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Corticosterone acts on the brain to inhibit adrenalectomy-induced adrenocorticotropin secretion

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Corticosterone Acts On The Brain To Inhibit Adrenalectomy-Induced Adrenocorticotropin Secretion

by

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This dissertation is dedicated to my grandparents

Lt. Col. Philip W. Reed, US Army Rt.	**Sadie F. Levin**
1895 - 1981	1896 - 1983

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Abstract

In the rat, adrenalectomy results in dramatic increases in both ACTH synthesis and secretion from the anterior pituitary. There is evidence that corticosteroids can exert feedback effects at hypothalamic and extrahypothalamic sites, as well as at the level of the pituitary. The present studies were undertaken to determine whether corticosterone acts at the brain or pituitary to normalize adrenalectomy-induced increases in plasma and pituitary ACTH.

In the first experiment, rats were either sham adrenalectomized or adrenalectomized and provided with subcutaneous pellets of corticosterone fused with cholesterol (0, 25, 50 or 75% corticosterone, by weight). Rats from each steroid treatment group were decapitated in the evening of the fourth, or morning of the fifth day following adrenal surgery. Morning plasma ACTH levels and thymus gland weight were restored to levels similar to those observed in sham adrenalectomized controls by treatment of adrenalectomized rats with 50% corticosterone pellets. This dose of corticosterone resulted in roughly constant plasma corticosterone levels of 5-9μg/dl. Treatment of adrenalectomized rats with 75% corticosterone pellets also restored morning plasma ACTH but additionally induced significant thymic atrophy, with respect to sham adrenalectomized controls.

In subsequent experiments rats were given three types of lesions which remove the normal hypothalamic input from the pituitary. In these lesioned rats, corticosterone feedback could be exerted only at the pituitary, while in sham lesioned controls feedback could be exerted at

the brain and at the pituitary. Some lesioned rats received constant infusions of exogenous hypothalamic releasing factors. In sham lesioned rats, corticosterone levels of 5-9µg/dl normalized morning plasma and pituitary ACTH levels and thymus gland weight after adrenalectomy. In lesioned rats lacking exogenous hypothalamic input, adrenalectomy did not induce increases in plasma or pituitary ACTH. In lesioned rats receiving exogenous CRF, corticosterone levels of 5-9µg/dl did not affect morning plasma or pituitary ACTH levels compared to adrenalectomy alone. Only when corticosterone levels were elevated to the range that produced thymic atrophy was feedback observed at the pituitary.

It was concluded that corticosterone normalizes morning plasma and pituitary ACTH after adrenalectomy by an action on the brain, and not the anterior pituitary.

Mary F. Dallman

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Chapter 1 - Background

There are three principle regulators of adrenocortical system activity; the circadian rhythm in basal activity, stress-induced activation, and glucocorticoid inhibitory feedback. The literature reviewed in this chapter was chosen in order to highlight the classic studies which established the importance of glucocorticcid feedback in the regulation of pituitary adrenocorticotropic hormone (ACTH) secretion, and to indicate that glucocorticoids have been shown to exert feedback effects at hypothalamic and extrahypothalamic sites in the brain, as well as on the pituitary. Finally, the effects of adrenalectomy on adrenocortical system activity are detailed.

Historical Perspective

In 1936, Hans Selye introduced a three stage, "general adaptation syndrome produced by diverse nocuous agents" (Selye, 1936). By observing the changes in the general state of health of a rat, and the weight and gross histologic character of various endocrine glands, Selye concluded that, during prolonged stress, the anterior pituitary ceases its production of gonadotropic hormones and prolactin, and favors the increased secretion of thyrotropic and adrenocorticotropic hormones.

This hypothesis was followed by the observations of Ingle and colleagues that the administration of "cortin" or adrenal cortical extract produced atrophy of the adrenal cortex (Ingle & Kendall, 1937; Ingle et al, 1938), which could be prevented by the simultaneous administration of a fraction of anterior pituitary extract which had

high adrenocorticotropic activity. Similarly, the increase in adrenal size which followed prolonged muscular exercise was abolished if animals were pretreated with adrenal cortical extract (Ingle, 1938). These results led Ingle to conclude that the anterior pituitary was in some manner sensitive to variations in the amount of "cortin" in the body fluids, and that increased physiologic requirements for cortin led to an increase in the output of adrenocorticotropic hormone from the anterior pituitary. Thus, the output of the pituitary would be suppressed when "cortin" was present in the body fluids in excess of physiologic requirements.

Following the observation that the decreases in adrenal cholesterol and ascorbic acid content in response to hemorrhage parallel the effects of exogenous ACTH administration (Sayers et al, 1945), the adrenal ascorbic acid depletion assay became a common method to assess the effect of various experimental manipulations on adrenocortical system activity. Sayers and Sayers (1947) found that this depletion occured rapidly (within 1 hour) and in proportion to the dose of ACTH or to the degree of stress. Also, they observed that the decrease in adrenal ascorbic acid content induced by various stresses (heat, cold, histamine, epinephrine, killed typhoid organisms) were blocked by pretreatment with adrenal cortical extract. These data, and the observation that adrenal cortical hormone pretreatment did not block the adrenal ascorbic acid depletion induced by exogenous ACTH, led Sayers and Sayers to postulate that stress increases pituitary adrenocorticotropic activity by increasing the requirement of peripheral tissues for cortical hormones, and that the anterior pituitary responds

to a resulting decrease in plasma levels of corticosteroids due to an increase in peripheral tissue cortical hormone "utilization", by increasing its rate of ACTH release.

At the same time that Sayers and colleagues proposed the corticosteroid utilization hypothesis, the role of the central nervous system in the secretory activity of the anterior pituitary was also being considered (Green & Harris, 1947; Harris, 1948). The anatomical studies of Green and Harris demonstrated that, while the nerve supply to the adenohypophysis was sparse, the possibility of neurohumoral transmission of stimuli to the glandular cells was supported by the existence of a dense vascular plexus in close approximation to nerve endings in the median eminence that was connected through a portal system of vessels to the pars distalis.

In support of this neurovascular hypothesis were the findings that, in rabbits, a lymphopenia induced by emotional stress was shown to be blocked by hypophysectomy and elicited by injections of pituitary extract into hypophysectomized animals (Colfer et al, 1950). Because incompletely hypophysectomized rabbits were found to have the lymphopenic response and vascular connections between the tuber cinereum and the remaining pituitary tissue, it was likely that the emctional stress stimulated the anterior pituitary via the hypophysial portal vessels. Also, electrical stimulation of the posterior part of the tuber cinereum or mammillary body produced a similar lymphopenia, while lesions in these areas, or in the zona tuberalis of the pituitary gland, abclished the lymphopenic response to emotional stress (deGroot & Harris, 1950).

Another line of evidence supporting the importance of the brain in the control of adrenocortical function came from the work of Gray and Munson (1951). They showed that adrenal cortical extract administered 10 seconds before histamine stress completely blocked the adrenal ascorbic acid depletion in response to this stress, while hormone injection 3 or 5 seconds after histamine partially blocked the response; adrenal cortical extract administered 10 seconds after histamine stress had no inhibitory effect. These results were among the first to demonstrate the rapidity with which the adrenccortical system responds to stress, and implied that something other than corticosteroid utilization had to be stimulating ACTH release. Using the experimental technique of cross-circulation (Brodish & Long, 1956), it was shown that the surgical stress of unilateral or bilateral adrenalectomy resulted in an increase in plasma ACTH. The rapidity of the ACTH response again implied that it was neurally mediated. Separately, it was shown that the injection of hypothalamc-hypophysial portal plasma from dogs into hypcphysectomized rats induced a greater degree of adrenal ascorbic acid depletion than did control injections of carotid artery plasma (Porter & Jcnes, 1956).

Using a modification of the adrenal asccrbic acid depletion assay, Sydnor and Sayers (1954) reported measurable plasma ACTH levels in adrenalectomized rats, while the levels in intact rats were below assay sensitivity. In this study, adrenalectomized rats were found to have prolonged plasma ACTH responses to stress, with respect to unoperated controls. Because there could nct be any corticosteroid "utilization" eliciting ACTH release in adrenalectomized animals, it was concluded

that, following the application of a stressful stimulus, a central neural mechanism induces an immediate acceleration of ACTH release, while adrenal cortical hormone utilization regulates the subsequent rate of ACTH discharge.

The various theories on the role and importance of corticosteroid feedback in the regulation of ACTH secretion were unified by Yates and colleagues (1961). Here, it was proposed that the rapid increases in plasma corticosteroid levels which follow many stimuli are caused by the reset of a negative feedback controller which operates at a variable set point. This theory predicted that a dose of corticosterone which was sufficient to produce an increment in plasma corticosterone equal to that produced by the stimulus itself, should prevent the release of ACTH following that stimulus. Experiments showed the theory to be correct; the corticosterone responses to histamine or laparotomy stress were completely blocked when an injection of corticosterone which mimicked the corticosteroid response to the stress was administered 15 seconds prior to the stress. When laparotomy was performed following doses of corticosterone which produced increments less than that observed after laparotomy, the subsequent corticosterone response was the same as after laparotomy alone. These results imply that the plasma corticostercid concentration is at all times under effective negative feedback control, and that the increase in the setpoint, cr reset, of the controller creates a signal which is sensed as an absolute drop in plasma corticosteroid levels.

The reset hypothesis generated controversy, however, when other groups reported that plasma corticosteroid responses to stress were not

related to the plasma corticosterone level at the time of the stress. Smelik reported that both the plasma corticosterone and the *in vitro* corticoidogenic responses to a novel environment or histamine were not reduced by pretreatment with corticosterone (Smelik, 1963a,b). Similarly, Hodges and Jones reported no inhibition of the plasma corticosterone response to sham adrenalectomy by corticosterone pretreatment (Hodges & Jones, 1963). These experiments differed from those of Yates et al, in that corticosterone pretreatments were given as intramuscular "infusions" 15 minutes, rather than 15 seconds, prior to stressing the animal. Both of these groups concluded that the inhibition observed by Yates et al was a function of the supraphysiologic steroid levels 15 seconds after intravenous corticosterone administration. These studies necessitated a more thorough characterization of the temporal aspects of corticosteroid feedback, and it is presently accepted that corticosteroid feedback is exerted over distinct time domains (Keller-Wood & Dallman, 1984).

Rapid, rate sensitive feedback was first postulated by Dallman and Yates (1969). These investigators reported that the plasma corticosterone response to histamine was inhibited when corticosterone was given 5 minutes or 15 seconds before histamine, but not when the steroid was presented 15 minutes before or two minutes after histamine. Thus, the rapid inhibitory effect of corticosterone occured while plasma corticosterone levels were increasing. These findings were confirmed by Kaneko and Hiroshige (1978), who found that fast, rate sensitive corticosteroid feedback is proportional to the rate of formation of hormone-receptor complex. The fast feedback effect of corticosterone

has been shown to be antagonized by steroids with hydroxyl groups at the 21 and 11-β positions (Jones & Hillhouse, 1976), and does not require protein synthesis (Abou-Samra et al, 1986).

Corticosterone exerts delayed feedback effects beginning 1-2 hours after steroid presentation. Dallman and Yates (1969) showed that corticosterone infusions 120, but not 10, 19 or 45 minutes before histamine, inhibited the corticosterone response to this stress. Steroids with hydroxyl groups at either the 21 or the 11-β position antagonize the delayed feedback effects of corticosterone (Jones & Hillhouse, 1976), and the mechanism of delayed corticosteroid feedback appears to depend on the duration of exposure to increased levels of corticosteroids (Keller-Wood & Dallman, 1984).

Sites of Corticosteroid Feedback

The site or sites through which corticosterone exerts feedback effects remains a subject of much controversy. To date the bulk of the experimental evidence is concentrated on corticosteroid feedback at the level of the anterior pituitary; however, this may be more the result of the ease with which this gland can be studied in the absence of the other levels of the adrenocortical system than the relative physiologic role of this feedback site. Both endogenous and synthetic corticosteroids have been shown to inhibit K^+-, hypothalamic extract-, phorbol ester-, 8-Br-cAMP-, vasopressin- and corticotropin-releasing factor (CRF)-induced ACTH secretion from pituitary segments, dispersed and cultured pituitary cells *in vitro* (Fleischer & Vale, 1968; Kraicer et al, 1969; Arimura et al, 1969; Sayers & Portanova, 1974; Yasuda et

al, 1976; Giguere et al, 1982; Vale et al, 1983; Bilezijian & Vale, 1983), and from the mouse pituitary tumor (AtT-20) cell line (Watanabe et al, 1973; Philips & Tashjian, 1982). Prolonged treatment with corticosteroids in vitro reduces the intracellular content of ACTH (Watanabe et al, 1973; Roberts et al, 1979). In the absence of changes in intracellular degradation or precursor processing rates (Roberts et al, 1979; Philips & Tashjian, 1982), this finding implies a direct inhibitory effect of corticosteroids on ACTH production. Corticosteroids decrease the level mRNA for the ACTH precursor, procpiomelanocortin (POMC) (Roberts et al, 1979) and have been shown by nuclear run-off assay to inhibit POMC gene transcription (Birnberg et al, 1983; Eberwine & Roberts, 1984).

The relative importance of the anterior pituitary as a site of corticosteroid feedback is more difficult to demcnstrate in vivo. Support stems from studies in which the pituitary is isolated from hypothalamic influences by means of hypothalamic lesions or drug pretreatment which selectively depresses hypothalamic function, and studies involving injections or implants of corticosteroids directly into the pituitary. For example, the compensatory adrenal hypertrophy in response to unilateral adrenalectomy was shown to be blocked by injections of exogenous hydrocortisone in rats with large hypothalamic lesions (McCann et al, 1958). Dexamethasone pretreatment has been shown to diminish the corticosteroid response to lysine vasopressin and hypothalamic extract in rats with similar lesions (DeWied, 1964), and to suppress plasma corticosterone levels in rats in which all forebrain structures, including the median eminence and stalk, were removed by

suction (Dunn & Critchlow, 1969). The ACTH response to CRF was reduced by corticosterone in basal hypothalamic-lesioned rats (Jones et al, 1977). Lesion studies are subject to scrutiny, however, because these large lesions usually disrupt the normal blood supply to the pitutary and often result in infarct; it is not known to what extent these changes may alter pituitary sensitivity to corticosteroid feedback.

When the endogenous secretion of corticotropin-releasing factors of hypothalamic origin is inhibited by nembutal/morphine or nembutal/-morphine/chlorpromazine pretreatment, dexamethasone has been shown to block the ACTH response to stalk-median eminence extract (Arimura et al, 1967), lysine and arginine vasopressin (Yates et al, 1971) and to CRF (Rivier et al, 1982). It is nearly certain that the pretreatment has effects of its own on the adrenocortical system; in the absence of dexamethasone, pretreated rats have blunted responses to CRF with respect to non-pretreated controls (Rivier et al, 1982). Finally, injections of dexamethasone directly into the anterior pituitary reduce the corticostercid response to scald and to median eminence extract (Russell et al, 1969). The limitation of injection or implant studies is that the spread of injectate or diffusion of dissolved implant may affect sites beyond the area in question, although in this study (Russell et al, 1969) injections of radioactive dexamethasone into the anterior pituitary were reported to not spread up into the brain.

The observations that corticosteroid pretreatment did not block the plasma corticosterone response to pitressin or hypothalamic extract (Leeman et al, 1962), reduced median eminence CRF bio- (Vernikos-Danellis, 1965) and immuno-activity (Suda et al, 1984), and prevented

the stress-induced increase in median eminence CRF bioactivity (Vernikos-Danellis, 1965; Takebe et al, 1971), argue that corticosteroids exert feedback effects at the level of the central nervous system. Further evidence stems from the work of Plotsky and colleagues, who have shown that dexamethasone pretreatment blocks hemorrhage-induced increases in hypophysial portal plasma immunoreactive CRF, and plasma corticosterone levels of 8-12μg/dl suppress immunoreactive CRF secretion into the hypophysial portal circulation following nitroprusside-induced hypotension (Plotsky & Vale, 1984; Plotsky et al, 1986). There is also abundant electrophysiologic evidence that corticosteroids act on the brain. By both multiple unit and single-cell recording techniques, (systemic) corticosterone has been shown to have excitatory effects on basal neural activity, and inhibitory effects on stimulated neural activity (Feldman et al, 1983; Mor et al, 1986). While these observations strongly imply a central component to corticosteroid feedback, they do not specify the site(s) at which these effects are exerted.

One of the earliest experimental approaches employed to isolate the site of central corticosteroid feedback was the use of steroid implants. Implants of cortisol or dexamethasone in the median eminence region decreased corticosterone content of adrenal venous (Endroczi et al, 1961; Bohus & Endroczi, 1964) and systemic blood (Corbin et al, 1965), in vitro corticosteroid production (Bohus & Strashmirov, 1970), resting ACTH (Bohus et al, 1968), adrenal weight (Corbin et al, 1965; Chowers et al, 1963, 1967), ether- and novel environment-induced ACTH secretion (Bohus & Strashmirov, 1970; Bohus et al, 1968), and inhibited the

compensatory adrenal growth (Davidson & Feldman, 1963; Feldman et al, 1966) and adrenal ascorbic acid depletion (Chowers et al, 1963) in response to unilateral adrenalectomy. That these effects were not due to diffusion to and action of steroid on the anterior pituitary is inferred from control studies in which similar implants placed directly into the anterior pituitary did not decrease adrenal weight (Chowers et al, 1963) or block the hypertrophy or ascorbic acid depletion in response to unilateral adrenalectomy (Chowers et al, 1963; Davidson & Feldman, 1963; Feldman et al, 1966). The lack of effect of pituitary implants was not a consisitent finding, however; anterior pituitary dexamethasone implants were found to decrease ether-induced ACTH secretion (Bohus & Strashmirov, 1970) as well as adrenal weight and pituitary ACTH content, and to increase stalk-median eminence CRF bioactivity (Chowers et al, 1967). The later finding may suggest the presence of short-loop, inhibitory feedback of ACTH on CRF transport to or degradation within axon terminals of the median eminence.

Microiontophoretically applied corticosteroids induce dose-dependent inhibiton of hypothalamic unit firing and alter the responsiveness of hypothalamic neurons to afferent stimuli (Feldman, 1981), arguing for a direct effect of corticosterone at the level of the hypothalamus. Corticosteroids have also been shown to inhibit the (bioactive) CRF secreted from hypothalami <u>in vitro</u> (Jones & Hillhouse, 1976; Jones et al, 1977; Vermes et al, 1977). Interestingly, while Jones and Hillhouse reported delayed hypothalamic feedback with basal and stress levels of corticosterone, they were able to demonstrate delayed feedback at the pituitary with levels of corticosterone secreted

normally only during stress. Corticosteroids may exert feedback effects at both the brain and the pituitary, and the site may vary depending on the concentration of circulating corticosterone.

Extrahypothalamic corticosteroid feedback could involve cell bodies and/or pathways of afferent input to the CRF neurons of the paraventricular nucleus. Early experiments in adrenalectomized rats demonstrated a high uptake and nuclear retention of radiolabeled corticosterone in the limbic areas, primarily the hippocampus and septum (McEwen et al, 1968; McEwen et al, 1969; McEwen & Plapinger, 1970; Knizley, 1972; Lemaire et al, 1974), which could be blocked by pretreatment with corticosterone (Stevens et al, 1971) but not by progesterone (Rhees et al, 1975a; Warembourg, 1975a). The uptake of labeled dexamethasone shows a disimilar distribution, with the heaviest accumulation of label observed in the anterior pituitary, and less label appearing in the brain over the ventricles and around endothelial cells of small blood vessels (DeKloet et al, 1975; Rhees et al, 1975b; Warembourg, 1975b; McEwen et al, 1976).

Studies of pituitary and hippocampal cytosol steroid binding imply that more than one population of corticosteroid-binding sites exist in these tissues. In the anterior pituitary, binding to cell nuclei is greater for dexamethasone than for corticosterone, while the opposite is true for binding to cytosolic macromolecules (DeKloet et al, 1975). Dexamethasone weakly competes for corticosterone bound to the cytosolic corticosterone-binding macromolecule in the pituitary, which resembles corticosterone-binding globulin (CBG) with respect to its physico-chemical properties and lack of affinity for DNA (DeKloet & McEwen,

1976a). Recently, CBG has been localized in the pituitary by immunocytochemistry (DeKloet et al, 1984).

When corticosteroid nuclear binding was studied following administration of dexamethasone or corticosterone in vivo, a stringent receptor specificity for corticosterone in hippocampus was revealed (DeKloet et al, 1975). When hippocampal cytosol corticosteroid binding is studied in vitro this specificity is abolished, and dexamethasone and corticosterone compete equally well in the displacement of bound dexamethasone (DeKloet & McEwen, 1976b). Thus, there appears to be more than one population of corticosteroid binding species in the hippocampus, as well as in the pituitary.

The significance of these hippocampal binding sites is a controversial area and has recently been reviewed (McEwen et al, 1986). The study of brain corticosteroid binding has been accelerated by the development of highly specific antiglucocorticoids which do not bind tc CBG and have high affinities for glucocorticoid-preferring sites (Chrousos et al, 1983). With these compounds, the binding specificities of two corticosteroid binding sites in hippocampal cytosol have been studied. The dexamethasone-preferring site, called the Type II, glucoccrticoid receptor (GR), has a high affinity for dexamethasone (~ 3nM) and a lower affinity for corticosterone (~10nM). When the synthetic antiglucocorticoid RU26988 is added to hippocampal cytosol, about 50% of the corticosterone binding sites remain (Veldhuis et al, 1982). These corticosterone-preferring, or Type I sites (CR), have a high affinity for corticosterone (<1nM) and a lower affinity for dexamethasone (~10nM). This remaining site also has a high affinity for

aldosterone (<1nM), and for deoxycorticosterone, and is believed to be a brain mineralocorticoid receptor (Krozowski & Funder, 1983; Coirini et al, 1983; Beaumont & Fanestil, 1983). Whether there are separate corticosterone- and aldosterone-preferring binding sites in hippocampus, in addition to GR, is not presently known.

Using monoclonal antibodies to the GR purified from rat liver cytosol, Fuxe and co-workers have localized GR-immunoreactive nerve cells in rat brain in the parvocellular paraventricular and anterior periventricular nuclei, ventral mediobasal hypothalamus, hippocampal regions CA1 and CA3, as well as in the catecholamine- and indolamine-containing cell groups of the lower brain stem, substantia gelatinosa of the spinal cord, spinal trigeminal nucleus and nucleus of the solitary tract (Fuxe et al, 1985a,b). In the paraventricular nucleus, GR are located in the majority of the CRF immunoreactive neurons (Agnati et al, 1985).

Recently, the regional distribution of GR and CR binding sites in rat brain has been determined (Reul & DeKloet, 1985). CR are located predominately in the subiculum and CA1 region of the hippocampus and in the dentate gyrus, and to a lesser extent in the CA3 region and ventral hippocampus and in the lateral septum. There is a paucity of CR in the paraventricular nucleus of the hypothalamus. In contrast, GR are localized in the nucleus of the solitary tract, central and cortical amygdalae, locus coeruleus, and paraventricular hypothalamic nuclei, as well as in the dentate gyrus and lateral septum, and conspicuously absent from the subiculum and CA1 regions of the hippocampus. For the most part, there is good agreement between the localization of GR by

cytosolic binding and monoclonal antibody-binding techniques.

CR and GR are also differentially occupied by increasing plasma cortcosterone levels; at approximately 1µg/dl corticosterone (normal morning levels), CR are 80% occupied while GR are only 10% occupied. At high corticosterone levels, normally observed during stress or briefly at the time of the circadian peak in plasma corticosterone, there is not much further occupation of CR, while GR are about 70% occupied (Reul & DeKloet, 1985). The significance of these binding data with respect to the regulation of ACTH secretion remains to be elucidated.

Electrical stimulation of the hippocampus causes an initial facilitation, followed by a delayed inhibition, of basal plasma corticosterone levels (Casady & Taylor, 1976). The hippocampal efferents from CA1-4, the regions with the highest density of corticosterone receptors (Reul & DeKloet, 1985), do not project directly to the hypothalamus, but end primarily in the septum (Swanson & Cowan, 1979). Fibers in the fornix which innervate the hypothalamus arise largely from the subiculum. The ventral subicular region gives rise to the fibers of the medial corticohypothalamic tract, which enters the region of the hypothalamus with the postcommissural fornix (Palkovits & Zaborsky, 1980). The majority of these fibers terminate within the rostral arcuate nucleus after passing through the medial retrochiasmatic area. Ventral hippocampectomy and fornix transection reduces the corticosteroid response to several stresses (Conforti & Feldman, 1976), and dexamethasone suppression of un-stimulated corticosterone levels and the plasma corticosterone response to ether stress is less in ventral and dorsal hippocampectomized animals (Feldman & Conforti, 1980). That

corticosteroids directly affect the function of cells in the hippocampus is inferred from observations that corticosteroids modulate the maximum velocity of GABA uptake in hippocampal synaptosomes (Miller et al, 1978), and corticosterone induces a decrease in the evoked population spike amplitude in hippocampal slices in vitro (Vidal et al, 1986).

Historically, the midbrain was one of the earliest sites to be investigated as an area of extrahypothalamic corticosteroid feedback, when it was observed that midbrain sectioned rats did not undergo adrenal ascorbic acid depletion in response to unilateral adrenalectomy or unilateral adrenalectomy plus laparotomy (Martini et al, 1960), and that lesions of the reticular formation also block compensatory adrenal hypertrophy following unilateral adrenalectomy (Sen & Sarangi, 1967). Cortisol or dexamethasone implants in the reticular formation decrease systemic (Corbin et al, 1965) and adrenal venous blood corticosteroid content (Endroczi et al, 1961), and decrease the ACTH response to a novel environment (Bohus et al, 1968), but do not inhibit the compensatory adrenal growth in response to unilateral adrenalectomy (Bohus & Endroczi, 1964). More recently, it has been reported that the laparotomy-induced increases in plasma ACTH can be blocked by concomitant brainstem stimulation in the region near the locus subcoeruleus (Rose et al, 1976).

Finally, the region of the amygdala has also been considered as an area of extrahypothalamic corticosteroid feedback. Electrical stimulation of the baso-medial region of the amygdaloid complex increases, while stimulation of the lateral amygdala results in a decrease in the corticosteroid content of adrenal venous effluent (Slusher & Hyde, 1961;

Redgate & Fahringer, 1973). McHugh and Smith also showed that amygdaloid stimulation increases plasma corticosteroid levels, and that this increase could be blocked by (systemic) pretreatment with cortisol (McHugh & Smith, 1967). Because these investigators observed that the same pretreatment did not inhibit the plasma corticosterone response to electrical stimulation of the tuberal hypothalamus, they concluded that the corticosteroid sensitive elements must be functionally located between the amygdaloid and hypothalamic areas stimulated. Amygdalectomy or lesions of the ventral amygdalo-hypothalamic pathway have been shown to block the corticosteroid response to leg break (Allen & Allen, 1974) and to sciatic nerve and olfactory stimulation (Feldman & Conforti, 1981), and to prevent adrenalectomy-induced ACTH hypersecretion (Allen & Allen, 1975).

Effects of CNS Lesions and Adrenalectomy

The techniques of CNS lesion placement and adrenalectomy have been employed by numerous investigators to study the operating characteristics of the adrenocortical system. With the isolation and purification of hypothalamic corticotropin-releasing factor (CRF; Vale et al, 1981), antisera were raised against this 41-amino acid peptide and the distribution of CRF-immunoreactive cells and fibers was determined (Swanson et al, 1983; Antoni et al, 1983b; Merchenthaler et al, 1984). CRF is now known to be synthesized in the cells in the parvocellular subdivisions of the paraventricular nucleus of the hypothalamus (PVN). The axons of these cells leave the PVN laterally, then turn ventrally in the lateral hypothalamus to approach the median

eminence in the lateral retrochiasmatic area, along the base of the brain. The vasopressin- and oxytocin-containing fibers of the magnocellular division of the PVN follow a similar route (Swanson & Sawchenko, 1983), but while the CRF-containing fibers terminate in the external zone of the median eminence, the vasopressinergic and oxytocinergic axons make up the internal zone of the median eminence and their terminations form the neurohypophysis.

A complete or anterior hypothalamic deafferentation knife cut usually begins behind the optic chiasm and extends to the mammillary region, functionally isolating the medial basal hypothalamus, including the median eminence, from the two main hypothalamic regulators of pituitary ACTH secretion, CRF and vasopressin. Halasz and colleagues were among the first to document the effects of partial and complete hypothalamic deafferentation on the adrenocortical system. Hypothalamic deafferentation results in diabetes insipidus, due to the transection of the supraoptico- and paraventriculo- hypophysial tracts (Halasz & Pupp, 1965), and the abolition of the normal diurnal plasma corticosterone rhythm (Halasz et al, 1967). Anterolateral hypothalamic deafferentation has been shown to greatly reduce the bioactive (Makara et al, 1979) and immunoreactive CRF content of the median eminence (Tilders et al, 1982).

Adrenalectomy, the removal of corticosteroid feedback from all corticosteroid feedback sites <u>in vivo</u>, profoundly affects the activity of the adrenocortical system. There is an immediate increase in plasma ACTH which persists for about two hours, after which circulating ACTH falls to a level slightly higher than that observed in sham operated controls. By 48 hours after adrenalectomy, plasma ACTH levels are

markedly elevated (Dallman et al, 1972; Buckingham & Hodges, 1974), and remain so for as long as the animal lacks corticosteroids. Pituitary ACTH content drops soon after adrenalectomy, returning to and then surpassing the levels of sham operated controls by 5-7 days after adrenalectomy (Gemzell et al, 1951). This triphasic response of pituitary ACTH content has been interpreted to indicate that, immediately following adrenalectomy, ACTH is secreted from the pituitary more rapidly than new peptide can be synthesized, resulting in a temporary depletion of pituitary ACTH stores. The observation that pituitary ACTH content continues to rise in the presence of elevated plasma ACTH levels implies that the rate of ACTH synthesis has also been increased by adrenalectomy. The morphologic changes of the pituitary in response to adrenalectomy also imply that ACTH synthesis has been increased; by 24 hours after adrenalectomy the size of (morphologically-identified) "ACTH-cells" has increased, and by the fifth postoperative day there is also an increase in nuclear size (Siperstein & Miller, 1973). The secretory granulation of "ACTH-cells" is greatly reduced at 24 hours, and then gradually increases (Siperstein & Miller, 1973; Westlund et al, 1985). In addition to an increase in corticotrope size, there is also immunocytochemical evidence for an increase in the number of ACTH-producing cells (Rappay & Makara, 1981). Pituitary POMC mRNA levels increase with adrenalectomy (Nakanishi et al, 1977; Schachter et al, 1982; Birnberg et al, 1983), as does the rate of POMC gene transcription (Birnberg et al, 1983; Eberwine & Roberts, 1984). It has recently been shown, using <u>in situ</u> hybridization techniques and complementary RNA probes for both heterologus nuclear and mature

messenger RNA for POMC, that adrenalectomy increases both the number of ACTH-synthesizing cells in the anterior pituitary, and the rate of ACTH synthesis within these cells (Fremeau et al, 1986).

The response of CRF bioactivity of stalk-median eminence extracts to adrenalectomy is similar to that of pituitary ACTH content (Vernikos-Danellis, 1965). Intially there is a decrease, followed by a repletion after 6 to 24 hours to levels not different from sham operated controls. Increases in stalk-median eminence bioactive CRF content above control levels have been reported from 5 to 7 days after adrenalectomy (Vernikos-Danellis, 1965; Hillhouse & Jones, 1976). The changes observed for immunoreactive CRF in the median eminence following adrenalectomy do not precisely follow the changes in bioactivity; median eminence immunoreactive CRF is decreased below control levels as early as 3 hours and up to 2 days after adrenalectomy, and the level of immunoreactive CRF in the median eminence is not reported tc be higher than control levels until 2 weeks after adrenalectomy (Suda et al, 1983). Adrenalectomy enhances CRF immunostaining in the hypothalamus (Swanson et al, 1983; Antoni et al, 1983b). Recently it has been reported that CRF mRNA levels in the hypothalamus also increase after adrenalectomy (Jingami et al, 1985; Young et al, 1986).

The differences between hypothalamic bio- and immuno-active CRF profiles following adrenalectomy probably stem from the ability of several peptides in addition to CRF to act as ACTH secretogogues. Vasopressin has long been considered to be an important regulator of ACTH secretion (McCann & Brobeck, 1954; McDonald & Weise, 1956), and has been shown to potentiate CRF-induced ACTH secretion both <u>in vivo</u> (Yates

et al, 1971; Rivier & Vale, 1983; DeBold et al, 1984; Rivier et al, 1984) and *in vitro* (Rivier et al, 1984; Knepel et al, 1984). The amount of electrical- or K^+-stimulated vasopressin release from the medial basal hypothalamus or isolated median eminence *in vitro* is greater from tissues taken from adrenalectomized rats (Knepel et al, 1984; Holmes et al, 1986). In response to adrenalectomy there is an increase in vasopressinergic fibers in the zona externa of the median eminence (Watkins et al, 1974; Stillman et al, 1977; Zimmerman et al, 1977), and the novel appearance of vasopressin immunostaining (Tramu et al, 1983; Sawchenko et al, 1984; Kiss et al, 1984), and vasopressin mRNA (Davis et al, 1986), in the parvocellular neurons of the PVN. The observation that CRF and vasopressin are contained in the same neurosecretory vesicles in terminals of the external zone of the median eminence after adrenalectomy (Whitnall et al, 1985) strongly supports the notion that vasopressin is secreted from terminals in the external zone of the median eminence and may act on the corticotropes of the anterior pituitary via the hypophysial portal vessels.

In every case, the effects of adrenalectomy have been shown to be reversible by glucocorticoid therapy. It is less clear, however, where glucocorticoids act in order to reverse these adrenalectomy-induced events. For example, it is likely that the increase in ACTH secretion following adrenalectomy results from an increase in CRF secretion into the hypophysial portal vessels, and not from the direct removal of corticosteroid feedback from the pituitary *per se*, since the compensatory hypersecretion of ACTH following adrenalectomy does not occur in animals that have undergone anterolateral hypothalamic

deafferentation (Allen et al, 1974). The increase in anterior pituitary POMC mRNA after adrenalectomy is also likely to be the result of increased CRF secretion from the hypothalamus. CRF has been shown to increase anterior pituitary POMC mRNA levels *in vivo* (Bruhn et al, 1984a) and *in vitro* (Affolter & Reisine, 1985), and, in rats whose anterior pituitary POMC mRNA content had been previously elevated by adrenalectomy, lesions of the paraventricular nuclei decreased anterior pituitary POMC mRNA (Bruhn et al, 1984b). Analogously, the increase in CRF synthesis and secretion after adrenalectomy may result from the removal of corticosteroid feedback from the CRF-producing neurons of the hypothalamus, or from cell bodies or fibers of afferent input to the paraventricular nuclei.

Rationale

While there is abundant evidence that corticosteroids may exert feedback effects at multiple levels in the adrenccortical system, there are few reports of the sensitivity of any possible feedback sites to physiologic increases in plasma corticosterone *in vivo*. The present studies were designed to determine the relative importance of corticosteroid feedback on the brain and anterior pituitary in the response of the adrenocortical system to adrenalectomy.

Lesions were made in order to isolate the pituitary *in vivo* from the normal hypothalamic influence and limit the site of corticosteroid feedback to the pituitary in lesioned animals. Rats were chosen for these studies because there are detailed stereotaxic atlases for rat brain microsurgery, facilitating histologic comparisons between subjects. Adrenalectomy was chosen as the stimulus for adrenocortical system activation because it is easily reproducible between animals. Some adrenalectomized rats were given pellets to fix their corticosterone levels, a technique we have extensively validated. Thus, both the site of corticosteroid feedback and the intensity of the corticosteroid feedback signal could be varied independently.

The specific aims of these studies were: 1) to characterize the effects of physiologic doses of corticosterone on plasma corticosterone and ACTH levels, and thymus gland and body weight in adrenalectomized rats, and 2) to determine the effectiveness of a range of corticosterone levels in inhibiting adrenalectomy-induced ACTH secretion in rats with only pituitary or with pituitary and brain corticosteroid feedback sites.

Chapter 2 - Characterization of the mathematical relationship between plasma ACTH and corticosterone in rats with constant corticosterone levels

Introduction

Corticosterone, the principle glucocorticoid in the rat, normally exhibits a marked diurnal rhythm. Plasma corticosterone levels are low in the morning, at the time of lights on in the animal room, when rats are at the end of their nocturnal active period. Plasma corticosterone levels begin to rise in the afternoon and peak around the time of lights off, when rats enter their noturnal period of eating, drinking and activity. Often, plasma corticosterone levels in intact rats are so low in the morning as to be indistinguishable from levels in adrenalectomized rats. Plasma ACTH also varies diurnally, although frequently this diurnal variation is not statistically significant. The marked circadian fluctuation in plasma corticosterone is accomplished by an increase in the sensitivity of the adrenal cortex to ACTH (Dallman et al, 1978; Kaneko et al, 1981), acting together with the (modest) evening increase in plasma ACTH. The circadian rhythm in ACTH is the result of circadian drive originating from the brain. Experimentally, lesions of the suprachiasmatic nuclei of the hypothalamus (SCN) have been shown to abolish the circadian rhythms in food and water intake and activity (Stephan & Zucker, 1972; Nagai et al, 1978), as well as in plasma and adrenal corticosterone (Moore & Eichler, 1972; Szafarczyk et al, 1979; Abe et al, 1979).

Because the studies presented in the following chapters relied

heavily on the use of corticosterone pellets to produce constant corticosterone levels, thereby avoiding the confounding effect of the adrenal response to ACTH stimulated by infusions of exogenous CRF, it was necessary to quantify the effects of constant corticosterone levels on the activity of the adrenocortical system. Rats were either sham adrenalectomized or adrenalectomized and given pellets of varying concentrations of corticosterone in cholesterol. Plasma ACTH and corticosterone levels were measured 5 days after pellet implantation, in the morning and evening. Two other corticosteroid-sensitive endpoints, body weight and thymus gland weight, were also measured. It has been well documented that adrenalectomy induces thymic enlargement (Dougherty, 1952), and that stress induces thymic atrophy (Selye, 1936).

Materials and Methods

Sixty adult male Sprague-Dawley rats (Bantin & Kingman, Fremont, CA) weighing 224-244g were housed two per cage in hanging wire basket cages in a temperature-, humidity-, and light-controlled room. Lights were on for 12 hours per day. Rats were provided with food and water ad libitum. Body weights and fluid intake were measured (to the nearest 2g) in the morning. Following adrenal surgery rats were provided with 0.5% saline for drinking fluid.

Following two days of baseline measurements of body weight and water intake, rats were sham adrenalectomized or adrenalectomized via the dorsal approach under ether anesthesia. At this time, adrenalectomized rats received pellets weighing approximately 100mg of wax (0% corticosterone pellets) or varying concentrations of

corticosterone in cholesterol (25, 50 or 75% corticosterone, by weight). Pellets were made by the method of Segaloff (1950), with modifications (Meyer et al, 1979; Akana et al, 1985). Briefly, a total of 1g of crystalline corticosterone and cholesterol (Sigma) were gently heated to a molten state over a bunsen burner. A warmed pasteur pipette was used to pipette the mixture into silicone molds (model 106, Ted Pella, Inc., Tustin CA) which had been previously coated lighlty with sterilized silicone diluent oil (General Electric RTV-910). The pellets were allowed to harden overnight, then removed, weighed, and cut down if necessary. The 0% corticosterone pellets were made of wax, rather than 100% cholesterol, because pure cholesterol pellets crumbled when removed from the molds. Pellets were inserted subcutaneously slightly rostral to the skin incision made for adrenalectomy, and the incisions were closed with wound clips.

On the evening of the fourth or the morning of the fifth day following adrenal surgery, rats were killed by decapitation. Trunk blood was collected into heparin-containing tubes and stored on ice until subsequent centrifugation and removal of plasma. Plasma ACTH was measured by radioimmunoassay (RIA) on 1ml extracted plasma samples (Dallman et al, 1974). At a working dilution of 1:7500, the ACTH antiserum cross-reacts 100% with $ACTH_{1-39}$, ACTH1-24 and ACTH11-24, <1% with β-MSH and ACTH25-39, and <.01% with ACTH1-19, $ACTH_{4-10}$ and α-MSH. Inter- and intra-assay coefficients of variation for the assay are 19.7 and 9.2%, respectively. Plasma corticosterone was also measured by RIA on 10µl heat-denatured plasma samples. The corticosterone antisera, at a working dilution of 1:10500, cross reacts 100% with

deoxycorticosterone and cortisol, 38% with progesterone, and <1% with testosterone, estradiol and dexamethasone. The intra- and inter-assay coefficients for the corticosterone assay were 10.8 and 11.1%, respectively. Thymus glands were collected from the group of rats killed in the morning. The glands were excised and placed in closed petri dishes on filter paper previously moistened with 0.9% non-sterile saline, and subsequently cleaned of fat and connective tissue and weighed (to the nearest milligram).

Data are presented as mean $\pm$ SE, and were analyzed by one- or two-way ANOVA (Zar, 1984). The ACTH and corticosterone values from pellet-bearing rats killed in the morning with plasma corticosterone levels of $\leq$6µg/dl were combined with similarly prepared rats with sham hypothalamic lesions (also with plasma corticosterone levels $\leq$6µg/dl and killed in the morning) to generate a data set of adequate size to quantitate the relationship between these two hormones. These individual data were ordered (ACTH values from animals with identical corticosterone levels were averaged) and subjected to a non-linear least squares analysis to determine the best fit to the Hill function: $Yx = Ak^p/(k^p + x^p)$. Here, "Y" and "x" represent the concentrations of ACTH and corticosterone in plasma, and "A", "k" and "p" are parameters which are estimated from the fitting. Only data with corticosterone levels $\leq$6µg/dl were used in order to optimize the fitting over the range of corticosterone values in which ACTH changes the most dramatically.

Results

Morning and evening plasma ACTH (in pg/ml) and corticosterone (in

μg/dl) measurements are shown in figure 2.1. In sham adrenalectomized rats, plasma corticosterone exhibited the expected diurnal rhythm. In adrenalectomized rats treated with 0% corticosterone pellets, plasma corticosterone levels were low. Plasma corticosterone levels increased with increasing corticosterone pellet concentrations. Plasma corticosterone levels in adrenalectomized rats treated with 50 and 75% corticosterone pellets were significantly different between morning and evening. Plasma ACTH levels also varied diurnally in sham adrenalectomized rats, although this trend was not significant. Plasma ACTH was significantly elevated in the morning and evening in adrenalectomized rats, with respect to sham operated controls, and adrenalectomized, non-replaced rats exhibited a significant rhythm in plasma ACTH. Treatment of adrenalectomized rats with 25% corticosterone pellets did not significantly reduce plasma ACTH levels in the morning or evening, while 50 and 75% corticosterone pellets restored morning and evening plasma ACTH to levels not statistically different from sham adrenalectomized rats at the same time of day.

The effect of these plasma corticosterone levels on plasma ACTH can be compared with their effects on the change in body weight (in grams per four days after adrenal surgery) and thymus gland wet weight (in milligrams per 100 grams body weight) in Table I. Adrenalectomized rats treated with 0, 25 or 75% corticosterone pellets gained significantly less weight than sham operated controls over the four days after adrenal surgery. Thymus gland wet weight increased with adrenalectomy, and was reduced by corticosterone replacement in a dose related manner. Thus, plasma corticosterone levels established by the 50% corticosterone

pellet were sufficient to restore morning and evening plasma ACTH levels to normal after adrenalectomy. Lower corticosterone levels were sufficient to normalize thymus gland wet weight, while no dose of corticosterone adequately restored body weight gain to normal in adrenalectomized rats.

The plasma ACTH data are plotted as a function of plasma corticosterone level for individual animals in figure 2.2. From this figure it is apparent that constant plasma corticosterone levels of 5μg/dl and higher are sufficient to normalize morning plasma ACTH levels in adrenalectomized rats to levels observed in sham operated rats (<50pg/ml). However, in the evening, these circulating corticosterone concentrations were not sufficient to normalize plasma ACTH to levels observed in sham operated rats ($\leq$100pg/ml).

In figure 2.3, the relationship between morning plasma ACTH levels and constant corticosterone concentrations is again illustrated. This figure includes the data from figure 2.2, as well as those of rats from a separate experiment which underwent sham hypothalamic lesions in addition to adrenalectomy and corticosterone pellet implantation. The curve superimposed on these data represents the best fit of the Hill function: $Y_{(x)} = Ak^p/(k^p+x^p)$. The parameters "A", "k" and "p" were estimated at: A=529$\pm$47 pg/ml (the theoretical plasma ACTH concentration when plasma corticosterone equals 0); k=1.6$\pm$.15 μg/dl (the concentration of corticosterone at which plasma ACTH levels are half-maximally reduced); p=3.8$\pm$1.2. The "p", or Hill coefficient, being significantly greater than 1 (by t-test), indicates that there is significant cooperativity to the inhibition of plasma ACTH by corticosterone.

Discussion

In this study, the effects of constant corticosterone concentrations on plasma ACTH, body weight gain, and thymus weight were measured. Plasma corticosterone concentrations of approximately 5μg/dl were sufficient to normalize morning plasma ACTH and thymus weight after adrenalectomy (table 1 and figure 2.1). By plotting the plasma ACTH and corticosterone values for individual rats (figure 2.2) it becomes apparent that constant corticosterone levels of up to 10μg/dl were not sufficient to normalize evening circulating ACTH levels after adrenalectomy to levels normally observed in sham adrenalectomized controls ($\leq$100pg/ml).

Previously, Akana et al (1985) showed that constant corticosterone levels of 4.5-7.4μg/dl adequately restore morning ACTH secretion and thymus gland weight after adrenalectomy. In that study (Akana et al, 1985), as well as in the present experiment (table 1) no dose of corticosterone completely restored the deficit in body weight gain induced by adrenalectomy. Previous data (Levin et al, 1984a,b) suggest that mineraloccrticoid treatment normalizes weight gain in adrenalectomized animals. The diurnal variations in plasma corticosterone in adrenalectomized rats receiving 50 and 75% corticosterone pellets remains unexplained. If constant plasma corticosterone levels influence the activity of hepatic steroid metabolism, then possibly there is a diurnal variation in the rate of corticosterone clearance or volume of distribution in rats with constant corticosterone levels. In a previous study a similar variation in plasma corticosterone levels was

reported in smaller rats treated with 40%, but not 10 or 20% corticosterone pellets (Akana et al, 1986). In intact rats, neither the clearance rate nor the volume of distribution of corticosterone varies between morning and evening (Kaneko et al, 1980).

Since this experiment was performed, the observation that constant corticosterone levels that are sufficient to maintain ACTH in the normal range in the morning are not adequate in the evening prompted us to undertake a detailed investigation of the phenomenon (Akana et al, 1986). In these studies we confirmed that there is a shift in the sensitivity of basal (morning), but not CRF- or histamine-induced ACTH secretion to inhibition by corticosterone between the times of the nadir (morning) and peak (evening) of the diurnal rhythm of activity in the adrenocortical system. We showed that this shift does not result from a change in the sensitivity of the corticotrope to CRF or from a change in the adrenocortical system sensitivity to histamine stress, and is unlikely to result from a change in the efficacy of corticosterone feedback as a function of the time of day. We proposed that the apparent shift in sensitivity of basal ACTH secretion to inhibition by corticosterone between the morning and the evening is the result of a shift in the feedback site from the pituitary in the morning, to the brain and the pituitary in the evening.

The least-squares analysis (figure 2.3) quantitates the relationship between morning plasma ACTH and constant circulating corticosterone. By factoring in a Hill coefficient greater than 1, the goodness-of-fit was increased by nearly 20%, indicating that cooperativity is a powerful aspect of the inhibition of adrenalectomy-

induced ACTH secretion by corticosterone. The results presented in the following chapters are evaluated against this whole-animal dose-response relationship.

Table I. Change in body weight (grams/4 days) and thymus gland wet weight (mg/100g body weight) in sham adrenalectomized (ShamADX) and adrenalectomized (ADX) rats with and without corticosterone (B) replacement

Group	% B pellet	Δ Body Weight	Thymus Weight
ShamADX	--	24 ± 2 (12)[1]	167 ± 14 (6)
ADX	0	11 ± 3 (12)*	253 ± 43 (6)*
ADX	25	11 ± 2 (12)*	224 ± 22 (6)*
ADX	50	12 ± 3 (12)	136 ± 29 (6)
ADX	75	-9 ± 3 (11)*	117 ± 17 (5)

[1]Numbers in parantheses refer to number of rats/group

*$p<.05$ versus ShamADX

Figure 2.1. Plasma ACTH (top, pg/ml) and corticosterone (bottom, µg/dl) in sham adrenalectomized (ShamADX) and adrenalectomized (ADX) rats with 0, 25, 50 or 75% corticosterone pellets. Bars represent mean values, and vertical lines represent SE. Open bars, morning samples; shaded bars, evening samples; N=6/group. Asterisks (*) indicate significant ($p<.05$) morning/evening difference. Daggers (†) indicate significant difference versus ShamADX at same time of day.

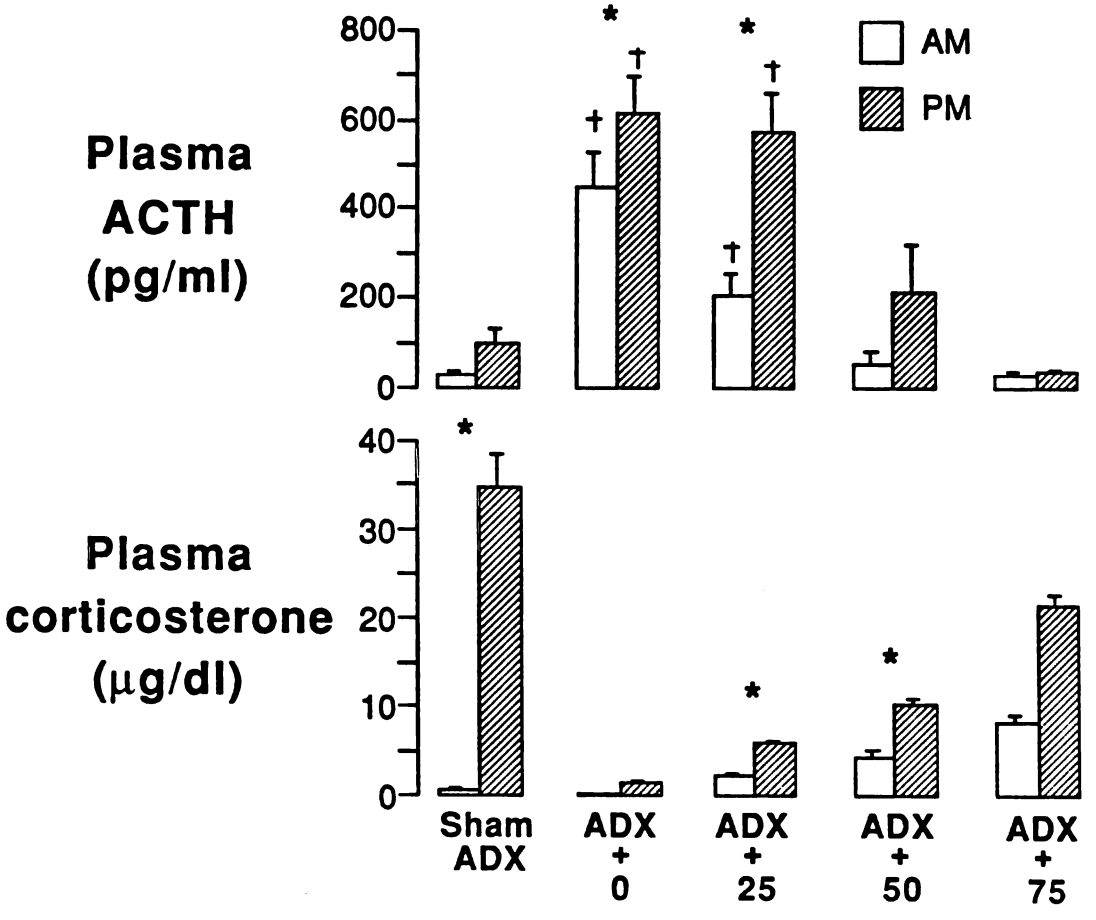

Plasma ACTH (pg/ml)
800
600
400
200
0
AM
PM
Plasma corticosterone (μg/dl)
40
30
20
10
0
Sham ADX
ADX + 0
ADX + 25
ADX + 50
ADX + 75

Figure 2.2. Plasma ACTH (pg/ml) as a function of plasma corticosterone (μg/dl) in adrenalectomized rats treated with pellets containing various concentrations of corticosterone. Squares represent evening samples; circles represent morning samples.

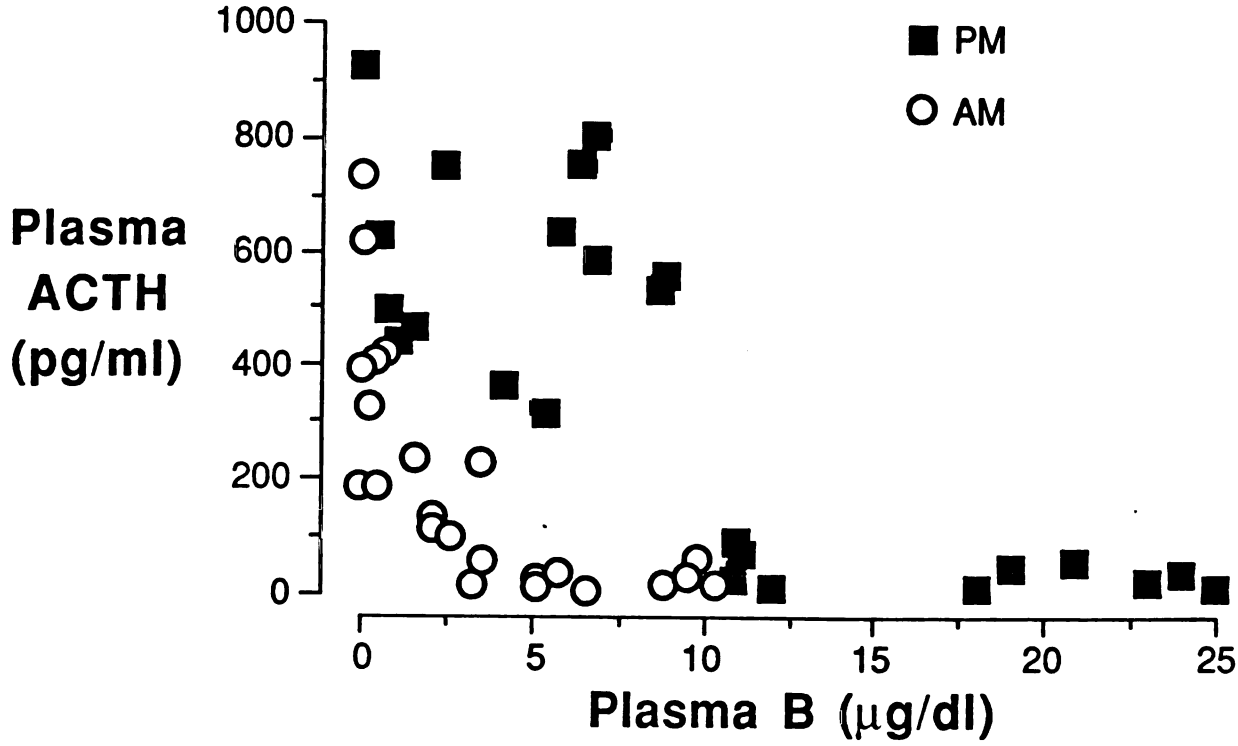
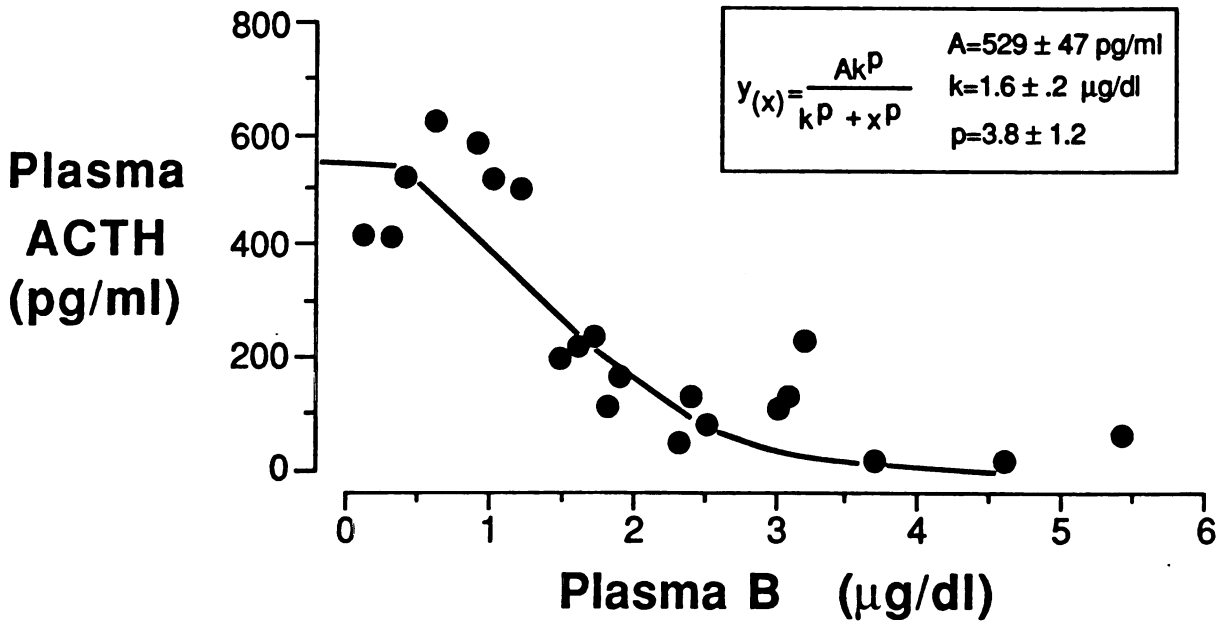

Plasma ACTH (pg/ml)
1000
800
600
400
200
0
PM
AM
0
5
10
15
20
25
Plasma B (µg/dl)

Figure 2.3. Non-linear least squares fit of Hill function to data from figures 2.2 and 4.1.


Plasma ACTH (pg/ml)
800
600
400
200
0
0
1
2
3
4
5
6
Plasma B (μg/dl)
$y_{(x)} = \frac{Ak^p}{k^p + x^p}$
A=529 ± 47 pg/ml
k=1.6 ± .2 μg/dl
p=3.8 ± 1.2

Chapter 3 - Corticosterone inhibition of plasma ACTH in rats with anterolateral hypothalamic deafferentations

Introduction

In the intact rat, adrenalectomy results in increases in ACTH synthesis and secretion (reviewed in chapter 1). In a previous study (Dallman et al, 1985) we attempted to quantitate the relative contribution of the removal of corticosteroid feedback from the pituitary *per se* to the ACTH response to adrenalectomy. Rats underwent anterior hypothalamic deafferentation; control rats were given sham lesions. Two days later half of the lesioned and sham lesioned rats were adrenalectomized, and half were sham adrenalectomized. The adrenalectomy-induced changes in plasma and anterior pituitary ACTH and anterior pituitary POMC mRNA were compared between sham lesioned animals (with brain and pituitary corticosteroid feedback sites) and lesioned animals (with only pituitary corticosteroid feedback sites). Since a previous study reported no increase in plasma ACTH in response to adrenalectomy following the removal of hypothalamic input (Allen et al, 1974), an additional set of lesioned rats (sham adrenalectomized and adrenalectomized) were given constant infusions of rCRF (10μg/d) beginning the day of adrenal surgery.

In sham lesioned rats, adrenalectomy resulted in a 12-fold increase in plasma ACTH, no change in pituitary ACTH content, and a 2.5-fold increase in pituitary POMC mRNA levels. In lesioned rats not receiving exogenous CRF, no increases in any of these variables were observed following adrenalectomy. In lesioned rats receiving exogenous CRF,

adrenalectomy was accompanied by a 2.2-fold increase in pituitary POMC mRNA, no change in pituitary ACTH content, and a 2.6-fold increase in plasma ACTH levels. The lack of an effect of the removal of corticosteroid feedback (adrenalectomy) in lesioned rats not receiving exogenous CRF led us to conclude that the corticotropes of the anterior pituitary cannot respond to adrenalectomy in the absence of hypothalamic drive. Because the increases in pituitary POMC mRNA were of a similar magnitude in the sham lesioned and lesioned plus CRF groups, we concluded that the increase in ACTH synthesis following adrenalectomy results from the removal of corticosteroid feedback from the pituitary, provided there is an increase in hypothalamic drive to the pituitary. The blunted increase in plasma ACTH following adrenalectomy in lesioned rats receiving exogenous CRF, with respect to sham lesioned rats, implied that the increase in plasma ACTH after adrenalectomy normally results from increased secretion of CRF, and an additional ACTH-releasing factor, that causes increased secretion but little synthesis of ACTH.

We were unable, however, to draw a strong conclusion as to the role of the pituitary <u>per se</u> in the ACTH response to adrenalectomy. As a consequence of the exogenous CRF infusion, plasma corticosterone levels in the sham adrenalectomized rats in the lesioned plus CRF group were >15μg/dl in the morning and evening. These high corticosterone levels produced significant thymic atrophy, with respect to sham lesioned controls, and indicated that the corticosteroid feedback signal in the lesioned plus CRF, sham adrenalectomized group was very different from that of the sham lesioned, sham adrenalectomized group. Consequently we

could not directly compare the effect of adrenalectomy between sham lesioned and lesioned plus CRF rats.

The present study made use of corticosterone pellets to fix the circulating corticosterone level in sham lesioned and lesioned rats in the range which normalizes body weight gain, thymus weight and plasma ACTH after adrenalectomy. The effect of the removal of a quantitatively similar corticosterone feedback signal could be compared between rats with only pituitary feedback and with brain and pituitary feedback. Because in the previous study the 10µg/d CRF infusion significantly elevated pituitary ACTH content, a lower dose (1µg/d CRF) was chosen for the present study. In order to investigate the role of an increase in vasopressin secretion into the hypophysial portal vessels following adrenalectomy, some lesioned rats received infusions of either 1µg/d vasopressin or 1µg/d each vasopressin and CRF.

Materials and Methods

Adult male Sprague-Dawley rats (Bantin and Kingman, Fremont, CA) were housed two per cage in hanging wire basket cages in a temperature-, light- and humidity-controlled room. Lights were on for 12 hours per day. Rats were provided with food and water *ad libitum*. After hypothalamic surgery, rats were housed singly. After adrenal surgery, rats were provided with 0.5% NaCl for drinking fluid. Fluid intake was measured daily, in the morning, to the nearest 2ml.

Anterolateral hypothalamic deafferentations or sham lesions were performed under pentobarbital anesthesia (50mg/kg, ip). Lesions were made with a Halasz knife with a radius of 1.8mm and a height of 2mm

(Dallman et al, 1985). The knife was lowered to the base of the skull on the midline, with the tip of the knife 1.3mm posterior to the bregma (Paxinos & Watson, 1982). Once the knife was in this position, the blade was rotated 90° and then drawn back 1.5mm, forming a posterior extension to the cut. This procedure was repeated on the opposite side of the brain, forming a symmetrical lesion. Sham lesions were made by lowering the knife to the base of the skull without rotating the blade.

Some of the lesioned animals markedly increased their water intake to compensate for the lesion-induced diabetes insipidus, while other lesioned rats consumed normal volumes similar to the sham lesioned controls (fluid intake of sham lesioned rats = 32±1ml/d, n=18). Lesioned rats consuming more than 90ml of water per day for the first two days after hypothalamic lesioning were considered to have complete lesions. Two days after hypothalamic surgery, lesioned rats which met this criterion, and all sham lesioned rats, were adrenalectomized under ether anesthesia. Lesioned rats consuming less than 90ml of water per day were removed from the study. Rats were provided with either wax or 40% corticosterone pellets, implanted subcutaneously slightly rostral to the adrenalectomy skin incision. At this time, lesioned rats were given miniosmotic pumps (Alza model 2001) preloaded to deliver either 1µg/d CRF, or 1µg/d vasopressin, or 1µg/d each vasopressin and CRF. The peptides were weighed out the day before the pumps were to be implanted, and dissolved in 1% acetic acid containing 10mg/ml bovine serum albumin and 1mg/ml ascorbate. The pumps were primed overnight at room temperature in 0.9% saline and were inserted subcutaneously adjacent to the corticosterone or wax pellet.

Five days after adrenal surgery (seven days after hypothalamic surgery), rats were decapitated under resting conditions within 2 hours of lights on. No more than 18 rats were killed on any day, and rats from each surgical group were included on each experiment day. Trunk blood was collected into heparin-containing tubes and stored on ice. After centrifugation, plasma was stored at -20C until subsequent measurement of ACTH and corticosterone by RIA. Thymus glands were excised and stored in closed petri dishes on filter paper saturated with 0.9% NaCl; the tissues were cleaned of fat and connective tissue and weighed to the nearest milligram.

Results

Thymus gland wet weights (in milligrams per 100 grams body weight) and plasma corticosterone levels (in µg/dl) are shown in Table II. Plasma corticosterone levels in adrenalectomized rats receiving wax (0% corticosterone) pellets were less than 1µg/dl. In adrenalectomized rats treated with 40% corticosterone pellets, plasma corticosterone levels ranged from 4.8-9.9µg/dl. Thymus gland weights were not different between sham lesioned and lesioned rats within corticosterone pellet treatment group.

The plasma ACTH results are plotted for each animal as a function of plasma corticosterone in figure 3.1. The stippled area in this figure indicates the relationship routinely observed between plasma ACTH and plasma corticosterone in the morning, mathematically derived in figure 2.3. Data from individual sham lesioned rats are illustrated by closed circles. In these rats, treatment with 40% corticosterone

pellets resulted in morning plasma ACTH levels in the normal range (<100pg/ml), while adrenalectomized rats not receiving corticosterone replacement had elevated plasma ACTH concentrations. Sham hypothalamic surgery appears to have no effect on the relationship between basal (morning) plasma ACTH and plasma corticosterone, because the ACTH levels in the sham lesioned rats in this experiment lie within the range observed in the previous experiment in non-lesioned rats.

Data from individual lesioned rats receiving 1µg/d vasopressin are illustrated by closed triangles. While this dose of vasopressin completely reversed the lesion-induced increase in fluid intake (data not shown), no stimulation of plasma ACTH was observed. All of the lesioned rats receiving 1µg/d vasopressin had plasma ACTH levels of <100pg/ml, irrespective of corticosterone treatment. In lesioned rats treated with either 1µg/d CRF (open circles) or 1µg/d each vasopressin and CRF (open triangles), plasma ACTH levels were elevated in both the 0% and 40% corticosterone pellet groups.

Discussion

In sham lesioned rats, adrenalectomy induced the expected increase in plasma ACTH. Treatment of sham lesioned, adrenalectomized rats with 40% corticosterone pellets reduced plasma ACTH to normal morning levels. When hypothalamic drive was replaced with a constant infusion of vasopressin, adrenalectomy did not result in an increase in plasma ACTH. This result was not unexpected, as vasopressin has been shown *in vitro* (Aguilera et al, 1983; Antoni et al, 1983a, Vale et al, 1983) and *in vivo* (Yates et al, 1971; Rivier & Vale, 1983) to be a relatively weak

ACTH secretogogue in the absence of CRF.

Unlike the findings in sham lesioned rats, circulating corticosterone levels of >5µg/dl did not normalize morning plasma ACTH levels in lesioned rats receiving CRF either alone or in combination with vasopressin. Because corticosterone could inhibit ACTH secretion only by an action on the pituitary in lesioned rats, this finding suggested that the pituitary is not the site at which corticosterone acts to inhibit adrenalectomy-induced ACTH secretion.

Corticosteroid feedback has been shown *in vitro* (Phillips & Tashjian, 1982) and *in vivo* (Kaneko & Hiroshige, 1978; Keller-Wood et al, 1984) to be exerted in a manner proportional to the dose of steroid administered, and independent of stimulus strength. If corticosterone acts at the pituitary to inhibit the plasma ACTH response to adrenalectomy, then the corticosterone concentration which suppressed the response to normal morning levels in sham lesioned rats would be expected to inhibit the same proportion of the plasma ACTH response to exogenous CRF (±vasopressin) in lesioned rats. Instead, no corticosteroid inhibition of plasma ACTH to exogenous ACTH-releasing factcrs was observed. These data suggested that the pituitary is less sensitive than the brain to corticosteroid feedback inhibition of the plasma ACTH response to adrenalectomy.

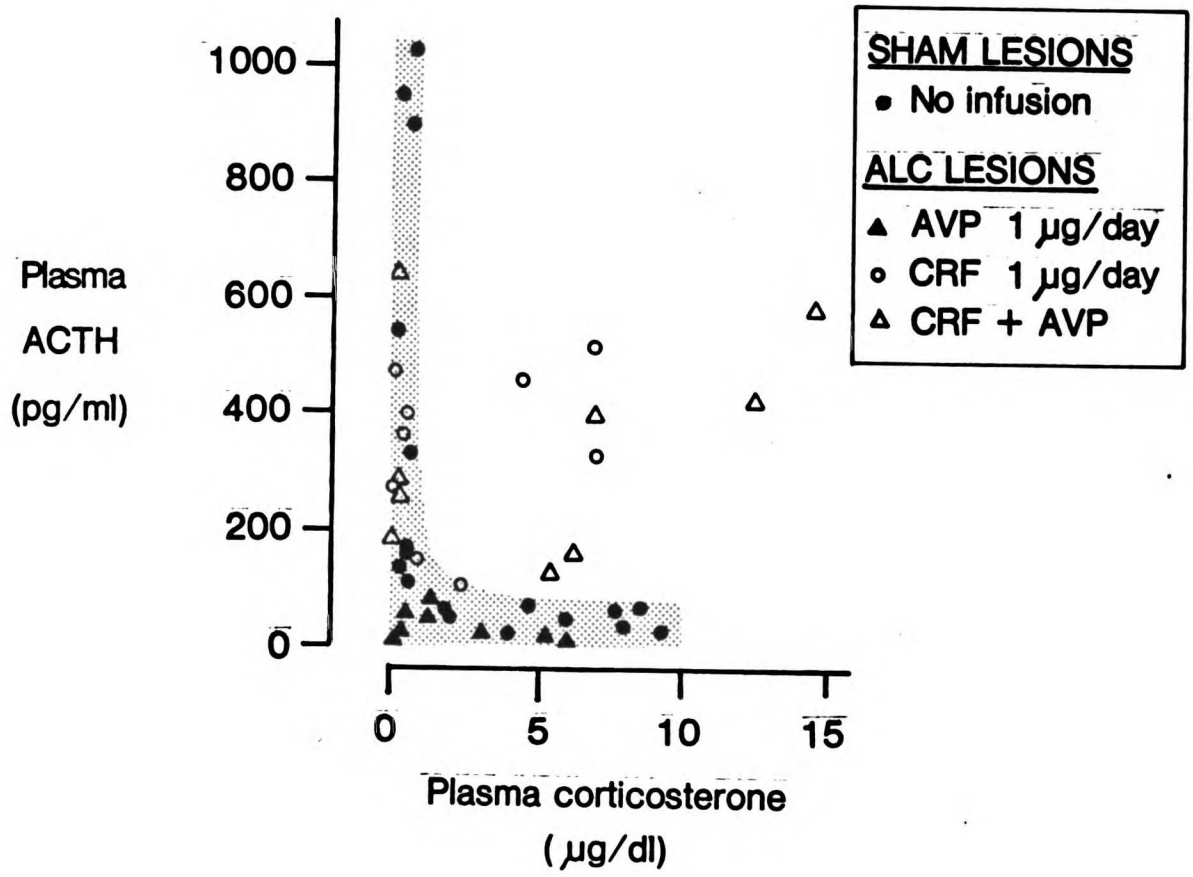
It is possible that the continuous infusions of releasing factors employed in this and the following experiments have altered the sensitivity of the corticotropes either to stimulation by CRF or to corticosteroid feedback. With respect to the first of these considerations, it has been shown that continuous exposure of

pituitaries to CRF *in vitro* results in decreases in CRF binding and ACTH responses to CRF (Holmes et al, 1984; Hoffman et al, 1985). *In vivo*, adrenalectomy is accompanied by a similar decrease in CRF binding to the pituitary, presumably due to an increase in CRF secretion (Wynn et al, 1985). At the same time, pituitaries from adrenalectomized rats are more sensitive to CRF when tested *in vitro* (Wynn et al, 1985). Prolonged stimulation with CRF results in sustained release of ACTH from neonatal rat pituitary explant cultures (Sato & Mains, 1986) and primary pituitary cell cultures (Vale et al, 1983), and elevated ACTH levels *in vivo* in man (Schopohl et al, 1986) and in rats (Rivier & Vale, 1985). Therefore, although desensitization to CRF is readily demonstrable *in vitro*, the *in vivo* evidence indicates that ACTH secretion continues despite chronic CRF stimulation. There are no reports in the literature which have addressed the question of a change in corticotrope sensitivity to corticosteroid feedback as a consequence of prolonged, continuous hypothalamic stimulation.

Table II. Thymus gland wet weights (mg/100g body weight) and plasma corticosterone (B) levels in lesioned (ALC) and sham lesioned rats

Surgical Group (N)	% B pellet	Plasma B (µg/dl)	Thymus Weight
Sham ALC (9)	0	0.6 ± 0.1	240 ± 25
" (9)	40	5.8 ± 0.9	154 ± 12
ALC + AVP (5)	0	0.8 ± 0.3	219 ± 15
" (3)	40	4.8 ± 0.9	164 ± 16
ALC + CRF (5)	0	0.4 ± 0.2	216 ± 18
" (4)	40	5.2 ± 1.1	145 ± 15
ALC + CRF+AVP (4)	0	0.3 ± 0.1	223 ± 50
" (5)	40	9.9 ± 2.2	156 ± 20

Figure 3.1. Plasma ACTH (pg/ml) as a function of plasma corticosterone (µg/dl) in sham lesioned (closed circles) and lesioned (anterolateral hypothalamic deafferentation, ALC) rats, 5 days after adrenalectomy and 0 or 40% corticosterone pellet implantation. At the time of adrenalectomy, lesioned rats received continous infusions of either 1µg/d CRF (open circles), 1µg/d vasopressin (closed triangles), or 1µg/d CRF and 1µg/d vasopressin (open triangles). Stippled area indicates the relationship between plasma ACTH and corticosterone in the morning in non-lesioned rats (see figure 2.3).


SHAM LESIONS
No infusion
ALC LESIONS
AVP 1 µg/day
CRF 1 µg/day
CRF + AVP
Plasma ACTH (pg/ml)
1000
800
600
400
200
0
0
5
10
15
Plasma corticosterone (µg/dl)

Chapter 4 - Corticosterone inhibition of CRF-stimulated ACTH secretion in rats with medial basal hypothalamic lesions

Introduction

The results of the previous experiment (Chapter 3) suggested that the pituitary is less sensitive than the brain with respect to corticosteroid feedback following adrenalectomy. Because no inhibition of ACTH release was observed with plasma corticosterone levels of 5-10μg/dl in the previous study, a wider range of corticosterone levels was tested in sham lesioned and lesioned rats in the present experiment. The normal hypothalamic input to the corticotropes was removed by large medial basal hypothalamic lesions, because this procedure yields a higher percentage of complete lesions than the more selective deafferentation procedure previously employed. Again, subcutaneous implants of corticosterone were used to fix the feedback level in sham lesioned and lesioned rats. In the present experiment a wide range of doses of CRF was employed, to better characterize the effectiveness of continuous subcutaneous infusions of CRF.

Materials and Methods

Adult male Sprague-Dawley rats (Bantin & Kingman, Fremont, CA) were housed two per cage in hanging wire basket cages in a temperature-, humidity- and light-controlled room, with food and water available *ad libitum*. Lights were on for 12 hours per day. Following hypothalamic surgery, rats were housed singly. Following adrenalectomy and pellet implantation, rats were provided with 0.5% saline for drinking fluid.

Body weights were recorded daily, in the morning.

For hypothalamic lesions, rats were anesthetized with pentobarbital (50mg/kg, ip) and mounted in a stereotaxic apparatus. The medial basal hypothalamus (MBH) was destroyed using a triangular Halasz-type knife with a width at the base of 3.5mm (Kartezi et al, 1982). The knife was lowered to the base of the skull on the midline with the base parallel to the midline and its most rostral tip 1.2mm posterior to the bregma (Paxinos & Watson, 1982). Once lowered to the base of the skull, the knife was rotated 360° twice in each direction. Sham lesions consisted of lowering the knife to the level of the thalamus without rotating the blade.

Two days after hypothalamic surgery, rats were adrenalectomized via the dorsal approach under ether anesthesia. Adrenal glands were stored in closed petri dishes on filter paper moistened with 0.9% saline; glands were subsequently cleaned of fat and connective tissue and weighed. Rats were provided with either wax pellets or pellets consisting of 20, 40 or 80% corticosterone, by weight, inserted subcutaneously slightly rostral to the skin incision. At this time, lesioned rats received mini-osmotic pumps preloaded to deliver either 1µg/d of an inactive fragment of rCRF (CRF_{3-41}) or .05 or 5µg/d synthetic rCRF41. The peptides were the generous gift of Dr. Jean Rivier of the Salk Institute, La Jolla, CA. Peptide diluent and pump priming and insertion procedures were as previously described.

Five days after adrenalectomy (7 days after hypothalamic surgery), rats were decapitated under resting conditions (within 30sec of cage opening) within 3h of lights on. Rats from each surgical and steroid

treated group were included on each day, and no more than 20 rats were killed on any day. Trunk blood was collected and processed as previously described for subsequent measurement of plasma ACTH and corticosterone by RIA. Pituitary glands were rapidly removed and anterior and neurointermediate lobes dissected. One-half anterior pituitary was homogenized in 1ml 0.1N HCl and stored at -20C for subsequent determinations of pituitary ACTH (by RIA) and protein content (Lowry et al, 1951). Thymus glands were removed, cleaned and weighed. Brains from lesioned rats were removed and fixed in neutral-buffered formalin (10%).

MBH lesions were verified on frozen sections of fixed material made in a cryostat. Brains were sectioned coronally in 40μm sections from the anterior commissure to the mammillary bodies and every fifth section was mounted and thionin stained.

Data were analyzed by one- and two-way ANOVA, or by Kruskal-Wallis nonparametric one-way analysis of variance, if variances were found to be heterogeneous by Bartlett's test (Zar, 1984). Multiple comparisons were made only when trends were found to be significant ($p<.05$) by the appropriate analysis of variance. Parametric multiple comparisons were made using Newman-Keuls' multiple range test, while nonparametric comparisons were made by the method of Dunn (Zar, 1984).

Results

Lesions of the MBH resulted in extensive damage to the hypothalamus. The greatest extent of the lesions was observed approximately 2mm posterior to the bregma, as noted by a hole

approximately 2mm wide and 1.5mm high centered on the midline, and the complete absence of the retrochiasmatic and anterior hypothalamic areas. The suprachiasmatic, arcuate and ventromedial nuclei were also damaged. In only 7 of the 40 brains for which data are reported was any portion of the median eminence intact, and in all cases this tissue was observed at least 3.3mm posterior to the bregma. Upon removal, the pituitaries of MBH lesioned rats appeared small and were often overlaid by blood clots. That the lesion disrupted the normal hypothalamic input to the pituitary was reflected in a signifcant reduction in adrenal gland wet weight in lesioned animals with respect to sham lesioned controls, 48 hours after lesion placement (adrenal weight in mg/100g body weight: Sham lesioned = 15.2±1.0; MBH lesioned = 9.8±0.2).

Body weight, plasma corticosterone level and thymus gland weight data are shown in Table III. Behaviorally, MBH lesioned rats were much more aggresive than sham lesioned controls. Lesioned rats required special handling at all times, and it was necessary to wear a protective glove in order to decapitate these rats. Sham lesioned rats did not require the use of a protective glove, and were always killed before lesioned rats, to prevent the excessive noise made by lesioned rats from disturbing the basal measurements in sham lesioned rats. Lesioned rats appeared hyperphagic, and were often found eating and drinking during the period of lights on, however, body weight was not significantly affected by hypothalamic surgery or adrenalectomy and corticosterone replacement (Table III). Plasma corticosterone levels in adrenalectomized, non-replaced rats were approximately 1μg/dl. Plasma corticosterone levels in the 20 and 40% corticosterone pellet groups did

not differ between sham lesioned and lesioned rats. The 80% corticosterone pellet resulted in a significantly higher corticosterone level in lesioned rats receiving 5µg/d CRF than in sham lesioned rats or lesioned rats receiving .05µg/d CRF.

Thymus gland weights in sham lesioned rats receiving 40% corticosterone pellets agree well with those previously reported in rats of similar body weight that had undergone sham hypothalamic lesioning and sham adrenalectomy (Dallman et al, 1985). In all three surgical groups, thymus glands were enlarged in the 0 and 20% corticosterone pellet groups with respect to the 40% corticosterone pellet group. The 80% corticosterone pellet treatment resulted in significant thymic atrophy in all three surgical groups.

In sham lesioned rats, ACTH levels were increased by adrenalectomy and reduced by corticosterone in a dose-related manner (Figure 4.1, upper left). The 20% corticosterone pellet reduced plasma ACTH, while the 40% corticosterone pellet restored plasma ACTH to normal morning levels. Plasma ACTH levels in sham lesioned rats receiving 80% corticosterone pellets were slightly but not significantly lower than those in the 40% corticosterone pellet group.

Pituitary ACTH concentration was also increased by adrenalectomy in sham lesioned rats, and was equally elevated in the 20% corticosterone pellet group (Figure 4.1, lower left). The 40% corticosterone pellet reduced pituitary ACTH concentration to levels normally observed in animals with adrenals (see chapter 5), whereas the 80% corticosterone pellet induced a slight but not significant further reduction. Total pituitary protein content was not affected by corticosterone over this

range of constant corticosterone concentrations.

In MBH lesioned rats receiving .05μg/d CRF, no increases in plasma or pituitary ACTH concentrations were observed following adrenalectomy (Figure 4.1, upper and lower right, broken line). Plasma and pituitary ACTH concentrations in these rats were not different from those in similarly lesioned rats receiving 1μg/d inactive CRF_{3-41} (data not shown). Plasma and pituitary ACTH concentrations in all lesioned rats receiving .05μg/d CRF or 1μg/d inactive CRF_{3-41} were not different from the levels observed in sham lesioned rats treated with 40% corticosterone pellets. Total pituitary protein content did not vary over this range of constant corticosterone concentrations. However, the pituitaries from lesioned rats receiving .05μg/d CRF contained significantly less protein than did the glands of sham lesioned rats (pituitary protein in μg/hemipituitary: Sham MBH = 625 $\pm$ 22; MBH lesion plus .05μg/d CRF = 368 $\pm$ 27).

The 5μg/d CRF dose significantly elevated plasma ACTH in adrenalectomized rats. In these lesioned rats, plasma ACTH levels were elevated in the 20 and 40% corticosterone pellet groups to a level similar to the 0% corticosterone pellet group (Figure 4.1, upper right, solid line). Only with the 80% corticosterone pellet treatment was plasma ACTH restored to normal morning levels. Neither pituitary protein content nor pituitary ACTH concentration (Figure 4.1, lower right, solid line) were significantly affected by corticosterone over this range of constant corticosterone concentrations. Although pituitary ACTH concentration was the same in the sham lesioned and MBH lesioned plus 5μg/d CRF groups, pituitaries from these lesioned rats

contained less total protein than did those of the sham lesioned controls (MBH lesion plus 5µg/d CRF = 370 ± 30µg protein/hemipituitary).

Discussion

In this study, the efficacy of a wide range of constant corticosterone levels in restoring plasma and pituitary ACTH to normal after adrenalectomy, in rats with and without large hypothalamic lesions was evaluated. These MBH lesions isolate the pituitary *in vivo* from the influence of corticotropin-releasing factors of hypothalamic origin. In lesioned rats, corticosterone feedback effects on plasma and pituitary ACTH concentrations can be exerted only at the pituitary. The results from lesioned rats are compared with those from similarly treated, sham lesioned controls, in which corticosteroid feedback is exerted at the brain and anterior pituitary. The concentration of corticosterone which normalized plasma and pituitary ACTH in sham lesioned rats was found to be insufficient in lesioned rats.

The corticosterone pellet compositions in this study were chosen to reflect the normal operating conditions of the adrenocortical system. All of the plasma corticosterone levels in Table III are found at some time during the course of the normal circadian excursion in plasma corticosterone in the rat. The important issue is not only the plasma corticosterone measurement at the time the rats were killed, but the constant nature of these corticosterone levels. In sham lesioned and lesioned rats, the 40% corticosterone pellet resulted in constant plasma corticosterone levels previously described (Akana et al, 1985) and independently validated (chapter 2) to restore thymus weight and morning

plasma ACTH after adrenalectomy. Plasma and pituitary ACTH levels were restored to normal in sham lesioned rats treated with 40% corticosterone pellets (Figure 4.1).

The significantly higher plasma corticosterone level in the MBH lesioned group receiving 5µg/d CRF with respect to the other two surgical groups is without explanation. Previously, acute MBH lesions have been shown to decrease the rate of corticosterone clearance and its volume of distribution (Kaneko et al, 1980). Since this effect was observed in the MBH lesioned group receiving 5µg/d CRF, but not in the group receiving either .05µg/d CRF, it is more likely that the elevated plasma corticosterone in the lesioned group receiving 5µg/d CRF is the result of the CRF infusion, rather than the MBH lesion.

Despite the thymic atrophy induced by the 80% corticosterone pellet regimen, no further suppression of plasma or pituitary ACTH was observed in sham lesioned rats with this (excessive) corticosterone replacement. We have previously suggested that the circadian nadir of adrenocortical system activity (in the morning for the rat) reflects the absence of stimulatory hypothalamic input to the anterior pituitary at this time of day (Dallman et al, 1987). The lack of a further reduction of morning plasma ACTH levels in sham lesioned rats treated with 80% corticosterone pellets is consistent with the recognized lack of inhibiton by corticosterone of non-stimulated ACTH release from pituitaries <u>in vitro</u> (Fleischer & Vale, 1968; Sayers & Portanova, 1974; Phillips & Tashjian, 1982; Widmaier & Dallman, 1984). Arguing against this conclusion is the observation that the plasma ACTH level in MBH lesioned rats not treated with exogenous CRF was been shown to be reduced by treatment with

dexamethasone (Dallman et al, 1987). This result may, however, reflect the differential sensitivy of the pituitary to the synthetic as opposed to the endogenous corticosteroid.

Plasma ACTH levels in lesioned rats receiving 1µg/d inactive CRF_{3-41} or .05µg/d CRF were not elevated in response to adrenalectomy. These results add further support to our previous conclusion (Dallman et al, 1985) that the corticotropes of the anterior pituitary cannot respond to the removal of the corticosteroid feedback signal (adrenalectomy) in the absence of hypothalamic drive. In addition, the basal (non-CRF stimulated) output of the isolated pituitary in vivo appears not to be steroid suppresible, since treatment of lesioned rats receiving either the inactive peptide or a low dose of CRF with 80% corticosterone pellets did not further reduce plasma ACTH levels. It is possible that higher doses of corticosterone, or dexamethasone, could accomplish such inhibition of basal secretion.

When lesioned rats were treated with continous infusions of 5µg/d CRF, plasma corticosterone levels of approximately 6µg/dl did not reduce plasma ACTH to normal morning levels. Only when plasma corticosterone levels were elevated to the range that induced significant thymic atrophy was corticosteroid feedback observed at the pituitary. These data are consistent with those of the previous experiment, and argue strongly that corticosteroid feedback, with respect to the restoration of plasma and pituitary ACTH after adrenalectomy, is exerted at the level of the brain and not the pituitary.

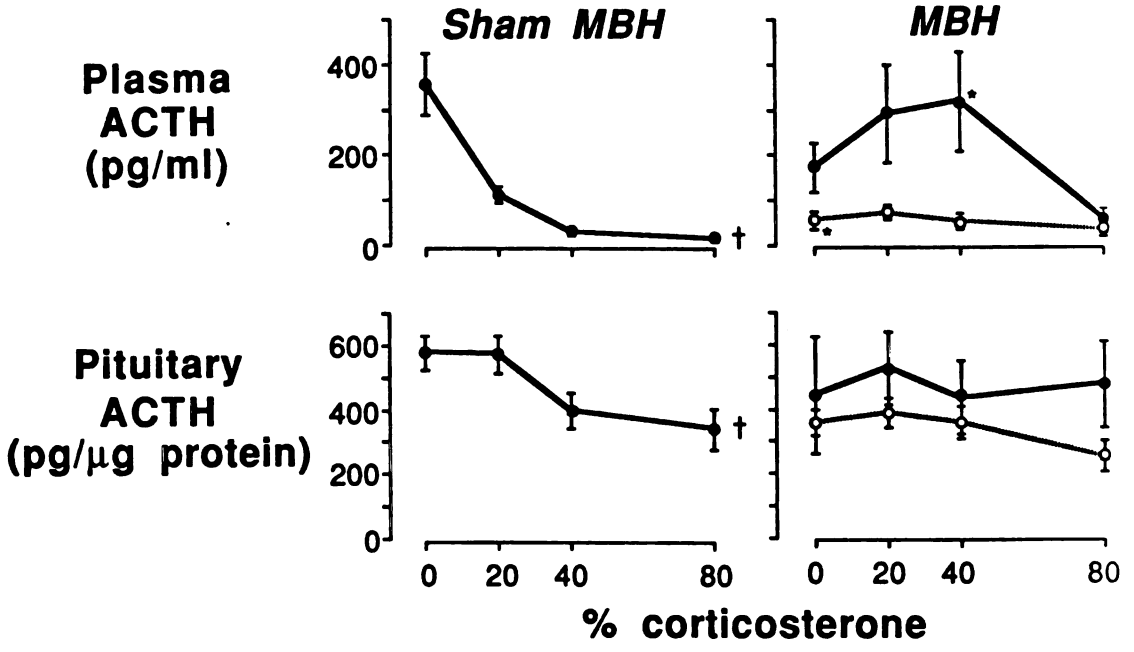
Table III. Body (BW) and thymus gland weight and plasma corticosterone (B) levels 7 days after hypothalamic surgery and 5 days after adrenalectomy with and without corticosterone replacement

Surgical Group	BW (g)	Plasma B (µg/dl)	Thymus Weight (mg)
Sham MBH (n=8/gp)			
0% B pellet	190±6	1.2±0.2	577±32
20% B pellet	204±9	2.5±0.2	599±26
40% B pellet	205±10	7.5±0.8	418±20
80% B pellet	190±4	13.9±1.4	115±20
MBH + .05µg/d CRF (n=5/gp)			
0% B pellet	195±8	0.5±0.2	536±31
20% B pellet	206±16	3.2±0.4	548±59
40% B pellet	195±13	7.6±0.9	275±34**
80% B pellet	204±25	17.5±3.2	101±28
MBH + 5µg/d CRF (n=5/gp)			
0% B pellet	198±6	0.3±0.1	485±35**
20% B pellet	215±10	2.1±0.4	465±33**
40% B pellet	193±11	6.0±0.9	323±22**
80% B pellet	180±13	30.3±4.7*	125±25

*$p<.05$ vs Sham MBH and MBH + .05µg/d CRF groups

**$p<.05$ vs Sham MBH within corticosterone pellet group

Figure 4.1. Plasma ACTH (pg/ml, top) and pituitary ACTH (pg/µg protein, bottom) concentrations in sham lesioned rats (Sham MBH, left, n=8/group) and rats with lesions of the medial basal hypothalamus (MBH, right, n=5/group), 5 days after adrenalectomy. All rats were adrenalectomized and treated with pellets composed of varying concentrations of corticosterone (0, 20, 40 or 80% corticosterone, by weight). MBH lesioned rats received constant infusions of either .05µg/d CRF (broken line) or 5µg/d CRF (solid line), beginning at the time of adrenalectomy. Points represent mean values and vertical lines represent the SE. Asterisks (*) represent significant difference versus Sham MBH at same corticosterone pellet concentration. Daggers (†) indicate a significant effect of corticosterone within surgical group.


Sham MBH
MBH
Plasma ACTH (pg/ml)
400
200
0
Pituitary ACTH (pg/μg protein)
600
400
200
0
†
*
0
20
40
80
% corticosterone

Chapter 5 - Corticosterone inhibition of CRF-stimulated ACTH secretion in rats with complete or partial lesions of the hypothalamic paraventricular nuclei

Introduction

In the previous experiment, total pituitary protein in MBH lesioned rats was significantly lower than in sham lesioned controls. It was possible that those results were representative of a damaged and/or regenerating pituitary, and did not reflect the response of a normal gland. Therefore, in an experiment of similar design, the normal hypothalamic input to the pituitary was removed by lesion of the paraventricular nuclei of the hypothalamus (PVN), using a modified Halasz-type knife (Makara et al, 1986). It was anticipated that this lesion, if complete, would destroy the cell bodies of both the medial (parvocellular) and lateral (magnocellular) subdivisions of the PVN, without disruption of the vasculature in the median eminence region.

Materials and Methods

Adult male Sprague-Dawley rats (Holtzman, Madison, WI) were housed two per cage in hanging wire basket cages in a temperature-, humidity- and light-controlled room with food and water available *ad libitum*. Lights were on for 12 hours per day. After hypothalamic surgery, rats were housed singly. After adrenal surgery, rats were provided with 0.5% saline for drinking fluid.

For hypothalamic surgery, rats were anesthesized with pentobarbital (50mg/kg, ip) and mounted in a stereotaxic appartus. The PVN were

lesioned using an Halasz-type knife designed to make an inverted cone-shaped lesion centered on the PVN (Makara et al, 1986). The radius of the kinfe was 1.8mm, and its horizontal blade was at 2mm above the tip. The knife was lowered to the base of the skull on the midline at 1.6mm posterior to the bregma, with the blade of the knife pointing rostrally. The lesion was made by rotating the blade 360° twice in each direction. Sham lesions consisted of lowering the knife to the base of the skull without rotating the blade.

Two days after hypothalamic surgery, rats were sham adrenalectomized or adrenalectomized via the dorsal approach under ether anesthsia. Adrenalectomized rats were provided with subcutaneous pellets consisting either of wax (0% corticosterone) or 40% corticosterone (in cholesterol). Lesioned rats were provided at this time with subcutaneous mini-osmotic pumps preloaded to deliver either 1 or 5µg/d CRF. A separate set of lesioned rats (0 and 40% corticosterone pellet-treated) was not provided with exogenous CRF. In addition to sham lesioned rats treated with 0 or 40% corticosterone pellets, a third group of sham lesioned rats underwent sham adrenalectomy, in order to compare the effects of the 40% corticosterone pellet with phasic corticosterone levels.

Five days after adrenal surgery and pellet and pump implantation, rats were decapitated in the morning under resting conditions, within three hours of lights on. No more than 16 rats were killed on any day, and rats from each surgical and steroid treated group were included on each day of sampling. Trunk blood was collected into heparin-containing tubes and stored on ice until subsequent centrifugation and plasma

collection. Plasma samples were stored at -20C until determinations of plasma corticosterone and ACTH were made, by RIA. Circulating CRF levels were measured by RIA on an additional plasma aliquot (Vale et al, 1983; Plotsky et al, 1985). Pituitaries were quickly removed and anterior and neurointermediate lobes dissected, and one-half anterior pituitary was homogenized in 1ml 0.1N HCl for subsequent determination of pituitary ACTH and protein content. Thymus glands were removed and placed in closed dishes on filter paper saturated with 0.9% saline and were weighed after being cleaned of fat and connective tissue.

Brains from lesioned rats were removed and fixed in 10% formalin containing 20g/L $CaCl_2$. PVN lesions were verified on frozen sections made in a cryostat. Brains were sectioned coronally in 30μm sections, starting slightly posterior to the anterior commissure and continuing 120μm past the region of visible damage, and every fourth section was mounted and thionin stained. PVN lesions were judged for completeness on the basis of destruction of PVN magnocellular neurons. A lesion was considered to be complete if no magnocellular neurons were intact. If magnocellular lesions were present in no more than three of the 24 sections examined per brain, then the lesion was scored as a partial. If magnocellular neurons were present in more than three of the sections examined, then the lesion was considered to be a miss. Lesions were scored without knowledge of ACTH data.

Data were analyzed by parametric and non-parametric analyses of variances and with multiple comparison tests as previously described (Chapter 4).

Results

PVN lesions, in addition to the medial, parvocellular regions of the PVN, also resulted in some damage to the dorsal aspect of the medial preoptic area and to the dorsomedial nucleus of the hypothalamus. Representative examples of complete, partial and missed lesions are shown in Figure 5.1. Of the 60 lesions for which data are reported, 13 were complete, 18 were partial, and 29 were missed lesions.

Behaviorally, PVN lesioned rats were much like MBH lesioned rats, and required special handling and the use of a protective glove for decapitation. As in the previous experiment, sham lesioned rats were always killed before PVN lesioned rats, to avoid disturbing the basal state of the sham lesioned rats. Plasma corticosterone levels and thymus gland weights of sham lesioned and lesioned rats are shown in Table IV. There was no significant effect of hypothalamic or adrenal surgery on body weight (data not shown). Plasma corticosterone levels in the sham adrenalectomized and adrenalectomized, non-replaced groups were approximately 1µg/dl. The 40% corticosterone pellet resulted in plasma corticosterone levels of approximately 7µg/dl in the sham lesioned group, and slightly lower levels in the lesioned groups. Lesioned rats treated with 0 or 5µg/d CRF and 40% corticosterone pellets had significantly lower plasma corticosterone levels than did sham lesioned rats receiving the same composition pellet.

In sham lesioned rats, the 40% corticosterone pellet resulted in a lowering of thymus gland weight, with respect to sham adrenalectomized rats, while adrenalectomy caused a significant increase in thymus weight (Table IV). Thymus gland weights in all of the PVN lesioned groups

receiving 40% corticosterone pellets were not different from that of the sham lesioned group treated with the same composition pellet. In none of the lesioned groups was adrenalectomy accompanied by an elevation in thymus gland weight above that of the sham lesioned, sham adrenalectomized group.

Plasma CRF levels were measured in some of the sham lesioned and lesioned rats. All plasma samples were determined to have >6pM immunoreactive CRF, although these readings are not believed to represent authentic tissue CRF (P.M. Plotsky, personal communication). In the sham lesioned groups, plasma CRF levels were 8.2 (n=2), 10.0±0.9 (n=3) and 7.9±0.1pM (n=3) in the sham adrenalectomized, adrenalectomized plus 0 or 40% corticosterone pellet groups, respectively. PVN lesioned rats receiving 0µg/d CRF had plasma CRF levels of 9.3±0.8pM (n=10), while lesioned rats receiving 1µg/d CRF had statistically similar plasma CRF levels of 12.7±1.3pM (n=11). There was no significant difference in plasma CRF levels between sham lesioned rats and lesioned rats receiving either 0 or 1µg/d CRF. The highest dose of CRF tested, 5µg/d, significantly elevated plasma CRF levels to 31.0±2.3pM (n=11).

Plasma ACTH results for sham lesioned and lesioned rats are shown in Figure 5.2. The data from lesioned rats receiving either 0 or 1µg/d CRF are combined (PVN+0,1µg/d CRF) because systemic CRF levels were not different in these two sets of animals (see above). Plasma ACTH levels in sham lesioned rats which were either sham adrenalectomized or adrenalectomized and treated with a 40% corticosterone pellet were low and not different (Figure 5.2, top). Adrenalectomy without corticosterone replacement significantly increased plasma ACTH levels in

sham adrenalectomized rats.

Eight of the PVN lesioned rats receiving 0 or 1μg/d CRF had complete lesions (Figure 5.2, middle). Of these eight, only one bore a 0% corticosterone pellet; consequently it was not possible to test for a statistically significant difference in the plasma ACTH response to adrenalectomy between sham lesioned rats and this lesioned rat. However, the plasma ACTH level in the completely lesioned, adrenalectomized rat was 5pg/ml, well below the range of ACTH levels measured in sham lesioned, adrenalectomized rats (574±98pg/ml). The remaining seven rats in this group (complete lesions, 0 or 1μg/d CRF) were treated with 40% corticosterone pellets. The plasma ACTH level of this group was not significantly different from sham lesioned rats treated with the same composition pellet.

Five of the PVN lesioned rats receiving 5μg/d CRF had complete lesions (Figure 5.2, bottom). Only one of these five rats bore a 0% corticosterone pellet, again precluding a statistical analysis of the plasma ACTH response to adrenalectomy in this group. This rat had a plasma ACTH level of 248pg/ml, well above the plasma ACTH level of sham lesioned rats which were either sham adrenalectomized (24±8pg/ml) or adrenalectomized and treated with 40% corticosterone pellets (24±9pg/ml). The remaining four rats in this group (complete lesions, 5μg/d CRF) were treated with 40% corticosterone pellets. The plasma ACTH level of this group (172±26pg/ml) was significantly higher than the sham lesioned group treated with the same dose of corticosterone.

Rats with partial or missed lesions receiving 0 or 1μg/d CRF had plasma ACTH responses to adrenalectomy equal to those of sham lesioned

controls (Figure 5.2, middle). Treatment of these lesioned rats with 40% corticosterone pellets resulted in a significant lowering of plasma ACTH values. However, the ACTH levels in these lesioned rats with 40% corticosterone pellets were greater than those of sham lesioned rats treated with the same composition corticosterone pellet.

Rats with partial or missed lesions receiving 5µg/d CRF also had plasma ACTH responses to adrenalectomy which were equal to those of sham lesioned controls (Figure 5.2, bottom). In the group with partial lesions, plasma ACTH was significantly reduced by the 40% corticosterone pellet treatment. In the group with missed lesions, corticosterone treatment did not reduce plasma ACTH levels.

Pituitary ACTH levels in sham and PVN lesioned rats are shown in Figure 5.3. Results from lesioned rats are subdivided with respect to the completeness of the lesion as in the previous figure. PVN lesions did not affect total pituitary protein content with respect to sham operated controls (data not shown). Pituitary ACTH concentration was increased by adrenalectomy in sham lesioned rats (Figure 5.3, top). Treatment of sham lesioned rats with 40% corticosterone pellets resulted in pituitary ACTH concentrations not different from sham adrenalectomized controls.

There were no significant differences in pituitary ACTH content between lesioned rats receiving 0 or 1µg/d CRF and sham lesioned rats, within corticosterone pellet group, regardless of the completeness of the lesion (Figure 5.3, middle). The one adrenalectomized rat in the complete lesion plus 0 or 1µg/d CRF group had a pituitary ACTH content of 288pg ACTH/µg protein, which is less than the level observed in sham

lesioned, adrenalectomized rats (666±109pg ACTH/μg protein).

In PVN lesioned rats receiving 5μg/d CRF there was no increase in pituitary ACTH content with adrenalectomy (Figure 5.3, bottom). The pituitary ACTH levels of lesioned rats receiving 5μg/d CRF and treated with 40% corticosterone pellets were not different from the group of sham lesioned rats treated with the same composition pellet.

Discussion

This study confirms the previous observations of the lack of an inhibitory effect of physiologic replacement doses of corticosterone on adrenalectomy-induced ACTH secretion in rats with only pituitary corticosteroid feedback sites. The advantages to the PVN lesions were that they circumvented the issue of a possibly damaged pituitary (as in the MBH lesions), and removed the normal hypothalamic input to the corticotropes without disturbing normal water balance (as in the anterolateral hypothalamic deafferentation procedure).

In sham lesioned controls, adrenalectomy resulted in the well-documented increases in plasma and pituitary ACTH concentrations. There was excellent agreement between sham adrenalectomized rats and adrenalectomized rats treated with 40% corticosterone pellets with respect to plasma and pituitary ACTH, indicating the physiologic nature of this replacement dose of corticosterone. Thymus weights in sham lesioned, 40% pellet treated rats were less than in the sham adrenalectomized reference group, although there was good agreement in thymus gland weight for rats treated with this composition pellet between sham lesioned and lesioned rats.

The additional information of (systemic) plasma CRF levels sheds considerable light on the sensitivity of the corticotropes *in vivo* to continuous infusions of CRF. Most reports on plasma CRF levels to date have indicated that the peptide is not measurable in peripheral plasma of normal man (Schurmeyer et al, 1984; Stalla et al, 1986) or rats (Plotsky et al, 1985). If the levels reported in the present study were authentic tissue CRF, then the levels would be expected to drop with PVN lesion, assuming the PVN was the sole or at least predominant source of this circulating CRF. Given the evidence that CRF is not detectable in the systemic circulation, along with the observation that CRF values did not decrease with PVN lesion, it is probably safe to assume that the immunoreactive substance measured in samples from sham lesioned rats and PVN lesioned rats not receiving exogenous CRF is not itself CRF. It is likely that the 1μg/d dose of CRF was below the threshold for stimulation of ACTH release, since (systemic) plasma CRF levels did not increase with this dose, and since no plasma ACTH response to adrenalectomy was observed when this dose of CRF was given to the rat with a complete PVN lesion. Despite the paucity of observations in the group with complete PVN lesion plus 0 or 1μg/d CRF, the result from the adrenalectomized, non-replaced rat is in complete agreement with the data presented previously (Chapter 4) and with our previous work (Dallman et al, 1985). Again it appears that the corticotropes cannot respond to adrenalectomy in the absence of hypothalamic drive.

When the normal hypothalamic drive was removed by complete PVN lesion and replaced with a continuous infusion of 5μg/d CRF, corticosterone levels which normalized adrenalectomy-induced increases

in plasma ACTH in sham lesioned rats were ineffective. This was the only CRF infusion rate tested which increased (systemic) CRF levels, and it was also the only dose which reliably increased plasma ACTH in adrenalectomized, MBH lesioned rats (Chapter 4). The change in plasma CRF levels between lesioned rats receiving 0 or 1µg/d CRF and 5µg/d CRF was approximately 20pM. This increase is less than that observed in hypophysial portal plasma following hemorrhage (Plotsky & Vale, 1984; Plotsky et al, 1985) or chemical adrenalectomy (Plotsky & Sawchenko, in press), and is slightly greater than estimates of the reliably effective concentration of CRF to release ACTH in vitro (Vale et al, 1983).

The results from rats with missed or partial PVN lesions show that plasma ACTH levels were increased by adrenalectomy to the same extent as in sham lesioned, adrenalectomized rats. This response is particularly impressive in the rats with missed lesions receiving sub-effective infusions of CRF (0 or 1µg/d), because it implies that relatively few PVN neurons are required to mount the full response. A similar observation was made by Makara and colleagues (Makara et al, 1981), who found that the ether-venesection stress- induced increment in corticosterone tended to be higher in rats with incomplete lesions of the PVN. In that study, lesions of >75% of the PVN brought about a maximal inhibition of the corticosterone response to this stress.

In rats with missed or partial lesions receiving 0 or 1 µg/d CRF, and in rats with partial lesions receiving 5µg/d CRF, plasma ACTH levels were partially reduced but not completely restored by the 40% corticosterone pellet treatment. One possible explanation for this finding is that these incomplete lesions have damaged a brain

corticosteroid feedback site, or a pathway from an extrahypothalamic corticosteroid feedback site to the PVN, such that the full inhibitory effecy of corticosterone on plasma ACTH cannot be exerted. In rats with missed lesions receiving 5μg/d CRF, however, the 40% corticosterone pellet treatment had no inhibitory effect on plasma ACTH. The ACTH level in this group was higher than in rats with complete lesions treated with the same doses of CRF and corticosterone. This observation supports the notion that the 5μg/d CRF dose was not so high as to "overwhelm" the pituitary, since the ACTH level in rats with endogenous and exogenous CRF (rats with missed lesions given 5μg/d CRF) was significantly greater than in rats with only exogenous CRF (rats with complete lesions given 5μg/d CRF).

Table IV. Plasma corticosterone (B) and thymus gland weight 5 days after sham adrenalectomy (ShamADX) or adrenalectomy without (ADX + 0% B pellet) or with (ADX + 40% B pellet) corticosterone replacement

Surgical Group (N)	Plasma B (µg/dl)	Thymus Weight (mg/100g BW)
Sham lesioned		
ShamADX (5)	0.8±0.1	199±22
ADX + 0% B pellet (7)	0.5±0.2	278±17
ADX + 40% B pellet (6)	6.9±0.5	132±22
PVN lesioned + 0µg/d CRF		
ADX + 0% B pellet (9)	0.5±0.1	208±16**
ADX + 40% B pellet (10)	4.4±0.4*	136±12
PVN lesioned + 1µg/d CRF		
ADX + 0% B pellet (10)	0.6±0.1	221±21**
ADX + 40% B pellet (10)	5.7±0.6*	133±15
PVN lesioned + 5µg/d CRF		
ADX + 0% B pellet (11)	0.5±0.1	216±14**
ADX + 40% B pellet (10)	5.1±0.5*	150±21

*$p<.05$ versus Sham lesioned, 40% B pellet group

**$p<.05$ versus Sham lesioned, 0% B pellet group

Figure 5.1. Representative examples of complete (top), partial (middle), and missed (bottom) PVN lesions. In the complete PVN lesion, the suprachiasmatic and supraoptic nuclei are apparent, while the region of the PVN is filled with glial cells and staining artifact. In the partial PVN lesion, the arrow points out a small cluster of remaining magnocellular neurons. In the missed PVN lesion, the PVN are clearly intact.

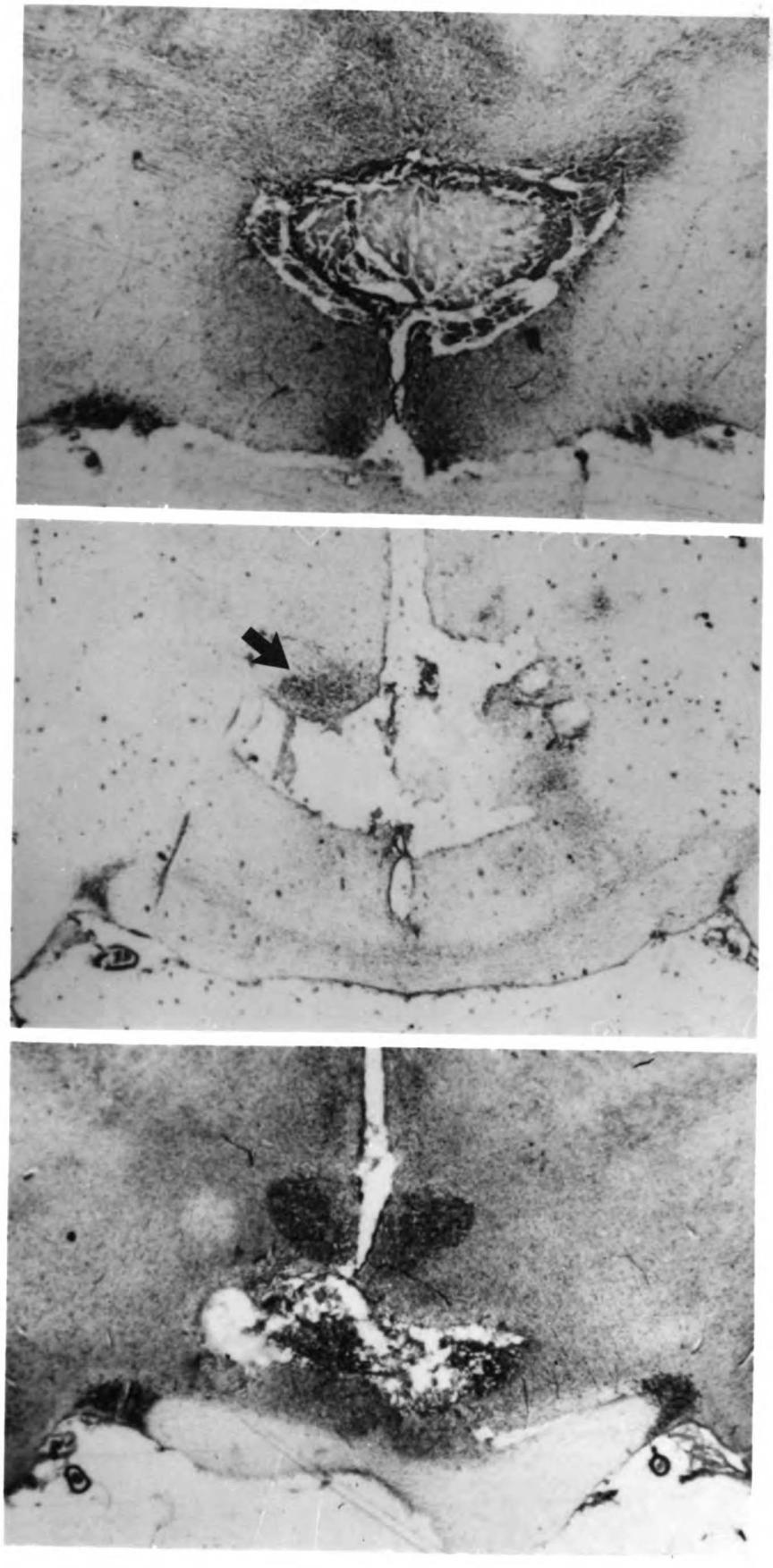


Figure 5.2 Plasma ACTH (pg/ml) in sham lesioned rats (Sham PVN, top) and rats with lesions of the paraventricular nuclei of the hypothalamus (PVN, middle and bottom), 5 days after adrenalectomy. Rats were either sham adrenalectomized (Sham Adx, solid bar) or adrenalectomized and treated with either 0 (open bars) or 40% corticosterone pellets (hatched bars). Lesioned rats received constant infusions of either 0 or 1 μg/d CRF (PVN+0,1μg CRF/d, middle), or 5μg/d CRF (PVN+5μg CRF/d, bottom), beginning at the time of adrenalectomy. Bars represent mean values, and vertical lines represent the SE. Numbers in parantheses refer to the number of rats per group. Asterisks (*) indicate a significant ($p<.05$) difference between lesioned and sham lesioned rats, within corticosterone pellet group. NS, not significant. NT, not tested.

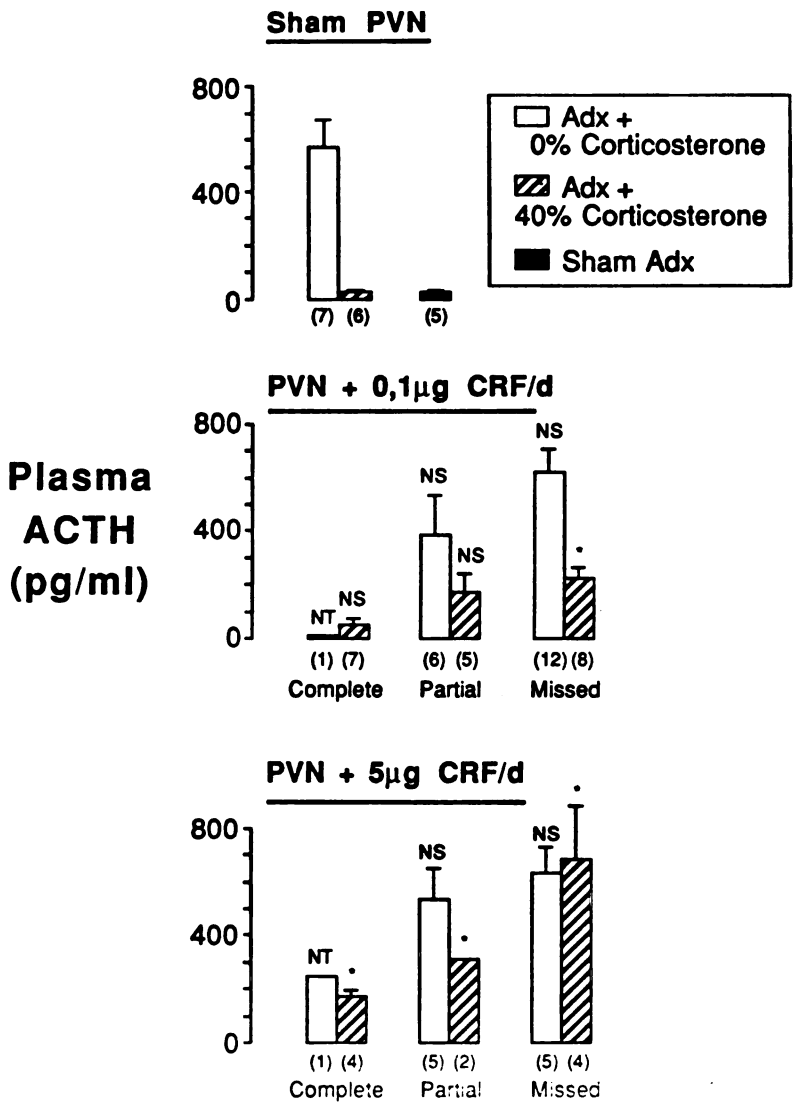
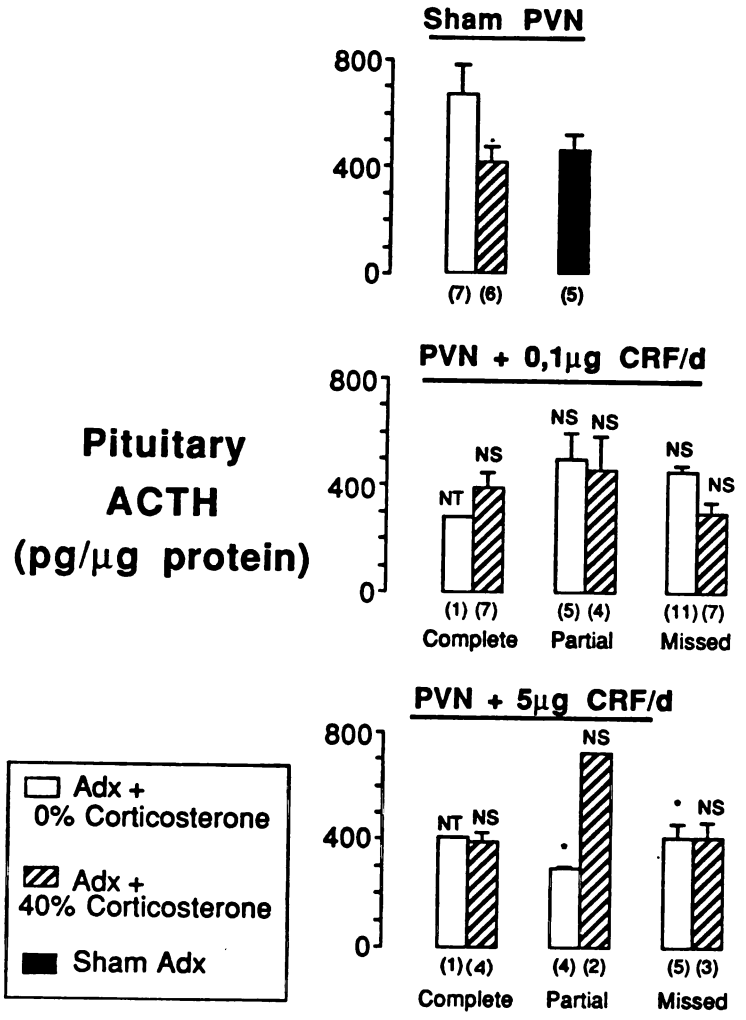

Sham PVN
800
400
0
(7) (6)
(5)
Adx + 0% Corticosterone
Adx + 40% Corticosterone
Sham Adx
PVN + 0,1µg CRF/d
Plasma ACTH (pg/ml)
NT
NS
NS
NS
NS
(1) (7)
(6) (5)
(12) (8)
Complete
Partial
Missed
PVN + 5µg CRF/d
NT
NS
NS
(1) (4)
(5) (2)
(5) (4)
Complete
Partial
Missed

Figure 5.3 Pituitary ACTH (pg/μg protein) in sham lesioned and PVN lesioned rats. Data and statistical notations are presented as in the previous figure.


Sham PVN
800
400
0
(7) (6)
(5)
PVN + 0,1µg CRF/d
Pituitary
ACTH
(pg/µg protein)
NT
NS
NS NS
NS
NS
(1) (7)
(5) (4)
(11) (7)
Complete
Partial
Missed
PVN + 5µg CRF/d
Adx + 0% Corticosterone
Adx + 40% Corticosterone
Sham Adx
(1) (4)
(4) (2)
(5) (3)
Complete
Partial
Missed

Chapter 6 - General Discussion and Conclusions

The studies presented in this thesis were designed to determine the relative importance of the brain versus the anterior pituitary in the corticosteroid feedback inhibition of adrenalectomy-induced ACTH secretion. Although the question of the site or sites through which corticosterone exerts feedback effects on ACTH secretion is not new, relatively few studies have addressed this question *in vivo*, and there are no reports concerned specifically with the site of action at which corticosterone acts to normalize morning plasma ACTH levels following adrenalectomy. The *in vivo* experimental approach is advantageous because it allows the hypothalamo-pituitary-adrenal axis to be manipulated and studied as a system, rather than broken into its components and studied in isolation. The studies presented in this thesis have shown for the first time that a dose of corticosterone which restores plasma ACTH to normal morning levels following adrenalectomy in sham lesioned rats is ineffective in inhibiting ACTH secretion stimulated to a similar degree by infusions of exogenous CRF in lesioned animals. Because sham lesioned rats had brain and pituitary corticosteroid feedback sites, while in lesioned rats corticosterone could only act on the pituitary, it is concluded that corticosterone inhibits adrenalectomy-induced ACTH secretion by an action solely on the brain.

In the first study, the effectiveness of a wide range of constant corticosterone levels on several corticosteroid-sensitive endpoints was tested. This study was necessary to determine an optimal corticosterone

replacement dose for subsequent experiments. Administration of 50% corticosterone pellets at the time of adrenalectomy resulted 5 days later in plasma corticosterone levels of approximatley 4µg/dl in the morning, and 10µg/dl in the evening. This corticosteroid feedback signal normalized morning, but not evening, plasma ACTH, as well as thymus gland weight, after adrenalectomy. These plasma corticosterone levels agree well with the previous observation that constant plasma corticcsterone levels of 4.5-7.4µg/dl are optimal to restore morning plasma ACTH and thymus gland and body weight after adrenalectomy (Akana et al, 1985).

It should be noted that there is a substantial difference between the constant plasma corticosterone levels afforded by implantation of subcutaneous corticosterone pellets and the phasic plasma corticosterone excursions observed in adrenal-intact rats. We have evaluated the physiologic relevance of the corticosterone pellets by comparing the inhibitory effects of constant plasma corticosterone replacement with those of phasic corticosterone replacement, accomplished by giving adrenalectomized rats corticosterone in the drinking water. This regimen results in phasic plasma corticosterone levels similar to adrenal-intact controls, because rats do most of their eating and therefore drinking beginning at the time of lights off. Basal morning plasma ACTH levels and thymus gland weight were shown to be normalized after adrenalectomy equally well by treatment of adrenalectomized rats with corticosterone in the drinking water or with subcutaneous corticosterone pellets which fix plasma corticosterone levels in the range of 4.5-7.4µg/dl (Akana et al, 1985). Constant plasma

corticosterone levels do not appear to affect the basal (non-stimulated) sensitivity of the adrenocortical system to corticosterone feedback. Therefore, the constant plasma corticosterone levels were considered to be a reasonable approximation of the endogenous corticosterone feedback signal, and were employed in the subsequent studies in order to control the corticosteroid feedback signal in rats receiving exogenous hypothalamic releasing factors.

While it has been shown repeatedly that glucocorticoids reverse adrenalectomy-induced activation of the hypothalamo-pituitary-adrenal axis, a great deal of this work has involved large doses of dexamethasone. For example, systemic dexamethasone treatment (100-480μg/d for up to 7 days) initiated at the time of adrenalectomy has been shown to block the increases in CRF and vasopressin immunostaining (Itoi et al, 1987), and hypothalamic CRF (Jingami et al, 1985) and vasopressin gene expression (Davis et al, 1986), again implying a central effect of glucocorticoids. Dexamethasone implants of approximately 70μg in the region of the PVN also block the adrenalectomy-induced increases in CRF and vasopressin immunostaining (Kovacs et al, 1986). There is, however, experimental evidence to suggest that the adrenocortical system is not equally sensitive to corticosterone and dexamethasone. In the study of Jingami et al (1985), changes in pituitary POMC mRNA levels in response to adrenalectomy were also measured. Dexamethasone treatment suppressed anterior pituitary POMC mRNA levels to 19% of the control values, while hypothalamic CRF mRNA levels were not suppressed below control levels. Similarly, systemic administration of large doses of dexamethasone has been shown

to result in decreases in pituitary and plasma ACTH concentrations without affecting hypothalamic CRF content (Carnes et al, 1987). These data imply that the pituitary is more sensitive than the brain to dexamethasone, possibly due to the presence of CBG in the pituitary which could prevent corticosterone, but not dexamethasone, from exerting inhibitory effects. These studies underscore the importance of the investigations in this thesis which employed physiologically relevant doses of corticosterone, the endogenous corticosteroid of the rat.

There were two main findings of the experiments presented in this thesis. First, adrenalectomy-induced ACTH hypersecretion is the result of increased hypothalamic input to the pituitary. This finding was observed in rats with anterolateral hypothalamic deafferentations receiving 1µg/d vasopressin, rats with medial basal hypothalamic lesions receiving .05µg/d CRF or 1µg/d inactive CRF_{3-41}, and rats with complete lesions of the PVN receiving 0 or 1 µg/d CRF. Along with similar results from previous work (Allen et al, 1974; Dallman et al, 1985), these data provide overwhelming support for the conclusion that the corticotropes of the anterior pituitary cannot respond to the removal of corticosteroid feedback in the absence of hypothalamic input.

The second principle finding of this thesis is that corticosterone inhibits adrenalectomy-induced ACTH hypersecretion by an action on the brain. When hypothalamic drive was replaced in lesioned rats with constant infusions of CRF, no inhibition of ACTH secretion was observed until constant plasma corticosterone levels were elevated to the range that produced thymic atrophy. The lack of an inhibitory effect of physiologic doses of corticosterone (in the range of 5-9µg/dl in these

studies) on ACTH secretion driven by exogenous hypothalamic input was observed in rats with anterolateral hypothalamic deafferentations receiving 1μg/d CRF (± 1μg/d vasopressin), rats with medial basal hypothalamic lesions receiving 5μg/d CRF, and rats with complete PVN lesions receiving 5μg/d CRF. These studies are unique because they demonstrate the lack of an inhibitory effect on the pituitary by physiologic doses of the endogenous glucocorticoid of the rat.

Previously, we reported corticosterone feedback effects on ACTH secretion at the level of the pituitary in rats with anterolateral hypothalamic deafferentations (Dallman et al, 1985). However, in those rats plasma corticosterone levels were high in the morning and evening (>15μg/dl) as a consequence of ACTH secretion driven by infusions of CRF. Further evidence that plasma corticosterone levels were elevated in those rats stems from the observations that adrenal gland weights were significantly increased, and thymus gland weights were significantly decreased, in those lesioned, adrenal-intact rats receiving 10μg/d CRF. The results presented in this thesis argue that, *in vivo*, corticosterone exerts feedback effects at the level of the pituitary only when corticosterone levels are elevated for a prolonged period of time.

Recently, Plotsky et al (1986) showed that hypotension-induced CRF secretion can be inhibited by a 2 hour infusion of corticosterone at a rate of 1.25μg/min, in rats whose endogenous corticosteroid secretion was pharmacologically blocked. Because measurement of CRF in the hypophysial portal vessels requires transection of the pituitary stalk, the corticosterone-induced decrease in CRF secretion is interpreted to

result from a central, as opposed to pituitary effect. This corticosterone infusion rate resulted in plasma corticosterone levels of approximately 12μg/dl by the end of the 2 hour steroid pretreatment. Thus, Plotsky et al have demonstrated that stress-induced CRF secretion can be inhibited by levels of corticosterone observed during stress. The interpretation of the data is limited, however, since these doses of corticosterone may have also exerted direct effects on the pituitary.

One of the main underlying assumptions of these studies is that the mode of CRF presentation (continous infusion) has not altered the sensitivity of the pituitary to corticosteroid feedback. Recently, it has been shown by push-pull cannulation that CRF levels in the median eminence peak episodically in conscious, freely moving and presumably unstressed rats (Ixart et al, 1987). There are no studies comparing the sensitivity of pituitaries to corticosterone feedback inhibition of ACTH release stimulated by constant versus pulsatile presentation of CRF. Despite the possibility of a qualitative difference in CRF secretion pattern, it appears that, 5 days after surgery, adrenalectomy stimulates an amount of ACTH secretion in sham lesioned rats which is quantitatively similar to the effect of infusion of 5μg/d CRF in rats with complete lesions of the PVN or medial basal hypothalamus. Therefore, the extent to which the pituitaries of sham lesioned and lesioned rats were stimulated was similar, and it is not inappropriate to compare the response to corticosterone between these two groups.

One inconsistency to be noted was that 1μg/d CRF failed to stimulate ACTH secretion in rats with complete PVN lesions, while the same dose of CRF significantly stimulated ACTH secretion in rats with

anterolateral hypothalamic deafferentations. It has previously been shown that neither of these lesions alter pituitary sensitivity to CRF (Stark et al, 1983). The most likely explanation for this finding is that some hypothalamic input to the pituitary remained in the rats with anterolateral hypothalamic deafferentations. The rats included in the anterolateral experiment were chosen on the basis of a significant increase in fluid intake, presumably due to lesion-induced diabetes insipidus, and not on histologic evidence of complete hypothalamic disconnection. Measurements of (systemic) plasma CRF levels in PVN lesioned rats revealed no increase in circulating CRF levels with the 1μg/d CRF infusion, implying that this dose of CRF probably does not stimulate ACTH secretion. This inconsistency underscores the importance of thorough histologic examination in lesion experiments.

These studies do not indicate at which site or sites corticosterone acts in the brain. It is tempting to speculate, with respect to the findings of this thesis, that corticosterone may be acting on the corticosterone-preferring receptor, possibly in the hippocampus, to normalize ACTH secretion after adrenalectomy. The observation that these receptors are occupied to a large extent by low circulating corticosterone levels (Reul & DeKloet, 1985), implies that these receptors would also be preferentially occupied by the low corticosterone levels which were shown here to normalize plasma ACTH in sham lesioned rats following adrenalectomy. The role of these receptors in the regulation of ACTH secretion will certainly be an area of active research in the future.

The significance of the findings of this thesis probably lies in

the advantage which central nervous system corticosteroid feedback offers to the homeostasis of the organism. It is difficult to imagine how the adrenocortical system could achieve the tight degree of regulation observed *in vivo* if pituitary feedback were the only way in which ACTH output were regulated. With the brain as the primary site of corticosteroid feedback it is easy to envision how input to the CRF neuron could be summated over several corticosteroid-sensitive pathways. In this way, corticosteroids could modify on a much larger scale both the amount and the nature of the ACTH-releasing stimulus secreted into the hypophysial portal vessels.

The mind which is free from passions is a citadel.

-Marcus Aurelius

References

1. Abe K, Kroning J, Greer M, Critchlow V 1979 Effect of destruction of the suprachiasmatic nuclei on the circadian rhythms in plasma corticosterone, body temperature, feeding and plasma thyrotropin. Neuroendocrinology 29:119

2. Abou-Samra A-B, Catt KJ, Aguilera G 1986 Biphasic inhibition of adrenocorticotropin release by corticosterone in cultured anterior pituitary cells. Endocrinology 119:972

3. Affolter H-U, Reisine T 1985 Corticotropin releasing factor increases proopiomelanocortin messenger RNA in mouse anterior pituitary tumor cells. J Biol Chem 260:15477

4. Agnati LF, Fuxe K, Yu Z-Y, Harfstrand A, Okret S, Wilkstrom A-C, Goldstein M, Zoli M, Vale W, Gustafsson J-A 1985 Morphometrical analysis of the distribution of corticotrophin releasing factor, glucocorticoid receptor and phenylethanolamine-N-methyl-transferase immunoreactive structures in the paraventricular nucleus of the rat. Neurosci Let 54:147

5. Aguilera G, Harwood JP, Wilson JX, Morell J, Brown JH, Catt KJ 1983 Mechanisms of action of corticotropin-releasing factor and other regulators of corticotropin release in rat pituitary cells. J Biol Chem 258:8039

6. Akana SF, Cascio CS, Du J-Z, Levin N, Dallman MF 1986 Reset of feedback in the adrenocortical system: an apparent shift in sensitivity of adrenocorticotropin to inhibition by corticosterone between morning and evening. Endocrinology 119:2325

7. Akana SF, Cascio CS, Shinsako J, Dallman MF 1985 Corticosterone:

narrow range required for normal body and thymus weight and ACTH. Am J Physiol 249:R527

8. Allen JP, Allen CF 1974 Role of the amygdaloid complexes in the stress-induced release of ACTH in the rat. Neuroendocrinology 15:220
9. Allen JP, Allen CF 1975 Amygdalar participation in tonic ACTH secretion in the rat. Neuroendocrinclogy 19:115
10. Allen CF, Allen JP, Greer MA 1974 Anterolateral hypothalamic deafferentation prevents compensatory hypersecretion of ACTH following adrenalectomy in the rat. Proc Soc Exp Biol Med 146:840
11. Antoni FA, Holmes MC, Jones MT 1983a Oxytocin as well as vasopressin potentiate ovine CRF in vitro. Peptides 4:411
12. Antoni FA, Palkovits M, Makara GB, Linton EA, Lowry PJ, Kiss JZ 1983b Immunoreactive corticotropin-releasing hormone in the hypothalamoinfundibular tract. Neuroendocrinology 36:415
13. Arimura A, Bowers CY, Schally AV, Saito M, Miller MC 1969 Effect of corticotropin-releasing factor, dexamethasone and actinomycin D on the release of ACTH from rat pituitaries in vivo and in vitro. Endocrinology 85:300
14. Arimura A, Saito T, Schally AV 1967 Assays for corticotropin-releasing factor (CRF) using rats treated with morphine, chlorpromazine, dexamethasone and nembutal. Endocrinology 81:235
15. Beaumont K, Fanestil DD 1983 Characterization of rat brain aldosterone receptors reveals high affinity for corticosterone. Endocrinology 113:2043
16. Bilezikjian LM, Vale WW 1983 Glucocorticoids inhibit corticotropin-releasing factor-induced production of adenosine 3',5'-monophosphate

in cultured anterior pituitary cells. Endocrinology 113:657

17. Birnberg NC, Lissitzky J-C, Hinman M, Herbert E 1983 Glucocorticoids regulate proopiomelanocortin gene expression in vivo at the levels of transcription and secretion. Proc Natl Acad Sci USA 80:6982

18. Bohus B, Endroczi E 1964 Effect of intracerebral implantation of hydrocortisone on adrenocortical secretion and adrenal weight after unilateral adrenalectomy. Acta Physiol Acad Sci Hung 25:11

19. Bohus B, Nyakas Cs, Lissak K 1968 Involvement of suprahypothalamic structures in the hormonal feedback action of corticosteroids. Acta Physiol Acad Sci Hung 34:1

20. Bohus B, Strashmirov D 1970 Localization and specificity of corticosteroid "feedback receptors" at the hypothalamo-hypophyseal level; comparative effects of various steroids implanted in the median eminence or anterior pituitary of the rat. Neuroendocrinology 6:197

21. Brodish A, Long CNH 1956 Changes in blood ACTH under various experimental conditions studied by means of a cross-circulation technique. Endocrinology 59:666

22. Bruhn TO, Sutton RE, Rivier CL, Vale WW 1984a Corticotropin-releasing factor regulates proopiomelanocortin messenger ribonucleic acid levels in vivo. Neuroendocrinology 39:170

23. Bruhn TO, Plotsky PM, Vale WW 1984b Effect of paraventricular lesions on corticotropin-releasing factor (CRF)-like immunoreactivity in the stalk median eminence: studies on the adrenocorticotropin response to ether stress and exogenous CRF. Endocrinology 114:57

24. Buckingham JC, Hodges JR 1974 Interrelationships of pituitary and plasma corticotrophin and plasma corticosterone in adrenalectomized and stressed, adrenalectomized rats. J Endocrinol 63:213

25. Carnes M, Barksdale CM, Kalin NH, Brownfield MS, Lent SJ 1987 Effects of dexamethasone on central and peripheral ACTH systems in the rat. Neuroendocrinology 45:160

26. Casady RL, Taylor AN 1976 Effect of electrical stimulation of the hippocampus upon corticosteroid levels in the freely-behaving, non-stressed rat. Neuroendocrinology 20:68

27. Chowers I, Conforti N, Feldman S 1967 Effects of corticosteroids on hypothalamic corticotropin releasing factor and pituitary ACTH content. Neuroendocrinology 2:193

28. Chowers I, Feldman S, Davidson JM 1963 Effects of intrahypothalamic crystalline steroids on acute ACTH secretion. Am J Physicl 205:671

29. Chrousos GP, Cutler GB Jr, Sauer M, Simons SS Jr, Loriaux DL 1983 Development of glucocorticoid antagonists. Pharmac Ther 20:263

30. Coirini H, Marusic ET, DeNicola AF, Rainbow TC, McEwen BS 1983 Identification of mineralocorticoid binding sites in rat brain by competition studies and density gradient centrifugation. Neuroendocrinology 37:354

31. Colfer HF, DeGroot J, Harris GW 1950 Pituitary gland and blood lymphocytes. J Physiol 111:328

32. Conforti N, Feldman S 1976 Effects of dorsal fornix section and hippocampectomy on adrenocortical response to sensory stimulation in the rat. Neuroendocrinology 22:1

33. Corbin A, Mangili G, Motta M, Martini L 1965 Effect of hypothalamic

and mesencephalic steroid implantations on ACTH feedback mechanisms. Endocrinology 76:811

34. Dallman MF, Akana SF, Cascio CS, Darlington DN, Jacobson L, Levin N 1987 Regulation of ACTH secretion: variations on a theme of B. Rec Prog Horm Res 43:113

35. Dallman MF, Demanicor D, Shinsako J 1974 Diminishing corticotrope capacity to release ACTH during sustained stimulation: the twenty-four hours after bilateral adrenalectomy in the rat. Endocrinology 95:65

36. Dallman MF, Engeland WC, Rose JC, Wilkinson CW, Shinsako J, Siedenburg F 1978 Nycthemeral rhythm in adrenal responsiveness to ACTH. Am J Physiol 235:R210

37. Dallman MF, Jones MT, Vernidos-Danellis J, Ganong WF 1972 Corticosteroid feedback control of ACTH secretion: rapid effects of bilateral adrenalectomy on plasma ACTH in the rat. Endocrinology 91:961

38. Dallman MF, Makara GB, Roberts JL, Levin N, Blum M 1985 Corticotrope response to removal of releasing factors and corticosteroids in vivo. Endocrinology 117:2190

39. Dallman MF, Yates FE 1969 Dynamic asymmetries in the corticostercid feedback path and distribution-metabolism-binding elements of the adrenocortical system. Annals NY Acad Sci 156:696

40. Davidson JM, Feldman S 1963 Cerebral involvement in the inhibition of ACTH secretion by hydrocortisone. Endocrinology 72:936

41. Davis LG, Arentzen R, Reid JM, Manning RW, Wolfson B, Lawrence KL, Baldino F Jr 1986 Glucocorticoid sensitivity of vasopressin mRNA

levels in the paraventricular nucleus of the rat. Proc Natl Acad Sci USA 83:1145

42. DeBold CR, Sheldon WR, DeCherney GS, Jackson RV, Alexander AN, Vale W, Rivier J, Orth DN 1984 Arginine vasopressin potentiates adrenocorticotropin release induced by corticotropin-releasing factor. J Clin Invest 73:533
43. DeGroot J, Harris GW 1950 Hypothalamic control of the anterior pituitary gland and blood lymphocytes. J Physiol 111:335
44. DeKloet ER, McEwen BS 1976a A putative glucocorticoid receptor and a transcortin-like macromolecule in pituitary cytosol. Biochim Biophy Acta 421:115
45. DeKloet ER, McEwen BS 1976b Differences between cytosol receptor complexes with corticosterone and dexamethasone in hippocampal tissue from rat brain. Biochim Biophy Acta 421:124
46. DeKloet ER, Voorhuis ThAM, Leunissen JLM, Koch B 1984 Intracellular CBG-like molecules in the rat pituitary. J Steroid Biochem 20:367
47. DeKloet R, Wallach G, McEwen BS 1975 Differences in corticosterone and dexamethasone binding to rat brain and pituitary. Endocrinology 96:598
48. DeWied D 1964 The site of the blocking action of dexamethasone on stress-induced pituitary ACTH release. J Endocrinol 29:29
49. Dougherty TF 1952 Effects of hormones on lymphatic tissue. Physiol Rev 32:379
50. Dunn J, Critchlow V 1969 Feedback suppression of pituitary-adrenal function in rats with pituitary islands. Life Sciences 8:9
51. Eberwine JH, Roberts JL 1984 Glucocorticoid regulation of pro-

opiomelanocortin gene transcription in the rat pituitary. J Biol Chem 259:2166

52. Endroczi E, Lissak K, Tekeres M 1961 Hormonal "feed-back" regulation of pituitary-adrenocortical activity. Acta Physiol Acad Sci Hung 18:291

53. Feldman S 1981 Electrophysiological effects of adrenocortical hormones on the brain. In Fuxe K, Gustafsson J-A, Wetterberg L (eds) Steroid hormone regulation of the brain. Pergamon Press, New York, p175

54. Feldman S, Conforti N 1980 Participation of the dorsal hippocampus in the glucocorticoid feedback effect on adrenocortical activity. Neuroendocrinology 30:52

55. Feldman S, Conforti N 1981 Effects of hypothalamic deafferentations on adrenocortical responses in the rat following hippocampal stimulation. Exp Brain Res 44:232

56. Feldman S, Conforti N, Davidson JM 1966 Adrenocortical responses in rats with corticosteroid and reserpine implants. Neuroendocrinology 1:228

57. Feldman S, Papir-Kricheli D, Dafny N 1983 Single-cell and multiunit activity in freely moving rats after corticosterone administration. Exp Neurol 80:427

58. Fleischer N, Vale W 1968 Inhibition of vasopressin-induced ACTH release from the pituitary by glucocorticoids in vitro. Endocrinology 83:1232

59. Fremeau RT Jr, Lundblad JR, Pritchett DB, Wilcox JN, Roberts JL 1986 Regulation of pro-opiomelanocortin gene transcription in individual

cell nuclei. Science 234:1265

60. Fuxe K, Harfstrand A, Agnati LF, Yu Z-Y, Cintra A, Wikstrom A-C, Okret S, Cantoni E, Gustafsson J-A 1985a Immunocytochemical studies on the localization of glucocorticoid receptor immunoreactive nerve cells in the lower brain stem and spinal cord of the male rat using a monoclonal antibody against rat liver glucocorticoid receptor. Neurosci Let 60:1

61. Fuxe K, Wikstrom A-C, Okret S, Agnati LF, Harfstrand A, Yu Z-Y, Granholm L, Zoli M, Vale W, Gustafsson J-A 1985b Mapping of glucocorticoid receptor immunoreactive neurons in the rat tel- and diencephalon using a monoclonal antibody against rat liver glucocorticoid receptor. Endocrinology 117:1803

62. Gemzell CA, VanDyke DC, Tobias CA, Evans HM 1951 Increase in the formation and secretion of ACTH following adrenalectomy. Endocrinology 49:325

63. Giguere V, Labrie F, Cote J, Coy DH, Sueiras-Diaz J, Schally AV 1983 Stimulation of cyclic AMP accumulation and corticotropin release by synthetic ovine corticotropin-releasing factor in rat anterior pituitary cells: site of glucocorticoid action. Proc Natl Acad Sci USA 79:3466

64. Gray WD, Munson PL 1951 The rapidity of the adrenocorticotropic response of the pituitary to the intravenous administration of histamine. Endocrinology 48:471

65. Green JD, Harris GW 1947 The neurovascular link between the neurohypophysis and the adenohypophysis. J Endocrinol 5:136

66. Halasz B, Pupp L 1965 Hormone secretion of the anterior pituitary

gland after physical interruption of all nervous pathways to the hypophysiotrophic area. Endocrinology 77:553

67. Halasz B, Slusher MA, Gorski RA 1967 Adrenocorticotrophic hormone secretion in rats after partial or total deafferentation of the medial basal hypothalamus. Neuroendocrinology 2:43

68. Harris GW 1948 Neural control of the pituitary gland. Physiol Rev 28:139

69. Hillhouse EW, Jones MT 1976 Effect of bilateral adrenalectomy and corticosteroid therapy on the secretion of corticotrophin-releasing factor activity from the hypothalamus of the rat in vitro. J Endocrinol 71:21

70. Hodges JR, Jones MT 1963 The effect of injected corticosterone on the release of adrenocorticotrophic hormone in rats exposed to acute stress. J Physiol 167:30

71. Hoffman AR, Ceda G, Reisine TP 1985 Corticotropin-releasing factor desensitization of adrenocorticotropic hormone release is augmented by arginine vasopressin. J Neurosci 5:234

72. Holmes MC, Antoni FA, Catt KJ, Aguilera G 1986 Predominant release of vasopressin vs. corticotropin-releasing factor from the isolated median eminence after adrenalectomy. Neuroendocrinology 43:245

73. Holmes MC, Antoni FA, Szentendrei T 1984 Pituitary receptors for corticotropin-releasing factor: no effect of vasopressin on binding or activation of adenylate cyclase. Neuroendocrinology 39:162

74. Ingle DJ 1938 The effects of administering large amounts of cortin on the adrenal cortices of normal and hypophysectomized rats. Am J Physiol 124:369

75. Ingle DJ, Higgins GM, Kendall EC 1938 Atrophy of the adrenal cortex in the rat produced by administration of large amounts of cortin. Anat Rec 71:363

76. Ingle DJ, Kendall EC 1937 Atrophy of the adrenal cortex of the rat produced by the administration of large amounts of cortin. Science 86:245

77. Itoi K, Mouri T, Takahashi K, Murakami O, Imai Y, Sasaki S, Yoshinaga K, Sasano N 1987 Suppression by glucocorticoid of the immunoreactivity of corticotropin-releasing factor and vasopressin in the paraventricular nucleus of rat hypothalamus. Neurosci Let 73:231

78. Ixart G, Barbanel G, Conte-Devolx B, Grino M, Oliver C, Assenmacher I 1987 Evidence for basal and stress-induced release of corticotropin releasing factor in the push-pull cannulated median eminence of conscious free-moving rats. Neurosci Let 74:85

79. Jingami H, Matsukura S, Numa S Imura H 1985 Effects of adrenalectomy and dexamethasone administration on the level of prepro-corticotropin-releasing factor messenger ribonucleic acid (mRNA) in the hypothalamus and adrenocorticotropin/β-lipotropin precursor mRNA in the pituitary in rats. Endocrinology 117:1314

80. Jones MT, Hillhouse EW 1976 Structure-activity relationship and the mode of action of corticosteroid feedback on the secretion of corticotrophin-releasing factor (corticoliberin). J Steroid Biochem 7:1189

81. Jones MT, Hillhouse EW, Burden JL 1977 Dynamics and mechanics of corticosteroid feedback at the hypothalamus and anterior pituitary

gland. J Endocrinol 73:405

82. Kaneko M, Hiroshige T 1978 Fast, rate-sensitive corticosteroid negative feedback during stress. Am J Physiol 234:R39

83. Kaneko M, Hiroshige T, Shinsako J, Dallman MF 1980 Diurnal changes in amplification of hormone rhythm in the adrenocortical system. Am J Physiol 239:R309

84. Kaneko M, Kaneko K, Shinsako J, Dallman MF 1981 Adrenal sensitivity to adrenocorticotropin varies diurnally. Endocrinology 109:70

85. Karteszi M, Dallman MF, Makara GB, Stark E 1982 Regulation of the adrenocortical response to insulin-induced hypoglycemia. Endocrinology 111:535

86. Keller-Wood ME, Dallman MF 1984 Corticosteroid inhibition of ACTH secretion. Endocrine Rev 5:1

87. Keller-Wood M, Shinsako J, Dallman MF 1984 Interaction between stimulus intensity and corticosteroid feedback in control of ACTH. Am J Physiol 247:E489

88. Kiss JZ, Mezey E, Skirboll L 1984 Corticotropin-releasing factor-immunoreactive neurons of the paraventricular nucleus become vasopressin positive after adrenalectomy. Proc Natl Acad Sci USA 81:1854

89. Knepel W, Nutto D, Meyer DK, Vlaskovska M 1984 Vasopressin release from rat medial basal hypothalamus *in vitro* after adrenalectomy or lesions of the paraventricular nuclei. Neurosci Let 48:321

90. Knizley H Jr 1972 The hippocampus and septal area as primary sites for corticosterone. J Neurochem 19:2737

91. Kovacs K, Kiss JZ, Makara GB 1986 Glucocorticoid implants around the

hypothalamic paraventricular nucleus prevent the increase of corticotropin- releasing factor and arginine vasopressin immunostaining induced by adrenalectomy. Neuroendocrinology 44:229

92. Kraicer J, Milligan JV, Gosbee JL, Conrad RG, Branson CM 1969 In vitro release of ACTH: effects of potassium, calcium and corticosterone. Endocrinology 85:1144

93. Krozowski ZS, Funder JW 1983 Renal mineralocorticoid receptors and hippocampal corticosterone-binding species have identical intrinsic steroid specificity. Proc Natl Acad Sci USA 80:6056

94. Leeman SE, Glenister DW, Yates FE 1962 Characterization of a calf hypothalamic extract with adrenocorticotropin-releasing properties: evidence for a central nervous system site for corticosteroid inhibition of adrenocorticotropin release. Endocrinology 70:249

95. Lemaire I, Dupont A, Bastarache E, Labrie F, Fortier C 1974 Some characteristics of corticosterone uptake by the dorsal hippocampus and the adenohypophysis in the rat. Canadian J Physiol Pharmacol 52:451

96. Levin N, Cascio C, Shinsako J, Dallman MF 1984a Steroid specificity of glucocorticoid-induced restoration of normal weight gain after adrenalectomy is compatible with an action on brain corticosteroid receptors. Program of The 7th International Congress of Endocrinology, Quebec City, Canada, July 1-7, 1984, p815

97. Levin N, Cascio C, Shinsako J, Dallman MF 1984b Specificity of adrenal steroid replacement on decreased body weight gain and plasma volume following adrenalectomy in rats. In: The neural and metabolic bases of feeding. Satellite symposium of The 14th Annual Meeting of

the Society for Neuroscience, Napa CA, October 7-9, 1984, p57

98. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193:265

99. McCann SM, Brobeck JR 1954 Evidence for a role of the supraopticohypophyseal system in regulation of adrenocorticotrophin secretion. Proc Soc Exp Biol Med 87:318

100. McCann SM, Fruit A, Fulford BD 1958 Studies on the loci of action of cortical hormones in inhibiting the release of adrenocorticotrophin. Endocrinology 63:29

101. McDonald RK, Weise VK 1956 Effect of arginine-vasopressin and lysine-vasopressin on plasma 17-hydroxycorticosteroid levels in man. Proc Soc Exp Biol Med 92:481

102. McEwen BS, DeKloet ER, Rostene W 1986 Adrenal steroid receptors and actions in the nervous system. Physiol Rev 66:1121

103. McEwen BS, DeKloet R, Wallach G 1976 Interactions *in vivo* and *in vitro* of corticoids and progesterone with cell nuclei and soluble macromolecules from rat brain regions and pituitary. Brain Res 105:129

104. McEwen BS, Plapinger L 1970 Association of 3H corticosterone-1,2 with macromolecules extracted from brain cell nuclei. Nature 226:263

105. McEwen BS, Weiss JM, Schwartz LS 1968 Selective retention of corticosterone by limbic structures in rat brain. Nature 220:911

106. McEwen BS, Weiss JM, Schwartz LS 1969 Uptake of corticosterone by rat brain and its concentration by certain limbic structures. Brain Res 16:227

107. McHugh PR, Smith GP 1967 Negative feedback in adrenocortical response to limbic stimulation in Macaca mulatta. Am J Physiol 213:1445

108. Makara GB, Stark E, Kapocs G, Antoni FA 1986 Long-term effects of hypothalamic paraventricular lesion on CRF content and stimulated ACTH secretion. Am J Physiol 250:E319

109. Makara GB, Stark E, Rappay G, Karteszi M, Palkovits M 1979 Changes in corticotrophin releasing factor of the stalk median eminence in rats with various cuts around the medial basal hypothalamus. J Endocrinol 83:165

110. Makara GB, Stark E, Karteszi M, Palkovits M, Rappay G 1981 Effects of paraventricular lesions on stimulated ACTH release and CRF in stalk-median eminence of the rat. Am J Physiol 240:E441

111. Martini L, Pecile A, Saito S, Tani F 1960 The effect of midbrain transection on ACTH release. Endocrinology 66:501

112. Merchenthaler I, Hynes MA, Vigh S, Schally AV, Petrusz P 1984 Corticotropin releasing factor (CRF): origin and course of afferent pathways to the median eminence (ME) of the rat hypothalamus. Neuroendocrinology 39:296

113. Meyer JS, Micco DJ, Stephenson BS, Krey LC, McEwen BS 1979 Subcutaneous implantation method for chronic glucocorticoid replacement therapy. Physiol Behav 22:867

114. Miller AL, Chaptal C, McEwewn BS, Peck EJ Jr 1978 Modulation of high affinity GABA uptake into hippocampal synaptosomes by glucocorticoids. Psychoneuroendocrinol 3:155

115. Moore RY, Eichler VB 1972 Loss of a circadian adrenal

corticosterone rhythm following suprachiasmatic lesions in the rat. Brain Res 42:201

116. Mor G, Saphier D, Feldman S 1986 Inhibition by corticosterone of paraventricular nucleus multiple-unit activity responses to sensory stimuli in freely moving rats. Exp Neurol 94:391

117. Nagai K, Nishio T, Nakagawa H, Nakamura S, Fukuda Y 1978 Effect of bilateral lesions of the suprachiasmatic nuclei on the circadian rhythm of food-intake. Brain Res 142:384

118. Nakanishi S, Kita T, Taii S, Imura H, Numa S 1977 Glucocorticoid effect on the level of corticotropin messenger RNA activity in rat pituitary. Proc Natl Acad Sci USA 74:3283

119. Palkovits M, Zaborszky L 1979 Neural connections of the hypothalamus. In Morgane PJ, Panksepp J (eds): Handbook of the hypothalamus. Marcel Dekker, Inc., New York, v1:375

120. Paxinos G, Watson C 1982 The rat brain in stereotaxic coordinates. Academic Press, Sydney.

121. Phillips M, Tashjian AH Jr 1982 Characterization of an early inhibitory effect of glucocorticoids on stimulated adrenocorticotropin and endorphin release from a clonal strain of mouse pituitary cells. Endocrinology 110:892

122. Plotsky PM, Bruhn TO, Vale W 1985 Evidence for multifactor regulation of the adrenocorticotropin secretory response to hemodynamic stimuli. Endocrinology 110:633

123. Plotsky PM, Otto S, Sapolsky RM 1986 Inhibition of immunoreactive corticotropin-releasing factor secretion into the hypophysial-portal circulation by delayed glucocorticoid feedback. Endocrin-

ology 119:1126

124. Plotsky PM, Sawchenko PE 1987 Hypophysial-portal plasma levels, median eminence content and immunohistochemical staining of corticotropin releasing factor, arginine vasopressin and oxytocin following pharmacological adrenalectomy. Endocrinology, in press

125. Plotsky PM, Vale W 1984 Hemorrhage-induced secretion of corticotropin-releasing factor-like immunoreactivity into the rat hypophysial portal circulation and its inhibition by glucocorticoids. Endocrinology 114:164

126. Porter JC, Jones JC 1956 Effects of plasma from hypophyseal-portal vessel blood on adrenal ascorbic acid. Endocrinology 58:62

127. Rappay G, Makara GB 1981 A quantitative approach to trace the corticotrophs in culture after adrenalectomy. Histochem 73:131

128. Redgate ES, Fahringer EE 1973 A comparison of the pituitary adrenal activity elicited by electrical stimulation of preoptic, amygdaloid and hypothalamic sites in the rat brain. Neuroendocrinology 12:334

129. Reul JMHM, DeKloet ER 1985 Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. Endocrinology 117:2505

130. Rhees RW, Grosser BI, Stevens W 1975a Effect of steroid competition and time on the uptake of [3H]corticosterone in the rat brain: an autoradiographic study. Brain Res 83:293

131. Rhees RW, Grosser BI, Stevens W 1975b The autoradiographic localization of [3H]dexamethasone in the brain and pituitary of the rat. Brain Res 100:151

132. Rivier C, Brownstein M, Spiess J, Rivier J, Vale W 1982 In vivo

corticotropin-releasing factor-induced secretion of adrenocorticotropin, β-endorphin, and corticosterone. Endocrinology 110:272

133. Rivier C, Rivier J, Mormede P, Vale W 1984 Studies of the nature of the interaction between vasopressin and corticotropin-releasing factor on adrenocorticotropin release in the rat. Endocrinology 115:882

134. Rivier C, Vale W 1983 Interaction of corticotropin-releasing factor and arginine vasopressin on adrenocorticotropin secretion in vivo. Endocrinology 113:939

135. Rivier C, Vale W 1985 Effect of the long-term administration of corticotropin-releasing factor on the pituitary-adrenal and pituitary-gonadal axis in the male rat. J Clin Invest 75:689

136. Roberts JL, Budarf ML, Baxter JD, Herbert E 1979 Selective reduction of proadrenocorticotropin/endorphin proteins and messenger ribonucleic acid activity in mouse piuitary tumor cells by glucocorticoids. Biochem 18:4907

137. Rose JC, Goldsmith PC, Holland FJ, Kaplan SL, Ganong WF 1976 Effect of electrical stimulation of the canine brain stem on the secretion of ACTH and growth hormone (GH). Neuroendocrinology 22:352

138. Russell SM, Dhariwal APS, McCann SM, Yates FE 1969 Inhibition by dexamethasone of the in vivo pituitary response to corticotropin-releasing factor (CRF). Endocrinology 85:512

139. Sato SM, Mains RE 1986 Regulation of adrenocorticotropin/endorphin-related peptide secretion in neonatal rat pituitary cultures. Endocrinology 119:793

140. Sawchenko PE, Swanson LW, Vale WW 1984 Co-expression of

corticotropin-releasing factor and vasopressin immunoreactivity in parvocellular neurosecretory neurons of the adrenalectomized rat. Proc Natl Acad Sci 81:1883

141. Sayers G, Portanova R 1974 Secretion of ACTH by isolated anterior pituitary cells: kinetics of stimulation by corticotropin-releasing factor and of inhibition by corticosterone. Endocrinology 94:1723

142. Sayers G, Sayers MA 1947 Regulation of pituitary adrenocorticotrophic activity during the response of the rat to acute stress. Endocrinology 40:265

143. Sayers G, Sayers MA, Liang T-Y, Long CNH 1945 The cholesterol and ascorbic acid content of the adrenal, liver, brain, and plasma following hemorrhage. Endocrinology 37:96

144. Schachter BS, Jonhson LK, Baxter JD, Roberts JL 1982 Differential regulation by glucocorticoids of proopiomelanocortin mRNA levels in the anterior and intermediate lobes of the rat pituitary. Endocrinology 110:1442

145. Schopohl J, Hauer A, Kaliebe T, Stalla GK, VonWerder K, Muller OA 1986 Repetitive and continuous administration of human corticotropin releasing factor to human subjects. Acta Endocrinol 112:157

146. Schurmeyer TH, Avgerinos PC, Gold PW, Gallucci WT, Tomai TP, Cutler GB Jr, Loriaux DL, Chrousos GP 1984 Human corticotropin-releasing factor in man: pharmacokinetic properties and dose-response of plasma adrenocorticotropin and cortisol secretion. J Clin Endocrinol Metab 59:1103

147. Segaloff A 1950 The gradation of effectiveness and absorption of

desoxycorticosterone acetate pellets by dilution with cholesterol. Endocrinology 47:320

148. Selye H 1936 A syndrome produced by diverse nocuous agents. Nature 138:32

149. Sen RN, Sarangi NN 1967 Effect of lesions in midbrain on ACTH release and control. Indian J Med Res 55:1072

150. Siperstein ER, Miller KJ 1973 Hypertrophy of the ACTH-producing cell following adrenalectomy: a quantitative electron microscopic study. Endocrinology 93:1257

151. Slusher MA, Hyde JE 1961 Effect of limbic stimulation on release of corticosteroids into the adrenal venous effluent of the cat. Endocrinology 69:1080

152. Smelik PG 1963a Relation between blood level of corticoids and their inhibiting effect on the hypophyseal stress response. Proc Soc Exp Biol Med 113:616

153. Smelik PG 1963b Failure to inhibit corticotrophin secretion by experimentally induced increases in corticoid levels. Acta Endocrinol 44:36

154. Stalla GK, Hartwimmer J, Schopohl J, VonWerder K, Muller A 1986 Intravenous application of ovine and human corticotropin releasing factor (CRF): ACTH, cortisol and CRF levels. Neuroendocrinology 42:1

155. Stark E, Karteszi M, Makara GB 1983 Responsiveness of adenohypophysial corticotrophs to hypothalamic (SME) extract remains unchanged in rats with hypothalamic lesions inhibiting CRF release. Neuroendocrinology 36:330

156. Stephan FK, Zucker I 1972 Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions. Proc Natl Acad Sci USA 69:1583

157. Stevens W, Grosser BI, Reed DJ 1971 Corticosterone-binding molecules in rat brain cytosols: regional distribution. Brain Res 35:602

158. Stillman MA, Recht LD, Rosario SL,Seif SM, Robinson AG, Zimmerman EA 1977 The effects of adrenalectomy and glucocorticoid replacement on vasopressin and vasopressin-neurophysin in the zona externa of the median eminence of the rat. Endocrinology 101:42

159. Suda T, Tomori N, Tozawa F, Mouri T, Demura H, Shizume K 1983 Effects of bilateral adrenalectomy on immunoreactive corticotropin-releasing factor in the rat median eminence and intermediate-posterior pituitary. Endocrinology 113:1182

160. Suda T, Tomori N, Tozawa F, Mouri T, Demura H, Shizume K 1984 Effect of dexamethasone on immunoreactive corticotropin-releasing factor in the rat median eminence and intermediate-posterior pituitary. Endocrinology 114:851

161. Swanson LW, Cown WM 1979 The connections of the septal region in the rat. J Comp Neurol 186:621

162. Swanson LW, Sawchenko PE 1983 Hypothalamic integration: organization of the paraventricular and supraoptic nuclei. Ann Rev Neurosci 6:269

163. Swanson LW, Sawchenko PE, Rivier J, Vale W 1983 Organization of ovine corticotropin-releasing factor immunoreactive cells and fibers in the rat brain: an immunohistochemical study.

Neuroendocrinology 36:165

164. Sydnor KL, Sayers G 1954 Blood and pituitary ACTH in intact and adrenalectomized rats after stress. Endocrinology 55:621

165. Szafarczyk A, Ixart G, Malaval F, Nouguier-Soule J, Assenmacher I 1979 Effects of lesions of the suprachiasmatic nuclei and of -chlorophenylalanine on the circadian rhythms of adrenocorticotrophic hormone and corticosterone in the plasma, and on locomotor activity of rats. J Endocrinol 83:1

166. Takebe K, Kunita H, Sakakura M, Horiuchi Y, Mashimo K 1971 Suppressive effect of dexamethasone on the rise of CRF activity in the median eminence induced by stress. Endocrinolgy 89:1014

167. Tilders FJH, Schipper J, Lowry PJ, Vermes I 1982 Effect of hypothalamus lesions on the presence of CRF-immunoreactive nerve terminals in the median eminence and on the pituitary-adrenal response to stress. Regulatory Peptides 5:77

168. Tramu G, Croix C, Pillez A 1983 Ability of the CRF immunoreactive neurons of the paraventricular nucleus to produce a vasopressin-like material. Neuroendocrinolcgy 37:467

169. Vale W, Spiess J, Rivier C, Rivier J 1981 Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and ß-endorphin. Science 213:1394

170. Vale W, Vaughan J, Smith M, Yamamoto G, Rivier J, Rivier C 1983 Effects of synthetic ovine corticotropin-releasing factor, glucocorticoids, catecholamines, neurohypophyseal peptides, and other substances on cultured corticotropic cells. Endocrinology 113:1121

171. Vale W, Vaughan J, Yamamoto G, Bruhn T, Douglas C, Dalton D, Rivier C, Rivier J 1983 Assay of corticotropin-releasing factor. In Conn PM (ed): Methods in enzymology. Academic Press, New York, 103H:565

172. Veldhuis HD, VanKoppen C, VanIttersum M, DeKloet ER 1982 Specificity of the adrenal steroid receptor system in rat hippocampus. Endocrinology 110:2044

173. Vermes I, Mulder GH, Smelik PG 1977 A superfusion system technique for the study of the sites of action of glucocorticoids in the rat hypothalamus-pituitary-adrenal system in vitro. II. Hypothalamus-pituitary cell-adrenal cell superfusion. Endocrinology 100:1153

174. Vernikos-Danellis J 1965 Effect of stress, adrenalectomy, hypophysectomy and hydrocortisone on the corticctropin-releasing activity of rat median eminence. Endocrinology 76:122

175. Videl C, Jordan W, Zieglgansberger W 1986 Corticosterone reduces the excitability of hippocampal pyramidal cells in vitro. Brain Res 383:54

176. Warembourg M 1975a Radioautographic study of the rat brain after injection of [1,2-3H]corticosterone. Brain Res 89:61

177. Warembourg M 1975b Radioautographic study of the rat brain and pituitary after injection of 3H dexamethasone. Cell Tiss Res 161:183

178. Watanabe H, Nicholson WE, Orth DN 1973 Inhibition of adrenocorticotropic hormone production by glucocorticoids in mouse pituitary tumor cells. Endocrinology 93:411

179. Watkins WB, Schwabedal P, Bock R 1974 Immunohistochemical demonstration of a CRF-associated neurophysin in the external zone

of the rat median eminence. Cell Tiss Res 152:411

180. Westlund KN, Aguilera G, Childs GV 1985 Quantification of morphological changes in pituitary corticotropes produced by *in vivo* corticotropin- releasing factor stimulation and adrenalectomy. Endocrinology 116:439

181. Whitnall MH, Mezey E, Gainer H 1985 Co-localization of corticotropin-releasing factor and vasopressin in median eminence neurosecretory vesicles. Nature 317:248

182. Widmaier EP, Dallman MF 1984 The effects of corticotropin-releasing factor on adrenocorticotropin secretion from perfused pituitaries *in vitro*: rapid inhibition by glucocorticoids. Endocrinology 115:2368

183. Wynn PC, Harwood JP, Catt KJ, Aguilera G 1985 Regulation of corticotropin-releasing factor (CRF) receptors in the rat pituitary gland: effects of adrenalectomy on CRF receptors and corticotroph responses. Endocrinology 116:1653

184. Yasuda N, Takebe K, Greer MA 1976 Studies on ACTH dynamics in cultured adenohypophyseal cells: effect of adrenalectomy or dexamethasone *in vivo*. Endocrinology 98:717

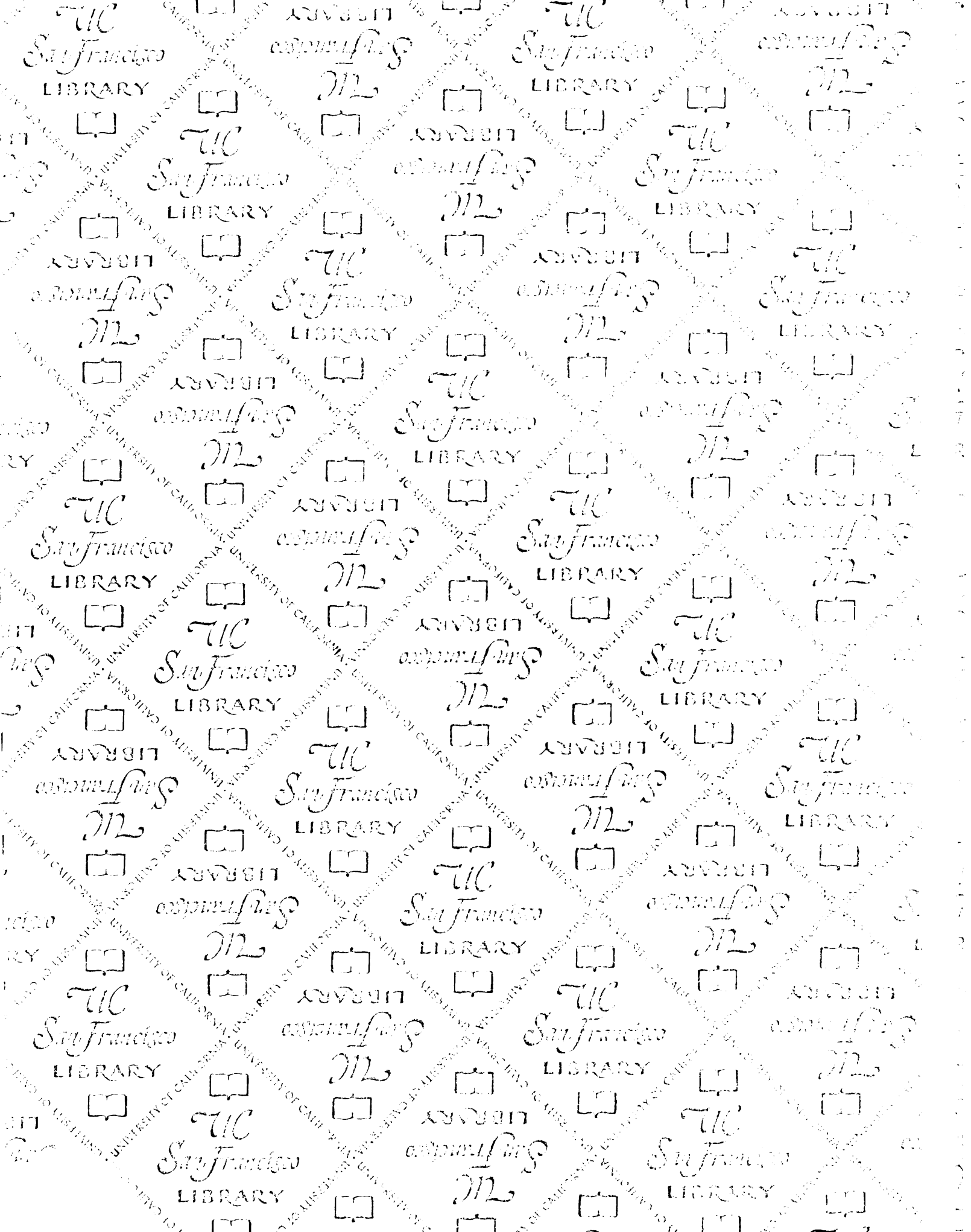
185. Yates FE, Leeman SE, Glenister DW, Dallman MF 1961 Interaction between plasma corticosterone concentration and adrenocorticotropin-releasing stimuli in the rat: evidence for the reset of an endocrine feedback control. Endocrinology 69:67

186. Yates FE, Russell SM, Dallman MF, Hedge GA, McCann SM, Dhariwal AS 1971 Potentiation by vasopressin of corticotropin release induced by corticotropin-releasing factor. Endocrinology 88:3

187. Young WS, Mezey E, Siegel RE 1986 Quantitative in situ hybridization histochemistry reveals increased level of corticotropin-releasing factor mRNA after adrenalectomy in rats. Neurosci Let 70:198

188. Zar JH 1984 Biostatistical analysis, 2nd edition. Prentice-Hall, Englewood Cliffs

189. Zimmerman EA, Stillman MA, Recht LD, Antunes JL, Carmel PW, Goldsmith PC 1977 Vasopressin and corticotropin-releasing factor: an axonal pathway to portal capillaries in the zona externa of the median eminence containing vasopressin and its interactions with adrenal corticoids. Ann NY Acad Sci 297:405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