Activation of the Locus Coeruleus Noradrenergic System by Intracoerulear Microinfusion of Corticotropin-Releasing Factor: Effects on Discharge Rate, Cortical Norepinephrine Levels and Cortical Electroencephalographic Activity

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ABSTRACT
Corticotropin-releasing factor (CRF) administered intracerebroventricular (i.c.v.) activates noradrenergic locus coeruleus (LC) neurons of halothane-anesthetized and unanesthetized rats. This study used a technique for microinfusing CRF into the LC from calibrated micropipettes to characterize and quantify the effects of locally administered CRF on LC discharge in halothane-anesthetized rats. CRF (3–100 ng) microinfusion into the LC increased discharge rate in a dose-dependent manner from 28 ± 8 to 105 ± 26% above preinfusion discharge rates. The CRF dose-response curve generated by local microinfusion was parallel to, and shifted approximately 200-fold to the left, of that generated by i.c.v. administration. Intracoerulear microinfusion of the CRF antagonist, [DPhe^{12},Nle^{21,38},CaMeLeu^{37}]hCRF(12–41), greatly attenuated LC activation produced by a maximally effective dose of i.c.v. administered CRF, suggesting that these effects are primarily due to actions within the LC. In rats in which both LC discharge rate and norepinephrine levels in prefrontal cortex were measured by in vivo microdialysis, CRF microinfused into the LC increased both endpoints. Finally, LC activation produced by CRF (60 ng) microinfusion into the LC was associated with cortical electroencephalographic activation. Taken together with previous anatomical and electrophysiological evidence for endogenous CRF interactions in the LC, our results support the hypothesis that CRF serves as an excitatory neurotransmitter in the LC, and suggest that its actions on LC neurons are translated to enhanced norepinephrine release and an impact on cortical targets.

The noradrenergic nucleus LC gives rise to a divergent efferent system that provides the major source of norepinephrine in the forebrain (Swanson and Hartman, 1976). The LC-noradrenergic projections have long been thought to play a role in the stress response based on electrophysiological and neurochemical indices of activation of this system by stressors. For example, stressors increased norepinephrine turnover (Cassens et al., 1981; Cassens et al., 1980; Korf et al., 1973; Thierry et al., 1968) and release (Abercrombie et al., 1988; Nisenbaum and Abercrombie, 1993; Nisenbaum et al., 1991; Smagin et al., 1994) in forebrain targets of the LC. Stress also increased tyrosine hydroxylase expression in LC neurons (Melia and Duman, 1991; Melia et al., 1992). Finally, many of the same stressors that elicit neurochemical indices of activation have also been demonstrated to increase discharge activity of LC neurons (Abercrombie and Jacobs, 1987; Elam et al., 1981, 1984, 1986; Morilak et al., 1987a, b).

Recently, anatomical and physiological studies designed to delineate afferents to the LC that regulate its activity have begun to identify potential mediators of LC activation by various stimuli, including stressors (see for review, Aston-Jones et al., 1991). CRF, the neurohormone that initiates adrenocorticotropic release from the anterior pituitary during stress (Vale et al., 1981), has been implicated as one potential mediator of LC activation. Thus, CRF fibers (Sakaknaka et al., 1987; Swanson et al., 1983; Valentino et al., 1992) and binding sites (DeSouza, 1987; DeSouza et al., 1985) have been visualized in the LC. More recent studies demonstrated synaptic contacts between CRF-immunoreactive terminals and LC dendrites (Van Bockstaele et al., 1996). CRF administered i.c.v. in doses that mimic other aspects of the stress response increased LC discharge rates of both anesthetized and unanesthetized rats (Valentino and Foote, 1987, 1988; Valentino et al., 1983). Moreover, LC activation by hypotensive stress (Curtis et al., 1994; Valentino et al., 1991) and low magnitudes of colon distention (Lechner et al., in press) were greatly attenuated by local administration of CRF antagonist.

ABBREVIATIONS: ACSF, artificial cerebrospinal fluid; CRF, corticotropin-releasing factor; DPheCRF_{12–41}, [DPhe^{12},Nle^{21,38},CaMeLeu^{37}]hCRF(12–41); EEG, electroencephalographic activity; i.c.v., intracerebroventricular; LC, locus coeruleus; PSA, power spectrum analysis; PSB, Pontamine sky blue.
nists into the LC. Taken together, these findings provide strong evidence that endogenous CRF may act as an excitatory neurotransmitter within the LC to mediate its activation by certain stimuli.

If CRF serves as an excitatory neurotransmitter within the LC, intracereoctxlear administration should increase discharge rates of LC neurons. The initial study that described the effects of i.c.v. administered CRF on LC neuronal activity demonstrated excitatory effects of local CRF application onto LC neurons (Valentino et al., 1983). However, these effects were only examined on a small number of neurons and were not quantified. Moreover, a recent study suggested that CRF injection into the LC decreases discharge rates of LC neurons (Borsody and Weiss, 1996). Our study used calibrated double barrel micropipettes to simultaneously record LC discharge and microinfuse known concentrations and volumes of CRF into the LC to more precisely characterize and quantify CRF effects on LC neurons. Additionally, in some of these rats norepinephrine levels in prefrontal cortex were measured by in vivo microdialysis, or cortical EEG was recorded, to determine whether the response of LC neurons to locally administered CRF was sufficient to impact on LC targets.

Methods

Surgery. The procedures used for recording LC discharge of halothane-anesthetized rats were similar to those previously reported (Valentino et al., 1983). Male Sprague-Dawley rats (Taconic Farms, Germantown, NY; approximately 300 g) were anesthetized with a 1 to 1.5% halothane-in-air mixture administered through a nose cone. Rats were positioned in a stereotaxic instrument using blunt ear bars, and the head was oriented at a 15° angle to the horizontal plane (nose down). Body temperature was maintained at 37 to 38°C with a small heating pad. The skull was exposed, and a 3.0-mm diameter hole, centered at 1.1 mm lateral to the midline and 3.5 to 3.7 mm caudal to the lambda suture point, was drilled over the cerebellum for approaching the LC. For experiments requiring i.c.v. administration of CRF, a hole was drilled 1.0 mm caudal to bregma and 1.5 mm lateral to midline for placement of a 26-gauge stainless steel cannula into the lateral ventricle, 5.6 mm ventral to skull surface. For experiments in which cortical EEG was recorded, a hole was drilled 3.0 mm rostral to bregma and 1.5 mm lateral to the midline and two small holes were drilled for skull screws. The dura was carefully removed using fine forceps and iridectomy scissors. For five rats in which mean arterial pressure was monitored, a catheter (PE 50) was inserted into the femoral artery and secured prior to positioning the rat in the stereotaxic.

Recording and microinfusion. Double barrel glass micropipettes were used to record single unit LC discharge and simultaneously microinfuse peptides or artificial cerebrospinal fluid (ACSF). The technique has been previously characterized and described in detail (Akaoa et al., 1992). Double barrel micropipettes consisted of a recording pipette glued using a photopolymerizing resin (Silux, 3 M Dental Products, St. Paul, MN) next to an infusion pipette (Fisher Scientific, Pittsburgh, PA). The recording pipette had a 2 to 4 μm diameter tip (4.7-7.0 MΩhm) and was filled with 2% Potamine Sky Blue (PSB) dye in 0.5 M sodium acetate. The infusion pipette (20-50 μm diameter tip) was angled at approximately 30 to 45° with its tip adjacent to the tip of the recording pipette but 100 to 150 μm dorsal. This was filled with a solution of either CRF (0.1–2.0 mg/ml), DPheCRF12-41 (0.33 mg/ml) or ACSF and connected by PE tubing to a source of solenoid-activated pneumatic pressure (PicSpritzer, General Valve, Inc., Fairfield, NJ). This infusion pipette was calibrated such that known volumes could be administered (1 mm displacement = 60 nl).

Bipolar EEG recording electrodes consisted of two adjacent 250-μm diameter wires insulated except at the tip and vertically separated by 1.5 mm. The EEG electrode was positioned ipsilateral to the LC recording electrode and lowered so that the shorter wire was approximately 100 μm below the brain surface. The electrode was affixed to the skull with screws using cranioplastic cement. EEG signals were amplified, filtered (0.1–60 Hz bandpass) and monitored on-line using a Cambridge Electronics Design 1401 data analysis system with Spike 2 software (Cambridge, UK).

The double barrel pipette was advanced toward the LC with a micromanipulator. Microelectrode signals were led from a preamplifier to filters and additional amplifiers. Impulse activity was monitored with an oscilloscope and loudspeaker to aid in localizing the LC. LC neurons were tentatively identified during recording by their spontaneous discharge rates (1–5 Hz), entirely positive, notched wave forms, 2 to 3 msec duration (in unfiltered trace), and biphasic excitatory-inhibitory responses to noxious stimuli (tail or paw pinch). When a stable, unitary action potential was isolated, an amplitude trigger was used to convert the occurrence of each action potential into a digital pulse.

LC spontaneous discharge rate was recorded for at least 9 min before microinfusion of peptides or ACSF into the LC. Microinfusions were made by applying small pulses of pressure (10–30 psi, 20–40 msec duration) to the calibrated infusion pipette at a frequency of 0.1 to 0.5 Hz to deliver a volume of 30–100 nl (60 nl for 60 ng, 100 nl for 100 and 200 ng and 30 nl for all other doses). The movement of solution through the calibrated pipette was observed through a microscope throughout the infusion. Injection of the entire volume at this rate usually required 1 to 3 min. LC activity was recorded for a period of at least an additional 15 min. In some experiments cortical EEG was recorded simultaneously before, during and after microinfusion. Only one dose was microinfused in an individual rat and only one cell was tested in an individual rat. In some experiments, the CRF antagonist, DPheCRF12-41 (10 ng in 30 nl) was microinfused into the LC using the same procedure as described above. LC discharge was then recorded for 9 min at which time CRF (3 or 10 μg in 3 or 5 μl, respectively) was injected into the lateral ventricle through the i.c.v. cannula over a period of 30 sec.

A dose-response curve for i.c.v. administered CRF was generated by administering a single-dose to an individual rat. For cumulative dose studies, LC discharge was recorded for at least 9 min and CRF (0.33 mg/ml) was administered in increasing doses at 9-min intervals. The doses administered were 0.33 μg, followed by 0.66 μg (for a total of 1 μg), followed by 2 μg (for a total of 3 μg).

Mean arterial pressure was continuously recorded through a pressure transducer connected to either a Micro-Med blood pressure analyzer (Micro-Med Inc., Louisville, KY) or a Cambridge Electronics Design 1401 data analysis system.

Microdialysis. The procedures for measuring cortical norepinephrine levels using microdialysis were similar to those described in a previous study (Lehmann et al., 1992). For experiments involving LC microinfusion and in vivo microdialysis, rats were first implanted with microdialysis cannula guides 1 wk before the experiment. Rats were anesthetized with pentobarbital (50 mg/kg) and positioned in a stereotaxic instrument. A hole centered at 3.4 mm rostral to bregma and 0.9 mm lateral to the midline was drilled for positioning a 25-gauge stainless steel cannula guide (Plastic Products, Ranoake, VA) in the prefrontal cortex. Two additional holes were drilled for skull screws. The guide was positioned 1.6 mm ventral to the skull surface and secured to the skull and skull screws with cranioplastic cement. A dummy cannula whose tip did not extend past the tip of the cannula guide was inserted. After surgery, rats were housed individually and allowed to recover for at least 1 wk before experimentation.

On the afternoon before the experiment, rats were placed in plexiglass cylinders (diameter 12 inch, height 15 inch; Instech, Plymouth Meeting, PA) with free access to food and water. The dummy cannula was removed and concentric microdialysis probes (Spectra/Por hollow fiber, molecular weight cutoff 6000, o.d. 250 μ, active length 3.0
mm) were inserted through the guide tube and extended 4.5 mm past the tip of the guide cannula. The probes were perfused with ACSF (147 mM NaCl; 1.2 mM CaCl₂, 0.9 mM MgCl₂, 4 mM KCl) at the rate of 0.1 μl/min. A swivel device was used to hold the probes and allow the animal free movement within the cylinder.

On the morning of the experiment, rats were anesthetized with a 1 to 1.5% halothane-in-air mixture administered through a nose cone, positioned in a stereotoxic instrument and prepared for LC recording and microinfusion as described above. Microdialysis probes were perfused for at least 1 hr with ACSF at 1.1 μl/min, and then switched to ACSF with 1.0 μM desipramine for the duration of the experiment. LC discharge activity was recorded and CRF (60 ng in 60 nl) or ACSF (60 nl) was microinfused as described above. Samples were collected at 20-min intervals for at least 2 hr before microinfusion, and thereafter for at least 80 min.

Norepinephrine was assayed by high-pressure liquid chromatography with electrochemical detection (HPLC-EC). The HPLC-EC consisted of a 150 × 2 mm Novapak C₁₈, 4 μm particle column (Waters, Milford, MA) maintained at 35°C. Mobile phase (0.02 M citrate, 1.6% methanol, 0.1 mM Na₂EDTA, 350 mg octane sulfonate, 25 mg sodium azide, pH 2.5–3.0 in 1 liter H₂O) was delivered at 0.3 ml/min by a Waters 510 pump. Norepinephrine was detected with a Waters 460 detector with glassy carbon electrode maintained at +0.65 V relative to a Ag/AgCl reference electrode. Concentrations were estimated from peak areas using a Waters Maxima 820 data station. To reduce variations in sample collection values, norepinephrine concentration was corrected for differences in individual probe recoveries (10–15%).

Histology. After the experiment, the recording site was marked by iontophoresis of PSB from the recording pipette (-15 μA, 10 min). In experiments in which CRF was administered i.c.v., 5 μl of neutral red dye were injected through the i.c.v. cannula. Rats received injections with pentobarbital (100 mg/kg) and were perfused transcardially with a 10% solution of paraformaldehyde. The brains were cut to sections with a 150 μm sections were cut and mounted on glass gelatinized slides and stained with neutral red for visualization of the PSB spot and tract made by the dialysis probe. Data from rats that had electrode placements outside of the LC, dialysis probe placements outside of prefrontal cortex or no neutral red throughout the ventricles were not used in the analysis (for example histology, see Valentino et al., 1983).

Analysis. LC discharge was recorded on-line on either an Apple 2E computer or Cambridge Electronics Design 1401 data analysis system using Spike 2 software. The mean LC discharge rate determined over three 3-min intervals before microinfusion was taken as the mean basal discharge rate and discharge rates were expressed as a percentage of this mean. ED₅₀,₉ were calculated by log-probit analysis.

EEG activity was recorded on-line using the Cambridge Electronics Design 1401 data analysis system with Spike 2 software. EEG segments (120 sec) before and after microinfusion (at the time of the peak CRF effect on LC discharge) were subject to fast Fourier transform and power spectrum analysis. The mean amplitude in different frequency bands was compared before and after microinfusion using the Wilcoxon matched-pairs signed-ranks test for correlated samples.

Dialysis data are plotted as a percentage of the mean baseline level in the presence of desipramine and analyzed using a repeated measures one-way analysis of variance with the Scheffe test for individual comparisons.

The mean arterial pressure prior to CRF administration was calculated by averaging the pressure each min for 10 min immediately before CRF administration. A repeated measures analysis of variance was performed using this mean basal pressure and pressures determined each minute after CRF administration (from 0–15 min).

Compounds. ovineCRF and DPheCRF₁₋₄ were generously supplied by Dr. Jean Rivier of the Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, La Jolla, CA. The peptides were dissolved in water to make a 1 mg/ml solution. Aliquots (10 μl) of this solution were concentrated using a Savant Speed Vac concentrator. The 10-μg aliquots were stored at -70°C and dissolved in ACSF on the day of the experiment. Desipramine HCl was obtained from Sigma Chemical Co. (St. Louis, MO).

Results

Effects of CRF on LC discharge rate. LC discharge rate recorded from 65 rats ranged from 0.7 to 4.0 Hz with a mean of 1.7 ± 0.1 Hz, comparable to previous reports in halothane-anesthetized rats (Curtis et al., 1994). Microinfusion of CRF (3–100 ng) into the LC produced a dose-dependent increase in LC discharge rate that usually began during (for 56% of neurons) or within 60 sec (for 24% of neurons) after the offset of the injection and peaked within 3 to 5 min (e.g., fig. 1). The latency of the effect was somewhat longer (between 2–4 min) for the remainder of the neurons and this longer latency was associated with the administration of relatively small doses of CRF (3 and 10 ng). The latency of this response was comparable to the latencies reported with microinfusion of other agents into the LC using this technique (Ennis and Shiple, 1992; Aston-Jones et al., 1992; Chiang and Aston-Jones, 1993). The duration of activation was at least 30 min, although it gradually declined during this time (fig. 1A). The increase in LC discharge rate was not accompanied by a change in mean arterial pressure (fig. 1A). In five rats in which mean arterial pressure was continuously monitored, the mean pressure before CRF administration was 95 ± 2 mm Hg and this was not significantly altered at any time up to 15 min after CRF administration [F(15,79) = 0.9, P > .1]. In contrast to CRF, ACSF (100 nl) microinfused into the LC did not alter discharge rate (fig. 1B, open diamond).

Figure 1B shows the dose-response relationships for CRF administered directly into the LC (open circles) vs. i.c.v. (closed circles). Significant increases in LC discharge rate were produced by microinfusion of 3 to 100 ng of CRF into the LC (P < .01 for all doses, Student’s t test for matched pairs). The maximum effect produced by microinfusion of CRF into the LC was approximately a 100% increase in discharge rate and this was produced by 30 to 100 ng CRF. The ED₅₀ of intracoerulear CRF determined by log-probit analysis was 5.7 ng.

Plots of the LC recording sites from experiments where CRF was microinfused into the LC are shown in figure 2. The excitatory effect of microinfused CRF did not exhibit any topographic specificity within the LC and was observed at different rostro-caudal levels and in both dorsal and ventral aspects of the LC.

Base-line LC discharge rate recorded in 47 rats that were administered CRF i.c.v. ranged from 0.6 to 3.4 Hz with a mean rate of 1.5 ± 0.1 Hz, that was not different than the base-line discharge rate recorded in the group of rats that were administered CRF into the LC (t = 1.5, P > .1, n = 112). The dose-response curve generated by administering a single dose of CRF i.c.v. to an individual rat (closed circles) was parallel to that generated by intracoerulear administration; slopes = 61 and 68, respectively. The maximum response produced by 10 μg CRF (i.c.v.) tended to be somewhat greater than that produced by intracoerulear CRF (60 ng), but was not statistically different; a 130 ± 8% (n = 4) vs. 105 ± 26% (n =
Fig. 1. Effects of CRF administered by microinfusion into the LC vs. i.c.v. on LC discharge rate. A, Recording of mean arterial blood pressure (top) and LC discharge rate (bottom) before and after microinfusion of CRF (30 ng in 30 nl) into the LC. The time of the infusion is indicated by the bar. The abscissa indicates time (sec). LC discharge rate began to increase immediately after the termination of the infusion, peaked at 200 sec after infusion, and remained elevated for 30 min. Blood pressure remained unaltered throughout and after the microinfusion. B, CRF dose-response curves. The abscissa indicates the dose of CRF (ng, log scale). The ordinate indicates the maximum increase produced by a dose of CRF expressed as a percentage of the mean rate determined over three 3-min intervals before CRF administration. Open circles represent the dose-response curve generated by intracoerulear infusion of CRF (n = 7–16 cells). Closed circles represent the dose-response curve generated by i.c.v. administration using the single-dose method (n = 4–6 cells). The open diamond on the ordinate indicates the effect produced by local infusion of ACSF (100 nl; n = 4). Closed triangles represent the mean effect of CRF (3 or 10 μg, i.c.v.; n = 11 and n = 5, respectively) in rats pretreated with DPheCRF12–41 (10 ng) microinfused into the LC (open symbols) vs. rats that were not pretreated (closed symbols). The abscissa indicates time in min. The ordinate indicates LC discharge rate expressed as a percentage of the mean rate determined over three 3-min intervals before injection. Each point is the mean of 4 (solid triangles), 5 (open triangles), 6 (solid circles) and 11 (open circles) rats. Vertical lines indicate ± 1 S.E.M.
9) increase in discharge rate, respectively (P = .6, Student’s t test for independent samples). The ED₅₀ for i.c.v. administered CRF was calculated by log-probit analysis to be 1.1 μg, making CRF approximately 193 times more potent when administered intracoerulearly compared to i.c.v. (fig. 1B). CRF was less effective when administered in increasing doses to the same rat (cumulative method). The effects associated with 0.3, 1.0 and 3.0 μg CRF administered i.c.v. in a cumulative manner to five rats were a 23 ± 5, 35 ± 2 and 57 ± 6% increase in discharge rate, respectively. Statistical analysis revealed that the effect produced by 3 μg CRF was significantly less when administered in a cumulative manner compared to a single dose (P < .01, Student’s t test for independent samples).

To ascertain whether LC activation produced by i.c.v. administered CRF was the result of direct effects within the LC, the ability of microinfusion of the CRF antagonist, DPheCRF₁₂₋₄₁, to antagonize a submaximal (3 μg) and maximally effective (10 μg) dose of i.c.v. administered CRF was determined. Local microinfusion of the CRF antagonist directly into the LC greatly decreased the magnitude of LC activation produced by both 3 and 10 μg i.c.v. administered CRF [fig. 1B, closed triangles and C; F(1,101) = 48, P < .001 and F(1,53) = 27, P < .002, respectively; two-way analysis of variance]. This is consistent with an earlier report of antagonism of 3 μg CRF (i.c.v.) by DPheCRF₁₂₋₄₁ administration into the LC (Curtis et al., 1994). However, i.c.v. administered CRF still produced a small, but statistically significant, increase in LC discharge rate in rats pretreated with DPheCRF₁₂₋₄₁ [for both the 3 and 10 μg dose, F(5,65) = 6.9, P < .0001 and F(5,29) = 8, P < .0003, respectively; one-way analysis of variance with repeated measures).

### Effects of CRF on norepinephrine levels in prefrontal cortex.

The mean base-line norepinephrine level in prefrontal cortex before desipramine was 2.5 ± 0.4 pg/20 μl (n = 10) and this stabilized at 4.9 ± 0.9 pg/20 μl after desipramine. The mean norepinephrine levels before microinfusion of CRF (n = 7) or ACSF (n = 3) into the LC were 5.3 ± 1.2 and 4.2 ± 0.4 pg/20 μl, respectively. Microinfusion of CRF (60 ng in 60 nl) increased the norepinephrine level by 37 ± 7% in the 20-min sample corresponding to the microinfusion (fig. 3A).

Norepinephrine levels in subsequent samples were comparable to base-line samples. In three of these rats in which LC recordings were sufficiently stable throughout the time period of the sample, LC discharge rate was increased by 95 ± 17% during the CRF microinfusion (fig. 3B). In contrast to CRF, microinfusion of ACSF into the LC did not alter norepinephrine levels (fig. 3A).

### Effects of CRF on cortical EEG.

Cortical EEG was recorded simultaneously with LC unit activity during CRF microinfusion in 7 rats. Figure 4 shows an example of the effects of intracoerulear CRF (60 ng in 60 nl) microinfusion on raw cortical EEG activity, the EEG power spectrum and LC discharge rate. CRF microinfusion into the LC was associated with EEG activation as indicated by a decrease in the power of low frequency activity (fig. 4A). This effect became apparent when the increase in LC discharge rate had peaked, and was of a shorter duration than the increase in LC discharge rate. The mean power in the 0 to 2 Hz and 2 to 4 Hz frequency bands was significantly decreased by CRF microinfusion, and this effect recovered by 10–12 min after the infusion (fig. 5).

### Discussion

Consistent with its effects after i.c.v. administration (Valentino and Foote, 1988; Valentino et al., 1983), CRF increased LC discharge rate after microinfusion into the LC in our study and was approximately 200 times more potent when administered directly into the LC compared to i.c.v. Moreover, microinfusion of a CRF antagonist into the LC greatly attenuated LC activation by a maximally effective dose of i.c.v. administered CRF. Taken together with recent ultrastructural evidence for CRF synapses on LC dendrites (Van Bockstaele et al., 1996), these results are consistent with the hypothesis that CRF serves as an excitatory neurotransmitter within the LC. The finding that increased LC discharge rate produced by CRF microinfusion was also associated with increased norepinephrine levels in prefrontal cortex and cortical EEG activation in the same rats indicates that LC activation by local infusion of CRF is sufficient to impact on cortical targets.

Previous studies demonstrated that CRF, administered i.c.v. in doses that mimic certain autonomic and behavioral responses to stress, also increased LC discharge rates of halothane-anesthetized rats (Valentino et al., 1983) and this effect was structurally specific, in that CRF analogues that do not elicit ACTH release were ineffective (Valentino and Foote, 1988; Valentino et al., 1983). The finding that this also occurred in unanesthetized rats and that CRF was somewhat more potent and efficacious in these rats indicated that this effect was not some artifact of anesthesia (Valentino and Foote, 1988). Taken with evidence for CRF innervation of the LC (Sakanaka et al., 1987; Swanson et al., 1983; Valentino et al., 1992; Van Bockstaele et al., 1996) and the presence of CRF binding sites in LC (DeSouza, 1987; DeSouza et al.,...
have supported this hypothesis. Thus, CRF microinfusion into the LC increased norepinephrine turnover (Butler et al., 1990) or release (Page and Abercrombie, 1995; Smagin et al., 1995; Schulz and Lehnert, 1996) in forebrain, similar to the effects of stressors. Stress-elicited increases in tyrosine hydroxylase expression in the LC were prevented by local microinfusion of CRF antagonists (Melia and Duman, 1991). Finally, LC activation by hypotensive stress (Curtis et al., 1994), and more recently by colon distention (Florin et al., 1995), was blocked or attenuated by microinfusion of CRF antagonists into the LC. Nonetheless, a crucial test of this hypothesis requires the demonstration that CRF administered into the LC increases discharge rates of these neurons. In contrast to the substantial data that support a direct excitatory action of CRF within the LC, a recent report suggested that CRF directly inhibits LC discharge (Borsody and Weiss, 1996).

**Technical considerations.** A critical aspect of this study, and one that may explain the discrepancies between these results and those of Borsody and Weiss (1996), lies in the method of CRF microinfusion. Our study used double-barrel micropipettes designed such that the microinfusion barrel was never more than 150 μm from the recording pipette, and compounds were infused by the application of small pressure pulses of short (msec) duration to minimize damage to parenchyma. This technique has been well characterized (Akaoka et al., 1992) and used to examine the effects of various agents on LC neurons, including parasympathomimetic agents (Ennis and Shipley, 1992), α1 adrenergic receptor antagonists (Aston-Jones et al., 1992), norepinephrine (Akaoka et al., 1992), excitatory amino acid agonists (Brun et al., 1993) and antagonists (Akaoka and Aston-Jones, 1991; Page et al., 1992) and GABA agonists and antagonists (Shiekhattar and Aston-Jones, 1992a). This technique has also been used to manipulate ion concentrations (Ca2+ or Mg2+) within the LC in vivo with resulting effects that were similar to those observed in the in vitro LC slice preparation (Shiekhattar et al., 1991; Shiekhattar and Aston-Jones, 1992b). Other studies involving discrete application of compounds to nuclei other than the LC, including GABA agonists, GABA antagonists and dopamine agonists to substance nigra neurons (Akaoka et al., 1992) and lidocaine, GABA and synaptic decouplers to neurons in the ventrolateral medulla (Chiang and Aston-Jones, 1993) have also utilized this technique. Finally, using this technique to microinfuse glutamate into the dorsal pons while recording bladder pressure, pontine neurons involved in the micturition reflex were mapped and found to be well localized to Barrington’s nucleus, as predicted by anatomical studies (Pavcovich and Valentino, 1995). In this study, relatively small changes in the position of the electrode (50–100 μm) could influence the bladder response to glutamate.

In contrast to our study, the study reporting inhibitory effects of CRF on LC activity used a 30 gauge stainless steel cannula to microinfuse peptides situated up to 300 μm dorsal to the recording pipette and the microinfusion was performed by manual movement of a syringe (Borsody and Weiss, 1996). The likelihood of damage to the parenchyma, including dendrites or terminals of afferents that may be the targets of CRF within the LC (see below), is much greater with this technique compared to that used in our study. Such damage
can also contribute to spread of peptide to regions outside of the nucleus.

Other methodological aspects that could contribute to the discrepancies between our study and that of Borsody and Weiss (1996) are the volumes of peptide microinfused and the use of cumulative vs. single-dose testing. In our study peptides were usually microinfused in a 30 nl volume, with the exception of supramaximally effective doses, and the volumes never exceeded 100 nl. The previous study used cumulative dosing, with total volumes in the range of 200 to 500 nl for CRF administration and 300 nl for administration of the CRF antagonist (see below). Evidence for the spread of peptide outside of the LC was suggested by the finding that similar effects on LC discharge were obtained with injections into the parabrachial nucleus. Although the magnitude of the inhibition was smaller when administered into the parabrachial nucleus vs. the LC, it is highly possible that such volumes of CRF spread to affect other nearby pontine nuclei that provide inhibitory input to the LC.

In our study each determination was based on the effects of a single dose on an individual cell in an individual rat. This was initially done because pilot data had been obtained suggesting that tachyphalaxis occurred with cumulative dosing. These pilot data were extended in our study and confirmed the phenomenon of tachyphalaxis with cumulative dosing. LC desensitization to CRF has also been observed in rats administered a single dose of CRF (3 μg, i.c.v.) and challenged with a second dose of CRF 24 to 96 hr later (Conti and Foote, 1995). Consistent with this, a single 30-min session of shock shifted the CRF dose response curve for LC activation to the right (Curtis et al., 1995). Desensitization that may occur with cumulative dosing could explain the decreased efficacy of i.c.v. administered CRF observed in a previous study (Borsody and Weiss, 1996) compared to our study. Although, this cannot explain the inhibitory effects observed after local administration of CRF, perhaps coupled with

Fig. 4. Example of the effect of CRF on LC discharge and cortical EEG. A, The top traces show raw EEG activity (60 sec) recorded from a halothane-anesthetized rat 1 min before (1), 4–5 min after (2) and 13–14 min after (3) CRF (60 ng in 60 nl) microinfusion into the LC. The bottom traces show a record of LC discharge rate during the same times. The abscissa indicates the time (sec). Note in A2 that when LC discharge is increased, cortical EEG becomes desynchronized. LC neuronal activity was lost in A3. B, Power spectrum analysis (PSAs) of the same EEG traces shown in A. The abscissa indicates the frequency (Hz) and the ordinate indicates the power. Note that power in the low frequency bands (0–4 Hz) is greatly decreased after CRF microinfusion, and that this effect recovers.

Fig. 5. Mean effect of CRF on cortical EEG. The abscissa indicates the frequency band. The ordinate indicates the amplitude (determined as area) in each band. Data were obtained from PSAs of 2 min of EEG activity. Bars represent the mean amplitude determined in seven rats 0 to 2 min before CRF (open), 1 to 7 min after CRF (hatched) and 7 to 11 min after CRF (closed). Vertical lines represent ± 1 S.E.M. The choice of 2-min segments to be analyzed after CRF was based on the time when LC activity peaked (if cellular recordings were available at this time) or when the EEG appeared to be altered according to visual inspection of the raw trace. This varied somewhat between rats and ranged from 1 to 7 min after CRF. *P < .05, **P < .01, Wilcoxon matched-pairs signed-ranks test.

Weiss (1996) are the volumes of peptide microinfused and the use of cumulative vs. single-dose testing. In our study peptides were usually microinfused in a 30 nl volume, with the exception of supramaximally effective doses, and the volumes never exceeded 100 nl. The previous study used cumulative dosing, with total volumes in the range of 200 to 500 nl for CRF administration and 300 nl for administration of the CRF antagonist (see below). Evidence for the spread of peptide outside of the LC was suggested by the finding that similar effects on LC discharge were obtained with injections into the parabrachial nucleus. Although the magnitude of the inhibition was smaller when administered into the parabrachial nucleus vs. the LC, it is highly possible that such volumes of CRF spread to affect other nearby pontine nuclei that provide inhibitory input to the LC.

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other factors such as parenchymal damage and spread of large volumes, desensitization could play an additional role.

Although the technique of CRF administration is the most obvious cause of discrepancies between the previous and current studies, other differences that may appear minor should not be discounted. These include the source of CRF used (Dr. Jean Rivier for our study vs. Bachem) and the source of rats (Taconic Farms for our study vs. rats bred at an institutional facility). That a difference in rat supplier may have an impact was recently shown in a study where LC projections to spinal cord in the Sprague Dawley rat were found to have very different termination patterns in Harlan vs. Sasco Sprague-Dawley rats (Clark and Proudfit, 1992).

Site of action of i.c.v. administered CRF. Although the effects of i.c.v. administered CRF were qualitatively similar to those observed with intracoerulear administration, it was possible that they were mediated by mechanisms other than, or in addition to, direct actions within the LC. In a previous study, it was determined that local microinfusion of a CRF antagonist prevented LC activation by 3 μg CRF administered i.c.v. (Curtis et al., 1994). This finding was extended in our study by the demonstration that a maximally effective dose (10 μg) of i.c.v. administered CRF could be antagonized by intracoerulear administration of a CRF antagonist. Importantly, the dose of D-PheCRF12–41 used in our study was previously demonstrated to be selective, in that it did not alter LC activation by excitatory amino acid inputs to the LC (Curtis et al., 1994). For the most part, these results are in agreement with those of Borsody and Weiss (1996), using α-helical CRF9–41, a CRF antagonist with partial agonist activity (Rainnie et al., 1992). However, in that study it was found that microinfusion of the antagonist into the ipsilateral parabrachial nucleus was somewhat more effective than microinfusion into the LC, and microinfusion into the contralateral parabrachial nucleus also attenuated LC activation by i.c.v. administered CRF. The dose (360 ng) and volume (300 nl) of α helical CRF9–41 used in that study could be considered excessive. Other studies have shown that 100 ng of this antagonist administered into the LC is sufficient to antagonize CRF-mediated effects in the LC and that when administered intracoerulear, α-helical CRF9–41 is only approximately 10 times less potent than D-PheCRF12–41 (Curtis et al., 1994; Page et al., 1993; Valentino et al., 1991). Considering the partial agonist activity of this antagonist and the doses and volumes used, the previous results need to be interpreted with caution. For example, it is possible that high concentrations of α-helical CRF9–41 achieved in the LC with intracoerulear injections had partial agonist activity (as suggested by the significant increase in LC discharge rate produced by these injections). In contrast, injections outside of the LC (parabrachial nucleus), would result in lower concentrations of the antagonist within the LC that may be below those having agonist activity. Evidence for this was suggested by the lack of an increase in LC discharge after antagonist infusion in the parabrachial nucleus and more effective antagonism (Borsody and Weiss, 1996).

Cellular targets of CRF action in the LC. Despite the evidence suggesting that CRF has excitatory effects within the LC, the cellular site of action has yet to be determined. A recent ultrastructural study examining morphological interactions between CRF-immunoreactive terminals and tyrosine hydroxylase-immunoreactive dendrites in the rostral ventromedial dendritic zone of the LC found evidence for both direct and indirect actions of CRF on LC neurons. Thus, CRF-immunoreactive terminals were observed to make synaptic contacts with tyrosine-hydroxylase-immunoreactive dendrites, and the majority of these synapses was asymmetric, consistent with an excitatory effect of CRF on LC neurons (Van Bockstaele et al., 1996). CRF terminals also contacted unlabeled dendrites, as well as unlabeled terminals that synapse with LC dendrites in this region (Van Bockstaele et al., 1996). CRF terminals were also apposed to glia that surrounded LC dendrites. Finally, CRF-immunoreactive large dense core vesicles were observed to be colocalized in terminals with small clear vesicles, indicative of colocalization with classical neurotransmitters. Based upon these morphological interactions, there are a number of possible mechanisms by which CRF could affect LC discharge, including a number of indirect mechanisms. The inability to detect mRNA for CRF binding sites in LC neurons (Potter et al., 1994) argues for indirect actions of CRF, perhaps on terminals of LC afferents whose cell bodies are distant from the LC. However, these studies too, should be interpreted with caution and regard to the technical limitations of detection.

Impact of LC activation by CRF on cortical targets. Our study indicated that LC activation by CRF microinfusions is translated to increased norepinephrine release in forebrain. These findings support previous studies which demonstrated that CRF microinfusion into the LC increases norepinephrine levels in prefrontal cortex (Smagin et al., 1995; Schulz and Lehner, 1996) and hippocampus (Page and Abercrombie, 1995). The study of hippocampal norepinephrine release showed dose-dependent effects of CRF microinfusion into the LC of unanesthetized rats with doses as low as 2 ng. The previous studies measuring cortical norepinephrine release used doses somewhat higher than our study. Importantly, the study by Smagin et al. (1995), demonstrated that in contrast to LC microinfusion, microinfusion of CRF into the nearby parabrachial nucleus did not increase norepinephrine levels in cortex, arguing against the possibility that diffusion to this nucleus is responsible for CRF activation of LC discharge. In our study, norepinephrine release was elevated only during the first 20-min sample after CRF administration, i.e., the peak of LC activation, but was not apparent in the next sample, although LC activity was still elevated. This may be a reflection of activation of only a limited number of LC neurons by the volume of CRF administered in this study.

Finally, our study provided evidence for cortical EEG activation produced by CRF microinfusion into the LC. This confirms a previous study in unanesthetized rats that used larger volumes and higher doses of CRF, and which did not simultaneously record LC discharge (De Sarro et al., 1992). Cortical EEG activation associated with LC activation by CRF was not surprising, as other studies using direct chemical manipulation of LC discharge have shown that changes in LC discharge rate are sufficient to affect cortical EEG. Thus, intracoerulear microinfusion of drugs that increase LC discharge rate, e.g., muscarinic agonists (Berridge and Foote, 1991), excitatory amino acids (Rispoli et al., 1994) and α2 adrenergic antagonists (De Sarro et al., 1987, 1988) activated the cortical EEG, and inhibitory agents (e.g., clonidine) had the opposite effect (Berridge et al., 1993; De Sarro et al., 1988). The possibility that LC activation by endogenous CRF...
during stress plays a role in stress-induced EEG activation, and thereby arousal, was suggested by the finding that EEG activation produced by hypotensive stress was prevented by microinfusion of clonidine or CRF antagonists into the LC (Page et al., 1993). As with cortical norepinephrine release, the effects on EEG in our study were not long lasting, and this may also be a reflection of activation of only a limited number of neurons by the volume of CRF administered in this study.

**Functional implications.** The putative neurotransmitter actions of CRF have been implicated in many autonomic and behavioral aspects of the stress response (for review see Dunn and Berridge, 1990; Owens and Nemeroff, 1991). In addition to the LC, physiological studies demonstrating that CRF alters activity of neurons in amygdala (Rainnie et al., 1992), hippocampus (Aldenhoff et al., 1983), complex of the solitary tract (Siggins, 1990), hypothalamus, cortex, thalamus and lateral septal area (Eberly et al., 1983) suggest that some of these effects may be mediated by neurotransmitter actions of CRF in these regions (for review see Siggins, 1990). Studies using microinjection of CRF or CRF antagonists into the LC have implicated CRF actions in the LC in some aspects of the stress response (for review see Valentino et al., 1993). Our own studies suggest that CRF effects on LC neurons may be important in maintaining or increasing arousal during stress (Page et al., 1993). Other studies have implicated CRF-LC interactions in anxiogenic behaviors (Butler et al., 1990; Swiergel et al., 1992). Findings from independent laboratories demonstrating that CRF microinfusion into the LC suppresses peripheral blood and spleen T lymphocyte mitogenic activity, suggest that LC activation by CRF mediates certain immunological responses to stress (Caroleo et al., 1993; Rassnick et al., 1994). Other reported consequences of CRF microinfusion into the LC include activation of the hypothalamic-pituitary axis (Butler et al., 1990; Rassnick et al., 1994) and increases in colonic motility (Monnikes et al., 1992). Thus, there is evidence for a role of CRF-LC interactions in endocrine, autonomic, behavioral and immunological responses to stressors. More systematic studies are required to determine whether the numerous effects discussed above are the result of direct CRF activation of LC neurons, and the overall importance of the LC-noradrenergic system in the constellation of responses to different stressors.

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**References**


