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# Involvement of corticotropin-releasing factor in chronic stress regulation of the brain noradrenergic system

(tyrosine hydroxylase/norepinephrine/locus coeruleus/depression/anxiety)

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ABSTRACT **Corticotropin-releasing factor (CRF) and norepinephrine (NE) mediate many hormonal, autonomic, and behavioral effects of acute stress, and it is possible that an interaction between these neurotransmitters could underlie neuronal adaptations in response to chronic stress. To test this hypothesis, the influence of chronically administered CRF and a specific CRF antagonist, α-helical CRF, on the induction of tyrosine hydroxylase, the rate-limiting enzyme in NE biosynthesis, was examined in the rat locus coeruleus (LC). We now report that administration of α-helical CRF specifically blocks the induction of tyrosine hydroxylase in response to a repeated intermittent stress paradigm involving foot shock and noise stress but has no effect on steady-state levels of the enzyme in nonstressed animals or on the induction of the enzyme in response to reserpine treatment. In addition, repeated administration of CRF alone for 5 days, like chronic stress, increases levels of tyrosine hydroxylase in LC. The results demonstrate that endogenous CRF is necessary for the induction of tyrosine hydroxylase in response to this stress paradigm and that exogenously administered CRF is sufficient for the regulation of this enzyme in nonstressed rats. These findings may prove important in elucidating mechanisms by which chronic stress triggers and sustains the biochemical alterations associated with some stress-related psychiatric disorders.**

The administration of corticotropin-releasing factor (CRF) initiates a constellation of changes typically seen in response to stress, including increased central and peripheral levels of catecholamine and catecholamine metabolites, increased plasma levels of adrenocorticotropin hormone (ACTH), and increased stress-related behaviors (1–5). Activation of the noradrenergic neurotransmitter system similarly increases stress-related behaviors, whereas inhibition of this system attenuates stress-induced changes in behavior (6–8). Recent studies suggest that some of the effects of acute stress may be mediated by CRF interactions with the locus coeruleus (LC), a near homogeneous nucleus containing approximately 50% of brain norepinephrine (NE) neurons (9, 10). CRF-immunoreactive fibers (11, 12) and receptors (13) have been localized in the LC, and acute and chronic stress increase immunoreactivity to CRF in this nucleus (14). Moreover, infusion of CRF directly into the LC increases catecholamine and catecholamine metabolite levels in the cerebral cortex, plasma corticosterone levels, and certain stress-related behaviors (15). Finally, CRF, like acute stress, increases the firing rate of LC neurons, whereas administration of a CRF antagonist blocks the activation of these neurons by acute stress (16, 17).

Given the above findings, it is possible that endogenous CRF may influence the biochemical adaptive responses of LC noradrenergic neurons to chronic stress. Chronic stress increases the firing rate of LC neurons (18) and increases levels of NE in this nucleus and its terminal fields (19–22). Chronic stress also increases the expression of tyrosine hydroxylase (TH) mRNA and protein in the LC, an effect that presumably underlies these neuronal adaptations (23–25). The present study examines the hypothesis that an increase in CRF activity underlies the induction of TH in response to chronic stress. The results show that administration of a CRF antagonist blocks the induction of TH in response to the repeated presentation of foot shock and noise stress and that CRF treatment increases the expression of TH in LC.

## METHODS

***In Vivo* Treatments and Stress Paradigms.** Male Sprague–Dawley rats (260–280 g) were anesthetized with Equithesin (3 ml/1 kg of body weight), and cannulae were implanted unilaterally (relative to lambda: −1.8 posterior, −1.5 lateral, −7.2 ventral) into the parabrachial nucleus, just lateral to the LC, for local infusion of vehicle or α-helical CRF (αh-CRF). This placement was chosen to avoid infusion-induced damage to the LC. All experiments were conducted between 5 and 10 days after surgery. In all cases, animals were sacrificed 18 hr after the last treatment, and the LC region was excised from 1.0-mm-thick coronal cross sections of brain by obtaining punches with a blunt 14-gauge syringe needle. Levels of immunoreactivity to TH were analyzed in right and left punches of LC taken from animals treated unilaterally with αh-CRF or vehicle to permit within-subject comparisons. The right and left LC punches from each CRF-treated rat were pooled and analyzed as one sample.

The first experiment assessed the ability of locally infused αh-CRF to block the induction of LC TH by repeated intermittent stress. Animals were unilaterally infused twice daily for 4 days with αh-CRF (5 μg in 1 μl of vehicle: artificial CSF with 0.1% bovine serum albumin) 10 min prior to 45 min of foot shock (10 1-mA 1-s duration shocks; interstimulus interval, ≈4 min) and 30 min of noise stress [95 decibels (dB)] beginning at 9:00 a.m. and 4:00 p.m., respectively. Unoperated, nonstressed animals were used for controls in this experiment. For foot shock stress, animals were placed into five separate aluminum and Plexiglas boxes (30 × 25 × 25 cm) having floors composed of 4.76-mm stainless steel bars spaced 19 mm apart. These boxes were located on two shelves within a 1 × 1 × 2 m darkened, ventilated, and sound-attenuating chamber. Foot shocks were generated by five Lehigh Valley constant-current shock generators (SGS-004) located outside of the chamber. Shock intensity was measured with a 1-kΩ resistor connected between adjacent bars. Current was defined as the rms voltage across the 1-kΩ

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Abbreviations: CRF, corticotropin-releasing factor; NE, norepinephrine; TH, tyrosine hydroxylase; LC, locus coeruleus; αh-CRF, α-helical CRF.

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resistor where mA = 0.707 × 0.5 × peak-to-peak voltage. Background noise of 55 dB was provided by a white-noise generator. For noise stress, animals were placed into separate Plexiglas and wire-mesh cages (8 × 15 × 15 cm) housed on two shelves in a darkened, ventilated, sound-attenuating chamber, and white noise was delivered (95 dB) through one speaker located on the door of the chamber ≈45 cm from each cage.

To determine whether CRF regulation of TH is phasic—i.e., in response to a perturbation of the system such as stress—or tonic, the effect of αh-CRF or vehicle on levels of TH in the LC of both stressed and nonstressed rats was examined. For 4 days rats were unilaterally infused (twice daily) with αh-CRF (5 μg in 1 μl) or vehicle and then half of the animals were returned to their home cages, while the other half were exposed to foot-shock stress (a.m.) and noise stress (p.m.) as described above.

Because αh-CRF is infused immediately lateral to the LC and repeated infusion produces tissue damage, the possibility exists that αh-CRF might block the stress-induced increase in LC TH indirectly by damaging LC neurons. To test this possibility, the ability of αh-CRF to block the induction of TH by reserpine was examined. Reserpine depletes catecholamine stores and is thought to increase TH expression in the LC by decreasing stimulation of inhibitory autoreceptors (26–28). Animals were unilaterally infused with αh-CRF (5 μg in 1 μl) or vehicle twice daily for 4 days as above; on day 3 all animals received an i.p. injection of reserpine (5 mg/kg), and one group of naive rats received vehicle alone.

To examine the effects of CRF in otherwise naive animals, rats received sham intraventricular infusions (relative to bregma: 1.2 lateral, −4.0 ventral) twice daily for 10 days and then were administered rat/human CRF (10 μg in 5 μl) or vehicle twice daily for 5 days. CRF was administered intraventricularly instead of locally to parallel previous studies demonstrating the activation of LC firing rate by intraventricular infusion of this peptide (15).

To determine the extent of tissue damage due to repeated infusion, four animals were infused with αh-CRF and four with vehicle exactly as described above. On day 5 these animals were anesthetized with chloral hydrate and intracardially perfused with 0.9% saline followed by 10% formalin. The brains were then stored for at least 2 days in 10% formalin/30% sucrose. Examination of cresyl violet stained 30-μm-thick coronal sections from these animals at ×10 and ×20 magnification revealed minor gliosis in the most lateral aspect of the infused LC in one αh-CRF- and one vehicle-treated rat; there was significant damage in the adjacent parabrachial nucleus throughout its rostral–caudal extent in each animal (data not shown).

**Immunoblotting of TH.** Isolated LC punches were homogenized in 2% SDS, and aliquots of LC containing equivalent amounts of protein (25–75 μg) were adjusted to contain 50 mM Tris (pH 6.7), 2% SDS, 4% (vol/vol) glycerol, 2% (vol/vol) 2-mercaptoethanol, with bromophenol blue as a marker. The samples were then subjected to one-dimensional SDS/polyacrylamide gel electrophoresis (with 7.5% acrylamide/0.4% methylenebisacrylamide in the resolving gels) and to immunoblot analysis exactly as described (29). A rabbit polyclonal antiserum raised against TH (1 μg/2 ml; from John Haycock, Louisiana State University Medical Center) and $^{125}$I-labeled goat anti-rabbit IgG (500 cpm/μl; New England Nuclear) were used. Filters were autoradiographed, and levels of TH were quantified by densitometric analysis of the autoradiogram and by counting bands excised from dried filters in a γ counter. Under the immunoblotting conditions used, levels of immunoreactivity to TH were linear over a 3-fold range of tissue concentration.

## RESULTS

To determine whether an increase in CRF activity underlies the induction of TH by chronic stress, the ability of locally administered αh-CRF, a CRF antagonist, to block this effect was examined. Unilaterally implanted animals were infused twice daily for 4 days with αh-CRF immediately lateral to the LC—once prior to foot-shock stress in the morning and once prior to noise stress in the evening. This discrete stressor paradigm produced a 76% increase in immunoreactivity to TH in the noninfused (contralateral) LC relative to experimentally naive animals. In contrast, the induction of TH by repeated stress was completely blocked in the αh-CRF-infused LC of the same animals (Fig. 1).

The next experiment examined the effect of repeated administration of αh-CRF on basal levels of TH in the LC.

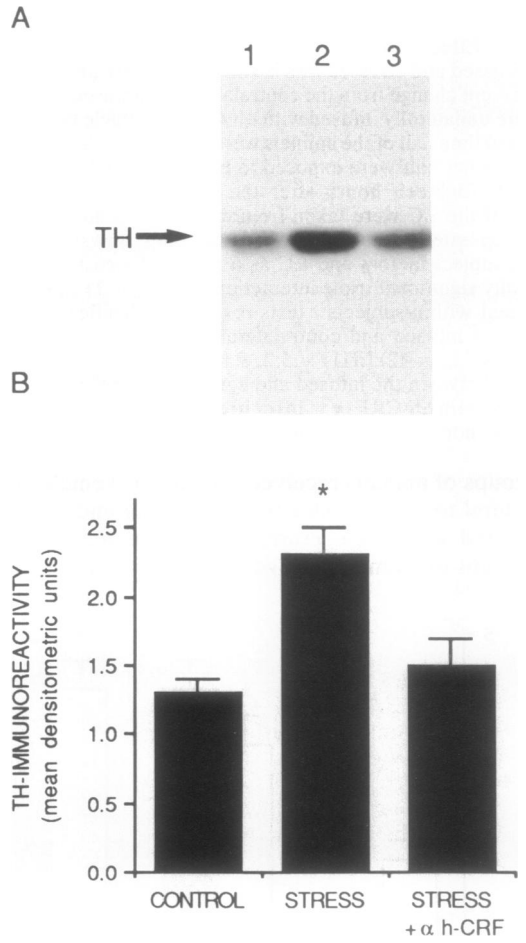


FIG. 1. (A) Levels of immunoreactivity to TH in the LC of control animals (lane 1) and the αh-CRF-infused LC (lane 3) and contralateral noninfused LC (lane 2) of repeatedly stressed animals. (B) Quantification of TH-immunoreactivity for these groups. Data are shown as the mean ± SEM for controls and for the infused and noninfused LC of stressed rats. Rats were infused twice daily for 4 days with αh-CRF lateral to the LC 10 min prior to foot shock or noise stress at 9:00 a.m. or 4:00 p.m., respectively. Eighteen hours after the last stressor, right and left punches of LC from each rat were analyzed for TH content for within-subject comparison. Immunolabeled TH was visualized by autoradiography, and levels of TH were quantified as described. Analysis of variance comparing TH levels in control animals ($n = 6$) and the infused ($n = 8$) and noninfused ($n = 9$) LC of stressed rats revealed a statistically significant difference [$F(2,20) = 7.58$, $P < 0.001$]. TH levels in the noninfused LC of stressed rats were significantly greater than TH levels in the contralateral infused LC of the same rats and experimentally naive animals [Scheffe test: $F(1,22) = 7.6$, $P < 0.005$; see asterisk]. In contrast, no significant difference was found between levels of TH in the LC of naive rats and the αh-CRF-infused LC of stressed rats.

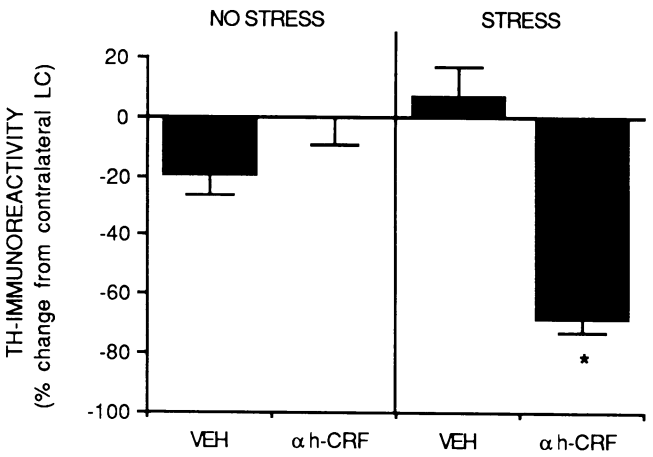


FIG. 2. Effect of αh-CRF or vehicle (VEH) on levels of TH in LC of nonstressed and stressed rats is shown. Data are presented as the mean percent change from the contralateral noninfused LC, ± SEM. Rats were unilaterally infused with αh-CRF or vehicle twice daily for 4 days, and then half of the animals were returned to their home cages while the other half were exposed to foot shock and noise stress as described. Eighteen hours after the last stressor, right and left punches of the LC were taken from each rat and analyzed for TH content separately. Analysis of variance comparing stress and drug between subject factors and LC as a repeated measure revealed a statistically significant triple interaction [$F(1,47) = 11.6$, $P < 0.001$]. Subsequent within-subjects $t$ tests revealed a significant difference between the infused and contralateral LC of stressed rats treated with αh-CRF ($n = 12$) [$t(11) = 5.2$, $P < 0.0003$; see asterisk] but no difference between the infused and contralateral LC of nonstressed rats treated with αh-CRF ($n = 14$) or in either vehicle-treated stressed ($n = 12$) or nonstressed rats ($n = 13$).

Two groups of animals received αh-CRF or vehicle immediately lateral to the LC once in the morning and once in the evening and were then returned to their home cages. Two other groups of animals received αh-CRF or vehicle prior to

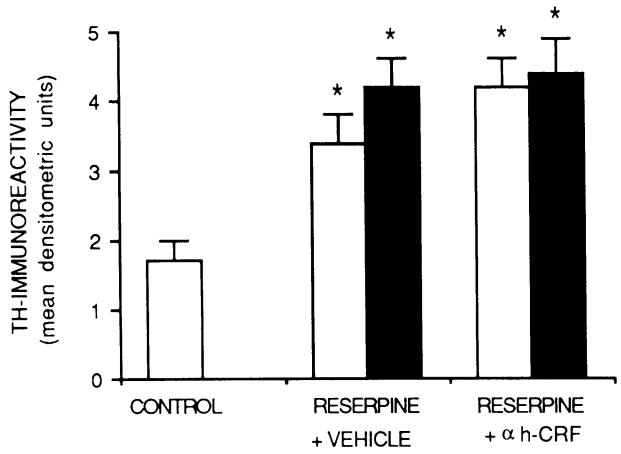


FIG. 3. Effect of αh-CRF or vehicle on levels of TH in the infused (■) and noninfused (□) LC of reserpine-treated animals. Data are presented as the mean ± SEM for each group. Animals were infused with αh-CRF ($n = 6$) or vehicle ($n = 6$) twice daily for 4 days. On day 3 all animals received i.p. injections of reserpine, and six naive rats received i.p. injections of vehicle. Analysis of variance using reserpine (vs. vehicle) and drug (vehicle vs. αh-CRF) between subject factors and LC as a repeated measure revealed no significant differences. A significant effect of reserpine was revealed with a between-subjects $t$ test when data were collapsed across drug and LC factors and compared to TH levels in vehicle-injected rats [$t(15) = 5.3$, $P < 0.0001$; see asterisks]. There was no significant difference in the magnitude of the TH response to reserpine among the four treated groups (infused and contralateral LC of vehicle- or αh-CRF-treated rats).

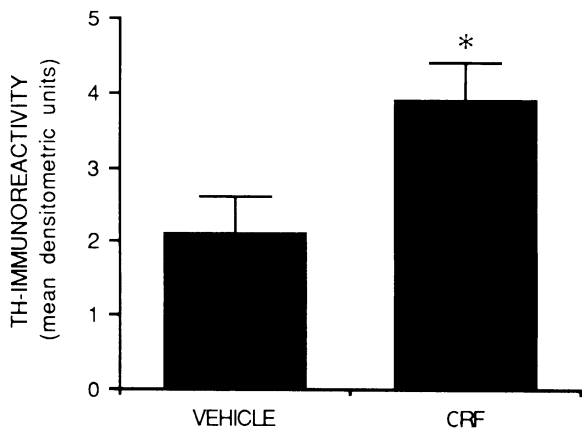


FIG. 4. Effect of repeated intraventricular infusion of CRF on levels of TH in LC. Data are presented as the mean ± SEM for each group. Cannulated animals were exposed to sham infusion procedures twice daily for 10 days prior to drug treatment. Animals were then infused with rat/human CRF ($n = 5$) or vehicle ($n = 7$) twice daily for 5 days. Animals were sacrificed on day 6, and the LCs were pooled from each rat and treated as a single sample. Repeated infusion with CRF significantly increased levels of TH in the LC to 186% of the vehicle-treated controls [$t(10) = 2.6$, $P < 0.02$; see asterisk].

stress to verify that the antagonist treatment was effective. In preliminary studies, no difference was found between levels of TH in the LC of naive animals and levels of TH in the noninfused LC of operated stressed and nonstressed rats, therefore the noninfused (contralateral) LC of each rat was used as a control in this experiment. Infusion of vehicle did not alter TH levels in the infused LC relative to the contralateral LC of either nonstressed or stressed rats, indicating that infusion *per se* did not damage LC neurons and thereby reduce levels of TH. As expected, infusion of αh-CRF caused a decrease in immunoreactivity to TH in the infused LC relative to the contralateral LC in stressed rats. However, αh-CRF did not alter levels of TH in nonstressed rats (Fig. 2). These results indicate that the influence of αh-CRF on the expression of TH in the LC is state dependent—i.e., antagonist treatment blocks the induction of TH by chronic stress but does not influence basal levels of the enzyme.

It is possible that repeated local infusion of αh-CRF might nonspecifically alter the response of TH in the LC to excitatory stimuli. However, the ability of reserpine to increase TH in the LC (26–28) was not altered in animals repeatedly treated with αh-CRF as above (Fig. 3). In addition, examination of nissl-stained coronal sections from αh-CRF-treated rats revealed only minor gliosis to the most lateral aspect of the LC (see *Methods*). These results indicate that chronic infusion of αh-CRF selectively blocks the effect of chronic stress on TH without compromising the functional or apparent histological integrity of LC neurons.

To determine whether the administration of exogenous CRF is sufficient to increase levels of TH in the LC, animals received intraventricular infusions of CRF or vehicle twice daily for 5 days. Chronic infusion of CRF, like chronic stress, markedly increased levels of immunoreactivity to TH to 186% of vehicle-treated controls (Fig. 4).

## DISCUSSION

Several authors have suggested a functional link between CRF and the LC/noradrenergic system (30–35). However, all of these studies have focused on the effects of acute stress or CRF on the NE system, whereas clinical and animal studies suggest a strong link between chronic stress and psychopathology. We have found that CRF is required for the induc-

tion of the rate-limiting NE biosynthetic enzyme, TH, by repeated administration of foot shock and noise stress and that CRF increases the expression of this enzyme in naive animals. These results show that interactions between these two systems may occur in part via regulation of TH by CRF. This could occur through CRF regulation of LC neuronal firing rate (36), which may be mediated by CRF regulation of intracellular second messengers such as cAMP (16, 37). Alternatively, CRF regulation of TH may result directly from an activation of such intracellular pathways.

The finding that αh-CRF blocked the induction of TH by repeated stress in the infused but not the contralateral LC of the same rats suggests that CRF receptors in the LC might mediate this effect since the right and left LC are separated by only 2 mm. Consistent with this, unilateral infusion of αh-CRF into a site more distal to the LC, the motor trigeminal nucleus, did not block the induction of TH in response to chronic stress (unpublished observations). However, at this point it is impossible to determine whether CRF stimulates receptors located on LC neurons or on terminals in the LC region.

The source of the CRF input to the LC, which mediates the effect of chronic stress on TH induction, is not yet known. Possible anatomical connections include direct projections from CRF containing neurons in the anterior paraventricular nucleus of the hypothalamus; the nucleus paragigantocellularis, a main excitatory afferent to the LC; the nucleus prepositus hypoglossi; the parabrachial nucleus; the lateral dorsal tegmental area (38, 39); or the amygdala (40), an afferent structure believed to be involved in aversive emotional states. Further studies are required to address these and alternative hypotheses.

While the present findings clearly indicate that endogenous CRF is necessary for the induction of TH by repeated intermittent foot shock and noise stress, these findings do not preclude the possibility that other neurotransmitter systems might contribute to the effects of different stressors on LC neuronal function. Recent studies have demonstrated a major excitatory amino acid pathway from the nucleus paragigantocellularis to the LC that might be involved in the relay of sensory information (41). Thus, it is conceivable that excitatory amino acids or other neurotransmitters known to regulate LC firing rate (see ref. 41) may participate in the activation of LC neurons by some stressors. However, the possibility that systems other than CRF might contribute to the induction of TH by the stress paradigm used in the present experiments is unlikely in the light of the complete blockade of this effect by αh-CRF.

The results of this study may have particular relevance to the etiology and treatment of a number of psychiatric disorders. Recent studies have shown altered levels of NE and its metabolites (42) and a hyperactivation of the CRF system in depressed patients (43–46). Alterations in the function of the NE and CRF neurotransmitter systems have also been reported in patients suffering from anxiety-related illnesses such as panic disorder and post-traumatic-stress disorder (47–51). The present findings suggest that the apparent dysregulation of CRF and NE in affective and anxiety disorders may be functionally related.

Finally, the results of this study raise the question as to whether inhibition of CRF actions in the LC may have therapeutic benefit. Virtually every class of antidepressant and many antipanic treatments decrease the expression of TH in the LC (52, 53) and block the induction of TH in response to chronic stress (54). Both effects are dependent upon chronic administration of antidepressants, in agreement with the treatment period required to observe the therapeutic actions of these agents in both depressed and panic patients. The ability of αh-CRF to block the noradrenergic response to chronic stress without pretreatment suggests that CRF antagonists might be efficacious as fast-acting antidepressants. The development of synthetic CRF antagonists capable of crossing the blood–brain barrier will allow these possibilities to be tested and may lead to the advancement of more specific and effective agents for the treatment of depression and anxiety disorders.

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