Discrete Local Application of Corticotropin-Releasing Factor Increases Locus Coeruleus Discharge and Extracellular Norepinephrine in Rat Hippocampus

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ABSTRACT The most prominent afferents impinging upon the noradrenergic neurons of the locus coeruleus (LC) utilize GABA and glutamate. However, peptide neurotransmitters such as galanin, neuropeptide Y, and corticotropin-releasing factor (CRF) have also been localized to LC afferents. The evidence for CRF modulation of LC activity was examined in the present studies. Specifically, the impact of local CRF administration on both LC-NE discharge characteristics and release of norepinephrine (NE) in hippocampus was determined. First, the ability of CRF microinfused into the LC area to increase NE efflux in the dorsal hippocampus was determined using in vivo microdialysis techniques in awake rats. CRF into the LC dose-dependently increased extracellular NE in the ipsilateral hippocampus. Second, a more detailed analysis was performed in halothane-anesthetized rats by characterizing the electrophysiological activity of LC-NE neurons in response to local application of CRF. Changes in the firing rate and pattern of single LC-NE neurons were measured while simultaneously monitoring the extracellular level of NE in hippocampus. A dose of 30 ng CRF applied directly into LC via pressure ejection elicited an 88% increase in the discharge rate of LC-NE neurons and increased the incidence of burst firing from 14% to 33%. This manipulation simultaneously increased extracellular NE in hippocampus by 63%. The CRF-induced increases in discharge rate of LC-NE neurons and extracellular NE efflux in hippocampus were prevented by prior i.c.v. administration of the CRF antagonist, d-PheCRF$_{12-41}$ (3 µg / 3 µl). The present findings demonstrate that CRF applied directly into the LC increases both the activity of LC-NE neurons and the release of NE in an LC terminal region. The shift in activity of LC-NE neurons to more burst-like firing in response to CRF may provide a means for enhanced release of NE in LC projection fields. This is the first report to demonstrate a dose-dependent increase in extracellular NE levels evoked by intra-LC infusion of CRF in unanesthetized animals. Synapse 33:304–313, 1999. © 1999 Wiley-Liss, Inc.

INTRODUCTION

The brainstem nucleus locus coeruleus (LC) is the major source of norepinephrine (NE) in the central nervous system (Swanson and Hartman, 1976). This highly divergent efferent system arises from a small cluster of neurons which receives a relatively restricted set of afferents. The two primary afferent inputs to the LC emanate from the nucleus prepositus hypoglossi (PrH) and the nucleus paragigantocellularis (PGi) (Aston-Jones et al., 1986; Luppi et al., 1995). Electrophysiological data show that the projection from the PrH is inhibitory and is the major source of GABA inputs to the LC (Ennis and Aston-Jones, 1987, 1989). In contrast, stimulation of the PGi results in predominantly excitatory responses of LC-NE neurons and this influence appears to be primarily glutamatergic in nature (Aston-Jones et
al., 1986; Ennis and Aston-j ones, 1988). Recent anatomical studies have revealed a limited number of additional minor inputs to the LC arising from the intermediate zone of the spinal cord, ventrolateral periaqueductal gray, Kolliker-Fuse nucleus, and various nuclei of the hypothalamus (Luppi et al., 1995). These minor inputs to the LC are diverse in neurochemical content, including peptides such as corticotropin-releasing factor (CRF), substance P, enkephalin, galanin, and neuropeptide Y, as well as serotonin and epinephrine (Cummings et al., 1983; Foote et al., 1983, for review; Sutin and Jacobowitz, 1988; Van Bockstaele et al., 1996).

A large body of research indicates that the LC plays a key role in the regulation of behavioral state, especially in the response to novel and/or potentially threatening stimuli, i.e., stressors (Abercrombie and J acobs, 1987a,b; A ston-j ones and Bloom, 1981a,b; Foote et al., 1980; Morilak et al., 1987a,b). LC-NE neurons respond to acutely presented sensory stimuli with a brief burst of activity followed by a period of inhibition; available data indicate that this response is mediated by release of glutamate in the LC. For example, activation of LC-NE neurons by sciatic nerve stimulation has been demonstrated to be mediated by glutamate-containing neurons in PGi (Chiang and A ston-j ones, 1993; Ennis and Aston-j ones, 1988). LC-NE neurons are also activated by a number of visceral and metabolic stimuli such as hypotension, hypoglycemia, and bladder or colon distention (Elam et al., 1984, 1986; Svensson, 1987). It has been shown that bladder distention activates LC-NE neurons via glutamate-mediated mechanisms (Page et al., 1992). Thus, afferent regulation of the LC by glutamate may be involved in phasic responses to acute or novel stimuli that would most likely be associated with orienting behaviors or moderate increases in arousal.

One of the minor peptide-containing afferents to the LC, utilizing CRF as a neurotransmitter, is now thought to also play an important role in the activation of LC-NE neurons in response to stress. CRF, a 41-amino-acid peptide, was first described as the hypothalamic neurohormone responsible for initiating the cascade of events associated with the stress response (Vale et al., 1981). However, this peptide is also likely to serve as a neurotransmitter in extrahypothalamic brain circuits to mediate behavioral and autonomic activation associated with stress. Stress increases CRF concentrations in the LC (Chappell et al., 1986). Moreover, local application of CRF in the LC produces behavioral activation in awake animals (Butler et al., 1990). Administration of CRF antagonists in a variety of studies has also proven effective in blocking stress-induced alterations in behavior. For instance, a reduction in stress-induced freezing was observed in animals that were treated with α-helical CRF9–41 (Tazi et al., 1987). Additionally, i.c.v. or bilateral LC infusion of α-helical CRF9–41 decreases shock-induced freezing, a reliable index of fear (Kalin et al., 1988; Swiergiel et al., 1992) and i.c.v. α-helical CRF9–41 attenuates immobilization stress-induced increases in extracellular NE levels in the prefrontal cortex (Shimizu et al., 1994).

Endogenous release of CRF has been implicated in the activation of LC-NE neurons induced by profound hypotension, as this effect can be blocked by prior local application of a CRF receptor antagonist in the LC (Page et al., 1993; Valentino et al., 1991). Furthermore, CRF is thought to play a role in the activation of LC-NE neurons observed in response to colon distention (Lechner et al., 1997). Administration of CRF i.c.v. results in a persistent elevation of discharge rate of LC-NE neurons following local microinfusion of CRF into the LC of anesthetized rats, which was accompanied by increases in cortical levels of NE and in EEG indices of arousal (Curtis et al., 1997). In other studies, both i.c.v. (Lavicky and Dunn, 1993) and local (Schulz and Lehnert, 1996; Smagin et al., 1995) infusion of CRF into LC have been shown to result in elevated levels of NE in LC terminal regions in anesthetized animals.

In the present studies, the dose-related effect of local CRF infusion into the LC on hippocampal NE efflux was assessed in awake, freely moving rats. In addition, the effects of discrete intra-LC application of CRF on the LC-NE neuron discharge rate and pattern as well as on hippocampal NE efflux were simultaneously monitored in anesthetized rats. The present results provide further evidence for an important role of CRF as a neurotransmitter in the LC. Furthermore, these studies utilized precise electrophysiological criteria to place infusions in the LC of awake animals and measured extracellular NE without the addition of reuptake inhibitors, allowing a more physiologically relevant situation in which to assess the effects of CRF on LC firing rate and release of NE. The finding that similar levels of NE were measured in awake and anesthetized rats allows some greater degree of certainty when interpreting the firing characteristics of LC neurons.

**MATERIALS AND METHODS**

**Animals**

Adult, male Sprague Dawley rats (Zivic-Miller Laboratories, Pittsburgh, PA) weighing 350–450 g were used. The animals were housed individually in plastic shoebox cages under conditions of constant temperature (21°C) and humidity (40%) on a 12:12-h light dark cycle (07:00 on and 19:00 off). Food and water were available ad libitum. After microdialysis probe implantation, rats were allowed to move freely about a 43 × 43 × 32 cm Plexiglas chamber or a cylindrical (30 cm
diameter) Plexiglas container. Animal procedures were conducted in accordance with the guidelines published in the NIH Guide for Care and Use of Laboratory Animals and all protocols were approved by the Rutgers University Institutional Animal Care and Use Committee.

**Microdialysis probe construction**

Vertical, concentric microdialysis probes were constructed as previously described (Abercrombie and Finlay, 1991). Briefly, a piece of fused silica capillary tubing (Polymicro Technologies, Phoenix, AZ) with i.d. = 73 µm and o.d. = 150 µm was threaded into a piece of PE-10 tubing (Clay Adams, Parsippany, NJ) through a small opening approximately 5 mm from the end. The silica was positioned to extend past the end of the PE tubing. A piece of dialysis membrane (o.d. = 250 µm, MW cutoff = 9,000; SpectraPor, Spectrum, Houston, TX) was glued in place within the lumen of the PE tubing over the exposed fused silica and the tip was sealed with epoxy. A portion of the membrane was coated with a thin layer of epoxy to leave an active area of 2 mm. The PE-10 tubing served as the inlet line while the fused silica served as the outlet line. In vitro recovery for dialysis probes was determined by placing them in a beaker of artificial cerebrospinal fluid (aCSF; consisting of 147 mM NaCl, 2.5 mM KCl, 1.3 mM CaCl2, 0.9 mM MgCl2; pH = 7.4). The aCSF was continually perfused through the probe at a rate of 1.5 µl/min. After a known concentration of NE standard was added to the bath, the concentration of NE in the perfusate was quantified and the percent recovery determined. Reported values for NE in dialysate samples are uncorrected because the in vitro and in vivo recovery rates may differ.

**Dialysate analysis**

The amount of NE in dialysates was determined with HPLC-EC (see Abercrombie and Finlay, 1991, for full description). Briefly, 20 µl samples were injected directly onto an HPLC, which consisted of an ESA 580 solvent delivery system (ESA Inc., Chelmsford, MA) and a Velosep RP-18 column (100 × 3.2 mm, 3 µm; Brownlee Labs, Foster City, CA). The mobile phase consisted of 60 mM sodium phosphate buffer (pH = 4.2) with 100 µM EDTA, 1.5 mM sodium octyl-sulfate, 3.5% (v/v) methanol. The flow rate through the system was 700 µl/min. The detection system utilized was an ESA 5100A electrochemical detector with three electrodes in series. The conditioning electrode was set at +270 mV. The applied potential of the second electrode was set at −250 mV, and the compounds of interest were quantified at a third electrode which was set at +270 mV. Peak heights were measured and compared to peak heights of 10⁻⁸ M standards. The sensitivity of this assay is ~0.5 pg of NE.

**Data analysis**

Microdialysis data are expressed as mean ± SEM. The effect of CRF microinfusion on hippocampal NE efflux was analyzed using one-way ANOVA with repeated measures over time (P < 0.05) coupled to Dunn’s post-hoc test for comparison of drug values to a baseline mean. The absolute amount of NE measured in dialysates (pg/20 µl sample) was used as the dependent variable for assessment of within-group effects; the value for the mean of the three final baseline samples (unanesthetized group) or one baseline sample (anesthetized group) and the values for four to five samples collected immediately after CRF infusion were included in the analyses. Between-group analyses of dose differences (unanesthetized group) or treatment differences (CRF vs. CRF + antagonist, anesthetized group) were conducted using two-way ANOVA with repeated measures over time (P < 0.05) coupled to Dunnnett’s post-hoc test for comparison of group means relative to a control mean. The level of significance for all post-hoc analyses was P < 0.01.

Electrophysiological data are expressed as mean ± SEM in Hz (spikes per sec). The effect of pressure ejection of CRF on LC-NE neuronal discharge rate was analyzed using one-way ANOVA with repeated measures over time (P < 0.05) coupled to Dunn’s post-hoc test for comparison of drug values to a baseline mean. Mean discharge rates, determined as averages over 3-min intervals, were used as the dependent variable for assessment of within-group effects; the mean value for the 6–9 min preceding CRF application and the values for three, 3-min intervals immediately after CRF application were analyzed. For analysis of firing pattern, bursts were empirically defined as two or more consecutive spikes occurring with an interspike interval of less than or equal to 100 msec and not greater than 125 msec. A random sample of pre-CRF activity and a post-CRF train containing the same number of spikes, taken during the peak effect of CRF, were subjected to this pattern analysis.

**Histology**

LC infusion sites (awake animals) were verified by infusion of 200 nl pontamine sky blue (PSB) dye through the cannula (see Fig. 1). Rats were then injected with a lethal dose of chloral hydrate and subsequently perfused transcardially with 10% buffered formalin. LC recording sites (anesthetized animals) were marked by iontophoresis of PSB dye through the recording pipette (~10 µA, 10 min). Neutral Red dye (3 µl) was infused through the i.c.v. cannula. Rats were then perfused transcardially with 10% buffered formalin. Frozen, 60 µm coronal sections were cut on a microtome, mounted on gelatinized slides, and stained with Neutral Red to examine placement of the dialysis probe and LC infusion/recording sites.
Experimental procedures

Studies in awake rats

Rats were anesthetized with chloral hydrate (400 mg/kg, i.p.) and placed in a stereotaxic instrument. The incisor bar was lowered to a 15° angle (nose down) and a small piece of skull, centered at 3.8 mm caudal and 1.2 mm lateral to the lambda suture point, was removed. The overlying dura was carefully excised for insertion of a recording electrode. Electrophysiological recordings were made from glass micropipettes filled with PSB in 0.5 M sodium acetate. Impulse activity was monitored on an oscilloscope and audiomonitor. LC-NE neurons can be tentatively identified by their spontaneous discharge rate (1–4 Hz), entirely positive, notched waveform (2–3 msec duration), and the biphasic response to contralateral hindpaw pinch (Nakamura, 1977). The coordinates where LC was encountered (relative to interaural zero) were noted and the recording pipette removed from the brain. Subsequently, a 26 g stainless steel guide cannula was lowered 200 µm lateral and 1.0–1.3 mm above the site at which the LC was localized using electrophysiological methods. The cannula was secured in place with dental acrylic and skull screws. A dummy cannula was placed inside the guide. The incisor bar was then repositioned so that the skull was flat for implantation of a microdialysis probe into the dentate gyrus of the dorsal hippocampus at the following coordinates: AP – 3.8 mm, ML 2.0 mm relative to bregma and 3.8 mm ventral to dura (Paxinos and Watson, 1986). The probe was cemented in place with dental acrylic and skull screws. Animals were allowed to recover at least 18 h before initiation of experiments. Dialysate samples were collected every 15 min and analyzed by HPLC-EC. Baseline values were determined from at least three consecutive samples that did not vary by more than 10%. Once stable baseline levels were achieved, the dummy cannula was removed from the guide cannula. A 30 g infusion cannula then was inserted, using care not to disturb the animal. CRF (2, 20, or 60 ng) or aCSF vehicle was infused for 2 min at a rate of 100 nl/min using an automated microliter syringe pump (Harvard Apparatus, South Natick, MA) and a 1 µl glass syringe (Hamilton, Reno, NV). The infusion cannula was left in place for an additional 2 min following the infusion, then was carefully removed.

Studies in anesthetized rats

Rats were anesthetized with chloral hydrate as above and placed in a stereotaxic instrument with the incisor bar set for flat skull. Microdialysis probes were carefully lowered into the hippocampus as described above and secured with dental acrylic and skull screws. In some experiments, a 26 g stainless steel guide cannula also was implanted i.c.v. Coordinates used for implantation of i.c.v. cannulae were (from bregma): AP – 1.0 mm, ML 1.5 mm, and 3.6 mm ventral to the skull surface with the head lowered at a 15° angle. Animals then were allowed to recover approximately 18 h, as it has

Fig. 1. Histological coronal section showing the LC infusion site from an experiment in a freely moving animal. Pontamine Sky Blue dye (200 nl) was administered through the infusion cannula prior to perfusion with 10% formalin. LC, locus coeruleus; MeV, mesencephalic nucleus of the trigeminus; IV, fourth ventricle. Medial is to the left.
been shown that extracellular levels of neurotransmitter measured through microdialysis probes are TTX-insensitive immediately following probe implantation (Westerink and De Vries, 1988). The following day, rats were reanesthetized with halothane (1.0-1.5% in air) and placed in a stereotaxic instrument. Anesthesia was maintained with a face mask. The incisor bar was lowered to a 15° angle (nose down) and a piece of the skull centered at 3.8 mm caudal and 1.2 mm lateral to the lambda suture point was removed. The overlying dura was carefully excised for insertion of a recording electrode (see above). A double-barrel, glass micropipette assembly was used to simultaneously record single unit activity of LC-NE neurons and to apply small volumes of CRF or aCSF vehicle via pressure ejection. The recording and infusion pipette were separated vertically by approximately 120 µm (Akaoka and Aston-Jones, 1991). The recording pipette had a 2-4 µm diameter tip and was filled with PSB in 0.5 M sodium acetate. The infusion pipette (25-50 µm tip) was bent at an angle of 30-45° with its tip adjacent to the recording pipette. The two pipettes were glued together with a photopolymerizing resin (Silux, 3M, St. Paul, MN). The infusion pipette was filled with either CRF (1 mg/ml) or aCSF and connected to a source of solenoid-activated pneumatic pressure (Picospritzer, General Valve, Fairfield, NJ). It was determined ex vivo that a 1 mm displacement of solution in the pipette corresponded to a volume of 60 nl (Akaoka et al., 1987; Page et al., 1992). In this way, small pulses of pressure (20-40 psi) applied to the drug-containing pipette were used to eject nl quantities of solution.

Extracellular single unit activity of LC-NE neurons was monitored with an audiomonitor and an oscilloscope. Data were recorded on magnetic tape and analyzed off-line or acquired directly on-line (Spike 2 software, CED, Cambridge, UK). Spontaneous discharge rate of LC-NE neurons was recorded for at least 12 min prior to drug infusion. Infusions were made by brief pressure pulses until approximately 30 nl of solution had been delivered; movement of the solution was monitored through a microscope. For experiments involving d-PheCRF12-41, i.c.v. administration of the antagonist (3 µg / 3 µl) preceded intra-LC CRF infusion by 6-7 min. LC-NE activity was recorded for the duration of stable recording for up to 60 min after CRF infusion.

RESULTS

Microinfusion of CRF into LC of awake rats: Effects on hippocampal NE efflux

CRF infused directly in the LC area of awake, freely moving rats resulted in significant increases in extracellular NE measured from the dorsal hippocampus (Fig. 2). In contrast, infusion into the LC of the aCSF vehicle had no significant effect on hippocampal NE efflux (F[5,20] = 0.9; P = 0.9; Fig. 2). The 2-ng dose of CRF increased extracellular NE by 31% from 1.6 ± 0.2 pg / 20 µl to 2.1 ± 0.2 pg / 20 µl in the first 15-min sample following infusion (F[5,25] = 3.55; P < 0.05). No consistent behavioral effect of CRF infusion was noted at this dose. Infusion of 20 ng CRF into LC produced an increase in extracellular NE of 61%, from 1.8 ± 0.2 pg / 20 µl to 2.9 ± 0.3 pg / 20 µl in the first postinfusion sample (F[5,20] = 5.92; P < 0.005). The 60-ng dose of CRF was associated with a 58% increase in NE efflux from 1.9 ± 0.2 pg / 20 µl to 3.0 ± 0.3 pg / 20 µl (F[5,20] =
5.20; P < 0.005). The magnitude of the CRF-induced increase in hippocampal NE efflux was not significantly different between the group receiving 20 ng CRF and that receiving 60 ng CRF; however, NE levels were significantly elevated for a longer duration in the latter group. Overt signs of behavioral arousal were also observed following infusion of 20 or 60 ng CRF into LC, although these were not quantified. Rats shifted from a quiet resting state to one of exploration with increased sniffing and rearing behavior. This “aroused” state usually lasted between 15–20 min postinfusion.

Pressure ejection of CRF into LC of anesthetized rats: Effects on LC-NE neuronal activity and hippocampal NE efflux

CRF (30 ng / 30 nl) applied into LC of anesthetized rats via pressure ejection resulted in an increase in spontaneous discharge rate of LC-NE neurons from a baseline level of 1.6 ± 0.3 Hz to 3.0 ± 0.6 Hz at 6 min postinfusion, an increase of 88% (F[3,11] = 10.2; P < 0.005; Fig. 3A). In two cases in which a stable single unit recording was maintained for a prolonged period of time, the increase in LC-NE neuronal discharge rate in response to CRF pressure ejection was observed to be approximately 45 min in duration. The CRF-induced increase in the spontaneous discharge rate of LC-NE neurons was blocked when the CRF antagonist d-PheCRF12–41 (3.0 µg / 3.0 µl, i.c.v.) was administered 6 min prior to pressure ejection of CRF into the LC (F[3,18] = 0.79; P = 0.5; Fig. 3A). Administration of d-PheCRF12–41 alone had no significant effect on the basal discharge rate of LC-NE neurons (data not shown). Further analysis of the single unit data revealed an increase in the incidence of burst-like firing of LC-NE neurons during CRF-induced activation. Bursts tended to consist of pairs of impulses or “doublets” (Fig. 4). The incidence of burst firing shifted from 13.8 ± 3.8% in the pre-CRF state to 32.8 ± 5.5% in the period of maximal CRF-induced increase in firing rate (F[1,3] = 55.5; P < 0.005; n = 4).

Basal levels of hippocampal NE efflux were similar in the anesthetized rats (1.7 ± 0.2 pg / 20 µl) compared to the unanesthetized rats (1.8 ± 0.2 pg / 20 µl). The pressure ejection of CRF into LC (30 ng / 30 nl) produced a 63% increase in extracellular NE in hippocampus of anesthetized rats, from 1.6 ± 0.2 pg / 20 µl to 2.6 ± 0.6 pg / 20 µl (F[6,20] = 5.06; P < 0.005; Fig. 3B). This increase in extracellular NE was evident by the second 15-min sample and persisted for approximately 45 min. The CRF-induced increase in extracellular NE level in hippocampus also was blocked when the CRF antagonist d-PheCRF12–41 (3.0 µg / 3.0 µl, i.c.v.) was administered 6 min prior to pressure ejection of CRF into the LC (F[5,27] = 0.71; P = 0.6; Fig. 3B).

DISCUSSION

Discrete microinjections of CRF into the LC of awake rats increased extracellular NE levels in an LC terminal field, the dorsal hippocampus. Additional studies performed in anesthetized rats showed that this effect is similar in both awake and anesthetized animals. The elevation of extracellular NE in response to intra-LC
application of CRF occurs concomitant with an increase in firing rate of LC-NE neurons. Furthermore, the CRF-induced increase in firing rate of LC-NE neurons was found to be associated with a shift to a firing pattern characterized by increased frequency of burst-like discharge. Increases in both LC-NE discharge and extracellular NE levels were prevented by prior administration of the CRF receptor antagonist, d-PheCRF_{12–41}, into the lateral ventricle. The present findings are in agreement with the proposed role of CRF as an extrahypothalamic neurotransmitter acting in the LC to mediate autonomic and behavioral activation during stress (Valentino et al., 1998) and lend support to the hypothesis that neurotransmitter output may be a function not only of rate but also of neuronal firing pattern (Gonon, 1988; Gonon and Buda, 1985; Suaud-Chagney et al., 1990; Page and Abercrombie, 1996a).

Increases in extracellular NE have previously been reported following both i.c.v. and local CRF administration into the LC. Our studies confirm these reports and extend these findings by using more precise electrophysiological techniques to ensure accurate placement of microinfusions. This is the first report to demonstrate a dose-dependent increase in extracellular NE levels evoked by intra-LC infusion of CRF in unanesthetized animals. A critical factor in the specificity of intracerebral infusions is the volume of the infusion. Larger infusions can result in diffusion of solution to surrounding tissue. Given the proximity of the IVth ventricle, this is especially important for intra-LC infusions. Infusions made in awake, freely moving animals were delivered through an indwelling cannula aimed at the lateral edge of the LC. In this case, precise cannula placement was guided by prior electrophysiological recordings and confirmed by infusion of dye at the end of the experiment. The volume of solution infused over a 2-min period was 200 nl, a comparatively small amount relative to an earlier report describing similar findings (Schulz and Lehnert, 1996). These authors infused 1 µl of solution through the cannula aimed towards the LC without electrophysiological verification. Although Smagin et al. (1995) reported increases in extracellular NE in the frontal cortex following CRF infusion into the LC using 200 nl, their study was performed in anesthetized animals without electrophysiological verification of infusion site and the experiments were performed the same day as the microdialysis probe implant. Furthermore, their study

Fig. 4. Pressure ejection of CRF (30 ng/30 nl) into LC of anesthetized rats induces an increase in the incidence of burst firing in LC-NE neurons. (A) Example spike trains from a single LC-NE neuron recorded before and after local application of CRF. As indicated in (B), CRF-induced activation of this LC-NE neuron resulted in a shift from a predominantly single spike firing mode (11% spikes occurring in bursts) to a more “burst-like” firing mode (35% spikes occurring in bursts). Bursts, defined as two or more consecutive spikes occurring with an interspike interval of less than or equal to 100 msec and not greater than 125 msec, are indicated by the rectangular boxes.
only examined the effects of 100 ng CRF infused into the LC. Moreover, careful histological verification of infusion sites was executed in the present experiments. Infusions which did not impinge on the LC or into areas in which LC dendrites extend were ineffective in eliciting increases in extracellular NE.

The greater increase and prolonged elevation of NE associated with higher doses of CRF infused into LC is consistent with the increase in discharge rate observed in anesthetized animals. Moreover, the results obtained in awake animals did not differ from those observed in the anesthetized preparation. A previous study by Curtis et al. (1997) described only a brief increase in NE in anesthetized rats detected in the sample following CRF infusion. Although similar methods were employed to apply CRF, i.e., pressure application, their study included the NE reuptake inhibitor desmethylmipramine (DMI) in the dialysis perfusate. Administration of DMI can affect basal levels of NE and may also increase the negative feedback regulation of release by autoreceptors located on LC-NE terminals (Thomas and Holman, 1991). The present experiments compared the effects of CRF administration in anesthetized and awake animals in the absence of uptake inhibition. The 30-ng dose of CRF induced the same magnitude increase in extracellular NE in anesthetized rats as the 20 and 60-ng dose in unanesthetized rats. Local infusion of CRF in anesthetized rats was achieved as described above with small pulses of pressure applied to the drug containing pipette adjacent to the recording pipette. This technique allows for even smaller volumes of solution to be administered directly onto LC-NE neurons, thus reducing the potential for diffusion outside the nucleus.

As described above, the CRF innervation of the LC region may be considered minor. The primary inputs arise from the paragigantocellularis (PGi) nucleus and the prepositus hypoglossi (PrH) nucleus (Aston-Jones et al., 1986). Additional minor inputs arise from the spinal cord, periaqueductal gray (PAG), lateral hypothalamus, and the amygdala (Aston-Jones et al., 1986; Luppi et al., 1995; Van Bockstaele et al., 1996). Valentino et al. (1991) have shown that a portion of neurons in the PGi projecting to the LC contains CRF-like immunoreactivity. An additional source of CRF input to the LC may arise from the neighboring pontine structure, Barrington’s nucleus (Valentino et al., 1991). Furthermore, a recent report from Van Bockstaele et al. (1996) described the ultrastructural localization of CRF-containing terminals arising from the amygdala impinging on both TH-labeled and unlabeled dendritic processes in the rostral portion of the LC, providing evidence for direct CRF innervation of NE neurons of the LC. The presence of CRF binding sites in the LC region (DeSouza, 1987; DeSouza et al., 1985) and the CRF innervation of the LC described by several different investigators (Cummings et al., 1983; Merchenthaler et al., 1982; Valentino et al., 1992) lends support for a neurotransmitter role of CRF in the LC. To date, however, localization of CRF receptors in the LC has been elusive. Two CRF receptor subtypes with different affinities for CRF have been characterized (Lovenberg et al., 1995). The distribution of these two subtypes is distinct. Given the very strong evidence provided by our electrophysiological and neurochemical data that CRF has a neurotransmitter role in the LC, it is possible that another, as yet uncharacterized, CRF receptor subtype is responsible for CRF effects in the LC.

The diverse nature of neurochemical inputs to the LC suggests the hypothesis that the firing rate of these neurons may be intricately regulated by selective recruitment of neurochemically distinct afferents depending on the nature of the stimulus (Page and Abercrombie, 1996a). For example, it appears that glutamate inputs to the LC may predominate in the activation of these NE neurons in response to stimuli that are phasic and/or less intense in nature, whereas CRF inputs might be engaged to activate LC-NE neurons by stimuli that are of a more intense and/or prolonged character (see Introduction). It is well known that catecholamine neurons can fire in different pattern modes. This has been most clearly demonstrated for dopamine (DA) neurons, which normally fire in a single spike mode but can switch to a mode comprised predominantly of burst activity (Grace and Bunney, 1984a,b). Moreover, activation of glutamate inputs appears to be responsible for burst firing of DA neurons (Charlety et al., 1991). It has been shown that burst firing results in greater release of neurotransmitter per pulse than single spike firing (Gonon, 1988; Gonon and Buda, 1985). This latter phenomenon has also been described for the noradrenergic system (Suau-Chagney et al., 1990). Thus, differences in the pattern of activation can be a means to modulate and modify signaling in a system such as the LC. In fact, a more recent study (Florin-Lechner et al., 1996) demonstrated that the noradrenergic LC system is indeed responsive to changes in the pattern and frequency of stimulation. Their results show a frequency-dependent increase in NE release and an even greater increase in NE release when the same number of stimuli were presented as bursts of three pulses which mimic phasic activity evoked by physiological stimuli.

Many studies have examined the effects of pharmacologic agents on the overall discharge rate of neurons without monitoring possible alterations in more complex firing characteristics. The present studies attempt to correlate patterns of activity associated with CRF-mediated activation of LC-NE neurons with changes in NE output. Further studies are required to compare the effects of activation of LC-NE neurons by various neurotransmitters on extracellular NE levels and LC discharge patterns. In a set of preliminary experi-
ments, we observed profound increases in LC-NE firing rate with a shift to high-frequency discharge characterized by very short interspike intervals (data not shown) following glutamate application by either brief pulses or slow continuous infusion of glutamate onto LC-NE neurons. In a related set of experiments, local application of the O2 antagonist, idazoxan, directly onto LC-NE neurons resulted in a 34% increase in discharge rate with no increase in burst firing (Page and Abercrombie, 1996b). Systemic idazoxan, on the other hand, resulted in an 82% increase in discharge and a concomitant increase in the incidence of burst firing. It is possible that this increase in burst firing results from stimulation of glutamatergic inputs to the LC arising from the PG.

In summary, the present results demonstrate a direct excitatory action of CRF on noradrenergic LC neurons in the brainstem that is associated with increased extracellular levels of NE in the hippocampus, an LC target region. This increase in NE release was shown to be dose-dependent and could be prevented with prior i.c.v. administration of a potent CRF antagonist. The elevated NE level is coincident with an increase in single unit activity, suggesting that activation of the LC is likely to be an essential mediator of some of the behavioral parameters associated with stress. The increase in burst-like firing of LC neurons during stimulation by CRF might be an effective way to potentiate the release of NE and thereby have a greater impact on target areas.

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