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Delayed Persistent Corticosterone Response to an Intracerebroventricular CRF/norepinephrine Injection in Sprague Dawley Rats

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By

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Abstract

The effects of acute intense stress on physiology have repeatedly indicated a well-characterized over-activation of hypothalamic corticotropin releasing factor (CRF) neurons causing hyperactivity of the hypothalamic pituitary adrenal (HPA) axis. Such over-activation has characteristically been observed in those patients suffering from affective disorders such as depression, making this an area of therapeutic concern. Evidence suggests that during the intense stress encounter, CRF, besides its involvement in the HPA axis, exhibits a central function at the nucleus locus coeruleus to cause a release of norepinephrine (NE). Consequently, NE has shown to have as a target a number of brain areas that in turn release CRF. This feed-forward mechanism may be the key to triggering the hyperactivity of the HPA axis. To study this phenomenon CRF was administered centrally with and without NE to experimental Sprague Dawley rats. The data shows a persistent elevation in plasma corticosterone only in those rats treated with a simultaneous injection of NE and CRF. These results are indicative of a relationship existing between these two potent neurochemicals, and continue to lend evidence for targeting this feed-forward phenomenon as a potential therapeutic target.

Introduction

The body's ability to return to homeostasis after a normal stress is encountered is a function that is altered in the hypothalamic-pituitary-adrenal (HPA) axis when a severe or chronic stressor is encountered (Arborelius et al., 1999). In an effort to develop an adequate animal model to study the effects of intense stressors, what has largely been accepted is that the stressor in question must cause sustained behavioral and visceral arousal. Previous stress research using Sprague Dawley rats in the presence of intense stressors has shown sustained abnormal behavior in the form of altered activity in the running wheel, body weight changes with regards to feeding behavior, and increased latency to drop from a suspended wire (Ottenweller et al., 1989). Corticosterone has been the most useful marker for studying the visceral response to stress induced changes in physiology. Previously what has been observed is a persistent elevated plasma corticosterone level in the morning for several days after the last stressor (Ottenweller et al., 1989, Servatius et al., 2000, 2001; Fleshner et al., 1995). Both the behavioral and visceral responses have been observed when rats were repeatedly exposed to a 3-day regimen of Inescapable shock. (Servatius et al.,1995). This sustained, elevated plasma corticosterone level is believed to represent a persistent overactivation of the HPA axis. Based on this evidence, elevated levels of corticosterone existing 24 hours after the removal of a repeated stressor is sufficient to characterize this phenomenon. (Ottenweller et al., 1991).

These experiments were conducted to explore the actions of the neurochemicals CRF and NE in developing this characteristic persistent HPA overactivation. These neurochemicals were delivered directly into the Cerebrospinal fluid (CSF) via intracerebroventricular (ICV) cannulae to preferentially target the central effects of the treatments.

Evidence has supported the notion that during intense stress, CRF is not only released at the level of the hypothalamus to govern the activities of the HPA axis, but also from extrahypothalamic neurons in other brain regions. (Owens et al., 1991). Contribution of these regions along with the secretions of CRF from the hypothalamus is believed to lead to the overactivation; however, what remains unclear is what causes such a release of CRF from these extrahypothalamic brain regions.

Along with CRF's actions in the HPA axis, it is also known that CRF exhibits a central role during stress to mobilize the Sympathetic Nervous System. (Brown et al., 1982). Evidence suggests that CRF may have an action at the nucleus locus coeruleus causing a release of NE, and that NE may be involved in triggering the release of CRF from extrahypothalamic brain regions such as the amygdala in a feed-forward mechanism (Valentino, Foote and Page, 1993).

This thesis reviews the Stress Response, the effects of CRF on the HPA axis, the effects of CRF on the Central Nervous System, and the adverse effects of stress on the HPA axis. In addition, it offers experimental evidence to support the hypothesis of a feedforward mechanism existing between NE and extrahypothalamic CRF producing brain regions, as well as the hypothesis that

the neurochemicals released from these brain regions contribute in a significant way to delayed and persistent HPA activation.

Background

Stress Response and its Implications in the HPA Axis

Stress has been defined as a nonspecific result of any demand upon the body or anything that causes an alteration of psychological homeostatic processes (Selye, 1980; Burchfield and Weiss, 1979). Historically stress has been measured in the laboratory setting by investigating the measurable effects it exhibits on the HPA axis. This system contains a series of neurohormonal peptides that serve in controlling various homeostatic functions throughout the body. CRF, a 41-amino-acid hypothalamic peptide has potent activating effects on the pituitary adrenal axis as shown by its ability to trigger the release of Adrenocorticotropin hormone (ACTH) from the anterior pituitary (Rivier et al., 1984). ACTH stimulates the adrenal cortex to release potent hormones called glucocorticoids that have wide spread implications throughout the body. For example, the glucorticoids elicit such varying effects as gluconeogenesis, hyperinsulinemia, lysis of lymphoid tissue, increased gastric secretion, and reduced inflammatory and antibody responses (Koob, 1990). Metabolic changes are not the only effects; behavioral changes such as increased alertness and attention in response to elevated glucocorticoid secretions have also been observed (De Weid, 1977). Typically the HPA axis has been studied in the Sprague Dawley Rat using the glucocorticoid corticosterone as an indicator of the result of stress. This hormone can be detected in the plasma by using radioimmunoassay (RIA) techniques.

Stress Response and its Implications in the CNS

The Central Nervous System (CNS) also exhibits measurable changes in response to stress. The nucleus locus coeruleus specifically is one brain area that becomes activated to produce NE in the medial prefrontal cortex in response to stressors (Koob, 1999). As CRF is known to be an active factor during the stress response, its presence and potent effects have made it an obvious candidate for involvement at the level of the nucleus locus coeruleus (Valentino Foote and Page, 1993; Fisher, 1987). This correlation has been extensively researched and explored revealing insight from a wide range of perspectives. Prevalent studies have been centered mainly in physiological and anatomical perspectives. One such study indicated that ICV injections of CRF produce elevations of plasma epinephrine, NE, and glucose all of which cause measurable and observable alterations on the Sympathetic nervous system (Brown et al., 1982). This evidence establishes a physiological explanation for the various sympathetic effects seen during the stress response, and ties CRF release to central structures such as the nucleus locus coeruleus. The central administration of CRF is an important factor in that this implies that the major targets of the administered CRF are central structures and the HPA axis directly. Also of interest is the fact that the end points of such studies take into account observable manifestations such as how oxygen consumption rate changes in response to the ICV administered CRF (Brown et al., 1982). Electrophysiological studies have also been conducted utilizing both ICV administered CRF and single-unit measurements of electrophysiological activity at the nucleus locus

coeruleus. These results indicated that CRF stimulation on the nucleus locus coeruleus increased the spontaneous discharge rate of locus coeruleus neurons in male rats. (Borsody and Weiss, 1996).

In addition to physiological evidence, anatomical explanations also offer insight. The basis being that if CRF is active on the nucleus locus coeruleus, than anatomically these neurons should be found in close proximity to CRF producing neurons. One study shows how CRF neurons from the hypothalamus are preferentially co-localized with excitatory axon terminals in the nucleus locus coeruleus region. Such studies apply immunohistochemical techniques and electron microscopy to offer visual evidence of the anatomical distribution of CRF terminals (Valentino et al., 2001). Another such study utilized anterograde tract tracing combined with immunoelectron microscopic detection to verify, not only the proximity of CRF neurons to the nucleus locus coeruleus, but also the origin of the fibers. These origins included contributions from the central nucleus of the amygdala and the bed nucleus of the stria terminalis, regions involved in coordinating emotional responses to external stressors (Van Bockstaele et al., 2001).

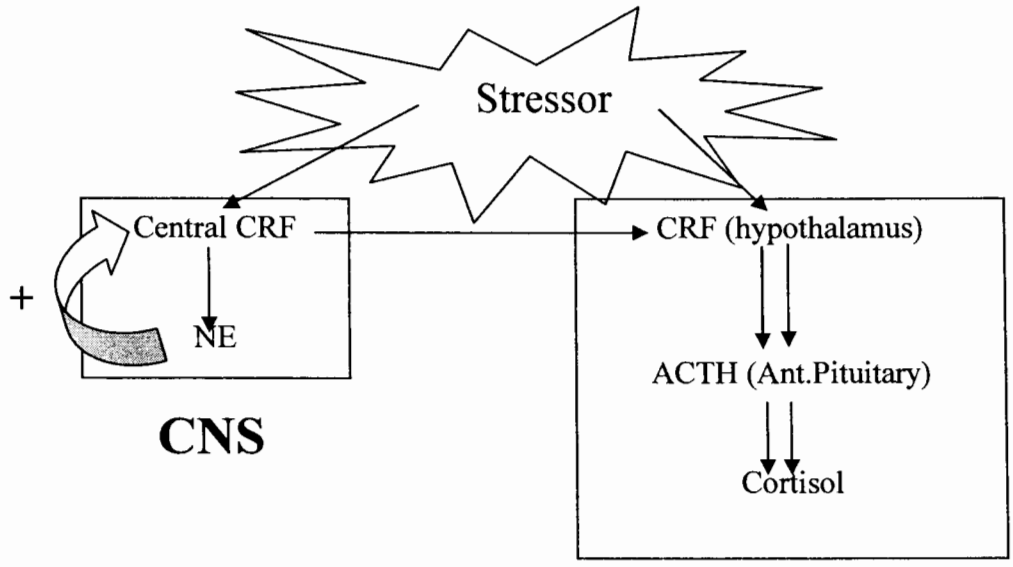
The subsequent actions of the catecholamines released from the Sympathetic Nervous System on the HPA axis also remains an area of constant transformation in light of new evidence. Previously the release of epinephrine and NE was considered inhibitory on the HPA axis. However, with the surfacing of new information regarding the central control of the HPA axis, the issues of excitation and inhibition have become more complicated than previously

accepted (Weiner and Ganong, 1978). The current hypothesis is that these catecholamines such as NE exhibit stimulation on other central CRF producing regions. The surfacing of such a hypothesis owes much to the observable overstimulation of the HPA axis in response to intense stress and the search for factors responsible for this effect. The first observable link is the definite and relatively fast mobilization of both the sympathetic nervous system and the HPA axis in response to stress. This feed-forward hypothesis speculates that an interaction between CRF and the locus coeruleus exists such that NE is produced in response to stress, and that NE in turn stimulates CRF release from the paraventricular nucleus of the hypothalamus as well as extrahypothalamic structures. These structures are namely the bed nucleus of the stria terminalis and the central nucleus of the amygdala (Koob, 1999). Such a system would suffice not only in initiating both systems, as both are known to become active during the general stress response, but also in maintaining a communicative link between both systems. The other implication is that such a system may possibly become overly active and vulnerable to considerable dysfunction in the presence of a substantial demand such as chronic stress. Numerous animal studies have been conducted specifically showing how the subsequent actions of the catecholamines could offer evidence for such a mechanism. The evidence for these claims lies mostly in studies set up to mimic or inhibit the neurological mechanisms involved. One such study offers an example by showing how the immediate release of stress-induced HPA hormones can be mimicked by ICV infusions of such catecholamines. Further, the same study showed how these

occurred even in rats whose catecholaminergic pathways entering the hypothalamus, (ventral noradrenergic ascending bundle) were blocked chemically (Szafarczyk et al., 1987). In another study, previously observed catecholamine stimulated ACTH release *in vivo* was found to be impaired in rats whose CRH activity was blocked by pharmacological, immunolological, or surgical procedures (Tilders et al., 1985). Such studies show the dependency of the HPA axis on central NE and CRF. Further studies have indicated the location of the actions of these catecholamines, specifically NE. Generally, the data shows a preferential targeting of not only the paraventricular nucleus of the hypothalamus, but also central structures such as the central nucleus of the amygdala, the bed nucleus of the stria termanilis, and areas within the hippocampus (Koob et al., 1990, 1999; Plotsky et al., 1987). The excitation of these regions due to NE along with the normal stimulation of the HPA axis due to stress supports the hypothesis that these mechanisms may cause the hyperactivation of the HPA axis. One specific proposition in question is the overactivation of the HPA axis to produce corticosterone in a delayed and persistent manner in response to high levels of stressors.

***Figure 1*:**

Overactivation of the HPA axis due to feed-forward stimulation between central CRF and NE from the nucleus locus coeruleus



Stress and Adverse Effects on the HPA

A persistent stress state has been previously referred to as the appearance of persistent alterations in physiology and behavior (Ottenweller et al., 1992; Servatius et al., 1994; Servatius and Shors, 1994). One such physiological alteration can be seen in the overactivation of the HPA axis in its production of corticosterone in response to Inescapable Shock (IS) in the Sprague Dawley rat long after the removal of the stressor. This phenomenon is observable during the morning trough period, normally the lowest point of the rat corticosterone circadian rhythm. (Ottenweller et al., 1992; Marti et al., 1993;

Servatius et al., 1994; Fleshner et al., 1995; Moldow et al., 2001). This peculiarity is of concern due to the many known human affective disorders existing because of such hormonal imbalances. The most well accepted finding among patients afflicted with psychiatric affective disorders is this very overactivation of corticotropin releasing hormone neurons that in turn cause hyperactivity of the HPA axis (Keck and Holsboer, 2001). Typically, some indicators of this overactivation in patients are basal hypercortisolemia, inappropriate HPA suppression by synthetic corticosteroid dexamethasone, and paradoxically increased ACTH and cortisol secretion in dexamethasone pretreated patients (Rybakowski and Twardowska, 1999; Holsboer, 2000; Holsboer and Barden, 1996). These physiological dysfunctions are directly related to behavioral alterations such as the psychopathology of anxiety disorders and depression. Furthermore, with continuing severe stress, actual physical damage to body organs can result (Koob, 1999).

Overview of experimental approach

The limitations posed in human experimentation have led to the necessity of establishing a suitable animal model for studying the persistent HPA overactivation. Previous experimentation has shown that one such suitable model for studying this phenomenon is carried out by using Inescapable Shock (IS) on the Sprague Dawley rat. Results from these experiments indicate a persistent elevation of plasma corticosterone twenty-four hours after the cessation of the IS (Ottenweller et al., 1992, 1994). Since basal hypercortisolemia has been established, this model serves as a base from which

to begin to ask questions as to the mechanisms of the neurochemicals involved. The purpose of these experiments was to reproduce the persistent elevations observed during IS by substituting IS with the neurochemicals in question. These neurochemicals, namely CRF and NE, were administered alone, and in combination, to better understand the mechanisms involved in producing persistent HPA overactivation. To ensure that these neuropeptides correctly mimicked the levels of stress induced by IS the dosage of each was comparable to the amounts reported in research articles involving the use of elevated stressors (Plotsky, 1987). To ensure that the central role of CRF was a factor, all injections were delivered in an ICV manner. Four experimental groups were used and are as follows: Vehicle, CRF, NE, and NE/CRF.

Materials and Methods

Thirty-two male Sprague Dawley rats (250-400 g) were obtained from Charles River (Wilmington, DE) and were housed individually in shoe-box type cages. The cages were placed inside light-tight, sound-attenuated, individually ventilated chambers. The lights were on from 0600-1800 h. Access to Purina Rodent Laboratory Chow and tap water was available ad lib throughout the experiments.

The rats were allowed to acclimate to these conditions for at least one week prior to surgical procedures. The rats were then weighed, stratified by weight, and randomly assigned to one of four groups: Vehicle(n=8), CRF(n= 8), NE(n=6), and CRF/NE(n=10).

All animals were anesthetized with Nembutol (50mg/kg of body weight intraperitoneally [IP]) and supplemental Ketamine (0.1cc doses of 100mg/ml solution) as needed. They were then placed in a stereotaxic instrument (Stoelting Physiology Research Instruments, Wooddale, IL) with toothbar 5mm above the interaural line. They were then implanted with a stainless-steel 23 gauge cannula aimed 1mm above the lateral ventricle. Coordinates for cannulae placement are as follows: Anterior to Posterior distance = - 0.6 bregma, Lateral = 1.3, and Depth = 3.5 cannulae, and 4.5 injector. Cannulae were fastened to the skull using supporting screws and acrylic. Briefly, powder and liquid acrylic (Crosslinked 444 acrylic Clear, and Cross-linked Flash Acrylic Liquid, Moltoid Co., Chicago, IL) were mixed to a glue-like consistency and placed around the

cannula and supporting screws. This was given time to harden. Rats were given one week for post-surgical recuperation.

The ICV infusions were administered as follows: Vehicle received 0.9% Normal Saline (Baxter, Deerfield, IL) at a dose of 1ul given at times 0, 30, and 60 minutes for a total of 3ul. The CRF group received 1ug CRF (Rat Corticotropin Releasing Factor, Sigma, St. Louis, MO) dissolved in 1ul of Sterile Saline given at times 0, 30, and 60 minutes for a total of 3ul. The NE group received 0.8ug NE (NE, USP, Sigma, St. Louis, MO) dissolved in 1ul PBS with pH 7.2-7.4. This injection was given only once. NE/CRF received 0.8ug NE/1ul PBS at time 0, and CRF 1ug/ul given at times 0, 30, and 60 minutes as described previously. All experiments were conducted beginning at 0800 to correspond to the trough of the circadian rhythm for plasma corticosterone. All injections were administered using a syringe microliter pump (Stoeling, Wood Dale, IL) and 50ul Hamilton Syringes (Fischer, Pittsburgh, PA). Thirty gauge needles (Fischer, Pittsburgh, PA) and PE-10 tubing (A. Diagger and Co, Vernon Hills, IL) attached to a 5.5mm injector (Plastics One, Roanoke, VA) was used to ensure correct dosing. The doses were delivered over a 30-60 second period. After delivery, the needle was left in place for a 1minute period and replaced by a wire stylet.

Tail bloods were taken at three specific time parameters as follows: First at 0800 (pre-injection), second ten minutes after last injection, (post-injection), and third at 0800 the next day (24-hours post-injection). The rats were gently removed from their cages and placed into a folded towel allowing only their tails to be exposed. The tip of the tail was then clipped using a surgical blade

(Fischer, Pittsburgh, PA), and the tail was gently massaged to release the blood. The blood was collected into heparinized capillary tubes (Fischer, Pittsburgh, PA), and one end was stoppered using hematoseal (Fischer, Pittsburgh, PA). The rats were then returned to their cages and the tubes were ready to be centrifuged. The tubes were centrifuged for at least four minutes, and plasma samples were stored frozen at -20°C until analyzed for corticosterone by radioimmunoassay as described previously (Ottenweller et al; 1989).

Verification of cannulae placement was conducted as follows: Methylene blue dye was injected into the cannula after all experimental procedures were completed. The dye was injected over a one-minute period at 10ul/min with a microliter pump and 50ul Hamilton syringe as previously described. Fifteen to thirty minutes were allowed for proper circulation before the rats were sacrificed. 0.2ml of Euthasol solution (Delmarva Laboratories, Midlothion, VA) was then administered IP, before removal of the brain. The head was removed via guillotine for brain removal. The skull was then removed from the region surrounding the brain with a bone cutter, and the brain was removed using a metal spatula. The brain was then placed in Formalyn Solution for storage. Brains were given at least seven days in the preservative before slicing. Slicing was done using either a Chryostat (Minotome Plus, Triangle Biomedical Sciences, Durham, NC), or a manual rat brain-slicing device. Slices were performed until the dye location was observed, and comparisons with an Atlas (Stereotaxic Atlas of the Rat Brain by Louis J Pellegrino and Anna J. Cushman,

Appleton-Century-Crofts, NY 1967) were made for confirmation of cannulae placement.

Statistical analysis

Plasma corticosterone was analyzed by analysis of variance (ANOVA). Post-injection values were analyzed separately using a One-Way ANOVA so that the stress level factor would be removed when analyzing the basal levels of plasma corticosterone. A Repeated Measures ANOVA was then conducted on the basal levels (pre-injection and 24 hours post-injection) to assess for any significant differences between injection (Veh, CRF, NE, NE/CRF) or time period (pre-injection, 24 hours post-injection). This was performed with one between-subject factor (injection), and one repeated measures within-subject factor (time period). A Duncan's Multiple Comparisons test was conducted on the Repeated Measures data for post-hoc analysis. $p<0.05$ was considered statistically significant.

Results

With regards to stress level plasma corticosterone, One-Way ANOVA revealed a significant main effect of injection, $F(3,31)=12.5$, $p=0.000026$. Post-hoc analysis (Duncan's) showed significant differences between vehicle and all other injection groups (See Figure 2; post-inj). NE/CRF demonstrated significant difference from both vehicle and NE, but not from CRF. This data demonstrates the effect of the injections on the immediate corticosterone levels and maintains that CRF and NE are potent stimulators of the HPA axis. With regards to the CRF/NE injection, the high elevations seen post-injection were not significantly different than the CRF dose levels, however since these plasma concentrations are typically not seen post stress, it remains of concern.

Repeated Measures ANOVA revealed a significant main effect of time period, $F(1,60)=4.25$, $p=0.05$. Post-hoc analysis (Duncan's) confirmed a difference between pre-injection and 24 hours post-injection time periods, and revealed the NE/CRF 24 hour post-injection group to be significantly different from all other basal level groups (Vehicle pre-injection, CRF pre-injection, NE pre-injection, NE/CRF pre-injection, Vehicle 24 hours post-injection, CRF 24 hours post-injection and NE 24 hours post-injection). Also of note, the NE/CRF pre-injection showed statistical difference only from NE/CRF 24 hours post-injection and no other basal level groups. This data demonstrates that the NE/CRF group was comparable to all other pre-injection groups and removes any uncertainty regarding stress factors outside of those relating to the injections (See Figure 2). With regards to the 24 hours post-injection levels, the data

demonstrates a significant elevation observed only in those rats treated with the simultaneous NE/CRF injection. This analysis suggests that the delayed persistent corticosterone effect is observed with the simultaneous injection only and is absent when either NE or CRF are delivered alone (See figure 2 post-inj).

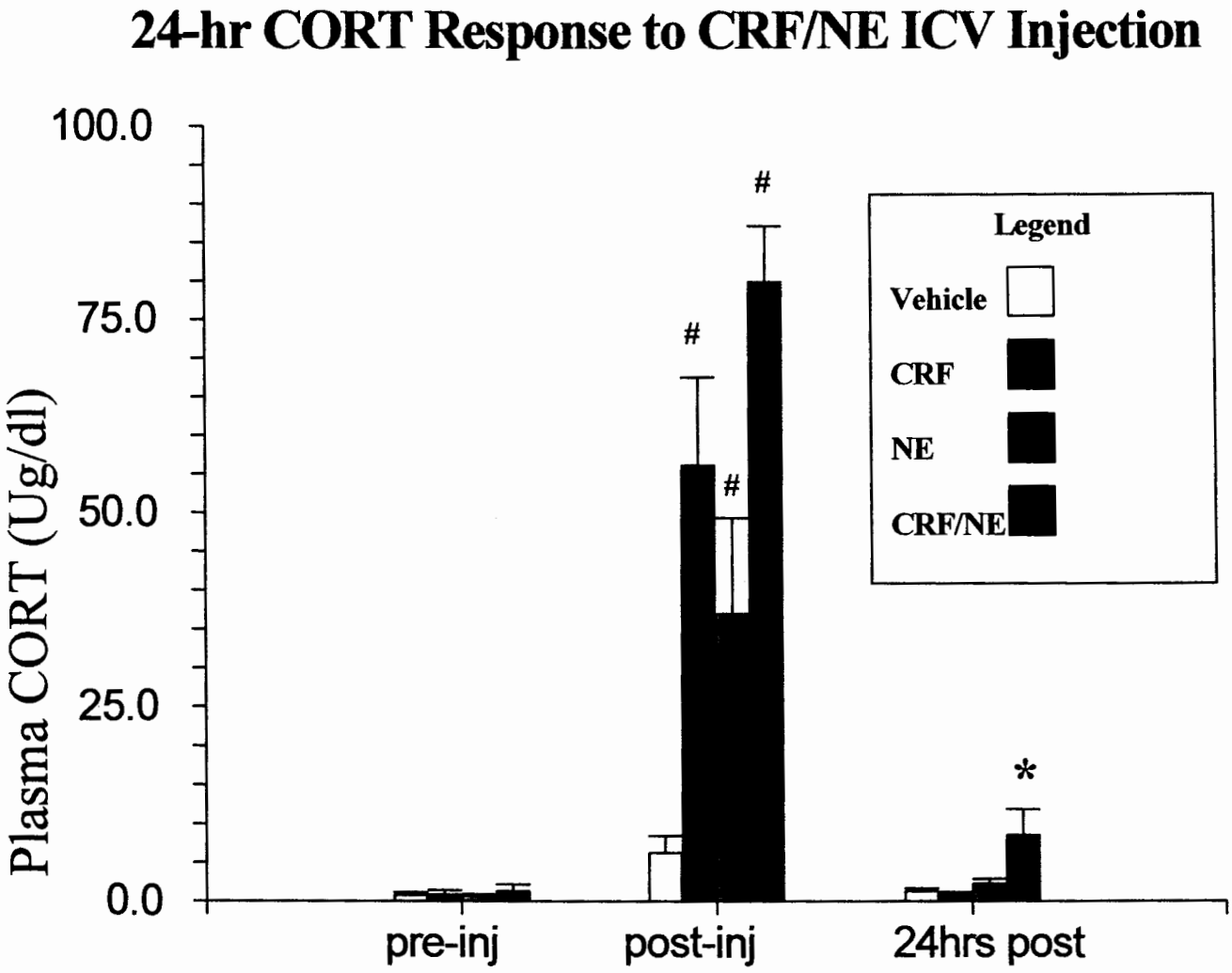


Figure 2:

The Effects of Various Intracerebroventricular Injections on Basal Morning Plasma Corticosterone (CORT) Concentrations in Adult Male Sprague Dawley Rats.

All data are presented as group means ± standard errors of the means (SEM). Asterisk indicates significant results of the Duncan's test denoting the 24-hour NE/CRF group as statistically different from all other basal level groups (pre-inj and 24hrs post). Note: Post-injection (post-inj) analysis was conducted separately. Duncan's test results indicated significant differences between vehicle post-inj and all other post-inj groups denoted by #. $P<0.05$ was considered statistically significant.

Table 1: *Plasma Corticosterone levels (ug/dl) represented as group means ± SEM*

	Pre-Injection	**Post-Injection**	**24hrs post-injection**
Vehicle	0.77 ± 0.91	06.24 ± 2.69	1.27 ± 0.72
CRF	0.82 ± 1.12	56.13 ± 11.23	0.95 ± 0.03
NE	0.66 ± 0.06	36.93 ± 12.32	2.31 ± 1.25
CRF/NE	1.27 ± 1.34	79.8 ± 7.00	8.53 ± 3.12

Discussion

Previous results using tail shock experimentation have shown characteristic rises in the trough levels of plasma corticosterone between 7-11ug/dl.(Ottenweller et al 1992; Servatius et al 1994; Fleshner et al 1995; Moldow et al 2001) The results of the current study indicate a comparable rise of 8.53 $\pm$ 3.12ug/dl seen only with concurrent ICV administration of CRF and NE. This data suggests that a simultaneous injection of CRF/NE suffices in mimicking the effects of the tailshock protocol previously described.(Ottenweller et al., 1989) The mechanism leading to this phenomenon still remains to be elucidated. The development of corticosterone dysfunction is of import for many reasons including the clinical impact observed with depression and other affective disorders (Yehuda, McFarlane and Shaley, 1989, Plotsky Owens and Nemeroff, 1998).

Under normal conditions the negative feedback mechanism for corticosterone is dependent mainly upon Mineralocorticoid receptors (MR). These are high affinity receptors responsible for negative feedback during the trough of the circadian rhythm, whereas Glucocorticoid receptors (GR) are low affinity receptors that are bound at the peak of the circadian rhythm and during the stress response. (DeKloet et al., 1998). In the presence of an intense stressor these receptors are hypothesized to become downregulated in regions such as the hippocampus and paraventricular nucleus of the hypthothalmus. This alteration could potentially result in a decreased sensitivity to the effects of elevated corticosterone with regards to negative feedback (DeKloet et al., 1998).

However, results regarding this hypothesis have been controversial. The following studies illuminate this point. In a study by Makino, GR mRNA levels were measured in the nucleus locus ceruleous in response to acute and repeated immobilization stress. What was observed was a stress dependant decrease in receptor mRNA where the repeated stress group demonstrated a larger decrease than the acute stress group (Makino, Smith and Gold, 2002). Contrary to this, findings in IS experiments demonstrated no changes in the amount of GR protein following the acute stressor (Deak et al., 2000). Deak suggests that the target cells in the brain and pituitary may have greater receptor occupancy and activation for up to several days following termination of the acute stressor, and that this may account for the long term consequences of acute stressor exposure (Deak et al., 2000). The determining factor between the two studies seems to be the intensity and length of the stressor applied. In comparing the two studies it appears that there is a progression from the over activated receptors to their eventual downregulation.

Another factor in question is the role of Corticosterone Binding Globulin (CBG), and the role it plays in the development of the delayed persistent response. What has been observed is a decreased level of CBG 24 hours following IS (Fleshner et al., 1995). CBG, or transcortin, is the carrier protein for corticosterone. It plays a regulatory role in corticosterone metabolism such that bound corticosterone is not able to cross the plasma membrane and bind to its intracellular receptors (Westphal, 1971). In the absence of transcortin, free corticosterone is available to exhibit its effect. Thus, the effect seen at 24 hours

post stressor could conceivably be attributed in part to the increased amount of free corticosterone. This finding reveals the complexity of the mechanism in question suggesting that multiple factors are at work and must be considered with regards to the observed elevation.

Also of interest is the role of the immediate acute rise in corticosterone following a stressor and the implications that these levels may have in the development of the elevated basal corticosterone levels observed. What has been observed using the IS protocol and metyrapone (blocks the synthesis of corticosterone), is that the delayed persistent rise in corticosterone would still occur even in the absence of the initial corticosterone response that reaches stress levels (Moldow et al., 2001). This finding suggests that the acute stress rise is not the factor responsible for the subsequent events noted. The GR antagonist, Mifepristone (RU-38486) has also been utilized in conjunction with IS experimentation. Results indicate that when administered prior to the stressor; the subsequent elevation of basal corticosterone at 24 hours is blocked (Moldow et al., 2005). These findings lend evidence suggesting that the importance in the mechanism lies in the reception of basal levels of corticosterone, and not the stress levels observed.

CRF has been implicated as one of the causal factors in the observation noted. The finding that CRF alone could not cause the subsequent observed rise is consistent with other experiments. What has been observed with regards to ICV CRF is the finding that some of the neurobehavioral aspects, such as exaggerated acoustic startle responses, and acquisition of the eyeblink

conditioned responses, model the behavioral changes observed after exposure to IS (Servatius et al., 2005). This finding recalls a critical aspect in the development of this model in that the behavioral aspects are as significant as the physiologic findings (Ottenweller et al., 1989). A similar protocol of ICV CRF was used in this experiment, and consistent with those findings, there is no evidence to suggest that ICV CRF caused the persistent elevated plasma corticosterone levels the following morning.

The influence of catecholamines on the HPA axis has historically been a topic of controversy. Pharmacological blockade of hypothalamic epinephrine has shown an increase of immunocytochemical staining intensity in median eminence CRF thus suggesting an inhibitory role for this catecholamine (Mezey et al 1984). This finding has been refuted by evidence suggesting a more excitatory role for catecholamines. Both central NE injection and electrical activation of catecholaminergic pathways have shown excitation of Immunoreactive CRF (irCRF) in the HPA axis; further, the NE ICV injection used in these experiments showed a dose dependent increase in irCRF when administered within a range of 1-5 nmol (Plotsky, 1987). A similar dose of ICV NE was used in this study, and consistent with their findings, acute HPA activation resulted immediately post injection. However, since the results observed during this study did not take into account the rise in irCRF, but instead the rise in plasma corticosterone this can be considered an indirect finding. Nonetheless this study is in agreement with the excitatory role previously reported for the effects of catecholamines on the HPA

axis. No further studies have been conducted considering the role of NE alone in developing the delayed persistent corticosterone effect.

One peculiarity lies within the extremely high elevations in plasma corticosterone represented by the NE/CRF, Post injection group. As the intent of this experiment was to mimic the persistent elevations in basal plasma corticosterone seen in Inescapable shock by using only the neurochemicals known to be involved, it seems that the goal was met however the means are controversial. Typically what can be seen Post Stress in IS are rises in mean plasma corticosterone from basal levels to 40-45 ug/dl (Moldow et al., 2001). This was previously considered to be in line with what an intense stressor would elicit. In this experiment, NE/CRF ICV injections caused a post-injection rise of 79.8 ug/dl. This exceeded the previously reported 45 ug/dl observed immediately Post-Stress in IS. This effect may be a dose dependent result that would need further evaluation as to the exact dosing that would maintain the delayed persistent corticosterone rise but keep the post-injection levels within the previously reported range. Also, with regards to RIA, this part of the standard represents the plateau after the steep slope. The values in this range lack precision and therefore, although we know the levels were high we can not be sure how elevated they really were. What remains evident is that regardless of the immediate effects, the long term elevations can only be seen in the NE/CRF treatment group.

Although the exact mechanism remains to be elucidated, what is apparent is that there is an intimate connection both anatomic and physiological between

the HPA axis and the nucleus locus coeruleus. Here the finding that only the simultaneous injection of the two most critical neurochemicals in their respective pathways could cause the phenomenon in question illuminates the interplay between the two regions.

What distinguishes this study is not only the neurochemicals involved, but the timing of delivery. The simultaneous presence of both CRF and NE may potentially closely replicate the way the body responds to intense stress in a natural environment and thus trigger the cascade towards the delayed persistent effect. What has been suggested is that a feedforeward mechanism may exist, and while the evidence continues to support this mechanism, the timing of events also seems to be a critical factor in the clarification of this event.

To further explore the complete mechanisms involved in causing the sustained corticosterone levels one proposition would be to conduct dose dependent ICV CRF delivery with a constant ICV NE variable to provide insight into the concentrations of CRF needed to elicit persistent elevations in corticosterone. Conceivably, once these concentrations are discovered, experimentation involving constant ICV NE and ICV CRF infusion variables with differing end times in tail blood procedures would give better insight as to how the rise occurs over time. This would yield the information necessary for developing a neurochemical model more reflective of how persistent corticosterone elevations occur as it would take into account any timely considerations to developing these persistent rises in corticosterone along the way.

Conclusion

Evidence maintains that extrahypothalamic CRF in addition to the normal release of CRF from the HPA axis explains the well-characterized persistent rise in corticosterone in response to intense stress. The surfacing of such information has led to persistent inquiries about mechanisms causing such a release of extrahypothalamic CRF. Animal models have provided substantial evidence in support of the release of CRF from central structures such as the central nucleus of the amygdala, the bed nucleus of the stria terminalis, and regions within the hippocampus during the stress encounter. The current hypothesis suggests that CRF may have an action at the nucleus locus coeruleus causing a release of NE, and that NE may be involved in triggering the release of CRF from extrahypothalamic brain regions in a feed-forward mechanism. One measurable indicator of CRF levels frequently studied in the lab setting is plasma corticosterone levels. The use of this indicator has allowed for the emergence of a suitable model for studying the effects of chronic stress. One such effect is the persistent overactivation of the HPA axis to produce corticosterone in response to intense stressors. This is a phenomenon that has been observed in patients with various affective disorders. The model consists of replacing the intense stressor with IS on the Sprague Dawley rat. Results from experiments implementing this procedure indicated a persistent elevation of plasma corticosterone twenty-four hours after the cessation of IS (Moldow et al., 2001). Using this criterion for delayed persistent corticosterone response this model served as a general starting point to study the actions of the neuropeptides

involved. The purpose of these experiments was to reproduce the observed delayed persistent corticosterone conditions by substituting IS with the neuropeptides in question. CRF and NE were administered alone, and in combination, to better understand the mechanisms involved in producing persistent HPA overactivation.

Having examined the effects of intense acute stressors on physiology, the most prominent finding among the results is that the delayed and persistent rise in corticosterone can be induced *in vivo* by simultaneous ICV injections of NE and CRF. One peculiarity lies within the extremely high elevations in plasma corticosterone represented by the NE/CRF, Post-injection group. As the intent of this experiment was to mimic the persistent elevations in basal plasma corticosterone seen in IS by using only the neurochemicals known to be involved, it seems that the goal was met; however the effects of the neurochemicals on corticosterone levels immediately post-injection remain in question.

Nonetheless, with these considerations in mind the data maintains a persistent elevation in plasma corticosterone only in those rats treated with a simultaneous injection of NE and CRF. These results are indicative of a relationship existing between these two potent neurochemicals in the development and sustenance of elevated corticosterone levels, thereby continuing to lend evidence for targeting this feed-forward phenomenon as a potential therapeutic target.

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