Social Stress Engages Opioid Regulation of Locus Coeruleus Norepinephrine Neurons and Induces a State of Cellular and Physical Opiate Dependence

Nayla N Chaijale^{*,1}, Andre L Curtis¹, Susan K Wood¹, Xiao-Yan Zhang¹, Seema Bhatnagar¹, Beverly AS Reyes², Elisabeth J Van Bockstaele² and Rita J Valentino¹

¹Division of Stress Neurobiology, Department of Anesthesiology, Abramson Research Center, The Children's Hospital of Philadelphia, Philadelphia, PA, USA; ²Department of Neurosurgery, Thomas Jefferson University, Philadelphia, PA, USA

Stress is implicated in diverse psychiatric disorders including substance abuse. The locus coeruleus–norepinephrine (LC–NE) system is a major stress response system that is also a point of intersection between stress neuromediators and endogenous opioids and so may be a site at which stress can influence drug-taking behaviors. As social stress is a common stressor for humans, this study characterized the enduring impact of repeated social stress on LC neuronal activity. Rats were exposed to five daily consecutive sessions of social stress using the resident-intruder model or control manipulation. LC discharge rate recorded 2 days after the last manipulation was decreased in stressed rats compared with controls. By 10 days after the last manipulation, LC rates were comparable between groups. Systemic administration of the opiate antagonist, naloxone, robustly increased LC discharge rate in a manner suggestive of opiate withdrawal, selectively in stressed rats when administered 2 or 10 days after the last manipulation. This was accompanied by behavioral signs of mild opiate withdrawal. Western blot and electron microscopic studies indicated that repeated social stress decreased corticotropin-releasing factor type 1 receptor and increased μ -opioid receptor levels in the LC. Together, the results suggest that repeated social stress engages endogenous opioid modulation of LC activity and induces signs of cellular and physical opiate dependence that endure after the stress. These cellular effects may predispose individuals with a history of repeated social stress to substance abuse behaviors.

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INTRODUCTION

In response to perceived life-threatening stimuli, endocrine, physiological, and behavioral responses are initiated in a coordinated manner to cope with the challenge (McEwen, 1998). This 'stress response' is adaptive and important for survival. However, repeated or chronic engagement of stress response systems can have pathological consequences and predispose to psychiatric disorders such as depression, anxiety, post-traumatic stress disorder, and substance abuse (Berenz and Coffey, 2012; Gold and Chrousos, 2002; Heim and Nemeroff, 2001; McEwen, 1998). The locus coeruleus (LC)–norepinephrine (NE) system is a stress response system that has been implicated in heightened arousal and cognitive aspects of the stress response (Valentino and Van Bockstaele, 2008). Excessive activation of the LC–NE system has been linked to hyperarousal symptoms such as sleep

disturbances, inability to concentrate, ruminations, and/or increased vigilance that are shared by stress-related psychiatric diseases (Southwick *et al*, 1999; Wong *et al*, 2000).

In response to acute stress, LC activity is co-regulated by the stress-related neuropeptide, corticotropin-releasing factor (CRF), and the endogenous opioid, enkephalin (for review, see Valentino and Van Bockstaele, 2008). CRF axon terminals synapse with LC dendrites and CRF increases LC neuronal discharge rate (Curtis *et al*, 1997; Jedema and Grace, 2004; Van Bockstaele *et al*, 1996). Enkephalin-containing axon terminals also synapse with LC dendrites (Van Bockstaele *et al*, 1995) and act on μ -opioid receptors (MORs) that are highly expressed by LC neurons to potentially inhibit activity (Mansour *et al*, 1994; Williams and North, 1984). Notably, although LC neurons are also innervated by the κ -opioid receptor agonist, dynorphin, κ -opioid receptors are predominantly localized in axon terminals (Kreibich *et al*, 2008). Consistent with this presynaptic localization, κ -opiate receptor agonists do not alter LC spontaneous activity (Kreibich *et al*, 2008). Recordings of LC neuronal activity during acute stress indicate that stressors release both CRF and enkephalin to co-regulate LC activity in opposing manners. The net overall effect is a

*Correspondence: Dr NN Chaijale, Division of Stress Neurobiology, Department of Anesthesiology, Abramson Research Center, Children's Hospital of Philadelphia, 3416 Civic Center Boulevard Suite 402, Philadelphia, PA 19104, USA, Tel: +1 215 590 0654, Fax: +1 215 590 3364, E-mail: chaijalen@email.chop.edu
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CRF-mediated excitation. However, removal of this influence with a CRF antagonist reveals a naloxone-mediated inhibition. This inhibition restrains the CRF activation and also facilitates a return toward baseline activity when the stressor is terminated (Curtis *et al*, 2001; Curtis *et al*, 2012).

In contrast to the well-described effects of acute stress on LC neuronal activity, there are few investigations of the effects of repeated or chronic stressors that more closely relate to psychopathology, on LC activity. One study demonstrated that repeated auditory stress desensitizes LC neurons to CRF and hypotensive stress (Conti and Foote, 1996), consistent with reported agonist- and stress-induced CRF type 1 receptor (CRF1) internalization (Reyes *et al*, 2006, 2008). A common stressor encountered by humans that has been implicated in psychiatric disease is social stress (Faravelli *et al*, 2012; Taylor *et al*, 2011). This has been modeled in rodents using the resident-intruder model, an ethologically relevant animal model of psychosocial stress that involves subjecting a male rat (intruder) to aggressive threats by a larger, unfamiliar male rat (resident) by placing it in the resident's home cage (Miczek, 1979). Although there are several modifications of this model, many studies demonstrate that repeated exposure to this stress produces endpoints of depression and anxiety in animal models and promotes drug self administration (Miczek *et al*, 2004; Rygula *et al*, 2005; Wood *et al*, 2010).

This study characterized the enduring effect of repeated social stress on rat LC activity using the resident-intruder model. LC neuronal discharge was recorded in the unanesthetized state before stress exposure and then 2 and 10 days after the final exposure. As social stress resulted in a decrease in LC rate, the role of endogenous opioids in this effect was further investigated.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats were used as intruders (275–300 g) and male Long-Evans retired breeders were used as residents (650–850 g). All rats were ordered from Charles River (Wilmington, MA) and were individually housed in a controlled environment (20 °C, 12-h light-dark cycle, lights on at 0700 hours). Food and water were available *ad libitum*. Experiments were performed in the light cycle at approximately 1000 hours. Care and use of animals was approved by the Children's Hospital of Philadelphia Institutional Animal Care and Use Committee and in accordance with NIH Guide for the Care and Use of Laboratory Animals.

Surgery

Rats were anesthetized in 2% isoflurane-air mixture, positioned in a stereotaxic instrument, and surgically prepared for localization of the LC and subsequent implantation of an electrode array consisting of eight Teflon insulated 50 μ m stainless-steel microwires (NB Labs, Denison, TX), as previously described (Curtis *et al*, 2012; Kreibich *et al*, 2008). The array was attached to a Microstar head stage that was connected to a 16-channel data acquisition system (AlphaLab; Alpha Omega; Nazareth Illit,

Israel). After detecting LC activity, the multiwire array was affixed to the skull and screws with dental cement. The scalp wound was sutured closed. Animals were allowed 3 days of recovery before the experimental manipulations.

Repeated Social Stress

After post-surgical recovery, rats were randomly assigned to either a social stress or control group. A baseline of LC neuronal activity was recorded on the first day (day 1) before any manipulation. Rats assigned for repeated social stress were exposed to the resident-intruder stress as in our previous study (Wood *et al*, 2010). Intruder rats were placed into the cage of the resident. A period of investigation followed by an aggressive encounter by the resident usually ensued. When the intruder either assumed a supine posture signaling subordination for 3 s (defeat) or 15 min had elapsed from the time when the intruder was placed into the cage, the intruder and resident were separated by a wire mesh partition to prevent further physical contact but allow for exposure to auditory and olfactory stimuli for the remainder of a 30-min session. Control rats were placed in a novel cage behind the partition for 30-min daily. All rats were returned to their home cage after each session. This was repeated daily for 5 consecutive days with the intruder being exposed to different residents each day. As previous studies in our laboratory demonstrated that rats cluster into two sub-populations based on their latency to assume defeat (Wood *et al*, 2010), the latency to defeat was noted for each subject.

Electrophysiological Recordings

All electrophysiological recordings were performed in the unanesthetized state in the home cage, in which rats had free movement. On the first experimental day, the implanted multiwire array was connected by cables to the data acquisition system and LC activity was recorded after a 10-min habituation period. Immediately after this baseline recording, the cable was disconnected and rats were exposed to 5 days of social stress or control manipulation. As the goal was to characterize the enduring effects of social stress on LC activity, neuronal recordings were obtained 2 days (day 7) or 10 days (day 15) after the last manipulation. To investigate the influence of endogenous opioids on LC activity, some rats were administered the opiate antagonist, naloxone (2 mg/kg, s.c.) 2 or 10 days after the last stress or control manipulation and LC activity was recorded before and after naloxone administration. In some experiments 2 days after the last manipulation, the selective CRF1 antagonist, NBI 30775 (10 mg/kg, s.c.) was administered 30 min before naloxone.

Electrophysiological Analysis

LC multiunit activity was recorded as continuous analog waveforms using the AlphaLab interfaced to a host computer (Alpha Omega, Alpharetta, GA), as previously described (Curtis *et al*, 2012; Kreibich *et al*, 2008). Multiunit activity on all eight wires (channels) was monitored in real-time and spike sorting of multiunit activity was done offline. The WaveMark template-matching algorithm in the

Spike2 software (Cambridge Electronic Design, CED, v7.09) 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 the principal component analysis and associated autocorrelograms (Figures 1a and b). Briefly, for principal component analysis, Spike2 generates a cluster of dots representing waveform events from a putative single unit in three-dimensional space. An ellipsoid representing 1.5 SD from the cluster centrality is generated with the cluster of waveform events. 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 or less was considered verification that those events were from a single unit. For each channel with LC activity, 2–5 single units were usually discriminated. LC activity was typically isolated on 2–4 channels in an individual rat. LC discharge rate from all units from all rats were averaged to obtain overall mean discharge rate from control and social stressed groups. In addition, to analyze the time course of the mean LC discharge rates, the discharge rates from all cells from each treatment group were averaged and plotted in 5-s bins.

Histology

At the end of the experiment, current was passed through the electrode (10 μ A, 20 s) and rats were perfused with 6% potassium ferrocyanide (in phosphate-buffered saline) to form a Prussian blue reaction product for identification of the electrode site. After perfusion, brains were removed and stored in -80°C . Frozen coronal sections (30 μm) were cut through the LC and thaw-mounted onto slides, stained with Neutral red and the recording site was identified by microscopic visualization of the Prussian blue reaction product (Figure 1c). Data from rats with electrode localizations outside of the LC were not used in the analysis of neuronal activity.

Behavioral Analysis

For rats that were administered naloxone, behavior was recorded before and after naloxone injection and quantified by a treatment-blind observer as the incidence of specific behaviors including: chewing/teeth chattering, body/head shakes, grooming, rearing, and burying/digging. Behavioral observations were carried out in each rat's home cage.

Western Blot

Protein levels of CRF1 and MOR in LC tissue were quantified by western blotting as previously described (Bangasser *et al*, 2010). Rats were killed by decapitation 24 h after the last manipulation and extracted brains were snap-frozen in isopentane and placed at -80°C until used. Brains were blocked and sectioned in a cryostat to the level of caudal LC (identified as -10 mm from Bregma using Paxinos and Watson Brain Atlas). The LC was micro-dissected bilaterally using a trephine (1 mm). These were pooled and stored at -80°C until use. Tissue samples were homogenized, centrifuged, and supernatant was collected as

Figure 1 Discrimination of LC units and histology. (a, b) An example of three waveforms identified by the WaveMark template is verified as events from a single unit by the principal component analysis (a) and associated autocorrelograms (b). The lack of overlap of the ellipsoids (clusters) (a) and the lack of spikes occurred during an absolute refractory period of 2 ms or less (b) verified that these waveforms represent three different units. (c) Histological verification of a recording site in LC. The photomicrograph shows a coronal section through the LC. Slides were stained with Neutral red and the recording site was identified by microscopic visualization of the Prussian blue reaction product within the LC nucleus (arrow). Scale bar (bottom left) represents 200 μm . LC, locus coeruleus nucleus; Me5, mesencephalic trigeminal nucleus.

previously described (Bangasser *et al*, 2010). Protein content was determined using the BCA method. Protein extracts (10 μg) were subjected to SDS-PAGE gel electrophoresis and transferred to polyvinylidene fluoride membranes (Immobilon-FL). Membranes were blocked with Odyssey Buffer (diluted in PBS 1:1) and incubated with the following primary antibodies: goat anti-CRF1/2 (1:500; Santa Cruz Biotech, Santa Cruz, CA), rabbit anti-MOR (1:1000; Invitrogen, Camarillo, CA), rabbit anti-GAPDH (1:5000, Sigma-Aldrich, St Louis, MO), or mouse anti- β -actin, (1:5000, Sigma-Aldrich). After rinses, membranes were incubated with the following fluorescent secondary antibodies: donkey anti-goat for CRF1/2 (1:5000, IRDye 800CW, LiCor), donkey anti-rabbit for detection of MOR (1:5000, IRDye 800CW, LiCor), donkey-anti rabbit for GAPDH (1:10 000, IRDye 680, LiCor), and donkey anti-mouse for β -actin (1:10 000, IRDye 680, LiCor). For loading controls, a fluorescent secondary antibody with a different infrared wavelength than the protein of interest was used for simultaneous detection of both primary antibodies. Membranes were scanned and Odyssey Infrared Imaging software quantified the integrated intensity of each band

and determined molecular weights based on Bio-Rad Precision Plus Protein Standards. The CRF1:GAPDH and MOR: β -actin ratios were calculated and the mean ratios were compared between groups using ANOVAs. Duplicate or triplicate samples were averaged for an individual rat.

Electron Microscopy

Different groups of rats were used for electron microscopic visualization of CRF1 and MOR following previously described protocols (Reyes *et al*, 2006; Xu *et al*, 2004). Twenty-four hours after the last manipulation, rats were anesthetized with sodium pentobarbital (60 mg/kg) and perfused transcardially with 50 ml of 3.8% acrolein (Electron Microscopy Sciences, Fort Washington, PA) and 200 ml of 2% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Brains were cut into 1–3 mm coronal slices, postfixed and sections (40 μ m) were cut through the LC using a Vibratome. The sections were incubated in rabbit anti-CRF1 antiserum (H-215; 1:200) or rabbit anti-MOR antiserum (1:2500) for 15–18 h at room temperature. The specificity of the CRF1 antibody has been previously characterized with blocking peptide and in CRF1 knockout mice (Bangasser *et al*, 2010; Reyes *et al*, 2006, 2008). Control sections were processed identically with the exception of the omission of the primary antisera. Sections were incubated in goat anti-rabbit IgG ultra-small conjugate (Electron Microscopy Sciences). Silver intensification of the gold particles was achieved using a silver enhancement kit (Amersham Biosciences). Sections were flat embedded in Epon 812 (Electron Microscopy Sciences) and thin sections (80–100 nm) were cut from the outer surface of the tissue, collected on grids, and counterstained with uranyl acetate and Reynolds lead citrate. Sections were examined with an electron microscope (Morgagni, Fei, Hillsboro, OR), and digital images were captured using the AMT advantage HR/HR-B CCD camera system (Advance Microscopy Techniques, Danvers, MA). Figures were assembled and adjusted for brightness and contrast in Adobe Photoshop.

For quantification, tissue sections from at least 3 rats per group with optimal preservation of ultrastructural morphology were used. The ratio of cytoplasmic to total silver grains in a dendrite was determined for each dendrite (at least 300 and 100 dendrites for CRF1 and MOR count, respectively) and the mean ratio was determined per rat. The average of the ratio for each rat in an experimental group was taken as the group mean and these were compared between groups by an ANOVA.

Statistical Analysis

A two-way ANOVA was used to analyze changes in mean LC discharge rates between groups. A repeated-measure two-way ANOVA was used to analyze the time course of LC activity and cellular and behavioral responses after naloxone injection. A one-way ANOVA was used to analyze western blot and electron microscopy results. For all data analyses, the Student–Newman–Keuls test was used for *post hoc* comparisons of means. An alpha level of $p < 0.05$ was the maximum threshold for statistical significance (Sigma Plot version 11.0, Systat Software, Chicago, IL).

Drugs

Naloxone (Sigma-Aldrich) was dissolved in saline (2 mg/ml), prepared fresh on each test day and administered subcutaneously in 2 mg/kg dose. NBI 30775 (Neurocrine Bioscience, San Diego, CA) was dissolved in a 10% solution of 0.1 M tartaric acid in sterile water and administered as a 10 mg/kg dose (s.c.). The dose and pre-treatment time was chosen based on previous studies (Caille *et al*, 1999; Funk *et al*, 2007).

RESULTS

Repeated Social Stress Decreases LC Activity

Previous studies using rats without intracranial implants demonstrated that exposure to resident-intruder stress results in two populations of intruder rats, defined by the latency to assume a subordinate posture (> 300 or < 300 s; Wood *et al*, 2010). In the present experiment, rats used for LC recordings did not cluster into two populations. Rather the latency to assume a supine posture was biased toward the longer duration (599 ± 66 ; $n = 13$).

Figure 2a shows the time course of the mean LC discharge rates for control and stressed rats ($n = 8$ rats per group), on day 1 ($n = 65$ cells and $n = 53$ cells for control rats and stressed rats, respectively) and day 7 (2 days after the final experimental manipulation; $n = 41$ cells in both, control and stressed rats). Discharge rates were stable and comparable between groups on day 1 (Figures 2a and c). Mean overall LC rates recorded on day 1, before the first experimental manipulation were not different between groups (Figure 2c). By 2 days after the last manipulation (day 7), LC neuronal activity was significantly decreased in stressed rats as compared with control rats (Figure 2a). Across the time course of the recording, a two-way repeated-measures ANOVA showed a group by time interaction ($F(120, 8940) = 4.43$; $p < 0.001$). A two-way ANOVA comparing the mean overall rates between groups on different days demonstrated a group by day interaction ($F(1, 197) = 11.51$, $p < 0.001$; Figure 2c).

Figure 2b shows the time course of the mean LC discharge rates for a different group of control and stressed rats ($n = 3$ rats per group) on day 1 ($n = 15$ cells and $n = 23$ cells for control and stressed rats, respectively) and day 15 ($n = 12$ cells and $n = 21$ cells for control and stressed rats, respectively). Discharge rates were stable and comparable between groups on day 1 (Figures 2b and d). Similarly, the overall mean LC rates recorded on day 1, before the first experimental manipulation were comparable (Figure 2d). On day 15, LC discharge rates were also similar between groups. Analysis of the entire time course of the recording revealed that there was no effect between the groups ($F(1, 2880) = 0.04$; $p = 0.84$; Figure 2b). A comparison of the overall mean rates on days 1 and 15 indicated that there was no effect of group or day ($F(1, 67) = 0.61$; $p = 0.44$; Figure 2d).

Naloxone Selectively Activates LC Neurons of Rats Exposed to Repeated Social Stress

Naloxone, administered on day 7, selectively increased LC discharge rates of stressed rats ($n = 21$ cells per 4 rats)

Figure 2 Effects of repeated social stress on LC discharge rate. (a) Time course of the mean LC discharge rates of control (open circles) and stressed rats (closed circles), determined over a 10-min recording on day 1 ($n = 65$ cells per 8 rats and $n = 53$ cells per 8 rats for control and stressed rats, respectively) and day 7 ($n = 41$ cells per 7 rats for control and stressed rats, respectively). The abscissae indicate time in second and the ordinates indicate mean LC discharge rate (Hz). (b) Time course of the mean LC discharge rates of control (open circles) and stressed rats (closed circles), determined over a 10-min recording on day 1 ($n = 15$ cells per 3 rats and $n = 23$ cells per 3 rats for control and stressed rats, respectively) and day 15 ($n = 12$ cells and $n = 21$ cells, for control and stressed rats, respectively). The abscissae and the ordinates as indicated in a. (c) Bar graphs showing the mean LC discharge rates on day 1 and on day 7 for control (open bars) and stressed (closed bars) rats, respectively. (d) Bar graphs showing the mean LC discharge rates on day 1 and on day 15 for control (open bars) and stressed (closed bars) rats, respectively. For all graphs, alpha values are represented as: *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$. Note that the seven rats used for day 1 and day 7 (a) are different from those rats used on panel b (day 1 and day 15).

compared with control rats (17 cells per 4 rats; Figures 3a1 and b1). The increased LC neuronal activity occurred immediately after naloxone injection and was maintained above the level of control rats for at least 90 s (Figure 3a1). A two-way repeated-measures ANOVA over the time course revealed an effect of stress and naloxone injection ($F(120, 3236) = 4.11$; $p < 0.001$). A comparison of the mean overall discharge rates before and during the 200 s after naloxone revealed an effect of stress and naloxone ($F(1, 36) = 28.9$, $p < 0.001$). Before naloxone, the mean discharge rate was lower in stressed compared with control rats and this increased following naloxone administration. In contrast, the mean discharge rates before and after naloxone were not different for control rats (Figure 3b1).

LC sensitivity to naloxone in rats exposed to repeated social stress endured at least 10 days after the last stress exposure (day 15, Figures 3a2 and b2). Figure 3a2 shows the

time course of the LC discharge rate recorded in control (12 cells per 3 rats) and social stressed rats (21 cells per 3 rats) on day 15, before and after naloxone injection. Naloxone increased LC neuronal activity selectively in stressed rats and this increase was maintained for nearly 5 min (Figure 3a2). A two-way repeated-measures ANOVA over the time course indicated an effect of stress and naloxone injection ($F(120, 3751) = 7.40$; $p < 0.001$). Similarly, a comparison of the mean rates before and after naloxone injection revealed an effect of stress and naloxone ($F(1, 31) = 38.11$, $p < 0.001$; Figure 3b2). Rates were comparable before naloxone and increased only for stressed rats after naloxone injection. Notably, the magnitude of the naloxone-induced increase was larger on day 15 compared with day 7 (Figures 3a and b).

To determine whether the naloxone-elicited increase in LC activity in stressed rats was due to an unmasking of a

Figure 3 Effects of naloxone (NLX) on LC discharge rate and behavior. (a1) Time course of the mean discharge rates of control (open circles, 17 cells per 4 rats) and stressed rats (closed circles, 21 cells per 4 rats) on day 7, before and after NLX. (a2) Time course of the mean discharge rates of control (open circles, 12 cells per 3 rats) and stressed rats (closed circles, 21 cells per 3 rats) on day 15, before and after NLX. In both, day 7 and day 15, NLX is administered at 200 s, as indicated by the arrow. (b1, b2) Bar graphs represent the mean LC discharge rate of control (white bars) and stress (black bars) rats, on day 7 and day 15, before and after NLX injection. (c) Mean incidence of opiate withdrawal-related behaviors observed before (pre-) and after (post-) NLX injection to control ($n = 6$) and stressed ($n = 5$) rats on day 7. (d) Mean incidence of opiate withdrawal-related behaviors observed before (pre-) and after (post-) NLX injection to control ($n = 5$) and stressed ($n = 5$) rats on day 15. For all graphs, asterisks represent *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$.

CRF tonic activation, the selective CRF1 antagonist, NBI 30775, was administered before naloxone administration in stressed rats on day 7. For these subjects, the mean baseline LC discharge rates on day 1 and day 7 were 2.0 ± 0.2 and 1.6 ± 0.1 Hz, respectively ($n = 12$ cells per 2 rats). Following NBI 30775 and before naloxone administration, LC rates were similar 1.4 ± 0.1 Hz. Naloxone still increased LC discharge rate in rats pre-treated with NBI 30775 to a mean of 3.4 ± 0.3 Hz ($F(1, 11) = 6.8$, $p < 0.05$), similar to the effect of naloxone on day 7 in rats not pre-treated with NBI 30775 (see Supplementary Material and Supplementary Figure S1).

Naloxone Elicits Opiate Withdrawal Signs Selectively in Rats Exposed to Repeated Social Stress

Naloxone administration on day 7 elicited behaviors indicative of opiate withdrawal in stressed rats, including chewing/teeth chattering ($F(1, 7) = 6.9$, $p < 0.01$) and body and headshakes ($F(1, 7) = 19.3$, $p < 0.01$). The incidence of these and other scored behaviors before naloxone administration was not different between groups ($p = 0.87$; Figure 3c). Similar signs were observed in rats that were pre-treated with NBI 30775. Although naloxone-elicited body and head shakes subsided in the stressed rats by

day 15, the number of episodes of chewing/teeth chattering behavior remained elevated in stressed compared with control rats ($F(1, 8) = 7.6$, $p < 0.05$; Figure 3d).

Repeated Social Stress Alters CRF1 and MOR Levels and Trafficking in the LC

Rats used for these studies were not implanted with electrodes. Examination of latency to defeat revealed both long and short latency populations (533.11 ± 49.8 and 218.33 ± 26.4 for long and short latency rats, respectively).

Repeated social stress increased MOR levels in the LC ($F(2, 23) = 5.1$, $p < 0.02$). This increase was selective to rats that were characterized by a relatively long latency to defeat (comparable to stressed rats with LC recordings; Figure 4a). In contrast to MOR, CRF1 protein levels decreased in the LC of rats exhibiting long latency to defeat ($F(2, 22) = 2.87$, $p < 0.05$) and tended to be decreased for short latency rats ($p = 0.09$; Figure 4b).

Figure 5a shows examples of immunogold labeled MOR in LC dendrites. Consistent with the results of the western blot experiments, quantification of the number of immunogold labeled MOR particles/dendrite revealed that this was greater in social stress rats that were characterized by a long latency to defeat (3.8 ± 0.1 , $n = 3$ rats) compared with control rats (2.4 ± 0.1 , $n = 3$ rats) and short latency rats (2.4 ± 0.1 , $n = 3$, $F(2, 8) = 52$, $p < 0.0005$). In addition, there was a greater ratio of cytoplasmic:total immunogold MOR labeling, indicative of MOR re-distribution (eg, internalization) in the social stress group characterized by long latency to defeat ($F(2, 8) = 8.7$, $p < 0.02$; Figure 5a, bar graph). Interestingly, this was not apparent in social stressed rats that were characterized by a short latency to defeat (Figure 5a, bar graph).

Figure 5b shows examples of CRF1 immunogold labeling in LC dendrites of control ($n = 8$ rats) and stressed rats. For rats exposed to social stress that were characterized by a long latency to defeat ($n = 7$ rats), there was no difference in the ratio of cytoplasmic:total CRF1 labeling. However, evidence for CRF1 internalization was apparent in social stress rats that were characterized by a short latency to defeat ($n = 7$ rats) as indicated by a greater cytoplasmic:total CRF1 ratio ($F(2, 21) = 8.7$, $p < 0.005$).

DISCUSSION

This study demonstrated that repeated social stress produces enduring changes in LC neuronal activity. In contrast to LC excitation that is associated with acute stress, repeated social stress produced an inhibition that was apparent at 48 h after stress termination and returned to baseline by 10 days. The naloxone-elicited LC activation that occurred selectively in stressed rats implicated endogenous opioids in this effect. Consistent with this, MOR levels were increased, particularly in the cytoplasmic compartment of LC neurons, as would be predicted by enkephalin release. The engagement of endogenous opioid regulation of the LC resulted in a state of cellular opioid dependence as indicated by the findings that naloxone increased LC discharge to rates greater than those seen in control rats and that naloxone-elicited LC activation also occurred at a time when LC discharge was no longer inhibited in stressed rats. The naloxone-elicited LC activation is reminiscent of that produced in animals that have been chronically administered morphine (Aston-Jones *et al*, 1997; Rasmussen *et al*, 1990; Valentino and Wehby, 1989). Naloxone also elicited physical signs of opiate withdrawal selectively in social stressed rats. Together, the findings

Figure 4 Western blot analysis of CRF receptor 1 (CRF1) and μ -opioid receptor (MOR) in the locus coeruleus (LC), in control rats or rats that went through repeated social stress and were classified as short (SL) or long latency (LL). (a) Representative blot showing protein expression of MOR (MW = 70 kDa) and β -actin (MW = 42 kDa). Bar graphs show the mean ratio of the integrated intensity of each band of MOR protein to the corresponding control band from the same sample. There was a difference of MOR expression in LC from LL ($n = 6$) rats compared with control ($n = 7$) rats ($F(2, 21) = 5.15$, $*p > 0.01$). SL ($n = 11$) rats did not show any differences from control rats ($p = 0.25$). (b) Representative blot showing protein expression of CRF1 receptor (MW = 52 kDa) and GAPDH (MW = 36 kDa). Bar graphs show the mean ratio of the integrated intensity of each band of CRF1 protein to the corresponding control band from the same sample. ANOVA analysis showed a decrease in CRF1 receptor expression between LL ($n = 6$) rats and control ($n = 8$) rats ($F(2, 22) = 2.87$, $*p > 0.05$). SL ($n = 11$) rats tended to be decreased as compared with control rats ($^{\#}p = 0.09$).

Figure 5 Electron microscopic evidence for repeated social stress-induced re-distribution of (a) μ -opioid receptor (MOR) and (b) CRF receptor 1 (CRF1) in rat LC. Panel a—top shows a dendrite from a control rat that contains immunogold-silver labeling for MOR (arrowheads) along the plasmalemma. MOR within the cytoplasmic compartment is depicted by an arrow. Panel a—center demonstrates MOR labeling in a dendrite from a rat exhibiting short latency behavior (SL). Immunogold-silver labeling for MOR (arrowheads) can be seen primarily along the plasmalemma, although some MOR-immunogold-silver particles are localized within the cytoplasmic compartment. Panel a—bottom illustrates that immunogold-silver labeling for MOR shifts from the plasmalemma to the cytoplasm in long latency rats (LL). Immunogold-silver labeling for MOR is shown within the cytoplasmic compartment as depicted by arrows, whereas arrowheads point to immunogold-silver labeling on the plasma membrane. Bar graph from panel a shows quantitative representation of the ratio of cytoplasmic to total immunogold-silver labeling of MOR in LC for control rats, SL rats and LL rats ($n=3$ rats per group, >100 dendrites per rat analyzed). ANOVA analysis for MOR internalization showed a difference between groups ($F(2, 6) = 8.71$; $*p < 0.05$), indicating that there was an increased percent internalization of MOR only in LL rats. Panel b—top shows a dendrite from a control rat that contains immunogold-silver labeling for CRF1 (arrowheads) along the plasmalemma. An arrow depicts CRF1 within the cytoplasmic compartment. Panel b—center illustrates that immunogold-silver labeling for CRF1 shifts from the plasmalemma to the cytoplasm in rats that exhibit short-latency behavior following exposure to repeated social stress. Immunogold-silver labeling for CRF1 is shown within the cytoplasmic compartment as depicted by arrows. A CRF1 is also found along the plasmalemma (arrowhead). A double headed arrowhead indicates an endosome-like vesicle associated with CRF1. Panel b—bottom shows a dendrite from a rat exhibiting long-latency behavior following exposure to repeated social stress. CRF1 distribution in LC from rats that exhibited long latency behavior was similar to CRF1 distribution in LC from control rats. Quantitative representation of the ratio of cytoplasmic:total CRF1 immunogold label (panel b, bar graph) indicated that CRF1 was internalized in SL ($n=7$) rats ($F(2, 19) = 8.812$; $*p < 0.01$), but not in LL ($n=7$) or control ($n=8$) rats (>300 dendrites per rat analyzed). Scale bars: $0.5 \mu\text{m}$.

suggest that repeated social stress induces an enduring opioid regulation of the brain LC-NE system and a state of cellular and physical opiate dependence. These neural changes may confer vulnerability to opiate abuse in subjects with a history of social stress.

Acute Stress and LC Activity

Acute stressors of different modalities activate the LC-NE system as indicated by recordings of LC neuronal activity,

c-fos expression in LC neurons, and NE release in LC-forebrain targets (Chan and Sawchenko, 1994; Curtis *et al*, 2012; Smagin *et al*, 1994). Evidence has implicated CRF as the neurotransmitter that mediates stress-elicited LC-NE activation. CRF axon terminals synapse with LC dendrites and intra-LC CRF administration mimics the effects of acute stressors on LC discharge (Curtis *et al*, 1997; Van Bockstaele *et al*, 1996). Administration of CRF antagonists directly into the LC prevents stress-induced activation of the LC-NE system and unmasks a naloxone-sensitive inhibition that is

apparent either during stress or immediately following stress termination (Curtis *et al*, 2001; Valentino *et al*, 1991). When both opiate and CRF receptors are blocked, LC neurons are non-responsive to acute stress, suggesting that these are the major afferents that regulate the LC-NE system during stress (Curtis *et al*, 2012). Together, these findings suggest a model whereby the LC is regulated in opposing manners by CRF and endogenous MOR ligands during acute stress (Valentino and Van Bockstaele, 2008). In this model, stress engages CRF afferents to the LC to bias activity to a high tonic state that promotes increased arousal and behavioral flexibility. At the same time, endogenous opioids are released to exert an inhibitory influence that restrains the excitation and facilitates recovery on stressor termination.

Repeated Social Stress and LC Activity

In contrast to LC activation by acute stressors, repeated social stress resulted in lower LC discharge rates compared with the control manipulation when measured 48 h after the last stress. A similar inhibition has been described with repeated auditory stress or repeated CRF when LC activity was recorded 24 h after the last stress (Conti and Foote, 1995, 1996). Given evidence that acute stressors engage an opioid inhibitory influence on the LC, this was a likely candidate for the inhibition seen after repeated social stress. The robust naloxone-elicited LC activation of rats exposed to repeated social stress confirmed a role for endogenous opioids in the relatively long-lasting inhibition of LC activity. The activation was not due to an unmasking of a CRF excitation because it occurred in rats that were pre-treated with a CRF antagonist. In addition, CRF receptors were downregulated in stressed rats. Naloxone-elicited LC activation likely represents a cellular withdrawal response, similar to that seen when morphine-dependent rats are administered an opiate antagonist (Aston-Jones *et al*, 1997; Rasmussen *et al*, 1990; Valentino and Wehby, 1989). The finding that naloxone still elicits a robust LC activation by 10 days after repeated stress, at a time when baseline LC neuronal discharge rates are no longer inhibited, indicates that repeated stress produces a state of cellular tolerance and dependence. The observation of naloxone-elicited physical signs resembling opiate withdrawal at both 2 and 10 days after the final stressor was consistent with this. These results are reminiscent of studies demonstrating that rats and mice exposed to repeated resident-intruder stress develop an analgesia that is cross-tolerant with morphine and that lasts for months (Miczek, 1991; Miczek *et al*, 1986). Similarly, in mice resident-intruder stress produced a state of opiate physical dependence as evidenced by naloxone-precipitated withdrawal jumping that was of a similar magnitude as that produced in morphine-dependent mice (Miczek *et al*, 1986). Together with the present findings, these studies underscore the ability of social stress to bring endogenous opioid systems online to a sufficient extent as to produce physical dependence.

LC Hyperactivity and Behavioral Withdrawal

In morphine-dependent rats, opiate antagonist administration directly onto LC neurons by iontophoresis or microinfusion

produces withdrawal hyperactivity (Aghajanian, 1978; Aston-Jones *et al*, 1997). Whether this effect is due to intrinsic mechanisms within LC neurons or to excitatory afferents has remained controversial (Akaoka and Aston-Jones, 1991; Rasmussen and Aghajanian, 1989). Currently, it is accepted that both intrinsic and extrinsic mechanisms contribute to the opiate antagonist-precipitated withdrawal hyperactivity of LC neurons of morphine-dependent animals. In this study using the microwire array, it was not feasible to directly inject naloxone into the LC so it is not known for certain whether naloxone-elicited LC activation of the stressed rats was the result of direct activation of LC neurons or activation of LC afferents. Similarly, the link between LC activation and withdrawal behaviors is not definitively established. Previous lesion studies suggested that the LC is not necessary for withdrawal behaviors (Caille *et al*, 1999; Delfs *et al*, 2000). However, other studies involving direct inhibition of LC activity or manipulation of cyclic AMP signaling in the LC provide evidence for its involvement in opiate withdrawal signs (Han *et al*, 2006; Mirzaii-Dizgah *et al*, 2008; Punch *et al*, 1997; Taylor *et al*, 1998). Even if not necessary, LC activation is sufficient to elicit opiate withdrawal signs as indicated by the findings that the antagonist is most potent at eliciting physical withdrawal signs when administered directly into the LC (Maldonado *et al*, 1992). Nonetheless, the finding that naloxone-precipitated LC activation is still very strong at 10 days after the last stressor but the withdrawal signs were weaker suggests the involvement of other circuits, at least with respect to head and body shakes.

Social Stress Effects on MOR and CRF1 in the LC

As rats from which LC recordings were obtained were only characterized by long latencies to exhibit defeat, the relationship between stress effects on neuronal activity and receptors can only be interpreted for this sub-population. Nonetheless, certain comparisons of stress-related receptor changes between long and short latency rats lead to interesting speculation. In general, the findings suggest that repeated social stress results in a decrease in CRF influence on the LC as a result of receptor down-regulation. The finding that CRF1 was downregulated in long latency rats without evidence of internalization may indicate that at this time CRF1 internalization and down-regulation had already occurred and, although fewer in number, the receptors were redistributed equally between the cytoplasm and plasma membrane. The observation that the short latency rats exhibited CRF1 internalization and only a tendency for CRF1 downregulation suggest that they lag behind the long latency rats in the processes of CRF1 internalization and downregulation. Importantly, the down-regulation of CRF1 could contribute to a shift from CRF excitatory influence to an opioid inhibitory regulation of the LC with repeated stress. The increase in MOR levels in LC detected by both western blot and electron microscopy and apparent MOR internalization in rats exposed to social stress that were characterized by a long latency to defeat would be consistent with an increased enkephalinergic drive to the LC in these rats. This could account for enhanced inhibitory tone of LC neurons and development of cellular dependence. That this is not seen in the short latency rats at

this time could also be due to a lag in the processes regulating MOR in response to social stress. Alternatively, these processes may be impaired in this sub-population and this may have a role in the different phenotype. These questions lie beyond the scope of the article but inspire much future research.

Clinical Implications

We previously suggested that disturbing the opioid–CRF balance that regulates the LC during stress has potential clinically relevant consequences (Valentino and Van Bockstaele, 2008). For example, chronic morphine administration sensitized LC neurons to CRF and stress and this was expressed as a hyperactive coping style (Xu et al, 2004). This study implied that chronic opiates could increase vulnerability to post-traumatic stress disorder, which is characterized by hyperarousal. Conversely, this study suggests that repeated social stress could predispose to opiate abuse by creating a state of cellular dependence where exogenous opiates would serve as negative reinforcers to alleviate withdrawal hyperactivity of the LC system. Although the social stress model used in this study has been demonstrated to promote self-administration of cocaine and other stimulants, effects on opiate self-administration have not been as well studied. One study indicated that social stress history did not influence heroin self-administration (Cruz et al, 2011). However, social stress effectively reinstates morphine conditioned place preference in rats to a similar extent as physical stress and cues related to morphine withdrawal share this effect (Ribeiro Do Couto et al, 2006). Notably in humans, a history of child abuse is associated with opiate abuse (Schiff et al, 2010). The present findings suggest that inducing a state of cellular dependence within the LC–NE system may be one mechanism by which repeated social stress increases vulnerability to opiate abuse.

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