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Persistent elevations of cerebrospinal fluid concentrations of corticotropin-releasing factor in adult nonhuman primates exposed to early-life stressors: Implications for the pathophysiology of mood and anxiety disorders

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ABSTRACT **There is increasing evidence for an important role of adverse early experience on the development of major psychiatric disorders in adulthood. Corticotropin-releasing factor (CRF), an endogenous neuropeptide, is the primary physiological regulator of the mammalian stress response. Grown nonhuman primates who were exposed as infants to adverse early rearing conditions were studied to determine if long-term alterations of CRF neuronal systems had occurred following the early stressor. In comparison to monkeys reared by mothers foraging under predictable conditions, infant monkeys raised by mothers foraging under unpredictable conditions exhibited persistently elevated cerebrospinal fluid (CSF) concentrations of CRF. Because hyperactivity of CRF-releasing neurons has been implicated in the pathophysiology of certain human affective and anxiety disorders, the present finding provides a potential neurobiological mechanism by which early-life stressors may contribute to adult psychopathology.**

Considerable evidence from genetic, neurochemical, pharmacological, and neuroanatomical studies obtained over the past three decades supports a biological basis for many of the major neuropsychiatric disorders (1). Prior to the modern era of biological psychiatry, psychoanalytic theory pioneered by Freud prevailed. Freud's theories emphasized the importance of early rearing conflicts in the pathogenesis of adult psychopathology (2). More recent studies indicate that psychosocial factors, including abuse and neglect in early life as well as untoward life events in adulthood, contribute to the development of mood and anxiety disorders (3, 4). An integration of biological and developmental approaches may illuminate further the role of adverse early life experience on subsequent neurodevelopment. Using random-assignment study designs, nonhuman primate models of psychopathology, produced by an unpredictable early rearing environment, provide for the experimental exclusion of genetic influences on development, a strategy rarely feasible in humans (5–9).

The present study presents evidence for persistent hyperactivity of corticotropin-releasing factor (CRF)-releasing neurons in the central nervous system (CNS) of grown nonhuman primates who, as infants, were reared by mothers exposed to environmental unpredictability. A neurochemically based hypothesis is proposed whereby emotionally adverse early experiences may antecede psychiatric disorders through the induction of persistently elevated neuronal release of CRF.

The CRF-containing parvocellular neurons of the hypothalamic paraventricular nucleus (PVN) represent the cephalic component of the hypothalamic–pituitary–adrenal (HPA) axis, which serves as the primary endocrine response of mammalian organisms to stress (10–12). Hypothalamic CRF release increases HPA axis activity by stimulating secretion of corticotropin [also called adrenocorticotropic hormone (ACTH)] and other pro-opiomelanocortin products from the anterior pituitary gland and, therefore, ultimately the secretion of cortisol, the major circulating glucocorticoid in primates. Outside of the PVN, radioimmunoassay and immunohistochemical studies indicate a widespread CNS distribution of CRF-containing cell bodies, terminals, and receptors (13). CRF and its receptors are found in many brain regions implicated in anxiety and depressive disorders—the prefrontal, cingulate, and insular cortices and the central nucleus of the amygdala, hippocampus, and periaqueductal grey. In the brain stem, CRF perikarya are present in the locus coeruleus, dorsal raphe nucleus, and ventral tegmental area, potentially modulating noradrenergic, serotonergic, and dopaminergic monoamine neurotransmitter systems. Thus, CRF-containing neurons in the CNS may well provide a primary mechanism by which mammalian organisms integrate not only endocrine but also behavioral, autonomic, and perhaps immunological responses to stress (13–16). Simultaneous measures of HPA axis activity and cerebrospinal fluid (CSF) CRF are asynchronous, providing further support for the distinction between nonendocrine versus endocrine functions of CNS CRF systems (17).

When centrally administered to laboratory animals, CRF produces many of the signs and symptoms of major depression and anxiety disorders. These include reduced food consumption, decreased sexual behavior, disrupted sleep, alterations in locomotor activity, and abnormal responses to novel stimuli (13, 14).

The complex link between stress and the pathophysiology of mood and anxiety disorders has prompted intensive study of the HPA axis activity and its central control (1). Depending on the mode of investigation, HPA axis hyperactivity is disproportionately evident in patients with mood disorders (18–20). The repeated demonstrations of elevations of CSF concentrations of CRF in drug-free depressive patients support the view that HPA axis activation in depression is a centrally driven endocrinopathy (21). Moreover, clinically effective antidepressants or electroconvulsive therapy reduces the activity of CRF neurons by measuring either CRF mRNA expression or electrophysiological responses in laboratory animals (22, 23) or

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Abbreviations: CRF, corticotropin-releasing factor; CNS, central nervous system; HPA, hypothalamic–pituitary–adrenal; VFD, variable foraging demand; LFD, consistently low foraging demand; HFD, consistently high foraging demand.

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CSF CRF concentrations in humans (24, 25). This concatenation of findings, taken together, suggest that close scrutiny of the developmental pathways leading to hyperactivity of CNS CRF systems in the adversely reared grown primate is warranted.

We report CSF CRF measurements in grown nonhuman primates reared during a period of infancy by mothers confronting various foraging conditions (5–7). The randomly assigned foraging conditions for the mothers included variable (or unpredictable) foraging demand (VFD), consistently low foraging demand (LFD), and consistently high (but predictable) foraging demand (HFD). From a behavioral standpoint, monkeys reared by VFD mothers, but not those reared by LFD or HFD mothers, exhibit stable anxiety/affective traits (5–7). In this respect, VFD bonnet macaques resemble highly reactive rhesus monkeys (26) and behaviorally inhibited children (27).

In addition to the behavioral findings, several years after rearing conditions were terminated, VFD-reared primates exhibited alterations in monoamine systems as manifested by behavioral hyperresponsivity to the noradrenergic probe yohimbine and hyporesponsivity to the serotonergic probe *m*CPP (*m*-chlorophenylpiperazine) in comparison to LFD-reared subjects (28).

In an effort to expand our knowledge regarding the neurobiology of adverse early rearing, we tested the hypothesis that primates reared under VFD conditions would demonstrate persistently elevated CSF CRF concentrations. By extrapolation to humans, this would provide indirect evidence for a neural substrate that enhances susceptibility to human anxiety and affective disorders following adverse early rearing experiences.

Procedures. Thirty bonnet macaques (*Macaca radiata*) served as subjects for the study. For 12 weeks beginning when the infants were ≈17 weeks old, 15 of the subjects (10 male and 5 female) were raised under VFD conditions, 8 under HFD conditions (5 male and 3 female), and 7 (4 male and 3 female) under LFD conditions. The mean age at the time of the cisternal CSF sampling in the VFD subjects was 2 years (SD = 0.5), whereas the mean ages of the HFD and LFD subjects were both 4 years, with all HFD and LFD subjects born within a few weeks of each other. There is no evidence for systematic age-related changes or gender-related changes in CSF CRF concentrations across this age range (Ned Kalin, University of Wisconsin, Madison), personal communication. Nevertheless, as the VFD subjects were significantly younger than the subjects of the other two groups and sex distribution was not controlled, a separate study to explore the possible role of age and gender effects on CSF CRF concentrations was carried out in 25 monkeys (11 males and 14 females). The control group monkeys were reared by their mothers in laboratory breeding groups under ad lib feeding conditions. These additional 25 animals had a mean age of 29.4 months (SD, 10.6; range, minimum = 12 months and maximum = 52 months), thus adequately spanning the age range of the differentially reared groups. The age range of the primates at the time of CSF sampling corresponded to that of peripubertal to young-adult phases of human development.

Rearing treatment was preceded by habituation to the rearing paradigm in which infants are required to remain within a nursery enclosed by contact-permissive mesh within their mother's pen. A full range of mother–infant behavioral patterns is permitted by the design. Following habituation to the nursery paradigm, differential treatment began. For the HFD mothers, this consisted of 12 weeks in which they were required to dig through clean wood-chip bedding to obtain their daily food ration. For the LFD mothers, abundant food items could simply be picked up from the pen floor during this same 12-week period. For the VFD mothers, foraging demand varied between low and high in two-week blocks over the 12-week rearing period, beginning with 2 weeks of low demand. The low-demand blocks were identical to the LFD condition. During the high-demand blocks, four mothers were required to dig for their food as in the HFD condition, whereas 11 mothers were required to perform a joystick task described previously (7) to earn small food pellets. Four infants of this latter group of 11 mothers were not housed in the nursery enclosure. Water was constantly available to all mothers. The infants had ad lib access to food and water in an area inaccessible to the mothers. Weekly body weight measurements and health checks revealed normal growth and health in all subjects. Assessment of "normal" patterns of development for the bonnet macaque has been developed over a 30-year period. Of note, the VFD-reared animals do not exhibit any of the overt characteristic behaviors of isolate rearing such as stereotypies, self-biting, or imaginary "fly-catching" (7).

The CSF sampling commenced with ketamine sedation (10 mg/kg of body weight) of the animal, which was administered within 2 min following entrance into a squeeze cage. The suboccipital area was shaved and prepped with povidone iodine solution. The animal was placed in a strictly symmetrical sitting position, and the neck was fully flexed so as to expose a small triangular depression directly below the occiput and superficial to the cisterna magna. By using a 24-gauge, 0.75-inch (1.9-cm) needle with a 3-ml syringe, the needle was advanced perpendicular to the surface. Once the dura had been penetrated, 1.5 ml of CSF was slowly withdrawn. The CSF was placed in Gant tubes and placed immediately in a −30°C freezer. The CSF was then stored at −70°C until analysis. All CSF sampling was performed between 1015 and 1130 hr to avoid diurnal confounds (29). Several hundred taps using this level of ketamine administration have been carried out over many years without any evidence of short- or long-term difficulty. Animals are observed until they are fully recovered following the tap and do not appear to be in pain or distress during or after the procedure.

Because capture activates the HPA axis, CSF cortisol concentrations were determined as a measure of HPA axis activity. CRF concentrations in CSF were measured by a well-characterized radioimmunoassay as described (21). The assay has a sensitivity of 2.5 pg per tube and intra- and interassay coefficients of variation of 3–6% and 10–13%, respectively. The laboratory personnel conducting the CRF radioimmunoassay were blind to the rearing status of the subjects' samples. CSF cortisol determinations were conducted under blind conditions by using a competitive protein-binding method of the Department of Analytic Psychopharmacology at the New York State Psychiatric Institute.

RESULTS

CSF CRF Concentrations. We first report group effects for the raw CSF CRF concentrations. VFD subjects exhibited significantly elevated CSF CRF concentrations (pg/ml); VFD = 101.1 ± 21.5 (SD); LFD = 76.8 ± 11.8; and HFD = 87.5 ± 21.5 ($F = 5.95$, df = 2, 27, $P < 0.007$). These data are illustrated in Fig. 1. Post hoc Newman–Keuls analysis revealed significant elevations in CSF CRF concentrations in the VFD subjects when that group was separately compared to both HFD ($P < 0.04$) and LFD ($P < 0.009$) groups. CSF CRF concentrations between the HFD and LFD groups were not distinguishable. Inspection of Fig. 1 reveals two outliers (>2 SD from their group mean)—one HFD with the second highest CRF and one VFD subject with the lowest CRF concentration. The group effect markedly increases ($F = 16.2$, df = 2, 25, $P < 0.00002$) when these two outliers are excluded. The two outliers are excluded from the age and sex control analyses ($N = 28$).

Ad lib reared females exhibited a moderate decline in CSF CRF concentrations with age ($r = 0.61$, df = 13, $P < 0.02$), whereas males showed no decline with age ($r = 0.16$, df = 10,

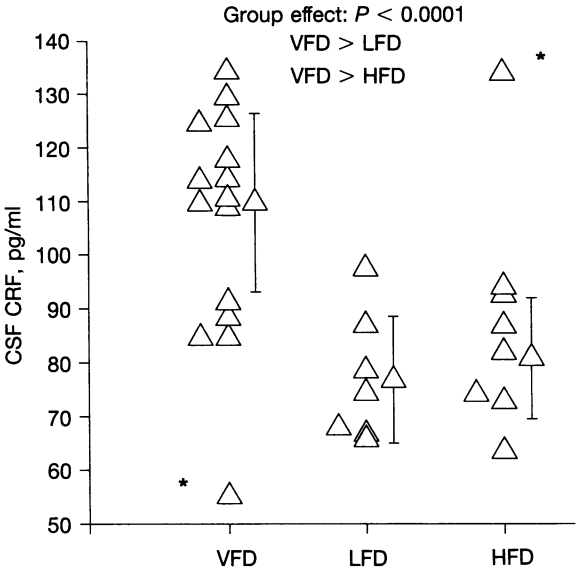

FIG. 1. CSF CRF concentrations in differentially reared primates. Scatterplot showing CSF CRF concentrations in grown bonnet macaques whose mothers were exposed to low, high, and variable foraging demands as infants. Pooled data are expressed as means ± SD. *, Not used for the determination of mean group concentrations.

$P < 0.64$). There was no overall gender difference or gender by age interaction for CSF CRF in the ad lib reared subjects.

In the first analysis, controlling for both sex and age, we calculated Z scores from CSF CRF values obtained from the ad lib reared cohort and calculated sex-specific expected Z scores for three age categories (24 months or less, 25–39 months, and 40 months or more) that spanned the ages of the differentially reared cohort. We then converted raw CSF CRF values generated from the differentially reared cohort into Z scores and computed for each subject the difference observed from its age and sex-expected Z score from its observed CSF CRF Z score. ANOVA was then applied to test between-group differences. Newman–Keuls post hoc tests were applied when ANOVA values were significant.

The overall group effect was unchanged with the VFD group exhibiting significantly higher CSF CRF concentrations than either the LFD or HFD groups; the latter two groups were not statistically distinguishable.

The LFD and HFD treatment groups were combined for analysis of gender effects, because of the relatively low N. For males, VFD subjects ($n = 10$) exhibited elevated CSF CRF concentrations in comparison to those of the combined group ($n = 8$) ($F = 8.98$; df = 2.15; $P < 0.003$). VFD females ($n = 4$) also demonstrated elevated CSF CRF concentrations in comparison to those of the combined group ($n = 6$), ($F = 15.02$; df = 1.8; $P < 0.005$). Thus, CSF CRF concentrations are significantly elevated following early rearing manipulation in both males and females.

Further analyses were performed that provide additional evidence sociating age and rearing effects with CSF CRF concentrations. The correlation between age and cisternal CRF correlation was significantly greater in the differentially reared group [$N = 28$; $r = -0.77$, $P < 0.001$] in comparison to the ad lib reared group [$n = 25$; $r = -0.24$; $P = 0.24$ (Fisher r to z comparison yields a χ^2 value of 7.2; $P < 0.007$)]. This analysis supports the view that the bivariate correlation between age and CSF CRF is significantly altered by manipulation of normative mother–infant rearing patterns in bonnet macaques.

In a further analysis, standardized CSF CRF Z scores for each differentially reared individual subject were calculated. Utilizing the 25 ad lib reared subjects, an algorithm describing the normative correlation of age with CSF CRF Z scores (CRF Z score = 0.7 − 0.02* age) was generated. For each differentially reared primate, an age-expected Z score was then calculated from the algorithm. The age-adjusted CSF CRF Z score for each subject was then computed from the difference between the age-expected versus observed Z score value.

By this methodology, the VFD group continued to demonstrate significantly elevated age-adjusted CSF CRF Z scores in comparison to the combined LFD/HFD group ($t = 4.0$; df = 26; $P < 0.001$). This last analysis provides additional support for the conclusion that the rearing effects recorded in this study were not the product of age.

CSF Cortisol Concentrations (See Fig. 2). Raw CSF cortisol concentrations were decreased in VFD subjects when compared with both the LFD and HFD groups ($F = 6.5$; df = 2, 27; $P < 0.005$) as follows: VFD = 2.82 μg/dl (SD = 0.85) versus HFD = 4.0 (SD = 0.9) (Neuman Keul post hoc; $P < 0.005$); and VFD versus LFD = 3.7 (SD = 0.8) (Neuman–Keul post hoc; $P < 0.05$). No correlation between CSF CRF and cortisol concentrations was noted, either within the VFD group alone, the entire differentially reared cohort, or the ad lib reared controls.

In the ad lib reared control subjects (three additional subjects were added), no sex effect or a significant age–sex interaction for CSF cortisol was observed. An age–gender control analysis, as described for CSF CRF, was not therefore performed. A significant reduction in CSF cortisol with increasing age ($r = -0.383$; df = 27; $P < 0.05$) was observed. When age was plotted versus cortisol, the slope associated with the subjects reared ad lib differed from the slope associated with the subjects reared differentially ($r = 0.34$; df = 29; $P = 0.065$) (Fisher r to z; $\chi^2 = 7.47$; $P < 0.006$). Thus, the rearing manipulation had significantly reversed the normative Bonnet decline of CSF cortisol with increasing age. In differentially reared subjects, because the young VFD subjects have aberrantly low CSF cortisol, the older HFD and LFD subjects appear to have relatively higher cortisol levels. Thus, a nearly significant positive correlation of CSF cortisol with age, the opposite direction to that observed in the ad lib reared group, is evident in the VFD-reared subjects.

Using the next age-control method, as presented for CSF CRF, we converted the cortisol values of the group reared ad lib to Z scores, thus generating an algorithm (ZCSFCORT = 1.12

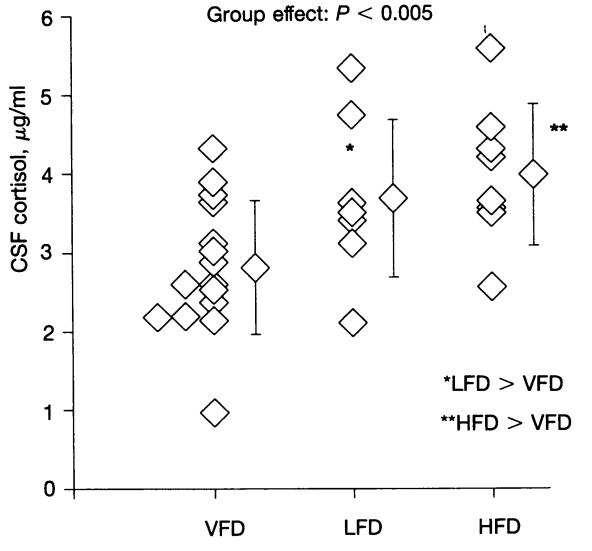

FIG. 2. CSF cortisol concentrations in differentially reared primates. Scatterplot showing CSF cortisol concentrations in grown bonnet macaques whose mothers were exposed to low, high, and variable foraging demands as infants. Pooled data are expressed as means ± SD.

− 0.037* age) by which to predict age-expected *Z*-score cortisol levels. The differentially reared CSF cortisols were converted to *Z* scores, and the *Z*-score difference between the subjects expected versus observed cortisol *Z* score was calculated. When age-corrected CSF cortisol concentrations were used, the VFD versus non-VFD subjects exhibited markedly lower CSF cortisol levels ($t = -5.628$; df = 28; $P = 0.000$)—a *t* value of 3.0 was observed with raw CSF cortisol measures. Thus, VFD-reared primates exhibit significantly lower CSF cortisol than non-VFD-reared subjects, an effect strengthened when a separate ad lib reared group is used as an age control.

Relationship Between Early Rearing, CSF CRF, and CSF Cortisol. To potentially provide further insight to the findings, we then performed a three variable linear regression analysis to assess the extent to which the dependent variable, rearing status (VFD versus non-VFD), could account for variance of the independent variables (CSF CRF and CSF cortisol). This analysis was performed with raw CSF CRF and CSF cortisol values and was then repeated using age-corrected *Z* scores, described above.

Using linear regression analysis, rearing status (VFD versus non-VFD) accounted for 68% ($R^2 = 0.68$; $F = 26.7$; df = 2, 25; $P < 0.00001$) of the variance as captured by the high CSF CRF and low CSF cortisol levels. Rearing status independently predicted ($P < 0.05$) both elevated CSF CRF values and decreased CSF cortisol values (β values = −0.68 and 0.35, respectively) in the VFD subjects. The outcome of the analysis was minimally changed when the age-corrected *Z*-score difference analysis was used (data not shown). Thus, VFD rearing status had a marked influence (literally accounting for two-thirds of the variance) in predicting grown primate CSF CRF and CSF cortisol concentrations.

DISCUSSION

The major finding of the study is that adverse early rearing in primates may lead to persistent overactivity of CSF CRF-containing neurons, reflected in elevated cisternal CSF CRF concentrations. The adversity of the early rearing is related to the unpredictability of foraging conditions imposed on the maternal–infant dyad as the HFD-reared group, which was engaged in high but predictably demanding foraging conditions, did not exhibit the elevated CSF CRF concentrations observed in the VFD group. Moreover, the HFD-reared group was not distinguishable from the LFD group by CSF CRF or cortisol concentrations. The inconsistent and erratic, sometimes dismissive, rearing behavior exhibited by mothers undergoing VFD is the putative mechanism resulting in a diminution of the infants' perception of a "security" of maternal attachment (5–8).

Because of the between-group age (VFD younger than non-VFD) confound, this data must be regarded with caution. Definitive proof for prolonged elevations of CSF CRF concentrations following adverse early rearing is best provided through the study of age-matched subjects. Because this was not available, the separate study of an ad lib reared age and sex control group was conducted, supporting our initial observation.

Paradoxically, elevated CSF CRF concentrations in the VFD group are accompanied by HPA axis suppression, to the extent reflected by lower CSF cortisol concentrations in the VFD when compared with those found either in the HFD or LFD groups. CSF cortisol measures of LFD and HFD groups, as was the case for CSF CRF, were indistinguishable. Again, the study of the relationship between age, sex, and CSF cortisol in a separate ad lib reared control group indicates that low cortisol in the VFD subjects was not an artifact of the VFD-reared groups' younger mean age. In fact, in the ad lib reared control group, younger age was associated with moderately higher CSF cortisol levels.

Nevertheless, without simultaneous plasma neuroendocrine measures, the study does not permit differentiation between altered acute HPA axis responses to capture in VFD subjects versus chronic HPA axis adaptation following adverse rearing. Plasma ACTH and cortisol concentrations, performed at the time of the CSF withdrawal, would have further enhanced the generality of the current findings and should be included in future studies. Moreover, to avoid the confound of blood contamination of CSF cortisol levels, documentation of the presence and degree of contamination by microscopically assessing the number of erythrocytes in the CSF samples should be performed.

Because of the relatively high CSF CRF and relatively low CSF cortisol in the VFD-reared primates, an inverse correlation between the two measures would be expected when considering the differentially reared cohort. The lack of significant or even nearly significant correlation between CSF CRF and CSF cortisol, either within the VFD-reared group, the differentially reared cohort, or the ad lib reared group, suggests that CNS CRF systems and the HPA axis may function quite independently, or more likely that CSF CRF largely reflects extrahypothalamic CRF neuronal system activity. Each of the CSF CRF and CSF cortisol concentrations provides significant variance to the linear regression model because of this lack of colinearity. The unusually high level of 68% of the variance still evident years after the initial experimental manipulation implies an intimate relationship between the early adverse experience and long-term CRF/HPA axis function.

Other limitations of the study include the absence of test–retest reliability for a given animal. Moreover, prospective data are the best means of determining the longitudinal course of these systems. In addition, primate studies directly connecting elevated CSF CRF concentrations induced by adverse early rearing with current behavioral patterns are lacking.

It is nevertheless of interest that VFD subjects, directly following the rearing manipulation, exhibit increased affective vulnerability to the challenges of novel environments and maternal separation. Moreover, VFD subjects are less social and more subordinate than LFD subjects as young adults (5–8). Excessive CRF receptor activation through development may, in part, explain the characteristic behavioral profile of VFD-rearing, analogous to certain longitudinal aspects of human affective and anxiety disorders.

These data therefore neurobiologically substantiate the view proposed by Freud (2) and are also congruent with studies by Brown and others (3, 4) and more recently by Kendler *et al.* (29), stressing the importance of early life experience on the development of psychopathology in adulthood.

Nevertheless, the findings raise further provocative questions. For instance, what are the biological mechanisms whereby VFD rearing induces prolonged CRF elevation? Indeed, the distinctive pattern of increased CSF CRF concentrations and reduced cortisol concentrations is precisely what is observed in patients with posttraumatic stress disorder (30, 31).

In the context of therapeutic implications, the question is raised as to whether the biological and behavioral sequelae of VFD-rearing, and by extrapolation to adverse early rearing in humans, are reversible. Most obvious is the potential utility for CRF receptor antagonists as novel and unique antidepressant or anxiolytic agents. Indeed, in preclinical studies, peptidergic CRF antagonists have been repeatedly demonstrated to exert antianxiety effects similar to those of clinically effective anxiolytics such as benzodiazepines (13, 14).

In addition to the implications of these findings to drug development is their relevance to two other relatively unexplored areas: (*i*) early intervention in children after exposure to abuse and neglect and (*ii*) the neurobiological effects of psychotherapy. Concerning the former, childhood sexual and physical abuse and neglect are, unfortunately, common in our society and frequently are associated with the development of

anxiety and/or affective disorders in adulthood (29). To prevent the putatively long-lasting neurobiological alterations associated with this abuse and neglect, early intervention may well be necessary. A related and virtually unexplored area is the potential salutary biological consequences of psychotherapeutic interventions in children and adults.

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