

Long-Term Consequences of Neonatal Rearing on Central Corticotropin-Releasing Factor Systems in Adult Male Rat Offspring

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In a series of studies on the long-term consequences of neonatal rearing, we compared hypothalamic and extrahypothalamic central corticotropin-releasing factor (CRF) systems in male rats reared under conditions of animal facility rearing, nonhandling (HMS0), handling with brief maternal separation for 15 min (HMS15), or handling with moderate maternal separation for 180 min (HMS180) daily from postnatal days 2–14. CRF-like immunoreactivity (CRF_{ir}) was elevated in lumbar cerebrospinal fluid of adult HMS180 and HMS0 rats relative to the other groups. In the paraventricular nucleus, central nucleus of the amygdala, bed nucleus of the stria terminalis, and locus coeruleus, CRF_{ir} and CRF mRNA levels were significantly elevated in HMS0 and HMS180 rats. Neonatal maternal separation was associated with regionally specific alterations in CRF receptor type 1 (CRF1) mRNA density in HMS180 rats. No rearing-associated differences in CRF2 α binding were apparent in either the lateral septum or the ventromedial hypothalamus. These findings indicate that early rearing conditions can permanently alter the developmental set-point of central CRF systems, and potentially influence the expression of behavioral and endocrine responses to stress throughout life, thereby providing a possible neurobiological substrate for the relationship between early life events and increased vulnerability for hypothalamic–pituitary–adrenal axis and coping skill alterations and the frequency of mood disorders in patients with a history of such experiences.

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INTRODUCTION

The nature of the early postnatal environment can stably alter behavioral and hypothalamic–pituitary–adrenal (HPA) axis responses to stressors in the rat (Denenberg, 1964; Levine, 1975; Bhatnagar *et al*, 1995; Meaney *et al*, 1996; Liu *et al*, 1997; Ladd *et al*, 1996, 2000), although the mechanisms underlying these effects remain unclear. Most notable among these behavioral effects are changes in responses to novelty. In response to a number of testing conditions, adult offspring reared under conditions of

neonatal maternal separation show increases, while neonatal handling exhibits decreases in behavioral fearfulness when compared to animals that were unhandled during the neonatal period (Caldji *et al*, 2000). Similarly, HPA axis responsivity to stressors is enhanced in animals exposed as neonates to repeated daily maternal separation for ≥ 180 min as compared to unhandled (Viau *et al*, 1993; Ladd *et al*, 1996) or animal facility reared rats (Ladd *et al*, 2000; Huot *et al*, 2001). These behavioral and endocrine responses to novelty may either be under independent control or tightly linked via alterations in novelty or stressor-sensitive neurocircuits.

Central corticotropin-releasing factor (CRF) systems have been strongly implicated in the expression of behavioral responses to stressors as well as in the regulation of HPA axis responsivity. Intracerebroventricular administration of CRF produces responses comparable to those of fear-inducing stimuli (Dunn and Berridge, 1990; Owens and Nemeroff, 1991) including increased locomotor activity in a

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familiar environment (reviewed in Koob *et al*, 1994), suppressed exploration in a novel environment (Britton *et al*, 1982; Sutton *et al*, 1982; Berridge and Dunn, 1987; Takahashi *et al*, 1989), enhanced stress-induced freezing (Sherman and Kalin, 1988) and grooming (Britton *et al*, 1984; Imaki *et al*, 1987), decreased sexual behavior (Sirinathsinghji *et al*, 1983), facilitation of acoustic startle (Swerdlow *et al*, 1986; Liang *et al*, 1992a,b), and decreased food intake (Krahn *et al*, 1986; Arase *et al*, 1988). These effects are blocked by administration of the CRF receptor antagonist α -helical CRF (Dunn and Berridge, 1990; Menzaghi *et al*, 1993). In addition, CRF administration has aversive properties, producing both taste and place aversions (Cador *et al*, 1992; Heinrichs *et al*, 1992a, 1993). Koob (1999) has proposed the existence of a feed-forward system between norepinephrine (NE) and CRF systems to coordinate the response to environmental challenges. Considerable pharmacologic, physiologic, and neuroanatomical evidence supports a CRF-NE interaction in the region of the locus coeruleus (Koob, 1999; Lejeune and Millan, 2003; Page and Valentino, 1994; Plotsky *et al*, 1989; Valentino *et al*, 1998).

Further evidence for the importance of the central CRF system is derived from studies of a transgenic CRF overexpression mouse model (Stenzel-Poore *et al*, 1994). These animals exhibit enhanced stress-induced suppression of exploration that can be reversed with intracerebroventricular administration of the CRF receptor antagonist α -helical CRF₉₋₄₁. These findings are consistent with the thesis that CRF overproduction contributes to the etiology and/or pathophysiology of mood disorders (Chrousos and Gold, 1992; Arborelius *et al*, 1999).

The effects of CRF on the expression of behavioral, endocrine, and autonomic responses to stress appear to be attributable to two major CRF systems: (a) the hypothalamic paraventricular nucleus (PVN) CRF neuronal system, which projects to the median eminence thus regulating adenohypophysial adrenocorticotropin (ACTH) secretion as well as to midbrain regions regulating sympathetic activity, and (b) CRF neurons in the central nucleus of the amygdala (CeA) and the bed nucleus of the stria terminalis (BNST), regions projecting to the PVN as well as to midbrain regions rich in noradrenergic cell bodies (Brown, 1989; Plotsky, 1991; Gray, 1993; Koob, 1999; Van Bockstaele *et al*, 2001).

Hofer (1994) has offered the concept that the mother or primary caregiver acts as a hidden regulator of the development of infant physiological regulatory systems. Thus, a further impetus for this research is the growing evidence for a link between early untoward life experiences, such as neglect or abuse, and increased vulnerability to HPA axis alterations and psychiatric diseases such as depression (Heim and Nemeroff, 2001; Heim *et al*, 2002, 2004). Accumulating data demonstrate increased neural CRF expression (Raadsheer *et al*, 1994, 1995) and hypersecretion (Banki *et al*, 1987; De Bellis *et al*, 1993; Geraciotti *et al*, 1992; Nemeroff *et al*, 1991) in some, but not all, patients with major depressive disorder. Many of these patients have a history of exposure to untoward life events occurring prior to their teenage years and often continuing into adulthood (Brown and Anderson, 1993; Mullen *et al*, 1996; McCauley *et al*, 1997; Heim *et al*, 2000; Kendler *et al*, 2002). This raises the question of whether early life trauma, rather than

depression *per se*, is associated with dysregulation of central CRF systems, which then results in secondary depression. In light of the pervasive effects of CRF on the organization of emotion and responses to stressors (Owens and Nemeroff, 1991; Koob *et al*, 1994), we have examined the status of hypothalamic and extrahypothalamic CRF neurocircuits in adult rats exposed as neonates to different rearing protocols associated with alterations in HPA axis function and behavioral responsiveness.

MATERIALS AND METHODS

Animals

Timed-pregnant Long Evans hooded rats were delivered on gestation day 12 to Emory University from Charles River (Crl:LE)BR; Charles River Laboratories, Portage, MI). Studies at McGill University were performed with rats obtained from an 'in-house' breeding colony derived from Charles River Laboratory Long Evans rats. All dams were housed in transparent, polypropylene cages (20 × 25 cm) with 2 cm bedding material and *ad libitum* access to food and water in a room with a 12:12 h light:dark cycle (lights on at 0700). The day of birth was considered postnatal day (PND) 0. Litters were standardized to 8–10 male pups per dam on PND2 and were exposed to one of the following rearing conditions from PND2 to PND14 inclusive: (a) animal facility rearing (AFR) consisted of brief handling for twice weekly cage changes beginning on PND4 with no other handling or separation, (b) nonhandled (HMS0) animals were not exposed to any handling or experimenter manipulation, including cage bedding changes, from PND2 to PND14, (c) handling with brief maternal separation for 15 min (HMS15) from PND2 to PND14, and (d) handling with moderate maternal separation for 180 min (HMS180) from PND2 to PND14. The HMS protocol was initiated in the morning between 0800 and 0900 by a gloved investigator. Each dam was removed from the home cage to an adjacent cage, then each litter was removed and placed as a group into a plastic cage (15 × 15 cm) that was transferred to an incubator in an adjacent room. These pup cages were lined with 3 cm of bedding material (wood shavings) and were placed into an incubator set to maintain an ambient temperature of 32 ± 0.5°C (PND2–5) or 30 ± 0.5°C (PND6–14). At the conclusion of the separation period, pups were returned to the maternity cage, rolled in the soiled home cage bedding material, and the dam was returned. In the course of normal mother–pup interactions in the laboratory rat, the mother is routinely off the litter for periods of 20–25 min (Jans and Woodside, 1990). Thus, the maternal separation protocol (HMS180), unlike handling and brief maternal separation (HMS15), represents deprivation of maternal care. Unless otherwise noted, the bedding material in all of the cages (eg maternity, transfer, pup isolation) was not changed from PND0 to at least PND15. According to Champagne *et al* (2003), gender composition of the litter does not significantly influence the frequency of maternal licking, grooming, or arched-back nursing and, thus, has little effect with respect to outcome measures of HPA development or CNS expression levels of stress-related genes. This is not to deny that specific aspects of maternal behavior toward male and female pups differ (Moore and

Morelli, 1979; Pryce *et al*, 2001). Rat pups were weaned on PND21–22 and housed in groups of 2–4 same rearing group animals per cage until testing. Food and water was available *ad libitum*. All experiments were carried out in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Blood Sampling and Airpuff Startle

Adults were tested for HPA axis responsiveness using airpuff startle on PND60–75. Jugular cannulae were implanted as previously described to allow for repeated sampling in freely moving animals (Thrivikraman *et al*, 2000), and a 4-day recovery period, during which time the rats were handled daily, was permitted before testing.

The blood sampling protocol began between 0900 and 1000 with airpuff startle initiated after obtaining a basal sample. Airpuff startle consisted of three blocks of three airpuffs each (3 s) from a compressed air source (Dust-Off; Fisher Scientific, Atlanta, GA) that was directed toward the side of the head from a distance of approximately 10 cm, with 1 min between each block (Engelmann *et al*, 1996). Blood (300 μ l/sample) was collected through the jugular cannula into 1.5 ml polyethylene tubes containing 10 μ l EDTA (100 mg/ml) at rest and at 5, 10, 15, 30, 45, and 60 min following airpuff startle. Blood samples were stored on ice, then centrifuged (12 500 g; 4°C; 12 min), plasma collected and frozen.

ACTH and Corticosterone Radioimmunoassay Analysis

Plasma ACTH and corticosterone (CORT) were assayed as previously described (Thrivikraman and Plotsky, 1993). For ACTH, 100 μ l plasma samples were used for the Allegro™ HS-ACTH kit (Nichols Institute, San Juan Capistrano, CA) with 50 μ l of iodinated tracer and one avidin-coated bead per tube. The ACTH assay had a sensitivity of 2 pg/ml and intra- and intercoefficients of variation of less than 10%. CORT was determined in 5 μ l plasma samples using the ImmunoChem™ Double Antibody kit (ICN Biomedicals, Costa Mesa, CA) with half of the trace and antibody volume. The CORT assay had a sensitivity of 1 ng/ml and intra- and inter coefficients of variation of less than 10%.

Lumbar Cerebrospinal Fluid Sampling

Intrathecal catheters were constructed of 32 ga polyurethane tubing (Micor, Allison Park, PA) of approximately 10.7 cm length as described by Sakura *et al* (1996). A 5 mm long stainless-steel tubing segment (27 ga) was fitted to a heat-flared segment of the polyurethane tubing. This assembly was cold sterilized and then a sterilized Teflon-coated stainless-steel wire stylet (0.003 in diameter) was inserted into the polyurethane catheter prior to placement.

The catheter was placed according to a modification of the technique described by Yaksh and Rudy (1976). Rats ($n = 10$ per rearing group) drawn from the same litters as animals in the other experiments were anesthetized as described above, then the back of the head and neck shaved, and disinfected with alcohol (70%). The rat was then placed in a stereotaxic headholder with the head flexed forward. A midline incision was made on the back of the neck. The

muscle was freed from the skull and retracted to reveal the cisternal membrane. This membrane was opened with a microknife and retracted with a dural microhook. The sterile catheter was inserted through this defect and gently passed caudally approximately 9 cm into the intrathecal space so that the tip rested in the Th11–L5 region. The dural defect was repaired with tissue glue (Vet-Bond; Henry Schein, Port Washington, NY) and a small piece of Gel-Foam. The cannula was anchored to the skull using jeweler's screws and cranioplastic cement and the skin incision was closed with stainless-steel wound clips. A recovery period of 5 days was permitted prior to sampling. Each rat was handled on a daily basis.

For sampling, the catheter was attached to a silastic extension line connected to a microsyringe pump (CMA Systems, Stockholm, Sweden). Cerebrospinal fluid (CSF) was withdrawn at the rate of 500 nl/min for 4 h into a replaceable loop of Teflon tubing immersed into an ice-bath. Upon completion of each collection, the CSF was expelled into a cold 500 nl centrifuge tube containing 1 μ l EDTA (100 mg/ml) and frozen at -80°C until assay.

Tissue Collection

Adult animals were killed by decapitation between 0900 and 1200 under low stress conditions when they were between PND100 and PND120. The skull was quickly opened, the brain removed, then rinsed and chilled in sterile saline on ice. Each brain was blocked ($\sim 300 \mu\text{m}$) in a coronal brain matrix (Bioanalytical Systems, West Lafayette, IN). The tissue slabs were placed on a glass plate on ice, then individual regions were obtained using 1 or 2 mm diameter stainless-steel punches according to the procedure of Palkovits and Brownstein (1988). Each punch was snap-frozen on dry ice and stored in 1.5 ml polyethylene microcentrifuge tube at -80°C until assay. Tissues from five to eight rats per group were collected for analysis.

CRF Radioimmunoassay

Tissue CRF-like immunoreactivity (CRF_{ir}) levels were determined using a modified version of the radioimmunoassay described by Plotsky *et al* (1985). Tissue punches homogenized by sonication in a solution composed 0.1 N HCl containing 0.001% β -mercaptoethanol, 0.05% ascorbic acid, 0.01% bacitran, and 0.001% phenylmethylsulfonyl fluoride. Samples were centrifuged at 12 000 g for 30 min at 4°C. The supernatant was removed, lyophilized, and frozen until assay. Samples were reconstituted in pH 7.0 phosphate buffer containing 0.1% bovine serum albumin (BSA) fraction-V, 0.1% Triton-X, and 0.01% sodium azide.

Antiserum rC70 (provided by W Vale, The Salk Institute, La Jolla, CA) was used at a final dilution of 1:500 000. Synthetic rat CRF_{1–41} (provided by J Rivier, The Salk Institute, La Jolla, CA) was used for assay standard and [^{125}I]Tyr-ovine CRF (DuPont-NEN, Boston, MA) was used for tracer. The limit of detection was 2 pg/tube with an IC₅₀ of 22 pg. The intra-assay coefficient of variation was 3.6%. Note that all tissue samples were run in the same assay.

In Situ Hybridization

Rats (PND100–120) were deeply anesthetized (0800–1200), decapitated, and brains removed and stored at -80°C . Serial coronal brain sections ($15\ \mu\text{m}$) were cut on a cryostat at -18°C and thaw mounted onto SuperFrost Plus slides (Fisher Scientific, Atlanta, GA) under RNase-free conditions and stored with Humi-Cap desiccant capsules (Gibco BRL Products, Grand Island, NY) at -80°C until assay. *In situ* hybridization was performed according to the procedures described by Simmons *et al* (1989). Briefly, slides were warmed in a stepwise manner to room temperature, postfixed in 4% paraformaldehyde (pH 7.4) for 20 min, and rinsed twice in 10 mM phosphate-buffered saline (PBS, pH 7.4) for 2 min. Next, the slides were treated for 15 min with proteinase K (Promega, Madison, WI) $10\ \mu\text{g}/\text{ml}$ in 0.1 M Tris with 50 mM EDTA followed by acetylation (10 min, 0.5% acetic anhydride in 0.1 M triethanolamine, pH 8.0), two rinses in $2 \times \text{SSC}$, and dehydrations through a graded ethanol series. The sections were then air-dried for at least 1 h prior to hybridization.

The CRF riboprobe was constructed from a 1.2 kb *EcoRI* fragment of a full-length rat CRF cDNA (provided by K Mayo, Northwestern University, Evanston, IL) subcloned into a pBluescript II-SK+ plasmid. The CRF1 riboprobe was constructed from a 1.3 kb *PstI*–*PstI* fragment containing the full-length coding region for the rat CRF1 receptor (provided by W Vale, The Salk Institute, La Jolla, CA), ligated into a pBluescript II-SK+ plasmid. The CRF2 α riboprobe was constructed from a 275 bp fragment, encoding the N-terminal region of the CRF2 α receptor, ligated into a pBluescript II-SK+ plasmid (provided by W Vale, The Salk Institute, La Jolla, CA). Radiolabeled antisense cRNAs were synthesized by incorporating either [^{35}S]UTP (DuPont-NEN) into the CRF probe or [^{35}S]CTP plus [^{35}S]UTP into the CRF1 and CRF2 receptor probes. The transcription reactions were performed utilizing the Ambion MAXIscript kit (Austin, TX) with SP6 (CRF), T7 (CRF1), or T3 (CRF2) RNA polymerases according to the instructions provided. Following transcription and removal of the cDNA template with 2 U DNase (Ambion), the cRNA probes were recovered through ethanol precipitation and/or gel filtration using a G-50 Sephadex Quick Spin column (Boehringer-Mannheim, Indianapolis, IN).

The brain sections were hybridized overnight at 58 – 60°C with 2×10^6 cpm of ^{35}S -labeled cRNA probe diluted into hybridization buffer (50% formamide, 10% dextran sulfate, 0.3 M NaCl, $1 \times$ Denhardt's solution, 10 mM Tris, 1 mM EDTA, 2 mg/ml yeast tRNA, 10 mM DTT) in humidified Nunc trays (Nalge Nunc Inc., Naperville, IL). The next day, slides were allowed to cool to room temperature before being washed four times in $4 \times \text{SSC}$ for 5 min on a rotating platform at 60 rpm. The sections were then treated with $250\ \mu\text{g}/\text{ml}$ RNase A (Promega, Madison, WI) for 30 min at 37°C . Subsequently, the slides underwent a series of SSC washes (supplemented with 1 mM DTT) with salt concentrations decreasing from $2 \times$ to $0.5 \times$, followed by a 60 min high-stringency wash with $0.1 \times \text{SSC} + 1\ \text{mM DTT}$ at 60°C , then dehydration through a graded ethanol series. The slides were air-dried for at least 1 h and then apposed to Kodak Biomax MR film (Rochester, NY) for 12 h to 12 days depending on the probe and brain region examined with ^{14}C

standards (Amersham, Piscataway, NJ). Controls were performed to establish specificity of the signal included hybridization with sense strand probes, as well as predigestion with RNase A.

CRF Receptor Autoradiography

Following a modification of the techniques of De Souza *et al* (1985), De Souza (1987), and Primus *et al* (1997), *in vitro* CRF receptor autoradiography was performed on $15\ \mu\text{m}$ rat brain sections mounted on SuperFrost Plus slides. Sections were fixed for 2 min in 0.1% paraformaldehyde followed by 15 min incubation in assay buffer (50 mM Tris, 10 mM MgCl_2 , 2 mM EGTA, 0.1% BSA, 0.1 mM bacitracin, and 0.1% aprotinin; pH 7.5) to remove endogenous CRF. Triplicate slides containing adjacent brain sections were incubated for 2 h at room temperature in one of three conditions: (a) 0.1 nM radiolabeled [^{125}I]sauvagine (DuPont-NEN) to determine total binding at both the CRF1 and CRF2 receptor subtypes, (b) 0.1 nM radiolabeled [^{125}I]sauvagine + $1\ \mu\text{M}$ CP-154,526 (CRF1 antagonist provided by Pfizer Inc., Groton, CT) to determine CRF2 α receptor-specific binding, or (c) 0.1 nM radiolabeled [^{125}I]sauvagine + $1\ \mu\text{M}$ unlabeled sauvagine (American Peptide Company, Sunnyvale, CA) to determine nonspecific binding. Following the incubation, unbound radioligand was removed by two 5 min rinses in ice-cold (4°C) PBS + 1% BSA on a rotating platform at 60 rpm, followed by two brief dips in ice-cold ddH_2O . Slides were then rapidly dried and apposed to Kodak Biomax MR film (Rochester, NY) with ^{125}I -microscale standards (Amersham, Piscataway, NJ) for 80–90 h.

Image Analysis

Images from the *in situ* hybridization and receptor autoradiography films were digitized using a Dage-MTI CCD72 camera (Sony Corp., Tokyo, Japan) and images were captured using a Frame Grabber card (Data Translation, S Natick, MA) equipped Quadra 950 computer. Semiquantitative analysis was performed using NIH Image (Wayne Rasband, NIH, Bethesda, MD). Optical densities were calibrated against ^{14}C standards (*in situ* hybridization films) or ^{125}I -microscale standards (receptor autoradiography films) and expressed in terms of nCi/g tissue equivalent. For the purpose of quantifying mRNA levels, specific signal was determined as the optical density of the region examined minus the optical density of neutral background present in the same brain section. For the purpose of quantifying CRF receptor level, CRF1 receptor-specific binding was calculated as total binding minus CRF2 receptor-specific binding, while CRF2 receptor-specific binding was calculated as CRF2 receptor-specific binding minus nonspecific binding. In all cases, 3–5 sections per region were matched for rostrocaudal level according to the atlas of Paxinos and Watson (1986) and used to produce a single value for each animal. Sections from five to eight rats per group were used for statistical analysis.

Statistical Analysis

Statistical comparisons were performed using the ANOVA with Tukey–Kramer multicomparison *post hoc* tests using

InStat 3 and Prism 4 (GraphPad Software, San Diego, CA). Data are presented as mean \pm SEM.

RESULTS

Basal and Stress-Induced ACTH and CORT

Basal plasma ACTH levels were similar among the rearing groups (AFR 19.94 ± 1.27 pg/ml; HMS0 19.33 ± 1.72 ; HMS15 18.74 ± 1.21 ; HMS180 19.18 ± 1.93). As shown in Figure 1a, presentation of an acute APS stressor was associated with a significant rearing-associated ($F_{3,216} = 69.51$, $p < 0.0001$) elevation in ACTH over time ($F_{6,216} = 586.60$, $p < 0.0001$) as determined by ANOVA corrected for repeated measures. In all four groups, acute APS caused a marked increase in mean plasma ACTH concentration, peaking at 10 min and returning toward baseline by 60 min poststress. The plasma ACTH peak was approximately 1.6-fold greater ($p < 0.001$) in HMS180 rats (263 ± 19 pg/ml) as compared to either HMS15 or AFR rats (148 ± 15 vs 165 ± 15 pg/ml, respec-

tively), which were not significantly different from one another. Peak ACTH levels in the HMS0 rats (219 ± 16 pg/ml) were significantly less ($p < 0.001$) than that observed in HMS180 rats, but significantly different from either HMS15 or AFR rats ($p < 0.001$).

Two-way ANOVA with repeated measures on time revealed significant effects for rearing ($F_{3,216} = 49.85$, $p < 0.0001$) and time ($F_{6,216} = 127.11$, $p < 0.0001$) on plasma CORT levels following an acute APS stressor (Figure 1b). However, no rearing-associated differences in basal trough CORT levels were observed. Plasma CORT levels peaked 15 min after initiation of APS in all of the groups. Peak levels at 15 min were not significantly different between AFR (143 ± 16 ng/ml) and HMS15 (137 ± 14 ng/ml) rats. Peak levels for HMS0 (203 ± 20 ng/ml) and HMS180 (300 ± 26 ng/ml) rats were significantly different ($p < 0.01$) from one another and from AFR and HMS15 rats ($p < 0.001$). While CORT returned toward initial levels in the AFR and HMS15 groups, it remained significantly elevated in the HMS0 and HMS180 groups.

Lumbar CSF CRF_{ir}

The distribution of morning CSF CRF_{ir} levels is shown in Figure 2. One-way ANOVA revealed a significant effect of rearing ($F_{3,36} = 15.19$, $p < 0.001$) on CSF CRF. Levels (mean \pm SEM) of the AFR (37 ± 3 pg/ml) and HMS15 (36 ± 2 pg/ml) groups were not significantly different from one another, but were greater than that of either the HMS0 (53 ± 4 pg/ml) or HMS180 (68 ± 6 pg/ml) groups.

CRF mRNA Expression

As illustrated in Figure 3, densitometry of X-ray films showed significant rearing-associated differences in the

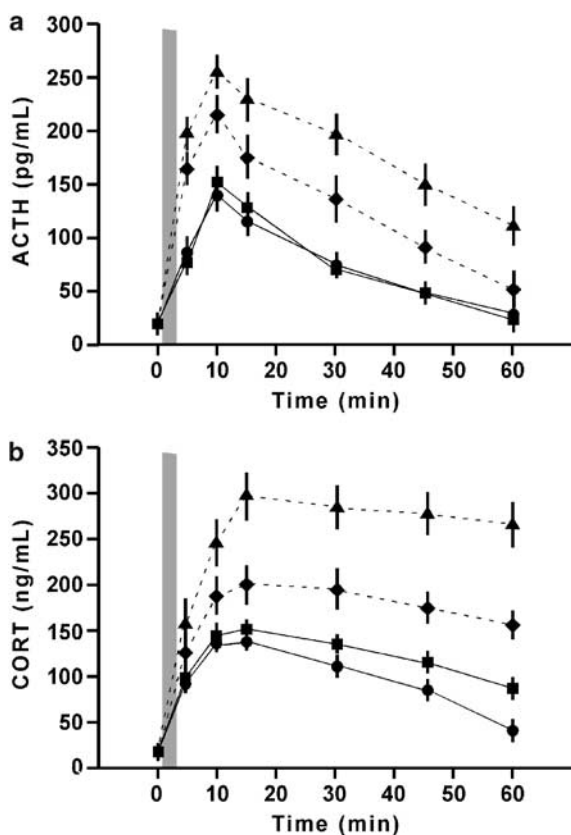


Figure 1 Effect of rearing condition on adult pituitary-adrenal responses to airpuff startle (APS). There were no rearing-associated differences in basal trough plasma ACTH (a) or CORT (b) levels. However, the response to airpuff startle (APS; denoted by gray area) showed a significant rearing effect for both ACTH ($F_{3,216} = 69.51$, $p < 0.0001$) and CORT ($F_{3,216} = 37.65$, $p < 0.0001$). Animal facility reared (AFR; squares) and handling-maternal separation 15 (HMS15; circles) groups were not significantly different from one another. However, both of these groups exhibited smaller responses than did the handling-maternal separation 0 (HMS0 or nonhandled; diamonds) or handling-maternal separation 180 (HMS180; triangles) groups. Each group consisted of 10 adult animals (PND75–90) selected from at least eight different litters. Data represent mean \pm SEM. See text for additional details.

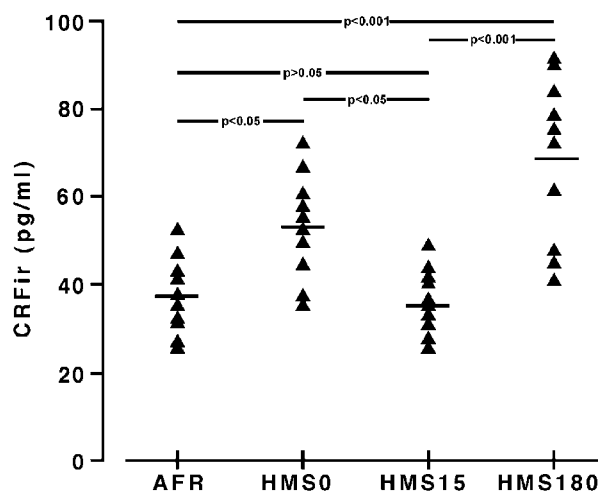


Figure 2 Lumbar CSF CRF_{ir} levels. Adult animals of the four rearing groups ($n = 10$ /group) were each instrumented with an indwelling intrathecal lumbar cannula (see text for details). After a 5-day recovery period, unanesthetized animals were sampled at the rate of 500 nl/min for 4 h beginning at 0800. Samples were radioimmunoassayed for CRF_{ir}. ANOVA confirmed a rearing effect ($p < 0.001$). Post hoc statistical comparisons by Tukey-Kramer are shown. AFR: animal facility reared; HMS0: handling-maternal separation 0 min or nonhandled; HMS15: handling-maternal separation 15 min; HMS180: handling-maternal separation 180 min.

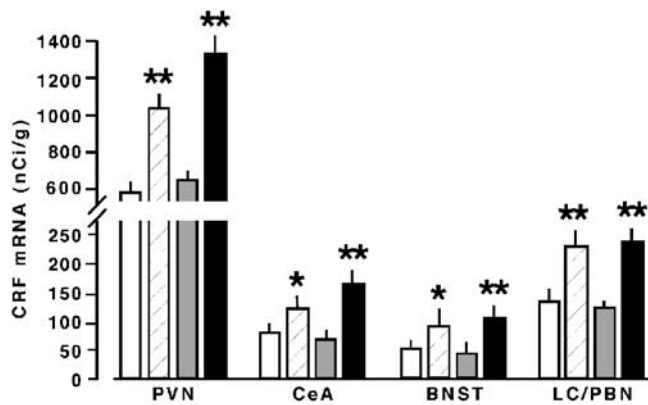


Figure 3 Densitometric analysis of CRF mRNA levels in the PVN, CeA, BNST, and LC/PBN of adult animals reared under conditions of common animal facility rearing (AFR; open bar), nonhandling (HMS0; striped bar), postnatal handling (HMS15; gray bar), and postnatal maternal separation (HMS180; black bar). Densitometry was performed on 3–4 rostrocaudally matched sections from each animal ($n=5$ rats/group). Levels were calibrated against ^{14}C standards run on each film. Experimental details and statistical analyses are presented in the text. Data represent mean \pm SEM. * $p<0.05$ or ** $p<0.01$ vs AFR.

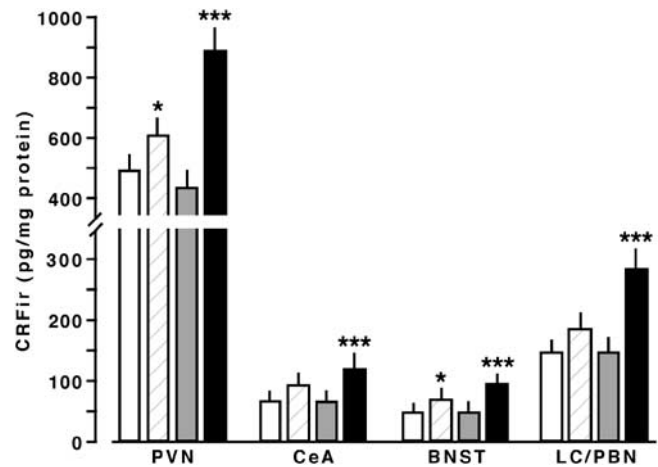


Figure 4 CRF content in the central nervous system of adult animals reared under conditions of common animal facility rearing (AFR; open bar), nonhandling (HMS0; striped bar), postnatal handling (HMS15; gray bar), and postnatal maternal separation (HMS180; black bar). Data represent mean \pm SEM. * $p<0.05$ or ** $p<0.01$ vs AFR ($n=8$ rats per group). Abbreviations are defined in Figure 3 legend. Experimental details and statistical analyses are presented in the text.

levels of CRF mRNA in the PVN ($F_{3,20}=11.35$, $p<0.01$), CeA ($F_{3,15}=6.79$, $p<0.05$), BNST ($F_{3,15}=5.82$, $p<0.05$), and locus coeruleus/parabrachial nucleus (LC/PBN; $F_{3,15}=4.45$, $p<0.05$). In each brain region, the HMS180 rats exhibited significantly greater levels of CRF mRNA than did the AFR animals. There were no significant differences in CRF mRNA density between AFR and HMS15 rats in any region examined. Adult CRF mRNA levels in the HMS180 rats were 2.1-fold (PVN, $p<0.001$), 1.8-fold (CeA, $p<0.001$), 2.2-fold (BNST, $p<0.001$), and 1.5-fold (LC/PBN, $p<0.001$) higher than in the AFR groups. Interestingly, CRF mRNA in the PVN of HMS0 rats was significantly greater than that of either AFR or HMS15 animals ($p<0.05$), but was significantly less than that of the HMS180 group.

CRF Content

CRF immunoreactivity was measured in tissue micropunch samples from the PVN, CeA, BNST, and LC/PBN. As shown in Figure 4, significant rearing-associated differences in the levels of CRF were evident in the PVN ($F_{3,28}=38.25$, $p<0.0001$), CeA ($F_{3,28}=15.05$, $p<0.0001$), BNST ($F_{3,28}=18.87$, $p<0.0001$), and LC/PBN ($F_{3,28}=42.22$, $p<0.0001$). As was found for CRF mRNA, CRF was significantly elevated relative to AFR and HMS15 groups in each brain region examined. There were no significant differences in CRF levels between AFR and HMS15 rats in any region examined. Adult CRF levels in the HMS180 rats were 1.80-fold (PVN, $p<0.001$), 1.80-fold (CeA, $p<0.001$), 1.92-fold (BNST, $p<0.001$), and 1.91-fold (LC/PBN, $p<0.001$) higher than in the AFR groups. The HMS0 group was significantly greater than AFR group in the PVN ($p<0.05$) and BNST ($p<0.05$). In contrast, the HMS0 rearing group was significantly less than the HMS180 group in the PVN ($p<0.001$), CeA ($p<0.01$), BNST ($p<0.05$), and LC ($p<0.001$).

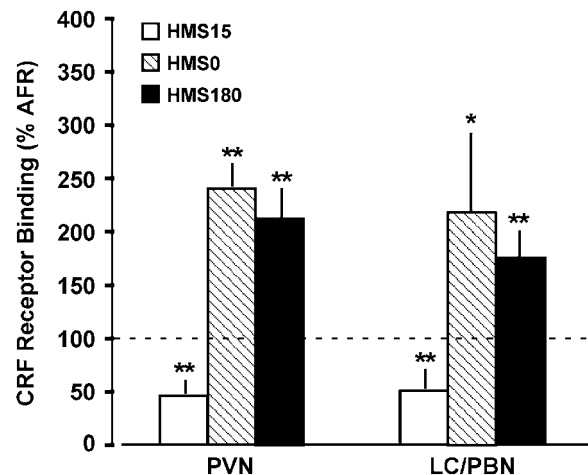


Figure 5 Total CRF receptor binding in the PVN and LC/PBN of handled (HMS15; open bars), nonhandled (HMS0; striped bars), and maternal separation (HMS180; black bars) animals expressed as a percentage of values from animal facility reared (AFR) rats. Brain regions were obtained from animals at postnatal day 100. * $p<0.01$ vs AFR.

CRF Receptors

Total CRF receptor binding was assessed across all rearing groups and the results expressed as the percent binding relative to AFR rats in Figure 5. Total CRF receptor binding was significantly higher in both HMS0 ($p<0.05$) and HMS180 ($p<0.01$) rats in the PVN and LC. In contrast, CRF receptor binding was significantly reduced ($p<0.01$) in these regions in the HMS15 rats relative to the AFR animals.

CRF2 α binding was assessed in the ventromedial hypothalamus (VMH; AFR 329 ± 37 nCi/g), lateral septum (LS; AFR 774 ± 69 nCi/g), and PVN (AFR 248 ± 36 nCi/g). No rearing-associated differences were apparent in CRF2 α binding in any of these regions (data not shown). Similarly,

no rearing-associated changes in CRF2 α mRNA were evident in the VMH (AFR 19 ± 2.1 nCi/g), LS (AFR 13 ± 1.6 nCi/g), or PVN (AFR 10 ± 1.2 nCi/g).

Evaluation of CRF1 binding revealed a significant rearing-associated effect in the PVN ($F_{3,16} = 24.31$, $p < 0.0001$), the frontal cortex ($F_{3,16} = 3.71$, $p = 0.03$), and the LC/PBN region ($F_{3,16} = 24.77$, $p < 0.0001$) as shown in Figure 6a. Rearing-associated differences in the basolateral nucleus of the amygdala (BLA) just failed to reach significance ($F_{3,16} = 3.07$, $p = 0.058$). No significant differences in CRF1 binding were detected between the AFR and HMS15 rearing groups in any region examined. In the PVN, CRF1 expression increased 2.1-fold in HMS180 ($p < 0.01$) and 1.8-fold in HMS0 rats ($p < 0.05$) vs other rearing groups. CRF1 binding also showed a 2.4-fold ($p < 0.01$) increase in the LC/PBN region vs HMSAFR and HMS15 rats. In contrast, HMS180 rats displayed a significant decrease in CRF1 binding in the FCTX ($p < 0.05$) as compared to the AFR group. The CRF1 binding in the HMS0 rats was intermediate between HMS180 and AFR rats and was not statistically different from HMS180 levels.

Binding changes were paralleled by changes in CRF1 mRNA in some of these brain regions (Figure 6b). Rearing-associated effects were found in the PVN ($F_{3,16} = 8.29$, $p < 0.005$), FCTX ($F_{3,16} = 6.05$, $p < 0.01$), and LC/PBN ($F_{3,16} = 28.45$, $p < 0.0001$), but not in the BLA ($F_{3,16} = 1.30$, $p > 0.05$). CRF1 mRNA was 2.2-fold ($p < 0.01$) greater in HMS180 than AFR rats and no significant differences were evident between AFR and HMS15 rats. A 2.7-fold ($p < 0.001$) increase was observed in the LC/PBN of HMS180 vs AFR rats with no differences between AFR and HMS15 animals.

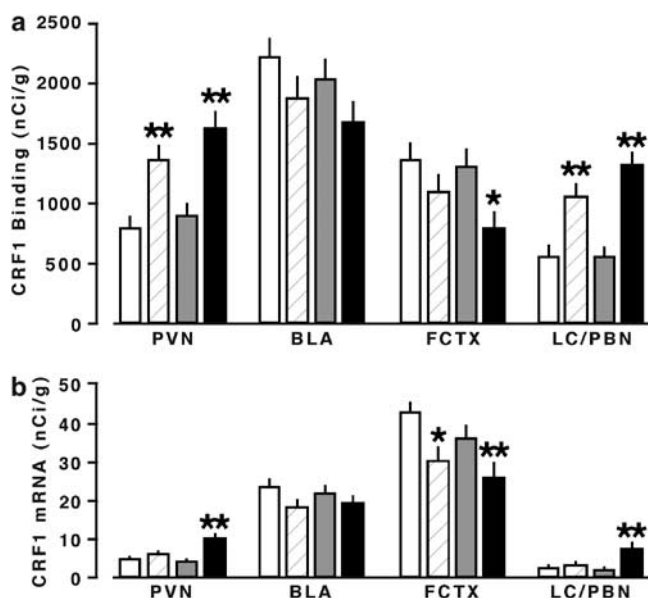


Figure 6 Rearing-associated differences in regional brain CRF1 receptor binding (a) and mRNA (b) in adult male rats. The rats were reared under conditions of animal facility rearing (AFR; open bar), nonhandling (HMS0; striped bar), postnatal handling (HMS15; gray bar), and postnatal maternal separation (HMS180; black bar) as described in the text. Experimental details and statistical analyses are presented in the text. PVN: paraventricular nucleus; BLA: basolateral nucleus of the amygdala; FCTX: frontal cortex; LC/PBN: locus coeruleus/parabrachial region. Data represent mean \pm SEM. * $p < 0.05$ or ** $p < 0.01$ vs AFR ($n = 5$ rats per group).

In contrast, there was a 40% decrease ($p < 0.01$) in CRF1 mRNA in the HMS180 rats vs the AFR animals.

DISCUSSION

Alterations in maternal behavior within the normal range (Champagne *et al*, 2003) as well as repeated exposure to moderate periods of maternal separation (Meaney *et al*, 1996; Ladd *et al*, 2000; Lehmann and Feldon, 2000) are associated with pronounced long-term changes in responsivity of the HPA axis in rats. As adults, animals exposed to HMS180 and, to a somewhat lesser extent, HMS0 exhibited increased ACTH and CORT responses to a psychological stressor and increased hypothalamic CRF_{ir} (Plotsky and Meaney, 1993). In the current study, we demonstrate rearing-associated elevations in CSF CRF_{ir}, increased CRF mRNA levels in the PVN, BNST, CeA, and the LC/PBN, and region-specific alterations in CRF receptor binding and mRNA. Thus, it appears that central CRF systems represent a major target of neonatal rearing conditions in the rat. In fact, this may represent a general response to a wide range of early life stressors in rodents (Dent *et al*, 2000; Ladd *et al*, 2000; Schmidt *et al*, 2002; Fenoglio *et al*, 2004), monkeys (Coplan *et al*, 1996), and humans (Raadsheer *et al*, 1994, 1995; Austin *et al*, 2003; Newport *et al*, 2003; Carpenter *et al*, 2004).

Adult HMS180 rats exhibit normal circadian trough ACTH and CORT levels, yet demonstrate profound hypersecretion in response to the psychological stressor of airpuff startle (Figure 1a and b). This pattern of hyper-responsiveness has also been reported in several non-human primate models of early adversity (Coplan *et al*, 1996; Smith *et al*, 2002) and in adult women with a history of prepubertal abuse (Heim *et al*, 2000, 2002; Heim and Nemeroff, 2001). Changes in nervous system CRF tone appear to be associated with these changes in HPA responsiveness, as illustrated by elevations in CSF CRF_{ir} in the adult HMS180 rats (Figure 2), in several primate models of early adversity (Coplan *et al*, 1996; Maestripieri *et al*, 2005), primates with fearful temperaments (Kalin *et al*, 2000), and in abused children as well as in adult women with a history of abuse (Heim and Nemeroff, 2001; Newport *et al*, 2003; Carpenter *et al*, 2004). Alternatively, we recently reported blunting of the diurnal cortisol rhythm in rhesus monkeys exposed to early adversity without accompanying changes in CSF CRF_{ir} (Sanchez *et al*, 2005). This difference may be developmentally related or represent a species difference.

Ladd *et al* (1996) reported that maternal separation increased CRF_{ir} in the LC and neighboring PBN. These structures appear to receive CRF projections from the CeA (see Gray, 1989; Van Bockstaele *et al*, 2001). Indeed, lesions to the CeA produce an 80–85% decrease in CRF_{ir} in the region of the LC (Koepler-Muly *et al*, 1993). The elevation of CSF CRF_{ir} in HMS180 rats is consistent with reports from clinical studies of depression and abuse (Banki *et al*, 1987; De Bellis *et al*, 1993; Geraciotti *et al*, 1992; Nemeroff *et al*, 1991; Newport *et al*, 2003). Taken together with our previous reports showing increased hypothalamic and median eminence CRF_{ir} in HMS180 animals (Plotsky and Meaney, 1993), these findings suggest that CRF gene

expression and peptide synthesis within the PVN and CeA-LC systems may represent a common target in animals exposed to what may be considered abnormal neonatal rearing conditions regardless of which end of the rearing spectrum they fall into—monotonous housing and care (ie HMS0) vs extended periods of neonatal maternal separation (ie HMS180). The observation that both the HMS180 and HMS0 rearing conditions result in a similar directionality of changes in these CRF systems is a conundrum that is difficult to reconcile (see below).

In contrast, animals exposed to either HMS15 or AFR rearing conditions exhibit comparable levels of CRF mRNA in the PVN and CeA as well as similar concentrations of CRF in the median eminence (also see Plotsky and Meaney, 1993; Viau *et al*, 1993) and LC. This is not unexpected as care protocols in most breeding colonies expose animals to repeated cage cleaning during the first 2 weeks of life that involve transferring the mother and the pups from the maternity cage to a clean, novel cage, and may therefore be considered as variant of the handling procedure (Greenberg and Bursdal, 1982). As we show here with respect to CRF and as we and others have previously reported, both routine animal husbandry protocols (eg AFR) and the HMS15 protocol produce similar neurobehavioral outcomes (Caldji *et al*, 2000; Pryce *et al*, 2001; Pryce and Feldon, 2003). However, Fenoglio *et al* (2004) recently reported that handling during PND2–9 was associated with a transient increase of CRF mRNA in the CeA by PND6, a transient increase of peptide levels in BNST, and a long-lasting decrease of CRF levels in PVN beginning by PND9. Importantly, these changes in CRF preceded changes in hippocampal GR expression by many weeks. It is noteworthy that a majority of the rodent neuroscience and behavioral literature is derived from studies on what are essentially animal facility reared rats; therefore, we propose that the AFR group is an appropriate comparison or colony control group for these types of experiments.

The HMS0 rats represent a problematical group with respect to their tendency to approximate the HMS180 group on many outcome variables such as enhanced behavioral fearfulness (Francis *et al*, 1999; Caldji *et al*, 2000), reduced hippocampal GR mRNA (Meaney *et al*, 1996; Avishai-Eliner *et al*, 2001), and enhanced PVN CRF mRNA (see Figure 3). The question of why the neonatal experiences of being either undisturbed (eg HMS0) or maternally separated (HMS180) exert a similar effect on the phenotype of the offspring awaits a definitive answer. This observation appears to contradict the ‘maternal mediation hypothesis’ (Denenberg *et al*, 1962; Denenberg, 1999), according to which differences in maternal care upon reunion of the dam with her pups may account for a significant portion of the neurobehavioral differences in the adult offspring (Suchecki *et al*, 1993; Meaney, 2001).

The environmental impoverishment inherent in standard laboratory rodent housing may render the HMS0 rats more fearful and reactive than HMS15 animals (Bors and Forrin, 1996; Rosenzweig and Bennett, 1996; Larsson *et al*, 2002). According to Macri *et al* (2004), the difference in neurobehavioral phenotype between HMS0 and handled offspring reflects adaptive plasticity in the HPA axis and behavioral function with respect to energy utilization and overall health. In this view, HMS0 rats live in a relatively

stress-free environment and, therefore, retain a highly reactive stress response while HMS15 rats that have been exposed to repetitive stressors adapt by downregulating HPA axis responsiveness. The enhanced responsiveness of the HMS180 rats, considered to be maladaptive, reflects disruption of normal pup homeostasis and in the intervals between nursing bouts as a result of the repeated, prolonged neonatal separations.

Disruption of the perceived safety of the nest area by the maternal separation protocol, as well as the actual physical separation of the dam from her pups, may elicit changes in behavior of the dam (Kalinichev *et al*, 2000; Fleming *et al*, 2002). In fact, we (Ladd *et al*, 2000; Huot *et al*, 2004) have proposed that in response to maternal separation, the quantity and/or quality of dam–pup interaction differs from that observed in AFR, HMS15, and HMS0 groups. Although each individual measure may exhibit only a small change, appropriate coordination of the sequence of maternal behavior may be altered. It should be noted that in a majority of the studies in which maternal behavior was measured, it was either the frequency and/or duration of the occurrence of particular maternal behaviors that was recorded (Champagne *et al*, 2003; Pryce *et al*, 2001; Stern, 1997). However, our own ongoing observations using Markov chain analysis indicate that the normally coordinated pattern of maternal behaviors may be disrupted in the HMS180 condition and that this disorganization of the sequencing of maternal behaviors may be equally or even more important than the frequency or duration of occurrence of isolated behaviors.

Differences in amygdaloid CRF expression are of interest considering the effects of early life events on behavioral responses to stress. The CeA has long been implicated in the expression of autonomic and behavioral responses to stress (reviewed in Davis, 1992; LeDoux, 1992, 1994; Gray, 1993). Lesions of the CeA have been found to block many of the behavioral effects associated with stress and the literature on the role of the amygdala in conditioned emotional responses is especially rich. Lesions of the CeA block the excitatory effects of both conditioned and unconditioned fear on startle responses (Hitchcock and Davis, 1986, 1989) and the development of conditioned emotional responses (van der Kar *et al*, 1991; LeDoux, 1994). Interestingly, both the central and the lateral nucleus of the amygdala have been found to be important substrates for the anxiolytic effects of benzodiazepines on stress-related behaviors.

These amygdaloid effects are at least partially mediated by CRF. Pich *et al* (1995), using *in vivo* microdialysis, reported an increase in extracellular CRF levels in the amygdala in response to stress and ethanol withdrawal. Microinjections of the CRF antagonist α -helical CRF_{9–41} into the CeA block the anxiogenic effects of ethanol withdrawal (Rassnick *et al*, 1993), fear-induced freezing (Swiergiel *et al*, 1993), and increased emotionality following social defeat (Heinrichs *et al*, 1992b). In contrast, CRF microinjections into the amygdala decrease exploratory behavior in an open-field test (Liang and Lee, 1988; Elkabir *et al*, 1990) and increase defensive aggression (Elkabir *et al*, 1990). Finally, lesions of the CeA block the excitatory effects of CRF on the acoustic startle reflex (Liang *et al*, 1992a). However, it must be noted that in attempting to extrapolate these observations to human psychopathology, one must take into account

species differences in the distribution of both CRF and its receptor subtypes (Sanchez *et al*, 1999, 2001). Thus, CRF acts at the level of the amygdala to mediate the expression of behavioral responses to fear and, predictably, the observed changes in this system in response to rearing condition altered anxiety-like behavior.

CRF also serves as a neurotransmitter/neuromodulator in the projection from the amygdala to the LC and neighboring PBN (Moga and Gray, 1989). The LC has long been considered as a major site for mediation of anxiety-like states in primates and rodents (see Schulkin *et al*, 1994 for review). This structure sends noradrenergic projections extending throughout multiple cortical and subcortical regions, including the prefrontal cortex, the hippocampus, the amygdala, and the hypothalamus (Foote *et al*, 1983). Stress increases CRF concentrations in the LC (Chappell *et al*, 1986) and LC injection of CRF is associated with increased catecholamine release in the hypothalamus and prefrontal cortex of freely moving rats (Lavicky and Dunn, 1993). Both stress and CRF infusion have been shown to increase the firing rate of LC neurons and to stimulate the autonomic nervous system (Brown, 1989). The effects on LC can be blocked by local injections of the CRF receptor antagonist α -helical CRF₉₋₄₁ (Valentino and Foote, 1988; Valentino *et al*, 1998). Microinjections of a CRF receptor antagonist into the LC also attenuate novelty-induced suppression of exploratory behavior in the defensive withdrawal paradigm (Butler *et al*, 1990), as well as stress-induced behavioral freezing (Swiergiel *et al*, 1993). In addition to changes in CRF expression, we found increased CRF receptor binding capacity in the pericoerulear region of both HMS180 and HMS0 rats (Figure 5). Both specific CRF1 receptor binding and mRNA were increased in HMS180 rats vs AFR and HMS15 rats (Figure 6). The CRF1 receptor subtype is associated with enhanced anxiogenic effects (Streckler and Holsboer, 1999). Taken together, these data suggest that changes in CRF tone to the LC and related areas may change the sensitivity (ie alter the set-point) of these pathways to stimuli that normally activate them.

These observations imply that there would be increased CRF-induced activation of the LC during stress in the HMS0 and HMS180 animals. At least two findings are consistent with this idea. In comparison to HMS15 rats, acute restraint stress in both HMS0 and HMS180 rats is associated with a greater increase in Fos-ir in the LC (Pearson *et al*, 1997) and larger increases in extracellular NE levels in the PVN (Liu *et al*, 2000). In a recent post-mortem study of depressed suicide victims, Austin *et al* (2003) observed increased CRF₁ in the LC as well as median and dorsal raphe neurons. Thus, we propose that the neonatal environment can regulate the expression of behavioral responses to stress, at least in part, by altering the set-point of the CeA/BNST-LC CRF-NE system during a developmentally sensitive window occurring during the 10–14 days immediately after birth.

The behavioral differences in stress responsiveness noted between AFR and HMS15 vs HMS0 and HMS180 animals parallel those found in HPA axis responsiveness. Maternal separation increases plasma ACTH and CORT responses to acute psychological stressors (ie stressors involving conditions that signal threat) as compared to HMS15 or AFR rats (see Meaney *et al*, 1996 for review). These differences in

HPA axis responsivity to stress are, in part, associated with differential expression of CRF, as well as arginine vasopressin (AVP) in parvocellular neurons of the PVN (Plotsky and Meaney, 1993; Viau *et al*, 1993; Bhatnagar *et al*, 1995). These neurons project to the external zone of the median eminence and provide the neurohumoral signal for the release of ACTH from pituitary corticotrophs (see Plotsky, 1991 for review). Thus, our current findings of increased PVN CRF mRNA expression as well as increased CRF peptide content in the median eminence terminals of HMS180 and HMS0 rats (Plotsky and Meaney, 1993) provide strong support for the thesis that the neuroendocrine CRF system and, thus, HPA axis responsivity, is sensitive to the early rearing environment. The upregulation of CRF1 receptors in the PVN of HMS180 rats without a change in CRF2 binding or mRNA suggests functional adaptation in this region. Previous reports of CRF- and stress-induced upregulation have appeared (Rivest *et al*, 1995; Imaki *et al*, 1996; Mansi *et al*, 1996). Remarkably similar endocrine findings have been reported in depressed women with a history of childhood sexual or physical abuse (Heim and Nemeroff, 2001; Heim *et al*, 2002).

The effects of neonatal rearing on the PVN median eminence and the CeA/BNST-LC neurocircuits are likely to ramify throughout the brain to modify additional neurotransmitter circuits, which together may contribute to differences in behavioral and neuroendocrine responsiveness to stressors. In addition to the LC, Ladd *et al* (1996) found that maternal separation increased CRF receptor binding in the raphe nuclei. This region also shows increased CRF₁ in depressed suicide victims (Austin *et al*, 2003). Due to the anatomical and functional relationships among these structures, these findings suggest that early experience-induced changes in CRF systems may lead to a cascade of adaptations including both the noradrenergic and serotonergic systems, thus rendering the individual more responsive to subsequent stressors.

It is likely that similar changes in these systems also contribute to human psychopathology. Indeed, in humans, abuse or neglect in early life has been repeatedly shown to be a major risk factor for depression and/or anxiety disorders in adulthood (Bifulco *et al*, 1991; Brown and Anderson, 1993; Mullen *et al*, 1996; McCauley *et al*, 1997; Weiss *et al*, 1999; Bulik *et al*, 2001; Molnar *et al*, 2001; Kendler *et al*, 2002). Hyperactivity of central CRF systems has been implicated in the expression of these affective disorders (Stout and Nemeroff, 1994; Heim and Nemeroff, 2001; Heim *et al*, 2002, 2004), and both noradrenergic and serotonergic systems appear to be involved in the development of affective disorders.

Overall, these findings provide further validation of neonatal maternal separation in rats as a model of vulnerability to an anxiety- and depression-like syndrome. Altered rearing conditions are strongly associated with persistent alterations in stress-responsive neurocircuits. This process, which may occur via altered methylation of target genes in a region-specific pattern (Weaver *et al*, 2004a, b), or other mechanisms serve to 'tune' stress-sensitive neurocircuits in an adaptive or maladaptive manner. Specifically, this study strengthens the linkage between early life environment and long-term alterations in central CRF systems. These findings may provide a mechanism whereby life events,

interacting with genetic predisposition, may increase vulnerability to pathology in later life.

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