250 ug/ml herring sperm DNA, and 200 ug/ml yeast RNA. Hybridization was performed in the same solution overnight at 60^0 C, with 5 x 10^6 cpm of in vitro transcribed ^{32}P-labeled cRNA probe added per ml of hybridization solution. After hybridization, filters were washed with 2X SSC/0.5% SDS for 5 minutes at RT, followed by two 30 minute washes with 0.5X SSC/0.5% SDS at 60^0 C. Filters were exposed to Hyperfilm (Amersham, Lake Forest,IL) with intensifying screens at -70^0 C for 12-15 days.

RNA transcription

A rat GR cDNA construct (Miesfeld, et al., 1986) was kindly provided by Dr. K. Yamamtoto, U.C. San Francisco. A 1072 bp AVA I/AVA I fragment, corresponding to the ligand binding domain and beginning of the 3' untranslated region (nts 1744-2815), was subcloned into a pGEM 3Z plasmid vector (Promega, Madison, WI). A 513 bp rat MR cDNA pGEM 4Z construct, corresponding to the ligand binding domain and beginning of the 3' untranslated region (nts 2809-3321), was kindly provided by Dr. R. Evans, The Salk Inst.(Arriza, et al., 1987).

To generate antisense RNA probes (262 bp for GR and 196 bp for MR) the vectors were first linearized with the restriction enzyme Dra I for GR, or Stu I for MR. The in vitro transcription protocol followed was a modification of that provided by the enzyme manufacturer (Promega, Madison, WI). The transcription reactions contained 40 mM Tris-HCl, pH 7.5; 6 mM $MgCl_2$; 2 mM spermidine; 10 mM NaCl; 10 mM dithiothreitol; 0.5 mM each of ATP, GTP, and UTP; 0.020 mM non-

radioactive CTP; 0.007 mM [alpha-^{32}P] CTP (800 Ci/mmol; Amersham, Arlington Heights,IL); 0.5 ug of linearized vector; 2 U/ul RNAsin; and 1-2 U/ul of the DNA dependent RNA polymerases, T7 or SP6, for GR or MR, respectively. After incubation for 1 hr at 37^{0}C, 10 U RNase-free DNase I was added and the incubation continued for 20 min to allow digestion of the template DNA. The reaction product was then phenol/chloroform/isoamyl alcohol (24:24:1) extracted, the aqueous phase subjected to spun column chromatography through Sephadex G-50 to remove unincorporated radioactivity, and then ethanol precipitated. These conditions resulted in radiolabeled antisense RNA probes with a specific activity of $>10^9$ cpm/ug. Sense strand RNAs were transcribed from the same constructs, by using the polymerases SP6 for GR or T7 for MR, following linearization with Pst I for GR or Eco R1 for MR. The concentration of non-radioactive CTP was 0.5 mM, with 0.5 uM of ^{32}P-CTP present. Aliquots of the transcribed RNAs were analyzed on 5% acrylamide, 7.5M urea gels to confirm their integrity. Only ^{32}P-labeled cRNA transcripts that were > 90% full length were used in subsequent assays. Synthesized sense strand RNA was >99% full length, and the yield was calculated by checking the 260 nm absorbance by UV spectroscopy. Dilutions of the sense strand RNA were used to generate standard curves.

RNase protection assay

Dilutions of in vitro transcribed sense strand RNA and 5-10 ug of sample RNA were hybridized in solution to a molar excess (100,000 cpm) of ^{32}-P labeled antisense RNA. Hybridization was performed for 16-20 h at 45^0 C in a 30 ul final

volume (80% formamide, 40mM PIPES, 1mM EDTA, 400mM NaCl; pH 6.7). Nonhybridized antisense RNA probe was digested with RNases A and T1 at final concentrations of 40 ug/ml and 2 ug/ml, respectively, in 10mM Tris, 300mM NaCl, 5mM EDTA; pH 7.5 for 1 h at 30^0 C. The RNA:RNA hybrids were purified by SDS/Proteinase K digestion (0.6%/290 ug/ml, respectively) for 15 min. at 37^0 C, followed by phenol/chloroform/isoamyl alcohol (24:24:1) extraction, and ethanol precipitation. RNA:RNA hybrids were resuspended in 80% formamide, 10mM TRIS, 10mM EDTA; pH 7.0, heat denatured at 90^0 C for 10 min., and then electrophoresed through 5% acrylamide, 7.5M urea gels at 300 V. Gels were fixed in 7% acetic acid for 10 min. to remove urea and then dried. Radioactivity in the dried gels was counted directly by a Betascope 6000 analyzer (Betagen, Waltham, MA). Values are expressed as femtomoles (fmol) protected probe/mg input RNA. Autoradiograms were obtained by exposure of the dried gels to Hyperfilm (Amersham, Lake Forest,IL) at -70^0 C for 1-7 days.

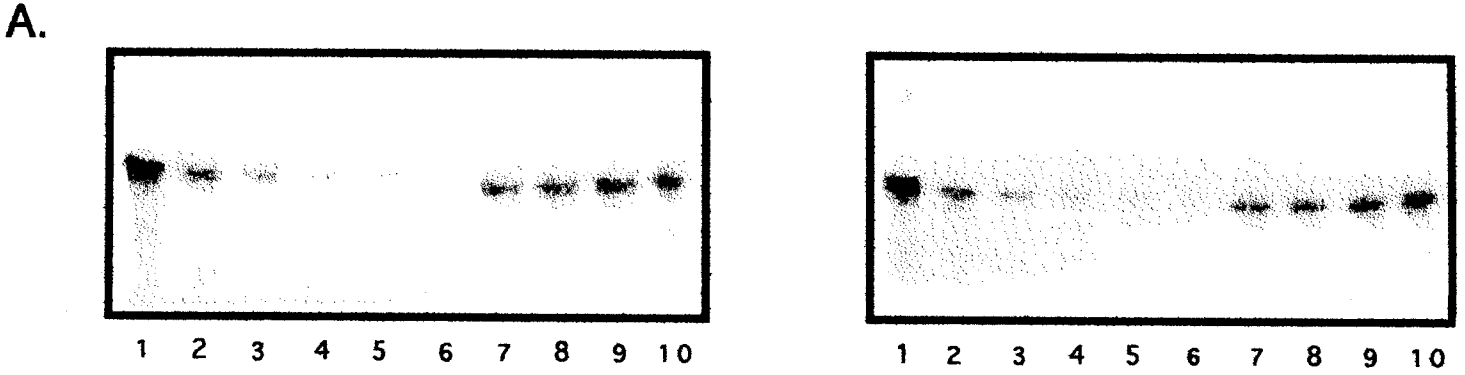
The amount of sense strand RNA used for the standard curves ranged from 1.3 to 135 attomoles (amol) and the curves generated were linear, with correlation coefficients consistently greater than 0.999. Validation of the assay is shown in figure 13A. Decreasing amounts of sense strand RNA (50, 25, 12.5, 5, 2.5 attomoles) protect decreasing amounts of radiolabeled probe (lanes 1-5). The absence of protected radiolabeled probe by t-RNA was used as a negative control (lane 6), and typical bands following protection by hippocampal RNA samples is seen in lanes 7-10. A typical standard curve (CPM in the protected band versus attomoles of sense

FIGURE 13. **A.** Decreasing amounts of in vitro transcribed MR or GR sense strand RNA and hippocampal RNA were hybridized in solution to 100,00 cpm of a ^{32}-P labeled MR or GR cRNA probe, respectively. The resulting hybrids were protected from RNase digestion and are shown visualized after acrylamide/urea gel electrophoresis and image analysis on a Betascope. Decreasing amounts of sense strand RNA (50, 25, 12.5, 5, 2.5 attomoles) protect decreasing amounts of radiolabeled probe (lanes 1-5). The absence of protected radiolabeled probe by t-RNA was used as a negative control (lane 6), and typical bands following protection by hippocampal RNA samples are seen in lanes 7-10.

B. Standard curves obtained when counts per min in the protected band are plotted against attomoles of sense RNA added.

C. Line drawings of the cDNAs for MR and GR. Boxed regions correspond to sequences translated into MR and GR proteins, respectively. The regions contained in the linearized vectors, $prMR_{EH}$ for MR and RBal 117 for GR, are shown below. The locations of the sequences transcribed in vitro into cRNA probes, 196 bp for MR and 262 bp for GR, are shown below the vectors.

MR & GR RNase Protection Assay Validation



RNA added) is shown in figure 13B.

Statistical analysis

All data were analyzed by a two-way or three-way analysis of variance followed by the Student-Newman-Keuls multiple comparisons test (Zar, 1984).

Experimental protocols

Exp 1: A timecourse of the effect of E on the levels of GR and MR mRNA

This study examined the steady state levels of GR and MR mRNA at various times (1, 7, 21 days) following E treatment. Female rats were bilaterally ovariectomized to reduce circulating E levels. Half of the animals then received a 0.5-cm Silastic capsule filled with 17 *B*-estradiol, while control rats were OVX and sham treated. Our previous studies have shown that this E treatment paradigm results in plasma E values of approximately 70-80 pg/ml (Handa and Rodriguez, 1991). Animals from each group were sacrificed 1, 7, and 21 days after surgery by decapitation. Aliquots of total RNA from the AP, HPOA, and HIPP were analyzed qualitatively by northern blot analysis to confirm the specificity of the cRNA probes. MR and GR mRNA levels in these tissues were quantitated using RNase protection assays. An additional group of OVX animals received an E capsule 20 days after surgery, and were subsequently sacrificed one day later. Control rats for this study were sham implanted 20 days after OVX.

Exp 2: Effect of E on the down-regulation of GR mRNA in the AP, HPOA, and HIPP

In this experiment we examined the effect of E on autologous GR mRNA regulation. Animals were OVX and E treated as described. For Exp 2., OVX and OVX + E treated rats were injected with either RU 28362 (40 ug/kg BW), DEX (40 ug/kg BW), or oil (0.2ml) daily for 4 days. Animals were sacrificed 24 hrs following the last injection, anterior pituitaries and brains removed, and rapidly dissected on ice. Total RNA was isolated from these tissues and levels of GR mRNA were determined by using the RNase protection assay.

Results

Northern blot analysis

Northern blot hybridization analysis of total RNA, using an MR specific probe, showed a single hybridizable band of approximately 6.0 Kb in all tissues examined (figure 14). This band was similar for both OVX and OVX + E groups. When a GR specific probe was used, a band of approximately 6.8 - 7 Kb was seen in all tissues examined (figure 14).

Exp 1: A timecourse of the effect of E on the levels of GR and MR mRNA

In the AP, GR mRNA levels were significantly lower ($p<0.01$) after E treatment, as compared to OVX values, at all time points examined (figure 15). In the hippocampus, GR mRNA levels were significantly lower after 1 and 21 days of E treatment, compared to the OVX values (figure 15). In addition, both of the

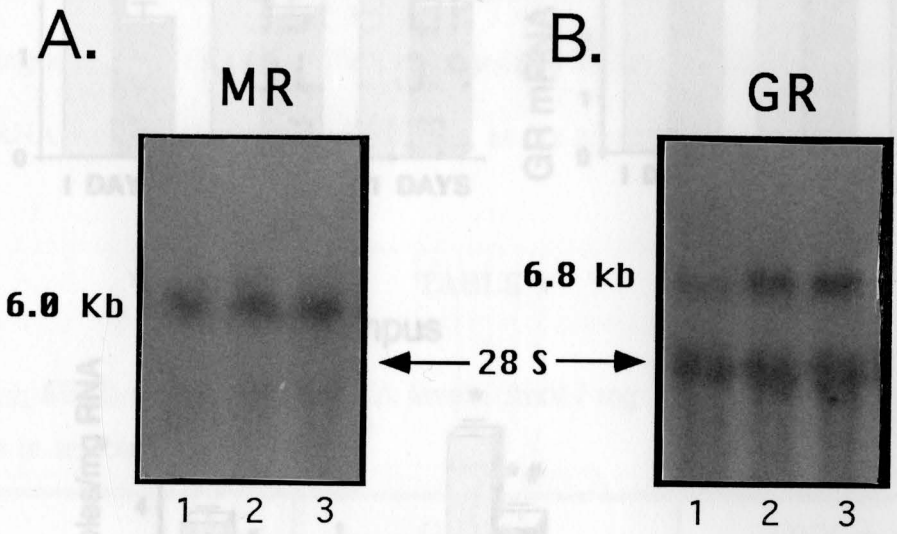


FIGURE 14. Northern blot analysis of MR and GR mRNAs in various tissues of female Sprague-Dawley rats. Aliquots of total RNA (20ug) isolated from the hippocampus (lanes 1-5) and the hypothalamic-preoptic area (lane 6), of OVX (lanes 2,3,5,6) and OVX + E (lanes 1,4) adult female rats were hybridized with cRNA probes transcribed in vitro from either $prMR_{EH}$ for MR or RBAL 117 for GR.

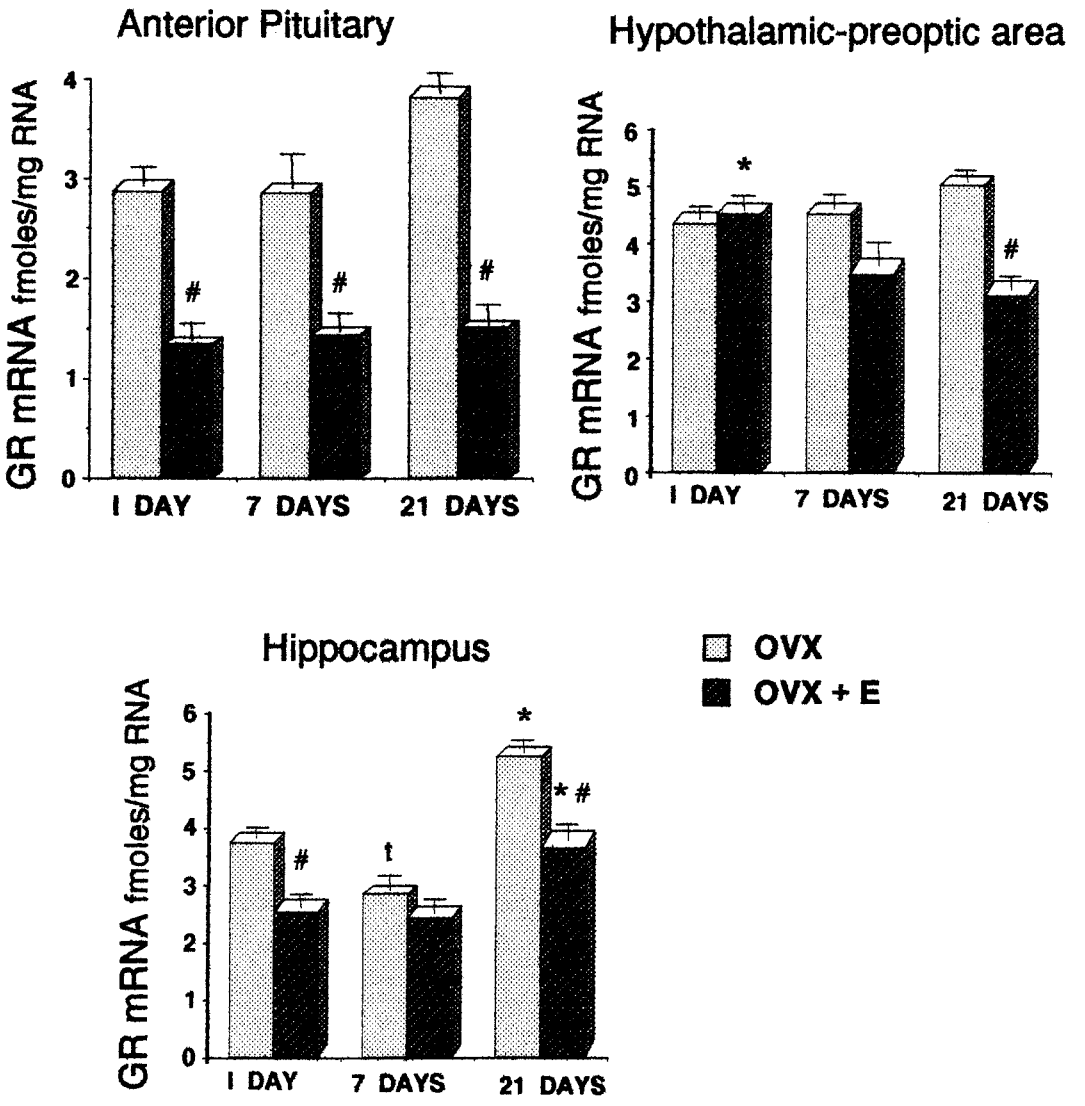


FIGURE 15. Concentrations of GR mRNA in the anterior pituitary gland (AP), hypothalamic-preoptic area (HPOA), and hippocampus (HIPP); 1, 7, or 21 days after OVX or OVX with E replacement (OVX + E). Each *bar* indicates the mean $\pm$ SEM of five to thirteen animals. Two-way ANOVA indicated significant treatment differences ($p < 0.002$) in all three tissues, significant time differences ($p < 0.005$) in the HPOA and HIPP, and a significant treatment by time interaction ($p < 0.005$) in the HPOA. #, Significant decrease ($p < 0.01$) from oil control values. *, Significant difference ($p < 0.05$) from other two time point values. t, Significant decrease from 1 day value.

hippocampal 21 day treatment groups (OVX and E treated) had significantly higher levels of GR mRNA ($p<0.05$) as compared to the other two timepoints. In the HPOA, levels of GR mRNA were significantly decreased below OVX values ($p<0.01$) only following 21 days of E treatment (figure 15). HPOA GR mRNA levels following just one day of E treatment were significantly higher than levels after 7 and 21 days of E treatment ($p<0.05$) (figure 15). One day of E treatment following long term (20 days) OVX also resulted in significantly decreased levels of GR mRNA from OVX controls ($p<0.01$), in the hippocampus (Table V).

TABLE V

Mean ($\pm$ SEM) MR and GR mRNA levels (fmol / mg RNA) in OVX and OVX + E animals in selected brain regions.

	OVX	OVX + E
Hippocampal GR	5.33 $\pm$ 0.19 (9)	3.94 $\pm$ 0.10[a] (7)
HPOA GR	5.03 $\pm$ 0.18 (11)	4.87 $\pm$ 0.14 (7)
Hippocampal MR	3.60 $\pm$ 0.20 (10)	2.47 $\pm$ 0.24[a] (7)
HPOA MR	0.63 $\pm$ 0.02 (11)	0.46 $\pm$ 0.01[a] (7)

OVX, 21 days after ovariectomy; OVX + E, 1 day of E, 20 days after ovariectomy; HPOA, hypothalamic-preoptic area. Number of rats per group are in parentheses.

[a] Value that is significantly ($P<0.05$) decreased from OVX value.

MR mRNA levels were significantly decreased after E treatment ($p<0.01$), as

compared to OVX values, at all time points and in all three tissues examined (anterior pituitary gland, HPOA, and hippocampus) (figure 16). In addition, MR mRNA levels in the hippocampus were significantly higher in the OVX group at 21 days ($p<0.05$) versus the other two timepoints (figure 16). One day of E treatment following long term (20 days) OVX also resulted in significantly decreased levels of MR mRNA from OVX controls ($p<0.01$), in the HIPP and HPOA (Table V).

Exp 2: Effect of E on the down-regulation of GR mRNA in the AP, HPOA, and HIPP

Analysis of GR mRNA levels by two way ANOVA revealed a significant treatment by injection interaction ($p<0.04$) in the hippocampus and HPOA. DEX administration significantly decreased levels of GR mRNA ($p<0.05$) in the hippocampus in both the E treated and control groups (figure 17). Administration of the GR specific agonist, RU 28362, significantly decreased the levels of hippocampal GR mRNA in the OVX control group ($p<0.05$), but not in the E treated animals. In the E treated group only DEX was able to down-regulate GR mRNA.

Treatment with DEX or RU 28362 significantly reduced GR mRNA levels in the HPOA ($p<0.05$) in OVX control animals, but not in the E treated animals (figure 17). E treatment significantly decreased HPOA levels of GR mRNA below OVX controls ($p<0.004$) in the oil treated group. No further decrease was found with either DEX or RU28362 treatment.

In the anterior pituitary gland, DEX treatment significantly elevated GR mRNA levels ($p<0.05$) in both E treated and control animals (figure 17). Levels of

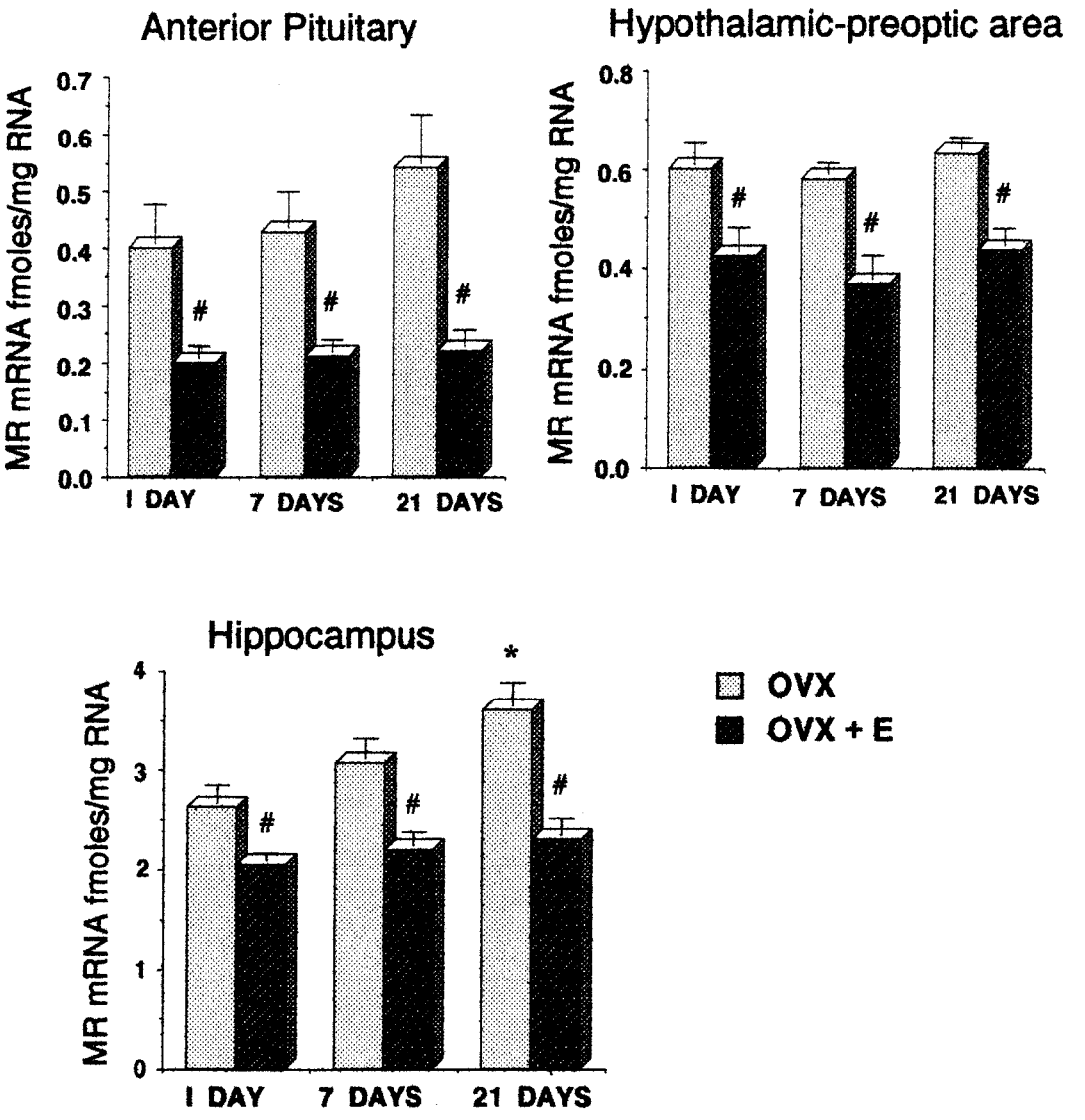


FIGURE 16. Concentrations of MR mRNA in the anterior pituitary gland (AP), hypothalamic-preoptic area (HPOA), and hippocampus (HIPP); 1, 7, or 21 days after OVX or OVX with E replacement (OVX + E). Each *bar* indicates the mean $\pm$ SEM of five to thirteen animals. Two-way ANOVA indicated significant treatment differences ($p < 0.0001$) in all three tissues, and significant time differences ($p < 0.005$) in the HIPP. #, Significant decrease ($p < 0.03$) from oil control values. *, Significant increase ($p < 0.05$) from 1 day value.

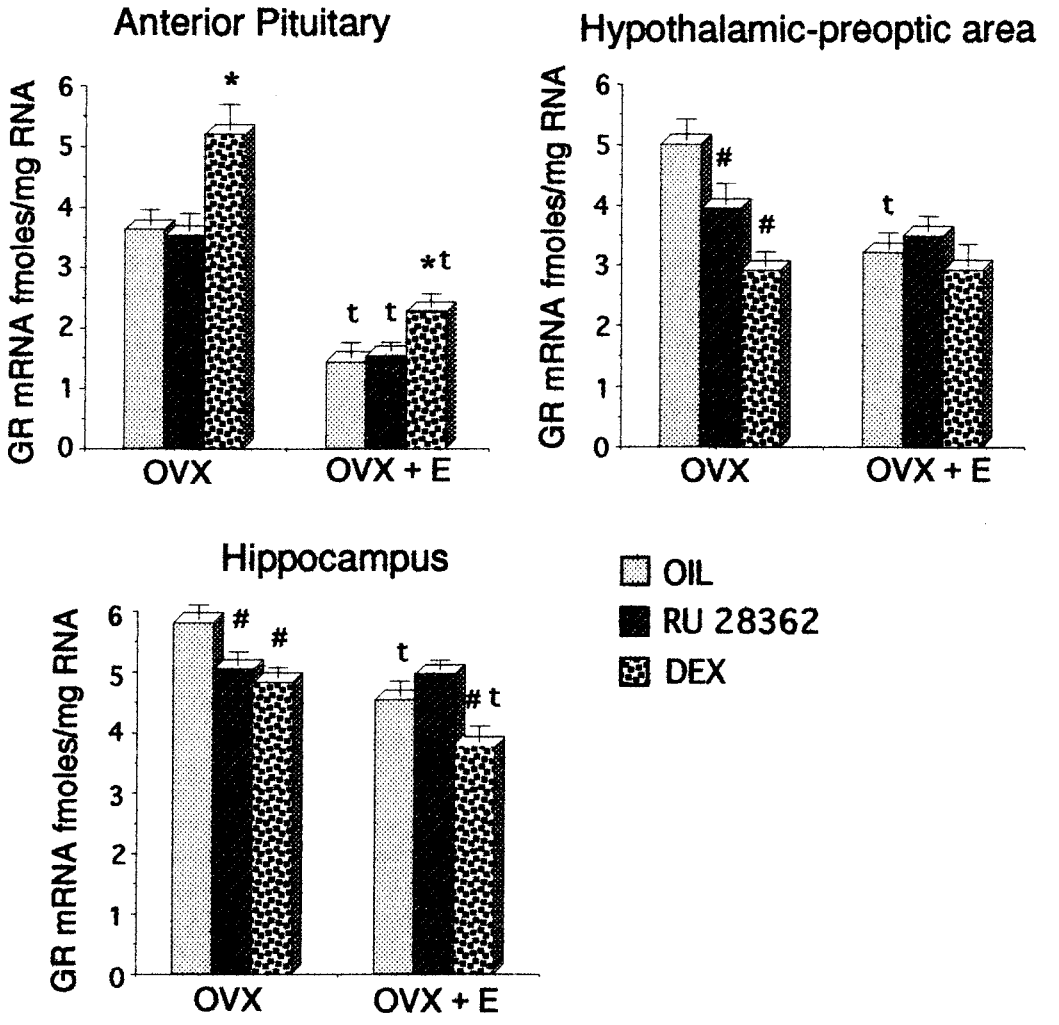


FIGURE 17. Concentrations of GR mRNA in the anterior pituitary gland (AP), hypothalamic-preoptic area (HPOA), and hippocampus (HIPP) after RU 28362 (RU) or DEX treatment. Animals were OVX for 17 days or OVX with E replacement (OVX + E). Animals were subsequently sc injected daily for 4 days with either RU 28362 or DEX (40 ug/100g BW in oil) or oil. Twenty-four hours after the last injection, animals were sacrificed, brains microdissected, and RNA isolated. Concentrations of GR mRNA were measured by RNase protection assays. Each *bar* indicates the mean $\pm$ SEM of six animals. Two-way ANOVA indicated significant treatment and injection differences ($p < 0.009$) in all three tissues, and significant treatment by injection differences ($p < 0.004$) in the HPOA and HIPP. *, Significant increase ($p < 0.05$) from oil control values. #, Significant decrease ($p < 0.05$) from oil control values. E treatment resulted in a significant decrease ($p < 0.009$) from OVX control values in all three injection groups in the AP, the OIL and DEX groups in the HIPP, and only the OIL injected group in the HPOA.

GR mRNA in all three E treated groups remained significantly decreased compared to OVX controls ($p < 0.002$).

Discussion

In this study we have demonstrated that E treatment decreases steady state levels of GR and MR mRNA in neuroendocrine tissues involved in regulating ACTH and CORT secretion. These data are consistent with previous studies which have shown that in the rat AP, ovariectomy increases GR mRNA levels, and this increase is prevented by E treatment (Peiffer, 1987). Ovariectomy increases GR mRNA levels in the hypothalamus, but not the hippocampus, when examined 14 days after OVX, and E treatment of intact female rats reduces GR mRNA levels to 65% of control values (Peiffer, 1991). Our finding, that GR mRNA is increased after 21 but not 1 or 7 days of OVX, suggests that the hippocampus may be more refractory to the removal of steroid hormone stimulation than other tissues such as the hypothalamus.

The results of a previous study suggested that E impaired GR mediated events, such as the feedback inhibition of ACTH secretion and GR down-regulation (Burgess and Handa, 1992). The lower levels of GR mRNA seen in the presence of E in the hypothalamus, hippocampus, and AP fit with a model of impaired CORT negative feedback due to a deficit in receptor function. While we previously reported no effect of E on MR or GR protein concentrations in the hypothalamus and hippocampus (Burgess and Handa, 1992), the E induced decreases in MR and GR mRNA suggest that E may be altering CORT receptor regulation. The decreased steady-state mRNA levels may result in decreased concentrations of functional receptor. At present it is

unknown whether there is a general decrease in mRNA concentration per cell across whole brain regions, or if there is a loss of message from a specific population of cells. Studies examining GR mRNA by in situ hybridization histochemistry would help to determine the anatomical specificity of this effect.

This apparent discrepancy between changes in mRNA but not protein concentrations in response to E is not unique. Differences in protein and mRNA levels have been suggested for GR, AR, and ER, based on a comparison of data across studies (Reul and DeKloet, 1985; Herman, et al., 1989a; Sar and Stumpf, 1975; Simmerly, 1990; Stumpf, et al., 1975; Toran-Allerand, et al., 1992). A dissociation of changes in concentrations of protein and mRNA has also been reported for GR, following chronic glucocorticoid treatments (Reul, et al., 1989; Sheppard, et al., 1990), as well as during development (Okret, et al., 1991). A possible explanation for the lack of correlation seen in the present study is that E may alter the translational efficiency of MR and GR mRNAs, or change the half-life of the proteins. An increase in the translational efficiency of the mRNA or the half-life of the protein would mean a smaller steady-state pool of mRNA would be necessary to maintain a constant level of that protein. The post-translational modifications of the protein resulting in its longer half-life may also result in its loss of function (Pratt, 1990; Beato, 1991). Decreased steady-state mRNA levels could also result from a change in the balance between mRNA synthesis and degradation.

We previously reported that E inhibited the autoregulation of hippocampal GR by RU28362 (Burgess and Handa, 1992). While this inhibition could result from a

post-translational effect of E on the protein, data from the present study show that E is inhibiting the down-regulation of hippocampal GR mRNA as well. We found a modest (18%) but significant reduction in hippocampal GR mRNA levels following four days of either DEX or RU 28362 treatment. In E treated animals, however, the down-regulation by RU 28362 was absent. This suggests that E is altering GR mediated transcriptional regulation of GR mRNA, further supporting our hypothesis that E is interfering with GR function.

The mechanism by which GR regulates its own mRNA is not presently known. However, recent studies have shown that GR may directly bind to sites within the coding region of the GR gene (Burnstein, et al., 1990; Alksnis, et al., 1991). E may be interfering with the ability of the ligand/GR complex to negatively regulate transcription. This could result from ER binding to DNA causing steric hindrance to the binding of GR to its glucocorticoid response element (GRE), or from E induced alterations in the availability of other transcription factors necessary for negative regulation of transcription (Diamond, et al., 1990).

The small decrease in GR mRNA observed following chronic DEX treatment is consistent with previous findings. While Sheppard et al. (1990) reported that 6 days of DEX administration did not alter hippocampal GR mRNA levels, differences based on route of administration and dosage could account for our finding a significant albeit small decrease following only 4 days of DEX.

The pattern of glucocorticoid regulation of GR mRNA seen in the HPOA was very similar to that reported by Peiffer et al. (1991). Dexamethasone and RU 28362

treatments resulted in 42% and 22% reductions, respectively, in GR mRNA levels in the absence of E. In the E treated animals, however, no reduction in GR mRNA levels below that associated with E treatment alone were observed. These data again support the concept that E is interfering with GR function, resulting in the impairment of GR mRNA autoregulation.

In contrast to brain tissue, the anterior pituitary showed no effect of E on GR mRNA autoregulation. In fact, we observed increases in GR mRNA levels following chronic DEX administration. These data are also consistent with the results of Sheppard et al. (1990). The fact that no changes in GR mRNA were detected following RU 28362 treatment, suggests that DEX-induced upregulation of GR mRNA may be due to MR occupancy. This supports the theory that GR and MR may interact in the regulation of receptor gene transcription (Evans and Arriza, 1989). Previous studies showing that the MR specific agonist aldosterone can down-regulate GR in the mouse brain, are consistent with an interacting regulatory system (Luttge, et al., 1989b).

The differential effect of E on GR mRNA down-regulation in the HIPP and HPOA, may be due to differences in receptor concentrations in those regions. MR has been shown to occur at much higher concentrations in the HIPP than in the HPOA (Reul and DeKloet, 1985). Thus, although E may interfere with GR function, the binding of DEX to MR could account for the down-regulation of GR mRNA observed in the HIPP-but not the HPOA-in the presence of E.

Although increases in circulating CORT levels following E treatment might be

a causal factor in the down-regulation of GR mRNA, the E-induced increase in basal CORT levels is far less than the diurnal rise in the evening and may not be of functional significance. In the HPOA, E attenuated any down-regulation of GR mRNA by either RU 28362 or DEX. The regulation of GR mRNA in the AP was in the opposite direction, DEX treatment resulted in an upregulation of GR mRNA. Thus, the HIPP is the only region we have examined where increased CORT levels are likely to play any role in the decrease seen in GR mRNA following E treatment.

Our finding that E treatment impairs the ability of GR to mediate down-regulation of its mRNA and protein suggests a decline in functional hippocampal GR. The physiological reasons for an effect of E on GR are not known. However, high concentrations of CORT have been shown to result in hippocampal cell loss (Sapolsky, et al., 1985). Since higher circulating CORT levels are a consequence of chronic E, the reduction in glucocorticoid receptor function in the hippocampus could be protective. In this study we also demonstrate a reduction in the steady state levels of GR and MR mRNAs, following E treatment, in tissues involved in mediating HPA regulation. This finding combined with estrogen's interference with GR function may explain the impairment of negative feedback inhibition by CORT, seen in the presence of estrogen.

CHAPTER V

Summary and General Discussion

A sex difference in the ACTH and CORT response to stress exists which is partially dependent on the presence of E. The studies in this dissertation have demonstrated that the presence of E increases the female rat's hormonal response to stress, in part, by impairing CORT negative feedback inhibition. Furthermore, E was shown to interfere with the autologous regulation of GR protein and mRNA. Overall, these studies demonstrate that the presence of E causes a disruption of GR function, which may be the means by which E impairs HPA axis negative feedback inhibition by CORT.

The precise mechanism by which E interferes with GR functioning is unknown. This discussion will postulate possible mechanisms by correlating recent findings describing estrogen-mediated molecular phenomena with those characterizing the molecular events mediating GR function. In addition, the possible consequences of this effect in terms of both normal and abnormal physiology/psychology will be discussed.

Mechanisms of Action

We have shown that E disrupts GR function in the context of both glucocorticoid suppression of the hormonal response to environmental perturbations,

and autologous regulation of GR protein and mRNA. In addition, E alters the regulation of both MR and GR mRNA, resulting in decreased levels relative to OVX controls. Whether E-induced changes in MR/GR mRNA regulation relate to E's impairment of GR-mediated functions as cause and/or effect is unknown. While one or more mechanisms may mediate these effects of E, an E-induced disruption of GR function probably reflects E interference with GR-mediated signal induction (trans-activation). General mechanisms, common to most GR-mediated events, by which E could be acting will be discussed first. These involve possible E-induced alterations in the ability of GR to mediate transcriptional regulation. Mechanisms specific to E's impairment of autologous GR regulation will also be discussed. These include potential ways that E could alter the posttranscriptional and posttranslational effects of glucocorticoids, such as changes in GR mRNA processing, stability, translation efficiency, and GR protein stability.

Transcriptional Regulation

Estrogen's impairment of GR-mediated transcriptional regulation could be mediated in several ways including: 1) direct regulation of expression of the glucocorticoid's target gene; 2) disruption of transcriptional regulation by steric hindrance to ligand-bound GR's binding to DNA, by blocking other transcription factors' binding and/or interactions, or by altering availability of other transcription factors (figure 18).

An effect of E on the glucocorticoid's genomic mechanism of action is supported by the following: 1) E's attenuation of GR-mediated suppression of the

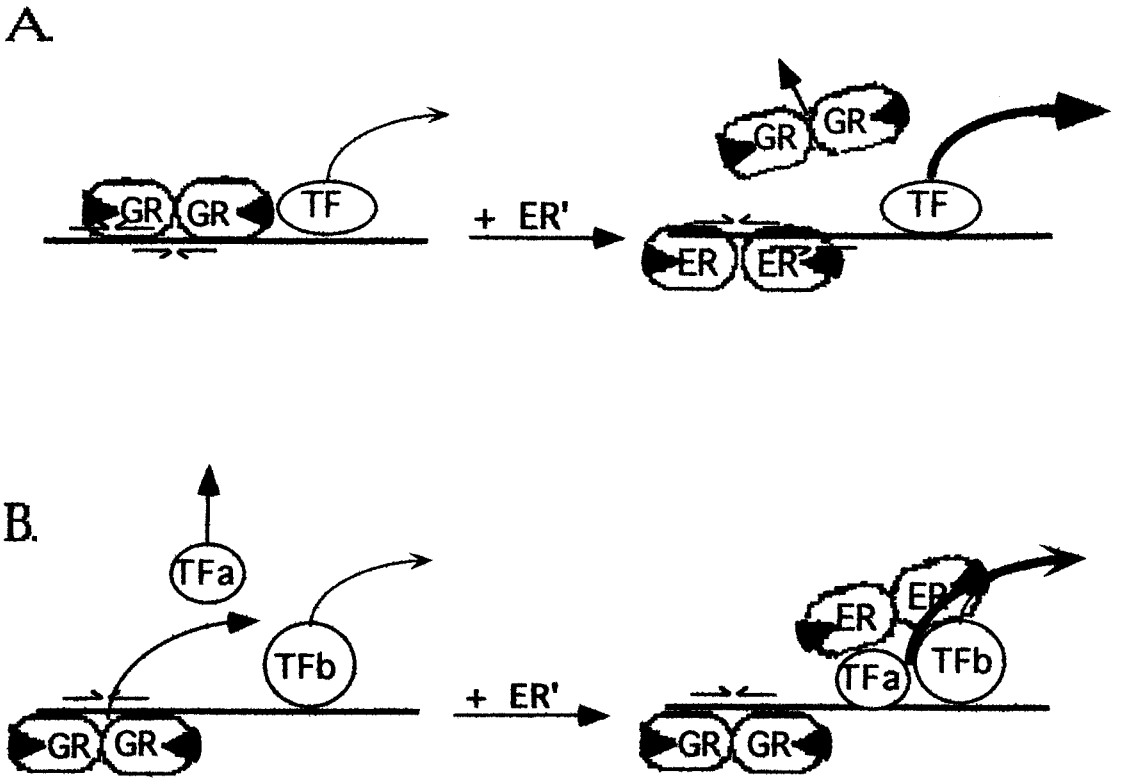


FIGURE 18. Possible mechanisms by which activated estrogen receptor (ER') could interfere with glucocorticoid receptor (GR) -mediated signal transduction (trans-activation). A. Steric hindrance by ER' of activated GR binding, preventing negative regulation. B. ER' stabilization of transcription factor (TF) interactions, preventing disruption by GR'. Arrow thickness indicates stength of transduction signal.

hormonal response to stress, occurring in the slow time domain; and 2) E's impairment of GR-mediated down-regulation of GR mRNA. The most straightforward way for E to achieve its effects is to regulate transcription of the same gene(s) regulated by activated GR, only in the opposite direction. A direct regulatory role for E would necessitate an estrogen response element (ERE) in a regulatory region of the glucocorticoid target genes. These targets include the CRH and POMC genes, as well as probably other still unknown genes, in the case of CORT suppression of the hormonal response to stress. The GR and MR genes are the obvious targets in autologous regulation. To date, no inspections for EREs have been reported for any of these genes.

Estrogen could also impair the glucocorticoid's genomic mechanism of action indirectly, through interference with the trans-activating function of GR. Numerous complex interactions between various trans-acting regulatory factors are involved in GR-mediated transcriptional regulation (Beato, 1989; Miesfeld, 1990). Possible ways that E could interfere with this regulation are shown in figure 18. The binding of the estrogen-bound estrogen receptor (ER) complex to the DNA, at either an undiscovered ERE or at a nonconsencus ERE (Martinez and Wahli, 1989), could result in steric hindrance of activated GR binding. This mechanism of steric hindrance appears to mediate glucocorticoid's negative regulation of expression, in several genes (Akerbloom, et al., 1988; Oro, et al., 1988; Sakai, et al., 1988; Guertin, et al., 1988; Drouin, et al., 1989). This concept of steric hindrance also applies to the binding of other transcriptional regulatory factors. Thus, while an

activated GR complex binds unimpaired, the binding of the activated ER complex to some other site in the regulatory region could prevent a necessary transcription factor's binding and subsequent interaction with the GR-transcriptional complex, thus precluding GR-mediated regulation (Fig. 18).

Evidence for transcriptional interference, between ER and GR, in Hela cells cotransfected with ER, GR, and GRE-containing reporter gene constructs has been presented (Meyer, et al., 1989). It was proposed that the interference was due to competition for mutual trans-acting regulatory factors, since the inhibition of GR trans-activation was E dependent and GR binding was not affected. Inhibition of transcription due to the sequestration of some transcriptional factor(s) by the activated GR, has been described in other systems, and termed 'squelching' (Ptashne, 1988; Gill and Ptashne, 1988). While this squelching in transfected cells results in transcriptional interference, no evidence has been presented yet for its occurrence under physiological conditions in vivo.

One class of nuclear regulatory factors involved in GR-mediated transcriptional regulation are the products of the immediate-early genes (IEGs). Transcription of these IEGs occurs rapidly and without de novo protein synthesis, resulting in the rapid appearance of their products following stimulation of a cell (Morgan and Curran, 1989, 1990). Various stimuli, including steroid hormones, as well as growth factors and neurotransmitters, evoke the expression of these genes (Murphy, et al., 1987; Fink, et al., 1988; Morgan and Curran, 1990; Hyder, et al., 1991). In particular, E has been shown to increase the expression of various nuclear regulatory transcription

factor genes, including c-*fos*, c-*jun*, and c-*myc*, both in the periphery and in the brain (Loose-Mitchell, et al., 1988; Weisz and Bresciani, 1988; Cattaneo and Maggi, 1990; Weisz, et al., 1990).

The levels of various nuclear regulatory proteins, including IEG products such as Fos and Jun, can profoundly affect GR-mediated transcriptional regulation (Yang-Yen, et al., 1990; Lucibello, et al., 1990; Jonat, et al., 1990; Diamond, et al., 1990; Touray, et al., 1991). It has recently been shown that the ratio of cJUN/cFOS can reverse the direction of GR-mediated hormone trans-activation (Diamond, et al., 1990). Thus, a GR-Jun/Jun interaction results in an active trans-activation function, while a GR-Fos/Jun interaction results in an inactive complex (Diamond, et al., 1990). E increases hippocampal cFOS expression (Cattaneo and Maggi, 1990), and decreases uterine cJUN expression (Lau, et al., 1990). If these effects on IEGs are coupled in certain neural tissues, this could lead to an increase in the Fos/Jun ratio and result in an impairment of GR function. Taken together, these findings suggest a potential mechanism by which E can impair GR-mediated signal transduction.

While these possible mechanisms of E action at the transcriptional level are compatible with my results, direct evidence awaits further studies examining the transcription rates and trans-activating factor regulation, in the presence of E.

Autologous Regulation of GR

In addition to the mechanisms outlined above, by which E could alter the transcriptional (decreased rates) effects of glucocorticoids on GR regulation, there are other ways E could alter the posttranscriptional and posttranslational effects of

glucocorticoids. Since these effects of glucocorticoids decrease GR protein and mRNA half-lives, possible mechanisms include changes in GR mRNA processing, stability, translation efficiency, and GR protein stability. Estrogen has been shown to increase the half-life of various mRNAs by increasing their stability (Nielson and Shapiro, 1990). E could accomplish this by: 1) transcriptional up-regulation of a factor which binds the mRNA, preventing degradation; 2) transcriptional down-regulation of a factor which targets the mRNA for degradation; 3) preventing the shortening or removal of the poly(A) tail; and 4) modifying the activity of a component of a mRNA degradative system (Nielson and Shapiro, 1990). Estrogen acting via one or more of this mechanisms could reverse the effects of glucocorticoids, which themselves may be acting through one or more of these mechanisms, but in the opposite direction (Vedeckis, et al., 1989).

The interactions among GR, tRNA, and other components of the translation system (Verdi and Campagnoni, 1990), may be responsible for decreasing GR mRNA translation efficiency. Changes in protein half-life are thought to be due to conformational changes induced after glucocorticoid binding and hsp90 dissociation (Pratt, 1990). Estrogen could potentially alter these GR-mediated actions by altering the environmental constituents, eg. increasing the amount of tRNA or other factors, or by direct binding of the activated ER to the GR. Increasing the machinery available for translation would be consistent with E's proliferative role in the reproductive tract. Whether similar effects mediate E's neurotrophic role in the brain is unknown. Direct binding of the ER to the GR has been postulated in the context of

the synergistic effects seen in cotransfected monkey kidney CV1 cells expressing GR and various ER mutants (Cato and Ponta, 1989).

Overall, a common denominator to most of the potential mechanisms by which E could effect disruption of GR function is that E-bound ER may act as an antagonist, directly or indirectly interfering with the glucocorticoid-bound GR's ability to regulate transcription, whether of its own gene, other trans-activating factors, or other factors involved in mediating non-transcriptional effects.

One effect of E that demands a mechanism independent of transcriptional interference concerns potential alterations in the efficacy of fast feedback inhibition by glucocorticoids. The documented rapid effects of E, presumably membrane mediated (Woolley and Timiras 1962a,b; Horvat, 1991; Wong and Moss, 1991), could interfere with the rapid effects of glucocorticoids, also presumably membrane mediated (Majewska, 1987; Lambert, et al., 1987; Hua and Chen, 1989), directly or indirectly. This could contribute to the sex differences reported in ACTH levels within the fast feedback time domain (Le Mevel et al., 1978).

Possible Consequences of E's Effects

Several consequences of an E-induced impairment in GR function could arise besides our reported decreases in glucocorticoid feedback inhibition. A shift in the balance of MR- and GR-mediated effects could result in the alteration of several neuronal characteristics known to be selective for CORT. Researchers have only recently begun to examine the CORT receptor subtypes mediating the actions of CORT affecting neurochemistry and behavior. These MR-mediated vs GR-mediated

characteristics are listed below in Table VI.

TABLE VI

Neuronal Effects of CORT Mediated by MR or GR

Receptor	*Characteristics*	*References*
MR	Decreases 5-HT induced AHP	Joels et al., '90
GR	Attenuates NE reduced AHP	Joels and DeKloet, '89
GR	Sensitization to repeated amphetamine administration	Rivet et al., '89 Piazza et al., '89
GR	Increased frontal cortex DA release	Imperato et al., '89
MR	Control of fear-motivated immobility	DeKloet, '91
GR	Extinction conditioned avoidance behavior	Kovacs et al., '77
MR	Improved information handling	Gerbec et al., 88
MR	Increases excitability, anxiety	DeKloet, '91; File, '90
GR	Decreases excitability, anxiolytic	DeKloet, '91; File, '90

Abbreviations:

MR, mineralocorticoid receptor

GR, glucocorticoid receptor

AHP, after-hyperpolarization

5-HT, 5-hydroxytryptamine

NE, norepinephrine

DA, dopamine

Correlations between the effects of E and a shift toward increased MR-mediated effects provides circumstantial support for our hypothesis. One of the best characterized effects of CORT mediated by the MR is a reduction in the 5-

hydroxytryptamine (5-HT)-induced afterhyperpolarization (AHP) in hippocampal neurons (Joels and DeKloet, 1989, 1990; Kerr, et al., 1989; Joels, et al., 1990). The AHP represents an intrinsic mechanism which protects hippocampal and other neurons against excessive stimulation (Nicoll, 1988). A reduction in the AHP, therefore represents a measure of increased excitability. Evidence for MR-mediation of this increased excitability is provided by studies demonstrating that low CORT levels are stimulatory, while high CORT levels override this effect and suppress excitability (Kerr, et al., 1989; Joels and DeKloet, 1989). Recently, MR specific agonists but not GR agonists were shown to reduce the 5-HT-induced AHP in the rat hippocampus Joels, et al., 1990).

In addition, GR-mediated actions attenuate the norepinephrine (NE)-induced decrease in AHP in hippocampal neurons, suppressing excitability (Madison and Nicoll, 1986; Joels and DeKloet, 1989). These findings further support the concept that MR mediates cellular excitability, while GR acts to suppress excitability, and correlate with reported sex differences in hippocampal pyramidal cell excitability (Teyler, 1980).

Another potential correlative effect of E and MR-mediated hippocampal excitability, concerns seizure activity. Estrogen has been shown to have an excitatory influence, resulting in lowered seizure thresholds (Woolley and Timiras, 1962a; Buterbaugh and Hudson, 1991). Furthermore, increased seizure frequency occurs during periods of high circulating E (Schachter, 1988).

Further evidence for a putative link between E and MR-mediated excitability

can be derived from behavioral correlations. MR activation results in improved avoidance behavior (DeKloet, 1991), while MR antagonists have anxiolytic (stress reducing) effects (File, 1990). Consequently, our proposed action of E, resulting in a disruption of GR function, could heighten the anxiety state. It has been suggested that anxiety and depressive disorders be combined and viewed as a general neurotic disorder, in which patients may exhibit one or more of the symptoms of anxiety and depression (Tyrer, 1986). In this context, our findings are consistent with the possible role of E in the etiology of depressive disorders, as inferred by the high incidence of clinical depression in females (Dohrenwend, et al., 1976; Weissman and Klerman, 1977).

Thus our findings are compatible with general neurotic disorders, whether the link is through an increase in MR-mediated neuronal effects, or through a dysfunction of the HPA axis, due to an impaired negative feedback mechanism or over-excitation. In further support of a putative role for GR in mediating anxiolytic effects, Pepin and Barden (1991) recently reported that during successful antidepressant therapy with desipramine, responsiveness to DEX suppression was restored. Furthermore, desipramine treatment was shown to increase both GR mRNA and binding in the mouse brain. Although restoration of DEX suppression occurred with the relief from depression, one must be careful not to draw a causal link. An effect of the antidepressant, just as the effect of E, on glucocorticoid suppression or even the larger picture of the overall HPA axis response, could be mediated indirectly by its effect on neurotransmitters or other neuromodulators.

Overall, the effects of E disrupting the normal functioning of GR may be viewed as a shift in the balance toward MR mediation of elevated CORT exposure. The data of Sapolsky et al. (1984, 1986) suggest that at least for the male rat, exposure to high CORT levels results in neuronal loss and subsequent HPA axis hyperactivation. Our findings suggest that in the female rat, although E exposure is elevating CORT levels, the effects of the elevated CORT exposure may be different. Consistent with this hypothesis are the findings of Brett, et al. (1983), demonstrating that in contrast to the elevation of plasma CORT found in aged vs young males in response to ether exposure, aged females exhibited a decrease in CORT levels relative to young controls. The lack of hypersecretion in the aged female may be due to two, not mutually exclusive, E-related events: 1) the CORT sensitive hippocampal neurons (as well as their MR and GR) involved in terminating the adrenocortical stress response (Sapolsky, et al., 1984) may have been spared by the GR's impaired function; and 2) the decline in circulating E levels, coincident with the loss of cyclicity (Lu, et al., 1979; Dudley, 1982). This suggests that E may play a protective role in the female, guarding against CORT overexposure and its deleterious consequences. In support of this, Uno et al. (1989) reported significant neuron loss in the hippocampal CA3 and CA4 fields in ulcerated (chronically stressed) male but not female vervet monkeys.

Another recent study demonstrated that while CORT and RU 28362, but not aldosterone, decreased the survival of primary hippocampal cultures, simultaneous E treatment prevented the decrease (Mizoguchi, et al., 1992). This provides evidence

that E may prevent hippocampal damage, specifically mediated by the GR.

Conclusions

The emerging picture of glucocorticoid regulation is a complex one. The players include not only the obvious - endogenous glucocorticoids, MR, and GR - but also regulatory influences that can alter their availability and activity. In addition to the classical corticosterone binding globulins (CBGs) in the circulation, CBG-like proteins are found in the anterior pituitary, potentially sequestering CORT, or perhaps acting as a sink for CORT. Further complexity is provided by the widespread localization of the microsomal enzyme 11*B*-hydroxysteroid dehydrogenase, which converts CORT to an inactive form, thus allowing for tissue specific control of CORT exposure. The ability of both the MR and the GR to not only bind the same ligand, but also to trans-activate the same target genes, suggests binary control of a glucocorticoid sensitive gene network. In certain instances, such as hippocampal neuronal excitability, the receptors appear to mediate antagonistic effects. While this is compatible with Selye's original pendulum hypothesis, revealing the underlying molecular mechanisms requires further study.

Autologous regulation of the CORT receptors is another area where interactions between the receptors can be demonstrated, but the physiological relevance is unsure. Although elegant in vitro studies have described the multiplicity of ways that the trans-activating function of GR can be affected, there is still the need to examine glucocorticoid regulation at the transcriptional level in neuronal tissues. While any theory of generalized MR-mediated excitability, combined with GR-

mediated suppressibility (at higher CORT concentrations) is attractive, more work is needed to substantiate it. Furthermore, care must be taken not to generalize any particular set of findings, as so much potential for tissue-specific regulation exists.

The capacity exists for estrogen to interact with and modify any or all of the components involved in mediating glucocorticoid's actions. Estrogen could alter levels of 11 B-HSD, just at it does CBGs, as well as impair the functioning the GR. The hippocampus in particular should provide an exciting arena for future studies, given the colocalization of MR and GR. Overall, further studies elucidating the probable effects of estrogen on other regulatory components of the HPA axis, as well as clarifying estrogen/glucocorticoid interactions at the molecular level are necessary.

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The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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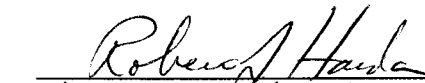
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