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The Role of Corticotropin-Releasing Factor in the Behavior and Proinflammatory Activity of Separated Guinea Pig Pups

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THE ROLE OF CORTICOTROPIN-RELEASING FACTOR IN THE BEHAVIOR AND
PROINFLAMMATORY ACTIVITY OF SEPARATED GUINEA PIG PUPS.

A thesis submitted in partial fulfillment of the
requirements for the degree of
Master of Science

By

VINCENT RASHAD ALEXANDER
B.S., Dillard University, 2008

2012
Wright State University

WRIGHT STATE UNIVERSITY
SCHOOL OF GRADUATE STUDIES

August 14, 2012

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY
SUPERVISION BY Vincent Rashad Alexander ENTITLED The Role of Corticotropin-
Releasing Factor in the Behavior and Proinflammatory Activity of Separated Guinea Pig
Pups
BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
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ABSTRACT

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The Role of Corticotropin-Releasing Factor in the Behavior and Proinflammatory Activity of Separated Guinea Pig Pups.

Isolation of guinea pig pups in a novel environment first produces active behaviors such as vocalizing and movement; over time, these behaviors wane and pups show characteristic passive responses similar to those produced by increased proinflammatory activity. Further, isolation of pups on two consecutive days has recently been shown to enhance those passive responses on the second day. Endogenous proinflammatory activity is thought to mediate the enhancement (sensitization). An injection of corticotropin-releasing factor (CRF) has been shown to increase passive behavior, possibly by increasing proinflammatory activity. The present study further investigated the role of CRF on proinflammatory activity and behavior during separation. In Experiment 1, pups were subcutaneously injected with 10 μ g of CRF or saline vehicle and then placed in a novel environment for 3 hr. CRF-injected pups exhibited more passive behavior and increased expression of the proinflammatory cytokine tumor necrosis factor alpha in the paraventricular nucleus of the hypothalamus when compared to saline injected pups. CRF increased plasma cortisol levels confirming that CRF activated the hypothalamic-pituitary-adrenal axis. In Experiment 2, pups were injected with either 75 μ g d-Phe₁₂₋₄₁(CRF₁₂₋₄₁), a corticotropin releasing factor antagonist, or saline

vehicle and separated for 3 hr on two consecutive days. CRF₁₂₋₄₁ increased active behaviors on Day 1. Passive behavior during separation was minimally affected by administration of the antagonist. In addition, passive responses increased from Day 1 to Day 2 in both the CRF₁₂₋₄₁ and saline groups. Together, these findings provide evidence that exogenous CRF increases passive behavior through a proinflammatory mechanism, but also raise questions about the role of endogenous CRF in the separation response.

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I. INTRODUCTION

Maternal Separation and Depression

The study of the association between prolonged infantile maternal separation and adulthood depression was pioneered by Rene Spitz and John Bowlby. Spitz (1945) found that children in institutions (hospitals/foundling homes) showed a decline in development shortly after birth, while similar aged children in nurseries showed an increase. Spitz suggested that while both groups had good food, safe housing, proper hygiene and sufficient medical care, it was the absence of adequate caregivers in the institutions that hindered development. The children initially cried incessantly then eventually quieted and displayed a reaction Spitz termed “anaclitic depression.” These responses were described by Bowlby (1969) as protest and despair. Together with Spitz’s data, Bowlby’s ideas led to the beginnings of the attachment theory.

The ideas of protest and despair are the two components of the attachment theory that is the focus of this paper. While in the protest stage, infants will try to re-establish a connection with their mother/maternal caregiver by crying. When this fails, the infant falls into the despair stage characterized by sadness and withdrawal from the environment. Persistent separation followed by reunion with the mother prompts the child to show negativism and hostile behavior towards her (Bowlby, 1970). This and other clues suggested that effects of separation might persist beyond the separation period. For example, Bowlby (1951) found previously separated children exhibited more delinquent

behavior than emotionally disturbed children. Based on such findings, Bowlby hypothesized that children who are separated from their mother/maternal caregiver will face permanent consequences: physical, intellectual, emotional and social.

More recent research has confirmed that early attachment disruption (separation/neglect/abuse) increases the chance of developing psychopathologies in adulthood. For instance, Hallstrom (1987) found that adults with major depression reported parental divorce or separation significantly more often than did controls. Cohen et al. (2001) found similar results in neglected children; they exhibited higher rates of depression in adulthood than their non-neglected counterparts. In another study, children that experienced neglect or death of their mother had higher rates of depression in adulthood (Harris et al., 1986). Childhood abuse has also been shown to produce similar long-term effects (see review Malinosky-Rummel & Hansen, 1993). The development of depression is thought to involve a sensitization of stress-related activity, which leads to increased activation of the hypothalamic-pituitary-adrenal (HPA) system (Gold et al., 1988). The HPA axis of women physically or sexually abused during childhood shows markedly enhanced activation in adulthood (Pariante & Lightman, 2008). When exposed to a psychosocial stressor, women with a history of depression exhibited a larger increase in adrenocorticotrophic hormone secretion (ACTH) and a much larger increase in cortisol secretion than non-depressed controls (Heim & Nemeroff, 2002). These findings suggest that early life stressors increase responsiveness of the HPA axis, which segues into depression in adulthood.

Animal models have proved to be valuable tools in studying the effects of maternal separation. The use of non-human primates and rodents allows researchers to manipulate controlled separation environments to produce depressive-like behaviors resembling those seen in humans. Monkeys show signs of protest and despair when separated. Kaufman and Rosenblum (1967) found that the immediate response to maternal separation in pigtail macaque infants was loud screams and attempts to re-establish maternal contact. When the infants could not achieve reunion, they hunched over, clasped their torso, and almost rolled into a ball. These behaviors were accompanied by social withdrawal. These responses suggested that attachment disruption produced depressive-like behaviors in monkeys. Hoff et al. (1994) found similar evidence in infant gorillas separated from their mothers and housed together with a sibling. Infant vocalizations and locomotion remained high for the first two days, but by the end of the second day the infants were holding themselves in fetal positions. During repetitive separations, infant rhesus monkeys displayed much higher rates of vocalizations and locomotion than self clasp on the first day (Suomi et al., 1983). Protest behaviors tended to decline over repeated separations. In contrast, the infant monkeys showed an increase in self clasping behavior with each separation. This increased “despair” provided evidence of behavioral sensitization. These results indicate that not only are protest and despair apparent in non-human primates, but so too is behavioral sensitization. Together these findings suggest that effects of attachment disruption like those seen in humans can also be seen in non-human primates.

Effects of maternal separation are also seen in rats and mice. Although the effects of prolonged separation are observed in altricial species such as these, they do not appear

to be due to an emotional attachment. Rat pups exhibit increased activity when separated from their mother as long as body temperature is maintained (Hofer, 1973). Removal of external warmth results in a decline in general activity levels of the pup (Hofer, 2006). These findings suggest that warmth provided by the mother normally maintains pup activity levels. In addition to body temperature, cardiac rate has also been observed to decline during separation. Hofer (2006) found that continuous infusion of milk into the separated pup's stomach maintained cardiac rate at normal levels. This supplied milk regulated the pup's heart rate. Separation of rat pups from their mother also leads to a lowering of growth hormone (GH) and ornithine decarboxylase (ODC) levels (Pauk et al., 1986). When stroked with a warm brush, a procedure used to mimic maternal stimulation, GH and ODC levels were preserved in separated pups. These findings suggest that specific forms of maternal stimulation regulate specific responses of the pup, and that no real emotional attachment exists. Although effects of maternal separation can be seen in rats and mice, they do not appear to be due to disruption of a specific attachment.

Guinea pigs show evidence of an attachment process that appears to approximate filial attachment in primate infants (Hennessy, 2003). Placing a pup into a test cage without its mother elicits an immediate elevation of plasma ACTH and cortisol levels (Hennessy, 1999). These responses coincide with increased active behaviors such as vocalizations and locomotor activity. Continued separation evokes a depressed-like, passive response characterized by crouching, piloerection, eye closure and lying down (Hennessy et al., 1995). Thus, the two stage, active/passive response is similar to that seen in primates (Hennessy, 1999). The passive response of the pups increases with

repeated separation from the mother (Hennessy et al., 2010). In a recent study, pups administered artificial cerebrospinal fluid (aCSF) prior to a first separation exhibited an increased passive response across days, while pups administered the anti-inflammatory cytokine interleukin-10 (IL-10) showed no sensitization (Hennessy et al., 2011b). These findings suggest proinflammatory factors contribute to the sensitization of depressive-like behavior.

Proinflammatory Activity During Separation

The immune system is usually recognized for its extremely specific properties (e.g., producing antibodies to combat antigens), but its initial response to invading bacteria or other pathogens is a nonspecific, inflammatory reaction, which is capable of occurring both locally (e.g., site of tissue damage) and systemically. The reaction, better known as the acute phase response (APR) or just “sickness,” is an innate immune response in which immune cells (e.g. macrophages, monocytes) release proinflammatory cytokines [e.g. interleukin-1 (IL-1), IL-6, and tumor necrosis factor alpha (TNF- α)] (Baumann & Gauldie, 1994). These peptide messengers induce the physiological components of sickness, including fever, shifts in the synthesis of liver proteins, and activation of the HPA axis. Although antigens are generally considered the stimulus for the APR, stressors can sometimes cause release of cytokines as well (Dantzer et al., 2008).

Cytokines can also produce behavioral changes known as “sickness” behaviors (Dantzer, 2004; Maier & Watkins, 1998). These behaviors include inactivity, sleepiness, warmth seeking, and postures to conserve body heat. These behaviors, similar to the

physiological mechanisms, appear to be adaptive such as supporting fever and conserving energy. Anyone who has experienced a viral or bacterial infection knows what it means to feel sick. Growing evidence supports the idea that symptoms of depression also result from proinflammatory cytokines (Yirmiya et al., 2000; Dantzer et al., 2008).

The sickness behaviors observed during the APR response are similar to the passive behaviors seen during separation in guinea pig pups. These similarities alone do not assure that the separation behaviors are true components of sickness. One could be more confident that this is the case if: (a) direct activation of the innate immune system in guinea pig pups produced the same passive behaviors as seen during separation, (b) the passive behaviors elicited by separation were reduced by anti-inflammatory agents, and (c) the passive behaviors seen during separation were accompanied by physiological responses of the APR (Hennessy et al., 2009). Investigators have provided evidence supporting each of these predictions. First, injecting pups with lipopolysaccharide (LPS), which is derived from the cell wall of gram negative bacteria, and induces proinflammatory activity, dose-dependently produces the same passive behavioral responses that are seen during separation (Hennessy et al., 2004).

Regarding the second prediction, intracerebroventricular (ICV) administration of alpha-melanocyte-stimulating hormone (α -MSH), a naturally occurring endogenous peptide hormone with a wide range of anti-inflammatory effects, resulted in pups spending less time exhibiting passive behaviors during a 3-hr separation than did pups administered saline (Schiml-Webb et al., 2006). Because the same dose of α -MSH reduced passive behaviors produced by injections of LPS (Hennessy et al., 2007), these

results suggest that the anti-inflammatory properties of α -MSH were responsible for reducing the passive behaviors produced by the separation.

Indomethacin is another anti-inflammatory that has been studied, but unlike α -MSH, it is not naturally occurring, but rather a drug used to reduce fever, pain, and inflammation. Injection of indomethacin also diminished the passive behaviors of pups during separation (Hennessy et al., 2007). In another study, the effects of IL-10 were explored. IL-10 is a naturally occurring cytokine secreted primarily by monocytes with robust anti-inflammatory properties. IL-10 has pleiotropic effects during the APR response and appears to regulate proinflammatory activity. Perkeybile et al. (2009) found that injections of IL-10 across five different doses reduced passive behaviors exhibited by pups during separation. Together, three different anti-inflammatory agents have decreased passive behaviors during separation.

Evidence for the third prediction has been provided by showing that along with behavioral changes, core temperature increases during separation, as does proinflammatory cytokine expression in the spleen (both are aspects of the APR) (Hennessy et al., 2007; Hennessy et al., 2010). Together this evidence provides reason to assume that passive behavioral responses of separated guinea pig pups are mediated in part by proinflammatory factors.

Corticotropin-Releasing Factor

The link between separation and sickness may involve the stress-responsive neuropeptide corticotropin-releasing factor (CRF). CRF is a 41-amino acid peptide

derived from a 191-amino acid prohormone and is found along with its high affinity binding sites in various regions of the central nervous system and periphery (Aguilera et al., 1987; Bruhn et al., 1982; Suda et al., 1984; Nakane et al., 1986). CRF is synthesized and secreted from the paraventricular nucleus (PVN) of the hypothalamus and is released at the median eminence by the parvocellular cells into the hypothalamo-hypophyseal portal system. From there, it is carried to the anterior lobe of the pituitary where it stimulates the release of the adrenocorticotrophic hormone (ACTH) and β -endorphin (Owens & Nemeroff, 1991; Rivier et al., 1982). This cascade appears to mediate the HPA “stress” response (Tilders & Berkenbosch, 1986; Lenz et al., 1987).

In addition to its endocrine-activating properties, central administration of CRF has been shown to produce other physiological and behavioral effects associated with stress. Central CRF administration produces a range of effects from activation of the sympathetic nervous system (Brown et al., 1982) to stimulation of central noradrenergic activity (Dunn & Berridge, 1987). Central injections of the hormone produce a rapid increase in body temperature (LeFeuvre et al. 1987) and increase metabolic rate (Rothwell, 1989). The behavioral effects of central CRF administration are observed in a variety of animals. For instance, in young adult rhesus monkeys ICV CRF has been found to very rapidly induce huddling, wall-facing, and self clasping while reducing locomotion and environmental exploration (Strome et al., 2002). In rats, central administration of CRF has been shown to have a range of effects on behavior including suppressed feeding (Morley & Levine, 1982), increased grooming (Morley & Levine, 1982), reduced exploratory behavior (Spardaro et al., 1990), potentiated acoustic startle (Swerdlow et al., 1989) and enhanced defensive withdrawal (Takahashi et al., 1989). Centrally

administered CRF also inhibits the sexual behavior of male rats (Sirinathsinghji, 1987). These behaviors are similar to those seen in rats exposed to stress.

Peripheral CRF may also affect behavior. Intravenous (IV) CRF in adult rhesus monkeys produced a number of behavioral changes including increased vocalizing and “lying down” behavior and decreased self grooming (Kalin et al., 1983). Behavioral measures of nociception were also found to decrease in adult rats given intracardial (IC) and IV CRF (Ayesta, & Nikolarakis, 1989; Hargreaves et al., 1987). Furthermore, Ayesta and Nikolarakis (1989) reported that rats exhibited “lying down” behavior similar to that of monkeys (Kalin et al., 1983) when given IC CRF.

Guinea pigs have also been shown to display behavioral effects of both central and peripheral administration of CRF. Isolated guinea pig pups exhibit a reduction in vocalizing when given ICV CRF by freehand injection (Hennessy et al., 1992). Peripheral CRF injections produce a similar behavioral change. That is subcutaneous (SC) CRF administration was found to inhibit the active behaviors of vocalizing and locomotor activity, and also to increase the passive responses of crouch, eye-close, and piloerection in guinea pig pups during separation (Hennessy et al., 1991; Becker & Hennessy, 1993; Hennessy et al., 1995). Injections of CRF elicit behavioral effects similar to those seen with increased proinflammatory activity: Passive behaviors increase while active behaviors decrease (Dunn & Berridge, 1990; Hennessy et al., 1991; Hennessy et al., 2007). It is important to note that with peripheral administration, CRF does not freely cross the blood-brain barrier (Martin et al., 1996). To produce behavioral effects, CRF could work in a number of ways, for example via circumventricular organs,

which lie outside the protection of the blood-brain barrier (Spardaro et al., 1990), or indirectly by activating peripheral proinflammatory activity (Dunn et al., 2005; Hagan et al., 1993; Leu & Singh, 1992; Paez Pereda et al., 1995; Singh & Leu 1990). Increased peripheral proinflammatory activity is capable of increasing proinflammatory activity in the central nervous system (Miller et al., 2009), which might produce increased passive behavioral outcomes (Hart, 1988; Hennessy et. al., 2009). Pups given IL-10 or α -MSH following CRF injection show reduced behavioral effects of CRF, indicating that passive behaviors induced by CRF appear mediated by proinflammatory activity (Schiml-Webb et al., 2009; Hennessy et al., 2011a).

Central administration of the non-specific CRF-receptor antagonist α -helical CRF₉₋₄₁ has been found to enhance active behaviors of separated guinea pig pups. When administered via indwelling cannula, the receptor antagonist increased vocalizations emitted by pups during the first 10 min of an isolation test, and increased line crossings during the last 10 min of the test (Hennessy et al., 1992). In a similar fashion, a peripherally administered a receptor antagonist D-Phe CRF₁₂₋₄₁ (CRF₁₂₋₄₁) also increased active behaviors. Guinea pig pups injected with the antagonist emitted more vocalizations and exhibited more line-crossing in a 60 min isolation test, than did saline-treated controls (Hennessy et al., 1997; McInturf & Hennessy 1996). McInturf and Hennessy (1996) also found that passive behavior was greater in pups receiving peripheral saline than in pups receiving CRF₁₂₋₄₁. Along with these findings, pups also had similar cortisol levels indicating a stress response was evident in both conditions. These findings show that both central and peripheral administration of CRF-receptor antagonist increases active behaviors, suggesting that endogenous CRF normally suppresses these behaviors.

Goals of the Present Study

The primary purpose of the current experiments was to further examine the role of peripheral CRF in the response of guinea pig pups to separation. In Experiment 1, I investigated the hypothesis that peripheral CRF administration produces behavioral changes by increasing central proinflammatory activity. It was predicted that peripherally injected CRF, at a dose that increases passive behavior, would increase central proinflammatory activity, resulting in increased expression of proinflammatory cytokines. Plasma cortisol levels following testing were also analyzed to assess effects on the HPA axis.

Experiment 2 examined behavioral effects of the inhibition of endogenous CRF by CRF₁₂₋₄₁ during separation. McInturf and Hennessy (1996) showed that when pups are given CRF₁₂₋₄₁ prior separation, their active behaviors increase. This provides evidence that the effects of endogenous CRF exert some degree of inhibition on active behavior. The study provided only preliminary results for passive behavior due to the short period (1 hr) of observation. With only a 60-min isolation test, passive behavior was just beginning to emerge towards the end of the test. When injected the antagonist, fewer pups exhibited two of the passive behaviors than when injected with saline (McInturf & Hennessy, 1996). Using a similar protocol as McInturf and Hennessy (1996), in the current Experiment I increased separation to 3 hr to provide a more complete examination of the behavioral effects of CRF₁₂₋₄₁ on passive behavior. A second purpose of Experiment 2 was to examine whether endogenous CRF plays a role in the sensitization of passive behavior that occurs with repeated separation. Using a similar

procedure as Hennessy et al. (2011b), I observed possible sensitization across 2 daily separations. Hennessy et al. (2011b) found that with administration of an anti-inflammatory prior to a first separation, sensitization was inhibited the next day. This indicates that proinflammatory activity the first day contributes to sensitization the next. It is hypothesized that endogenous CRF is involved in the initiation of proinflammatory activity that induces sensitization. If so, then inhibiting CRF on Day 1 of separation should decrease sensitization the next day.

II. GENERAL METHOD

Animals and General Testing Procedures

Guinea pigs (*Cavia porcellus*) of the Hartley strain were bred in our laboratory. After birth, the mother and her litter were kept in a plastic cage with a metal wire front with food and water freely available. The colony room was maintained on a 12/12 hour light/dark cycle (lights on at 0700).

For testing, the experimental animal was transported in a carrying cage from the colony room to the observation room located in the same laboratory suite. Here, the pup was placed alone into a clear plastic cage (47 x 24 x 20 cm) on an outline that divided the cage floor into fourths. A slotted lid was placed on the cage to prevent escape. No more than one pup from a litter was assigned to a particular experimental condition. After each test, all cages and lids used were cleaned with detergent and air dried.

Behavioral Observation

An observer sat behind a one-way glass and recorded active and passive behaviors of the pups. Behaviors were scored during minutes 0-30, 60-90, and 150-180 of the 3-hour separation. Active behaviors recorded were the “whistle” vocalization and locomotor activity. A microphone placed above the test cage transmitted the vocalizations into the observation room via headphones where they were scored on a hand counter. Locomotor activity was scored when all four of the pup’s feet crossed any of the lines dividing the cage into fourths. The passive behaviors were eye-closure (when the pup had either one or both eyes completely or near completely closed for at least 1 second), piloerection (when visible over most of the body surface), and crouch (body hunched down with head lowered and feet tucked beneath). Crouch sometimes

transitioned into lying down when the pup's body was supported by the cage floor. Crouch, including lying down, eye-closure, and piloerection observed in the same 60-s interval was scored as "full passive" behavior. Our primary measure of passive behavior was the full passive response. All scoring was done by a trained observer (inter-observer reliability of at least 85%).

Data Analysis

Analysis of variance (ANOVA) was the preferred method of analysis with Newman-Keuls for paired, post-hoc comparisons. When data violated assumptions for parametric tests, nonparametrics were used. The Mann-Whitney *U* Test was used for between subject comparisons and the Wilcoxin Signed Rank Test was used for within subject comparisons. A significance level $p < 0.05$ (2-tailed unless otherwise noted) was accepted throughout.

III. EXPERIMENT 1

Experiment 1 determined if an injection of CRF of a dose/route known to produce passive behavior would also increase proinflammatory cytokine expression in the PVN. Plasma cortisol was also assessed in order to estimate the effect on HPA activity.

Method

A total of 39 animals were used: 13 pups were assigned to the CRF condition (7 male, 6 female), 14 pups to the saline control condition (7 male, 7 female), and 12 pups to the undisturbed control condition (6 male, 6 female). The undisturbed group was used to assess baseline levels of the physiological measures. Pups were tested between postnatal days 20-23. The pups were removed from the home cage and received a subcutaneous (SC) injection of either 10 μ g CRF in saline or saline vehicle in a .2 mL volume. This dose of CRF has been shown to suppress active behavior and to increase the passive, depressive-like response (e.g. Hennessy et al., 2011a). After injection, the animals were returned to the home cage for 60 min prior to testing. Testing occurred between 0700hr and 01300hr. Following testing, animals were anesthetized with CO₂ for 1-min and then decapitated. Trunk blood was collected in a test tube and placed on ice until centrifugation to separate plasma, which was stored at -20 °C until assayed for cortisol. A commercial RIA kit (Siemens “Coat-a-Count”) was used with plasma diluted 1:5. Intra-assay variability was calculated to be 9.6%. All samples were collected within 2 min of the onset of disturbance.

The brain was quickly removed and brain tissue was dissected on a cold plate to remove a slice containing the PVN. The slice was placed in 750 μ L of RNAlater and refrigerated for at least 24 hours, but no longer than a week. After refrigeration, the PVN

was carefully removed and placed in a micro-centrifuge tube containing 25 μ L of RNAlater and stored at -20 °C until cytokine (IL-1 β , IL-6, TNF- α) analysis. Total cellular RNA was extracted from the tissue as was described in Blandino Jr. et al., (2009). Total RNA yield and purity were determined using the Experion Automated Electrophoresis System (Bio-Rad). Synthesis of cDNA was performed on 0.1-1.0 μ g of normalized total RNA using the QuantiTect $\text{\textcircled{R}}$ Reverse Transcription Kit (Cat No. 205313, Qiagen, Valencia, CA), which included a DNase treatment step. All cDNA was stored at -20°C until time of assay.

Reverse-transcription polymerase chain reaction (RT-PCR)

Probed cDNA amplification was performed in a 1741 μ l reaction consisting of 900 μ L IQ SYBR Green Supermix (BioRad), 9 μ L primer, 40 μ l cDNA template and 792 μ L RNase-free water run in duplicate in a 96-well plate (BioRad) and captured in real-time using the iQ5 real-time PCR detection system (BioRad). Following a 3-min hot start (95 °C), samples endured denaturation for 30 s at 95 °C, annealing for 30 s at 60 °C and were extended for 30 s at 72 °C for 50 cycles. For melt curve analysis, samples underwent 0.5 °C changes every 15 s ranging from 55 °C to 95 °C. A distinct peak expressed as the negative first derivative of the change in fluorescence as a function of temperature demonstrated primer specificity to the target gene. All PCR data were adjusted relative to ultimate control groups for the experiment and adjusted relative to the housekeeper gene (β -actin).

Results

Passive Behavior. The two-way (Group x Sex) between-subjects ANOVA for the full passive response yielded only one significant effect, a main effect of Group, $F(1, 20)$

= 12.61, $p < 0.05$ (Fig. 1). Animals injected with CRF exhibited the full passive response during more 1-minute intervals than did pups in the saline condition.

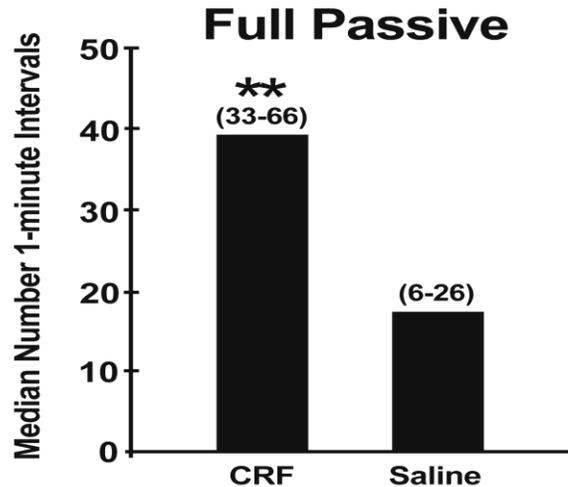


Figure 1. Median number of 1-min intervals of full passive exhibited by pups across groups during separation. Interquartile ranges are noted above each bar. **Indicates the significant main effect of group ($p < 0.05$).

Active Behavior. There were no significant effects between groups or between males and females with the Mann-Whitney U tests for vocalizing or line-crossing. However, as Figure 2 indicates, the median level of vocalizing (Fig. 2a) for saline-injected pups was 6-fold higher than that of pups injected with CRF and the median level of line-crossing (Fig. 2b) was twice as high as in the CRF condition. It appears that the lack of statistical significance can be traced to three animals in the saline condition that exhibited no vocalizations or line crossings.

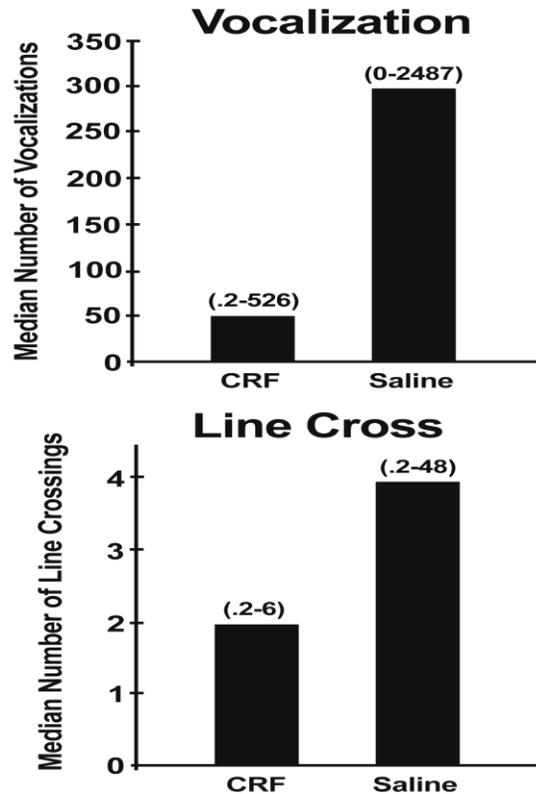


Figure 2. (a) The upper panel illustrates the median number of vocalizations across groups undergoing separation. Interquartile ranges are noted above each bar. (b) The lower panel illustrates the median number of line crossings across groups undergoing separation. Interquartile ranges are noted above each bar.

Physiology. For cortisol, the between-subjects ANOVA yielded a significant effect of Group, $F(2, 33) = 77.38, p < 0.01$ (Fig. 3). Animals injected with CRF had higher plasma levels of cortisol than did saline-injected and non-injected animals (p 's < 0.01). Initial ANOVAs of IL-1 β , IL-6, and TNF- α expression were non-significant, though the factor of Group approached significance for TNF- α ($p < 0.07$, Fig. 4). Since

the two control groups were similar, they then were pooled for further analysis. Between-subjects ANOVA yielded a significant effect, $F(1, 33) = 6.49, p < 0.05$ (Fig. 5), indicating that the CRF injection increased TNF- α expression in the PVN.

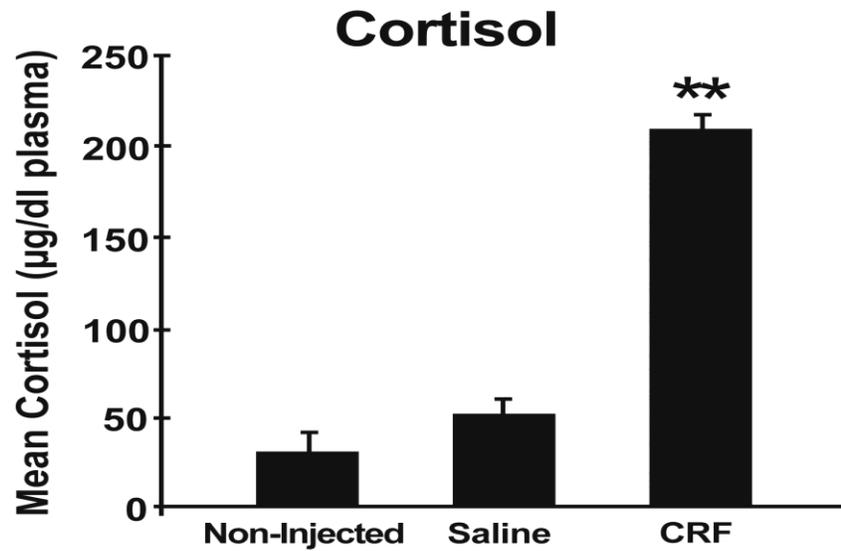


Figure 3. Mean cortisol of all groups. Vertical lines represent standard errors of the means. ** Indicates significant effect of group ($p < 0.01$).

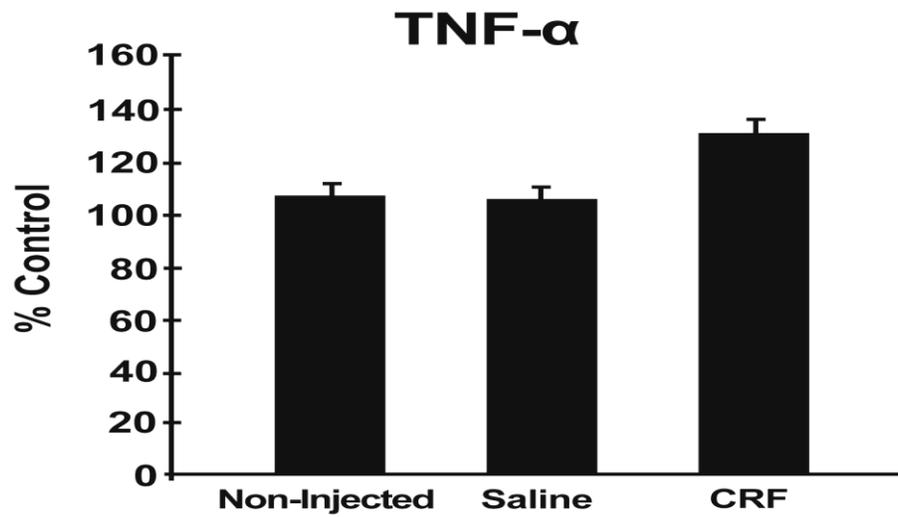


Figure 4. Initial ANOVA of TNF- α for all groups. Vertical lines represent standard errors of the % control. Groups approached significance ($p < 0.07$).

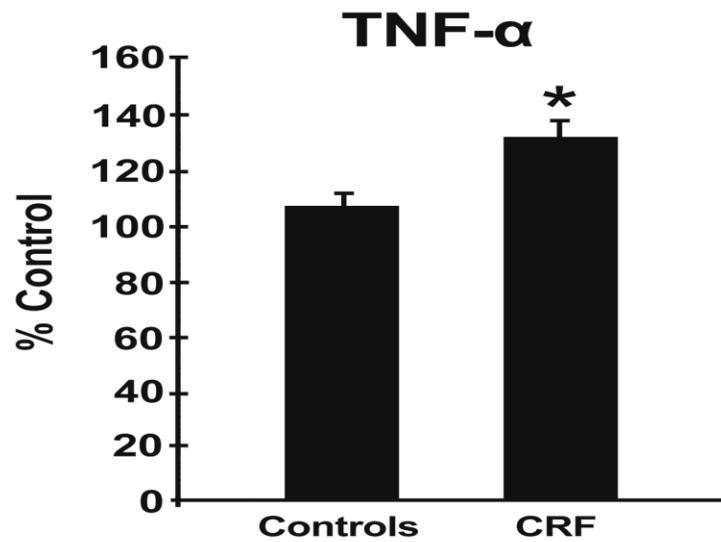


Figure 5. ANOVA of the CRF and Control groups after pooling. Vertical lines represent standard errors of the % control. * Indicates significant effect of group ($p < 0.05$).

IV. EXPERIMENT 2

Experiment 2 assessed the role of endogenous CRF in the transition from active to passive behavior during separation, as well as in behavioral sensitization with repeated separation. This was done by examining whether inhibiting CRF activity during an initial separation would: (a) increase active and decrease passive responses during that separation; and (b) inhibit sensitization of behavior the following day. A non-specific CRF-receptor antagonist was used to inhibit CRF activity.

Method

A total of 24 animals were used: 12 pups (6 male, 6 female) were assigned to either the d-Phe₁₂₋₄₁ corticotropin releasing factor antagonist (CRF₁₂₋₄₁) or saline groups. The pups were tested on two consecutive days. On the first day (postnatal days 20-23), the pup was injected with either 75 µg of CRF₁₂₋₄₁ in saline, or just the saline vehicle, in a .2 mL volume and then immediately separated for 3 hr. Pups were then returned to their home cage and twenty four hours after the first separation, both groups received an injection of saline and were separated for a second 3-hr period. Testing occurred between 1100 and 1500 hr.

Results

Passive Behavior. The 2 (Group) x 2 (Sex) x 2 (Day) repeated measures ANOVA yielded only a significant main effect of Day for full passive response, $F(1, 20) = 9.73, p < 0.05$ (Fig. 6). Animals spent more time exhibiting passive, depressive-like behavior on day 2 than on day 1. This sensitization was not affected by prior injection of CRF₁₂₋₄₁. Further, passive behavior on Day 1 was not significantly affected by injection of the antagonist. In an earlier paper (McInturf & Hennessy, 1996) in which the antagonist

reduced passive behavior, passive behavior was examined only on 1 day, and the individual components of crouch, eye-close, and piloerection were observed rather than the full passive response. Eye-closure and piloerection were reduced in that study. Therefore, to test the replication, comparisons between groups for crouch, eye-closure and piloerection on Day 1 were conducted with *t*-tests. Vehicle-treated animals were found to show more piloerection than pups receiving CRF₁₂₋₄₁ ($t(22) = 1.85; p < 0.05$, 1-tailed test, Fig. 7).

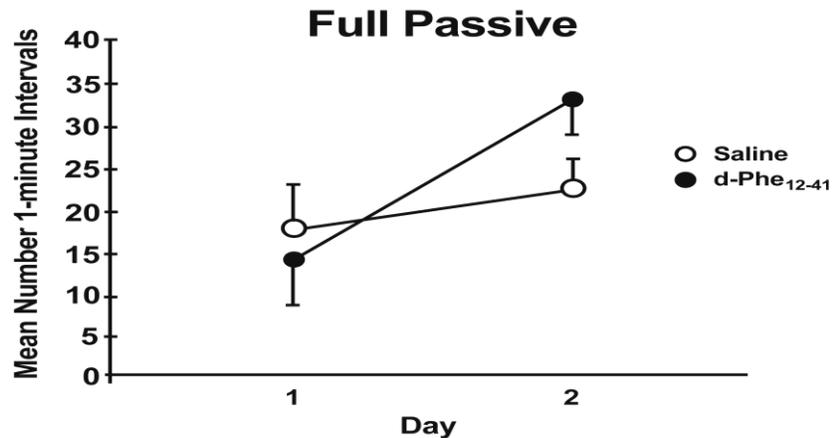


Figure 6. Mean number of 1-min intervals of full passive depressive-like behavior exhibited by animals across groups during consecutive daily separations. Vertical lines represent standard errors of the means.

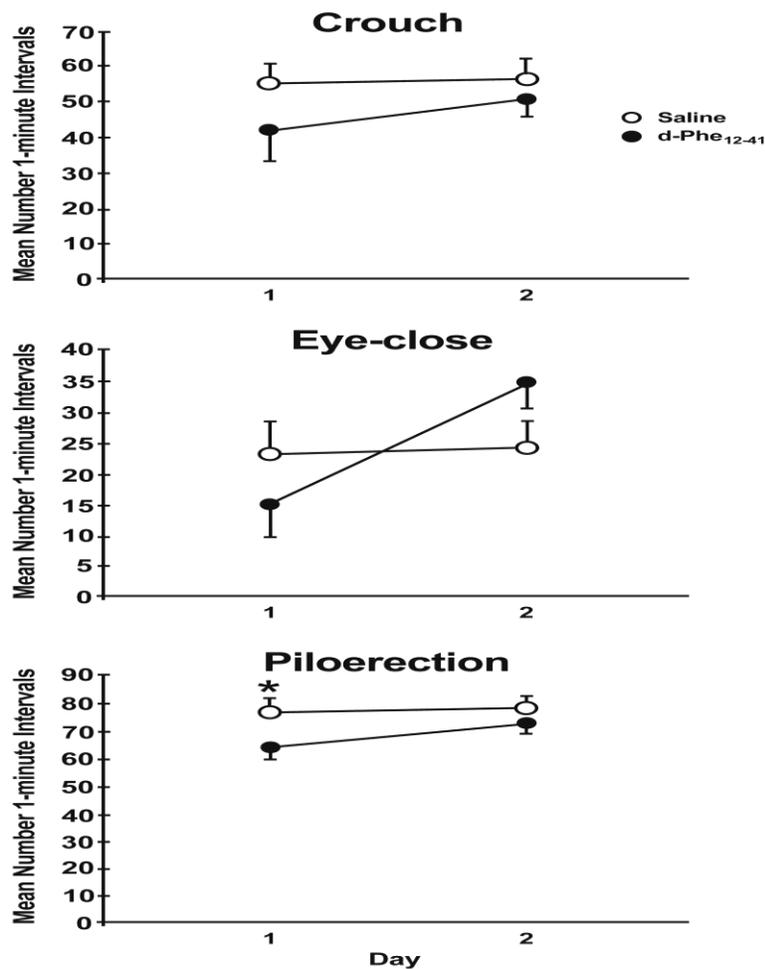


Figure 7. All comparisons between groups done with t-tests. The upper panel illustrates the mean number of 1-min intervals of crouch behavior exhibited by animals across groups during consecutive daily separations. The middle panel illustrates the mean number of 1-min intervals of eye-close behavior exhibited by animals across groups during consecutive daily separations. The lower panel illustrates the mean number of 1-min intervals of piloerection behavior exhibited by animals across groups during consecutive daily separations. Vertical lines represent standard errors of means. * Indicates significance of group on Day 1 ($p < 0.05$).

Active Behavior. The between groups Mann-Whitney U test showed that CRF₁₂₋₄₁ injection produced a large increase of vocalizations and line-crossings relative to saline on Day 1 (p 's < 0.05 ; Fig. 8). Active behavior in the CRF₁₂₋₄₁ group did not decline during testing a day after injection with the antagonist; in fact, it appeared to further increase, though this effect was not significant for either vocalizing or line-crossing. However, the modest increase in vocalizing and line-crossing from Day 1 to 2 in the saline group was significant (vocalizing: $p < 0.05$; line-crossing $p < 0.01$). On Day 2, the difference between groups approached significance ($p < 0.07$ for both).

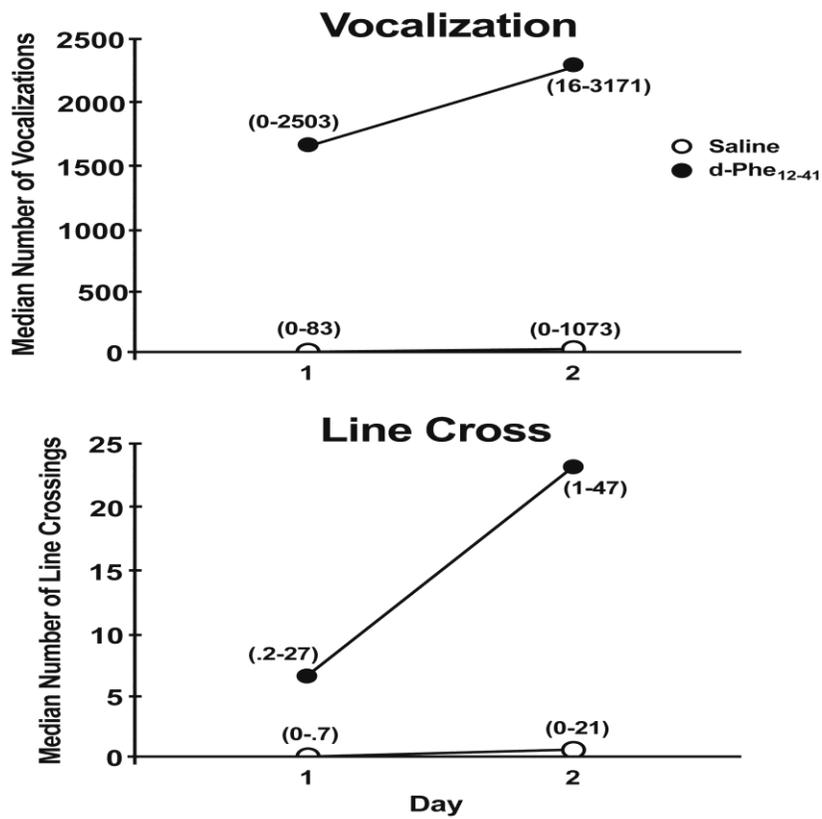


Figure 8. The upper panel illustrates the median number of vocalizations emitted by pups during consecutive daily separations. The bottom panel illustrates the median number of line crossings emitted by pups during consecutive daily separations. Noted on each panel are interquartile ranges for each group on each Day.

V. DISCUSSION

In Experiment 1, CRF was administered peripherally to determine its effect on behavior and proinflammatory activity. CRF was found to significantly increase passive behavior and proinflammatory activity. CRF was found to significantly increase passive behavior in separated guinea pig pups. Active behaviors in CRF- injected pups were observed to non-significantly decline during separation. The lack of significance may have been due to three saline-treated pups that exhibited no vocalizations or line-crossings. These findings support previous results that increased CRF activity increases passive behaviors and decreases active behaviors in separated pups (Hennessy et al., 1997, McInturf & Hennessy, 1996). There also was evidence that peripheral administration of CRF increased proinflammatory activity. Although initial ANOVAs yielded no significant effects on cytokine expression in the PVN, ANOVAs with control groups pooled provided evidence that CRF significantly increased expression of proinflammatory cytokine TNF- α , thus indicating that proinflammatory activity increases with heightened CRF activity. Finally, a marked increase in cortisol levels confirmed that CRF injection potentially activated the HPA axis.

Experiment 2 had two purposes: (a) assessing the role of endogenous CRF on active and passive behavior using CRF₁₂₋₄₁ during separation and (b) examining if CRF₁₂₋₄₁ would inhibit sensitization the following day. The antagonist increased active behaviors in pups on Day 1. In contrast, the full passive response on Day 1 was unaffected by administration of the antagonist. Yet further examination of the individual passive behaviors showed that the antagonist reduced levels of piloerection during this first test, thereby offering a partial replication of the findings of McInturf and Hennessy (1996). The full passive response increased from Day 1 to Day 2 in all pups, replicating the

previously observed sensitization (Hennessy et al., 2011b). However, sensitization was seen both in pups administered CRF₁₂₋₄₁ and in those given saline. This suggests that sensitization was not mediated by an increase in endogenous CRF. If in fact endogenous CRF did play a role in sensitization, then CRF₁₂₋₄₁ should have inhibited CRF, which would then be expected to reduce sensitization on Day 2. Though unexpected, increased active behaviors seen by pups given the antagonist on Day 1 showed no reduction on Day 2. This suggests that temporarily disrupting CRF activity somehow had a persisting effect on the mechanisms underlying vocalizations and locomotor activity.

CRF has been shown to be an effective agent to produce passive behavior in stressed animals. As was found in this and earlier studies (Hennessy et al., 1995), guinea pig pups injected with CRF show heightened passive behavior during separation. Passive behaviors seen are similar to those experienced with “sickness”, suggesting that proinflammatory activity is involved. Further, passive behaviors seen in pups injected with CRF or a proinflammatory agent are similar (Hennessy et al., 2009). An increase in the expression of the proinflammatory cytokine TNF- α in the PVN in the current study provides direct support that peripheral administration of CRF induces central proinflammatory activity. In addition, the emission of passive behavior produced by injection of CRF in earlier studies was reduced following administration of IL-10 or α -MSH (Hennessy et al., 2011a; Schiml-Webb et al., 2009). In all, these findings show that both increased proinflammatory activity and increased CRF can be linked to the depressive-like response in guinea pig pups.

Patients administered proinflammatory cytokines for chemotherapy had higher rates of depressive symptoms (Kronfol, 2002). Depressed patients also have high levels of circulating proinflammatory factors (Raison et al., 2006). Results such as these provide the basis for the cytokine hypothesis of depression (see Schiepers et al., 2005). Others have suggested a central role for CRF in depression (Gold et al., 1988). Our results are consistent with both of these hypotheses. However, the results of Experiment 1 say more about the effects of exogenous CRF than about how naturally occurring CRF might affect proinflammatory activity and mood.

Endogenous CRF in earlier studies has been found to reduce active behaviors during separation. Isolated guinea pig pups were found to vocalize more and exhibit more locomotor activity when given CRF₁₂₋₄₁ than when given saline (Hennessy et al., 1997). In rats similar findings are evident. Berridge and Dunn (1987) found that α -helical CRF₉₋₄₁ (ahCRF) can antagonize restraint-induced behavioral changes. Using the same antagonist Insel and Harbaugh (1989) reported that isolated rat pups exhibit increased ultrasonic vocalizations. These findings together support the hypothesis that endogenous CRF works to suppress active behaviors seen in pups during separation. Similar to previous findings, Experiment 2 in this study showed that the administration of CRF₁₂₋₄₁ increased vocalizations and locomotor activity. Passive behavioral results from Experiment 2 were not as clear. There was no difference between groups in full passive behavior the first day. Further, both groups of animals were found to show significantly more full passive behavior on Day 2. However, replicating an earlier finding from McInturf and Hennessy (1996), saline-treated pups exhibited significantly more piloerection. This finding could be taken as evidence for the hypothesis that endogenous

CRF promotes passive behavior. However, the effect was minimal and could have simply been due to the fact that pups vocalizing and moving around about the cage cannot also show crouch, piloerection and eye-close behaviors.

In conclusion, our results from Experiment 1 replicate previous findings that peripheral administration of CRF prior to separation does produce increased passive behavior. Peripheral CRF increased proinflammatory activity in pups continuing to link passive behaviors during isolation to behaviors seen during “sickness.” Cytokine expression in the PVN after peripheral injection of CRF provides evidence proinflammatory activity increases and is involved in the passive behavioral response. These findings together with increased cortisol levels are consistent with past results and indicate that exogenous CRF can induce the second behavioral stage of separation via a proinflammatory mechanism. Experiment 2 raises questions about the role of endogenous CRF. The data collected show that (a) there were not clear reductions in passive behavior following administration of CRF₁₂₋₄₁ and (b) sensitization of passive behavior, which previously has been found to be mediated by proinflammatory activity was not affected by the antagonist. Endogenous CRF in the current study does appear to reduce active behaviors during separation because upon injection of the antagonist these behaviors increased, agreeing with past results. Thus, although endogenous CRF does appear involved in the transition from the active to passive stage of responsiveness, there was minimal support for endogenous CRF inducing passive behavior by increasing proinflammatory activity. Together, these findings suggest that more research is needed to clearly identify the effect of endogenous CRF on behavior during separation.

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