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# Predator stress engages corticotropin-releasing factor and opioid systems to alter the operating mode of locus coeruleus norepinephrine neurons

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ABSTRACT

The norepinephrine nucleus, locus coeruleus (LC), has been implicated in cognitive aspects of the stress response, in part through its regulation by the stress-related neuropeptide, corticotropin-releasing factor (CRF). LC neurons discharge in tonic and phasic modes that differentially modulate attention and behavior. Here, the effects of exposure to an ethologically relevant stressor, predator odor, on spontaneous (tonic) and auditory-evoked (phasic) LC discharge were characterized in unanesthetized rats. Similar to the effects of CRF, stressor presentation increased tonic LC discharge and decreased phasic auditory-evoked discharge, thereby decreasing the signal-to-noise ratio of the sensory response. This stress-induced shift in LC discharge toward a high tonic mode was prevented by a CRF antagonist. Moreover, CRF antagonism during stress unmasked a large decrease in tonic discharge rate that was opioid mediated because it was prevented by pretreatment with the opiate antagonist, naloxone. Elimination of both CRF and opioid influences with an antagonist combination rendered LC activity unaffected by the stressor. These results demonstrate that both CRF and opioid afferents are engaged during stress to fine-tune LC activity. The predominant CRF influence shifts the operational mode of LC activity toward a high tonic state that is thought to facilitate behavioral flexibility and may be adaptive in coping with the stressor. Simultaneously, stress engages an opposing opioid influence that restrains the CRF influence and may facilitate recovery toward pre-stress levels of activity. Changes in the balance of CRF:opioid regulation of the LC could have consequences for stress vulnerability.

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## 1. Introduction

### 1.1. Attributes of the locus coeruleus

The locus coeruleus (LC) is the primary source of the widespread norepinephrine innervation of the forebrain (Jones and Moore, 1977; Swanson and Hartman, 1976). The physiological attributes of LC neurons that were initially characterized implicated this system in arousal and vigilance. For example, LC neurons discharge spontaneously and their frequency is positively correlated to behavioral and electroencephalographic (EEG) indices of arousal (Aston-Jones and Bloom, 1981a). LC neurons are also phasically activated by salient sensory stimuli and this activation precedes behavioral responses to the stimuli, implying a role for the LC in shifting attention (Aston-Jones and Bloom, 1981b; Berridge and Foote, 1991; Bouret and Sara, 2005; Foote et al., 1980). It has recently been proposed that by shifting between tonic and phasic modes of discharge the LC facilitates different behavioral outcomes (Aston-Jones and Cohen, 2005). The phasically-activated LC, characterized by synchronously driven discharge, has been associated with focusing attention and maintaining ongoing behavioral tasks. In contrast, a high tonic mode of discharge, which is spontaneous and asynchronous, is associated with scanning the environment and going off-task. The ability of LC neurons to switch between tonic and phasic modes of discharge would facilitate rapid behavioral adjustments in response to environmental challenges.

### 1.2. The LC and stress

Many of the same stressors that engage the hypothalamic-pituitary-adrenal axis activate the LC-NE system as indicated by early immediate gene expression and forebrain norepinephrine

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release (Cullinan et al., 1995; Finlay et al., 1997; Jordan et al., 1994; Kawahara et al., 2000; Kwon et al., 2006; Ma and Morilak, 2005; Pacak et al., 1995; Smagin et al., 1994, 1997). Because of technical limitations, fewer studies have recorded LC discharge during stress and most of these have examined the effect of physiological stressors on tonic (i.e., spontaneous), but not phasic LC activity (Abercrombie and Jacobs, 1987; Curtis et al., 1993; Morilak et al., 1987a, b). Studies from our laboratory using hypotensive stress or pharmacological stress produced by administration of corticotropin-releasing factor (CRF) support the idea that stress increases LC discharge and moreover suggest that stress biases the mode of LC discharge away from phasic activity toward a high tonic state (Valentino and Foote, 1987, 1988; Valentino and Wehby, 1988a). This would suggest that in addition to increasing arousal, LC activation during stress functions to facilitate a disengagement from ongoing behavior and to promote behavioral flexibility in response to challenging environmental conditions, an important cognitive limb of the stress response. Consistent with this, CRF administration into the LC, but not the lateral ventricle, facilitated extradimensional set shifting (Snyder et al., 2012).

The present study characterized LC discharge in response to an ethologically relevant stimulus, predator odor. The stimulus chosen was a chemical component of fox feces that has been reported to produce a concentration-dependent activation of the immediate early gene, c-fos in LC neurons as well as in LC afferents (Day et al., 2004). To best evaluate the impact of this stressor on LC discharge characteristics both tonic and phasic discharge were quantified during trials of auditory stimulus presentation. The roles of CRF and endogenous opioids in regulating LC activity in the response to the stressor were assessed in pharmacological antagonist studies.

## 2. Methods

### 2.1. Animals

The subjects were adult male Sprague–Dawley rats (320–360 g; Charles River, Wilmington, MA) housed three to a cage in a controlled environment (12 h light/dark cycle, lights on at 0700 h). Food and water were available ad libitum. Care and use of animals was approved by the Children's Hospital of Philadelphia Institutional Animal Care and Use Committee, and was in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering, reduce the number of animals used and to utilize alternatives to in vivo techniques.

### 2.2. Surgery for microelectrode implantation

Rats were anesthetized with an isoflurane-air mixture, positioned in a stereotaxic frame and surgically prepared for localization of LC with a glass micropipette and subsequent implantation of a microwire electrode array in LC as previously reported (Kreibich et al., 2008). Body temperature was maintained at 37.5 °C by a feedback controlled heating unit. A hole (4 mm) was drilled in the skull centered at 3.7 mm caudal and 1.2 mm lateral to lambda for approaching the LC. For experiments in which agents were administered into the lateral ventricle another hole (2 mm diameter) was drilled for implantation of an intracerebroventricular (i.c.v.) cannula guide as previously described (Howard et al., 2008). Additionally, five holes were drilled to insert skull screws for fixing the microwire electrode array to the skull with dental cement.

Neuronal recordings with glass micropipettes (2–4 μm diameter tip, 4–7 MOhm) filled with 0.5 M sodium acetate buffer were used to initially localize the LC. These were advanced toward the LC with a micromanipulator. Neuronal signals were amplified, filtered and monitored with an oscilloscope and a loudspeaker. LC neurons were tentatively identified during recording by their spontaneous discharge rates (0.5–5 Hz), entirely positive, notched waveforms (2–3 ms duration), and biphasic excitation–inhibition responses to contralateral hindpaw or tail pinch. Trajectories where LC units were encountered with the glass micropipette for at least 400 microns (dorsal-ventral penetration) were targeted for implantation with the microwire electrode array. The microwire array (NB Labs, Denison, TX) consisted of 8 Teflon insulated stainless steel wires (50 μm diameter) that were gathered in a circular bundle (7–8 mm long) and cut to produce bare wire tips for recording. A ground wire from the array encircled a skull screw and was in contact with brain tissue through another hole drilled next to the anchoring skull screw. The multiwire array was attached to a Microstar head stage and connected to a 16-channel data acquisition system (AlphaLab; Alpha Omega; Nazareth Illit, Israel). Accurate placement was aided by recording neuronal activity through the multiwire array during the implantation procedure. After detecting LC activity, the multiwire array was affixed to the skull and screws with dental cement. For experiments in which agents were administered into the lateral ventricle, an intracerebroventricular (i.c.v.) cannula guide was implanted as previously described (Howard et al., 2008). The scalp wound was sutured closed. Post-operative recovery was three days.

### 2.3. Electrophysiological recordings in unanesthetized rats

For the first 2 days after the postoperative period, rats were habituated to the recording chamber (30 cm long, 22 cm wide, 25 cm high) in which they had free movement. During these sessions, the Microstar cables were connected to the multiwire array for 1 h to assess detection of LC waveforms. In the recording chamber, two depot caps were taped to the inner surface of opposing walls 4 cm above the bedding covered floor. Each depot cap held tissue paper (1.0 cm square) to which 2,5-dihydro-2,4,5-trimethlythiazoline (TMT, 300 μmol in 40 μl), vehicle (water, 40 μl) or butyric acid (600 μmol in 40 μl) were applied using a pipetter. The concentration of TMT chosen was one that has been demonstrated to increase c-fos expression in the LC and in LC afferents, such as the central nucleus of the amygdala (Day et al., 2004). Butyric acid was used as an odorant control and the concentration was based on approximating the same number of volatile molecules as produced by TMT (300 μmol) (Hotsenpiller and Williams, 1997). The depot caps were replaced after each experiment and taped in position before the rat was placed in the chamber. For the first study (Study 1), LC activity was recorded during a 15-min baseline period that included two segments of spontaneous activity (5 min each), one before and one after a 5-min period of auditory stimulation (3 kHz tone, 50 ms duration, 80 db intensity, presented at 0.25 Hz, 50 presentations). After the baseline recording, exposure to vehicle was initiated by pipette application of 40 μl to tissue paper in the depot caps. Immediately after application, another trial of auditory stimulation was initiated followed by a final segment (5 min) of spontaneous activity. Then the rat was returned to the home cage. On the following day (Day Two of Study-1) the same rat was returned to the recording chamber. After recording baseline activity as described above, TMT was applied by pipette to tissue paper in the caps. The trial of auditory stimulation was repeated and the rat was then returned to the home cage. In a separate experiment, LC activity was recorded before and during exposure to butyric acid. These rats were only exposed on a single day (no pre-exposure to water).

For the second Study (Study 2) all rats experienced only one recording session. Each rat had a cannula guide implanted for injection of artificial cerebrospinal fluid (ACSF, 3 μl, i.c.v.), the CRF antagonist, $DPheCRF_{12-41}$ (3 μg in 3 μl ACSF, i.c.v.), naloxone (10 μg in 3 μl ACSF, i.c.v.) or a combination of $DPheCRF_{12-41}$ (3 μg) and naloxone (10 μg in 3 μl ACSF, i.c.v.). The dose of $DPheCRF_{12-41}$ chosen is that which prevents LC activation by CRF and hypotensive stress (Curtis et al., 1994). In these experiments the baseline recording period (15 min) was the same as that described for Study 1 but was followed by injection of an antagonist or ACSF. Exposure to TMT by pipetter application to the depot caps began 8–10 min after i.c.v. injections and a trial of auditory stimulation was initiated immediately after TMT application. Additional experiments assessed the effects of $DPheCRF_{12-41}$ or naloxone alone on LC spontaneous and auditory-evoked discharge in the absence of TMT exposure.

### 2.4. Data analysis

Putative LC multiunit activity was recorded as continuous analog waveforms using the AlphaLab interfaced to a host computer. Extracellular unit waveforms were amplified at a gain up to 25,000 with a bandwidth of 800 Hz to 1.4 kHz. Multiunit activity on all eight wires (channels) was monitored in real time simultaneously during experiments and spike sorting of multiunit activity was done offline. The WaveMark template-matching algorithm in the Spike2 software (Cambridge Electronic Design, CED) was used to discriminate putative LC single-unit waveforms. A set of waveforms identified by the WaveMark template is verified as events from a single unit by analyses of principal component clusters and associated autocorrelograms (Fig. 1). For principal component clusters, Spike2 generates a cluster of dots representing waveform events from a putative single unit in three-dimensional space. An ellipsoid representing 2 standard deviations (in three-dimensional space) from the cluster centrality is generated with the cluster of waveform events (Fig. 1b). The lack of overlap of any two ellipsoids was considered verification that the clusters were events from separate single units. An autocorrelogram of a set of waveform events in which no spikes occurred during an absolute refractory period of 2 ms was considered verification that those events were from a single unit (Fig. 1c). For each channel with LC activity, two to three single units were usually discriminated. LC activity was typically isolated on 2–4 channels in an individual rat.

Peristimulus time histograms (PSTHs) were generated offline from activity recorded during the period of repeated auditory stimulation. Discriminated LC unit discharge was pooled in 8 ms bins beginning 0.5 s before to 1.5 s after the auditory stimulation. Tonic and evoked LC activity were quantified from PSTHs as described previously (Valentino and Foote, 1987). Briefly, the histogram was divided into different time components and the discharge rate for each component was

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