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Social Stress Engages Neurochemically-Distinct Afferents to the Rat Locus Coeruleus Depending on Coping Strategy

Social stress, LC afferents and coping style

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BR performed immunohistochemistry, analyzed data, created figures, edited manuscript; GZ performed tracer injections and resident-intruder stress and analyzed data; CF assisted with immunohistochemistry; EJV designed and interpreted research, edited manuscript, created figures; RJV designed and interpreted research and wrote paper.

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Abstract

Stress increases vulnerability to psychiatric disorders, partly by affecting brain monoamine systems, such as the locus coeruleus (LC)-norepinephrine system. During stress, LC activity is co-regulated by corticotropin-releasing factor (CRF) and endogenous opioids. This study identified neural circuitry that regulates LC activity of intruder rats during the resident-intruder model of social stress. LC afferents were retrogradely labeled with Fluorogold (FG) and rats were subjected to one or five daily exposures to an aggressive resident. Sections through the nucleus paragigantocellularis (PGi) and central amygdalar nucleus (CNA), major sources of enkephalin (ENK) and CRF LC afferents respectively, were immunocytochemically processed to detect c-fos, FG and CRF or ENK. In response to a single exposure, intruder rats assumed defeat with a relatively short latency (SL). LC neurons, PGI-ENK LC afferents and CNA-CRF LC afferents were activated in these rats as indicated by increased c-fos expression. With repeated stress rats exhibited either a SL or long latency (LL) to defeat and these strategies were associated with distinct patterns of neuronal activation. In SL rats, LC neurons were activated, as were CNA-CRF LC afferents but not PGI-ENK LC afferents. LL rats had an opposite pattern, maintaining activation of PGi-ENK LC afferents but not CNA-CRF LC afferents or LC neurons. Together, these results indicate that the establishment of different coping strategies to social stress is associated with changes in the circuitry that regulates activity of the brain norepinephrine system. This may underlie differential vulnerability to the consequences of social stress that characterize these different coping strategies.

Significance

Social stress has been linked to psychiatric disorders, in part through activation of the locus coeruleus (LC)-norepinephrine system. This study identified circuits that are engaged during acute and repeated social stress to regulate this system. It was found that the establishment of different coping strategies with repeated social stress was associated with distinctions in stress-activated circuitry. In rats that resisted defeat, inhibitory enkephalin afferents to the LC were engaged, whereas in rats that are biased towards subordination, excitatory corticotropin-releasing factor inputs to the LC were engaged. The engagement of different circuits with opposing actions may underlie distinctions in the consequences of social stress in subjects with different coping strategies.

Introduction

Successful adaptation to stressors requires the coordination of multiple stress response systems that mount adaptive endocrine, autonomic, immunological, and behavioral responses. Whereas the immediate activation of these systems in response to acute stress is adaptive, continuous or chronic activation can have pathological consequences (McEwen, 1998; de Kloet et al., 2005). The locus coeruleus-norepinephrine (LC-NE) system is a major brain stress response system. Activation of the LC-NE system is important in maintaining arousal and cognitive flexibility in response to acute stress (Berridge and Waterhouse, 2003; Valentino and Van Bockstaele, 2008). However, repeated or prolonged activation of this system has been implicated in arousal-related symptoms of stress-related psychiatric disorders, including

93 depression and post-traumatic stress disorder (Chappell et al., 1986; Southwick et al.,
94 1999; Wong et al., 2000). Identifying the neuromediators and circuitry that regulate LC
95 activity during repeated stress is integral to our understanding of the arousal and
96 cognitive domains of stress-related psychiatric diseases.

97 LC dendrites receive convergent synaptic input from separate axon terminals
98 containing the stress-related neuropeptide, corticotropin-releasing factor (CRF) and
99 enkephalin (ENK) (Tjoumakaris et al., 2003). CRF and ENK afferents to the LC derive
100 from different nuclei, with the central nucleus of the amygdala (CNA) being a primary
101 source of CRF and the medullary nucleus paragigantocellularis (PGi) being a primary
102 source of ENK (Drolet et al., 1992; Van Bockstaele et al., 1998; Tjoumakaris et al.,
103 2003). CRF and ENK have opposing excitatory and inhibitory effects on LC neuronal
104 activity, respectively and these are both engaged during acute stress (Valentino and
105 Van Bockstaele, 2008, 2015). Although the predominant influence on LC activity during
106 acute stress is CRF-induced excitation, antagonizing CRF reveals an underlying opioid
107 inhibition that is thought to restrain LC activation and facilitate recovery to baseline
108 activity after stressor termination (Curtis and Valentino, 2008; Valentino and Van
109 Bockstaele, 2008; Curtis et al., 2012).

110 For humans, stressors of a social nature are common and this can be modeled
111 by the rodent resident-intruder stress in which an "intruder" rat is placed into the cage of
112 a "resident" rat, which is typically larger and sufficiently aggressive such that an attack
113 on the intruder ensues (Koolhaas et al., 1997). Repeated resident-intruder stress
114 produces hypothalamic-pituitary-adrenal axis dysfunction, decreased social interaction,
115 anxiety, anhedonia and self-administration of drugs of abuse in intruders (Tornatzky and

Miczek, 1994; Miczek et al., 2004; Rygula et al., 2005; Wood et al., 2010; Wood et al., 2012). Importantly, there is substantial individual variability in the magnitude of these consequences and in rats this has been associated with coping style (Krishnan et al., 2007; Wood et al., 2010). Repeated exposure of rats to resident-intruder stress results in the emergence of two distinct populations based on the latency to assume the subordinate defeat posture (Wood et al., 2010). The latency has a bimodal distribution, indicating that these are distinct populations rather than simply extremes of a normally distributed population. Rats that exhibit defeat with a relatively short latency (SL rats) show increased anhedonia, immobility in the forced swim test, decreased heart rate variability, and hypothalamic-pituitary-adrenal dysregulation similar to that reported in depression, compared to stressed rats that exhibit a long latency and resist defeat (LL rats) (Wood et al., 2010; Wood et al., 2012; Wood et al., 2015). Interestingly, the initial exposure to the stress results in relatively rapid onsets to defeat, whereas the LL phenotype develops with repeated exposures (Wood et al., 2010).

Given the decreased vulnerability of LL rats to rodent endpoints of stress-related psychiatric disorders, identifying neurobiological substrates and circuitry that distinguish the SL and LL phenotypes may reveal the basis for different stress coping strategies as well as vulnerability to stress-related pathology. In this regard, administration of CRF antagonists prior to repeated resident-intruder stress was demonstrated to bias coping strategy towards the LL phenotype and to prevent certain consequences of repeated resident-intruder stress, underscoring a potential role for CRF as a link between coping strategy and vulnerability to the pathological consequences of social stress (Wood et al., 2012). This study used a functional neuroanatomical approach to determine whether

afferent regulation of the LC differed between rats with different coping strategies to repeated stress. Retrograde tract tracing from the LC was combined with immunohistochemistry to detect c-fos, a marker of neuronal activation, and either CRF or ENK in LC afferents of rats exposed to repeated resident-intruder stress. To determine how this differed from rats exposed to an acute resident-intruder stress before the divergence of different coping strategies, similar approaches were applied to rats exposed to a single session of resident-intruder stress.

Materials and Methods

Experimental animals. All rats were ordered from Charles River (Wilmington, MA) and housed in a controlled environment (20°C, 12-h light-dark cycle). Food and water were available *ad libitum*. Adult male Long-Evans retired breeder rats (650-850g) were used as residents and adult male Sprague-Dawley rats (250-300g) were used as intruders (or matched controls). Intruders were initially pair-housed. After the first experimental manipulation, they were housed individually. Residents were housed individually. Experiments were performed during the light cycle. All animal procedures were performed in accordance with the Author's Institutional Animal Care and Use Committee regulations.

Fluorogold injection into the LC. Intruder rats and matched controls were anesthetized with a 2% isoflurane-air mixture, positioned in a stereotaxic instrument and surgically prepared for electrophysiological localization of the LC using a glass micropipette. Micropipettes (15–20 μm diameter tip) were backfilled with a solution of

2% Fluorogold (FG; Fluorochrome, Inc., Englewood, CO) in 0.9% sterile saline. Microelectrode signals were led from a preamplifier to filters and additional amplifiers. Impulse activity was monitored on an oscilloscope and with a speaker to aid in localizing the LC. When neuronal activity characteristic of the LC was localized (spontaneous discharge rate of 1-4 Hz, entirely positive notched waveform of 2-3 ms duration in unfiltered trace and biphasic response to tail or paw pinch), the micropipette was repositioned until the core of the LC was located, after which FG was iontophoresed (5 μA, 7-s duty cycle, 15 min). The micropipette was then kept in place for 10 min to prevent leakage. A dense CRF terminal field exists in the dorsolateral peri-LC where CRF axon terminals synapse with LC dendrites. The major source of these terminals is the central nucleus of the amygdala, which does not project to the LC core (Aston-Jones et al., 1986; Van Bockstaele et al., 1998). To assure labeling of this major CRF afferent, after iontophoresing FG into the LC core the pipette was repositioned to the dorsolateral peri-LC for an additional 15 min application of FG followed by a 10 min post-iontophoretic period. The micropipette was removed, the scalp wound was sutured and rats were allowed to recover 3 days before experimental manipulations.

Social Stress. Sprague Dawley rats were randomly assigned to intruder (n=7) or matched control (n=7) groups. Intruder rats were placed into the cage of the resident. After a brief period of investigation an aggressive encounter by the resident typically ensued. A mesh wire partition was placed between the intruder and resident either immediately after the intruder assumed a supine posture signaling subordination for 3 sec (defeat) or after 15 min had elapsed from the time when the intruder was placed into the cage if no defeat occurred. The partition prevented further physical contact, but

allowed for exposure to visual, auditory and olfactory cues for the remainder of a 30-min session. For repeated social stress, this was repeated for 5 consecutive days, with intruders being exposed to a different resident on each day. The latency to assume the subordinate posture was recorded for each rat for each exposure to resident-intruder stress. The mean latency over the 5 days was calculated for each rat and analyzed by a cluster analysis across the group (JMP 9.0.0, SAS Institute; www.jmp.com). Control rats were placed in a novel cage for 30 min daily, with the final 15 min behind the wire partition. All rats were returned to their home cage following each session.

In a separate experiment rats (n=8) were injected with FG in the LC as described above. They then received either a single exposure to resident-intruder stress (n=4) or control manipulation (n=4). Latency to defeat was recorded in the stressed rats as described above.

Immunohistochemistry. Rats were anesthetized with 5% isofluorane and transcardially perfused with 2% heparinized saline, followed by 4% paraformaldehyde, 90 min after the last experimental manipulation. The brain was removed and postfixed in 4% formaldehyde overnight at 4ºC and stored in sucrose solutions of 10% and 20% for 1 hour each, followed by 30% sucrose for 48-72 h in 0.1 M phosphate buffer (PB) containing 0.1% sodium azide at 4ºC. One side of the brain was notched to verify tissue orientation following sectioning. Frozen 40 µm-thick sections were cut in the coronal plane in a series of 4 using a freezing microtome and collected in 0.1 M PB. Every fourth section through the rostro-caudal extent of the LC was collected and processed for immunoperoxidase detection of the extent of the FG injection site. Likewise, serial coronal sections through the PGi and CNA were processed for immunoperoxidase

visualization of FG to evaluate the magnitude of retrograde labeling. These sections were washed in 0.1M Tris buffered saline (TBS; pH 7.6) and incubated in 0.5% bovine serum albumin (BSA) in TBS for 30 min. Subsequently, sections were incubated in 0.5% BSA and 0.25% Triton X-100 in 0.1 M TBS for 30 min and rinsed extensively in 0.1 M TBS. Sections were incubated in rabbit anti-FG (1:2,000; Chemicon International Inc., Temecula, CA) for 15-18 h at room temperature. They were then rinsed and incubated in biotinylated donkey anti-rabbit (1:400; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 30 min followed by rinses in 0.1 M TBS. Subsequently, sections were incubated for 30-minute in avidin-biotin complex (Vector Laboratories, Burlingame, CA, USA). FG was visualized by reaction with 3,3'-diaminobenzidine and 30% hydrogen peroxide in TBS. Sections were collected, dehydrated and coverslipped for light microscopic analysis of FG immunoreactivity.

A series of sections through the rostro-caudal segment of the PGi was processed for immunofluorescent visualization of FG, c-fos and ENK and a series of sections through the CNA was processed for immunofluorescent visualization of FG, c-fos and CRF. Only cases with the most restricted placement of FG and optimal retrograde labeling were used in the analysis. Free-floating sections were rinsed extensively in 0.1 M PB followed by rinses in 0.1 M TBS. Sections were then incubated in 0.5% BSA in 0.1 M TBS for 30 min, and rinsed in 0.1 M TBS. Following rinses, sections containing the PGi were incubated overnight at room temperature in a solution containing guinea pig anti-FG (1:2000; Protos Biotech Corp., New York, NY), rabbit anti-c-fos (1:3000; Calbiochem,) and mouse anti-ENK (1:100; Fitzgerald Laboratories, Concord, MA) in 0.1M TBS with 0.1% BSA and 0.25% Triton X-100. Likewise, tissue sections from the

CNA were incubated overnight at room temperature in rabbit anti-FG (1:2,000; Chemicon International Inc.), mouse anti-c-fos (1:100; Santa Cruz Biotechnology Inc., Santa Cruz, CA) and guinea pig anti-CRF (1:2,000; Peninsula Laboratories, San Carlos, CA). Sections were then washed in 0.1 M TBS and sections containing the PGi were incubated in a secondary antibody cocktail containing fluorescein isothiocyanate (FITC) donkey anti-rabbit (1:200; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA), tetramethyl rhodamine isothiocyanate (TRITC) donkey anti-mouse (1:200; Jackson ImmunoResearch) and AlexaFluor 647 donkey anti-guinea pig (1:200; Jackson ImmunoResearch) antibodies prepared in 0.1 % BSA and 0.25% Triton X-100 in 0.1 M TBS for 2 h in the dark. Sections from the CNA were incubated in a secondary antibody cocktail containing FITC donkey anti-mouse (1:200; Jackson ImmunoResearch Laboratories Inc.), TRITC donkey anti-guinea pig (1:200; Jackson ImmunoResearch) and AlexaFluor 647 donkey anti-rabbit (1:200; Jackson ImmunoResearch) antibodies. Following incubation with the secondary antibodies, the tissue sections were washed thoroughly in 0.1 M TBS, mounted on slides and allowed to dry in complete darkness. The slides were dehydrated in a series of alcohols, soaked in xylene and coverslipped using DPX (Sigma-Aldrich Inc.). Sections were visualized using an Olympus 1X81 laser microscope (Olympus, Hatagaya, Shibuya-Ku, Tokyo, Japan) and images captured using Olympus Fluoview ASW FV1000 program (Olympus, Hatagaya, Shibuya-Ku, Tokyo, Japan).

As an indication of stress-induced LC activation c-fos-immunoreactivity was also quantified in every fourth section through the LC. Free-floating sections were rinsed extensively in 0.1 M PB followed by rinses in 0.1 M TBS. Sections were then incubated

in 0.5% bovine serum albumin (BSA) in 0.1M TBS for 30 min, and rinsed in 0.1 M TBS for 10 min, three times. Following rinses, the sections were incubated overnight in a rabbit monoclonal antibody for c-fos (1:3,000; Calbiochem) in 0.1% BSA and 0.25% Triton X-100 in 0.1M TBS. Incubation time was 15-18 h in a rotary shaker at room temperature. The following day, sections were rinsed three times in 0.1 M TBS and incubated in biotinylated donkey anti-rabbit (1:400; Jackson ImmunoResearch Laboratories, Inc.) for 30 min followed by rinses in 0.1 M TBS. A 30-minute incubation of avidin-biotin complex (Vector Laboratories, Burlingame, CA, USA) followed. For all incubations and washes, sections were continuously agitated with a rotary shaker. C-fos was visualized by a 4-minute reaction in 22 mg of 3,3'-diaminobenzidine (Sigma-Aldrich Inc.) and 10 µl of 30% hydrogen peroxide in 100 ml of 0.1 M TBS. A total of 6 sections were analyzed per rat and the mean number per group was obtained by determining the average c-fos immunoreactive cells per section per rat. The mean number per rat was determined for comparison between groups.

Data analysis. Sections from each rat were first examined for accurate and localized FG injections determined from the brightfield images (Olympus BX51, Tokyo, Japan). Images were captured using Spot Advanced software (Diagnostic Instruments Inc., Sterling Heights, MI). Only animals with satisfactory injection sites were utilized for data analysis. The criteria for defining satisfactory injection sites included the extent of diffusion of injections, proximity of injections to the target nucleus and retrograde labeling in the PGi and CNA. The area of the PGi that was analyzed extends from the anterior pole of the lateral reticular nucleus to the level of the caudal third of the facial nucleus, as previously described (Andrezik et al., 1981)(Plate 62 to Plate 68; Paxinos

and Watson, 1997). The area of the CNA analyzed is located in the medial part of the amygdaloid complex which is bounded laterally by the basolateral amygdaloid nucleus, lateral amygdaloid nucleus and amygdaloid intermedullary gray, medially by primary substantia innominata and medial amygdaloid nucleus, dorsally by the basal nucleus and interstitial nucleus of the posterior limb of the anterior commissure and ventrally by the intercalated amygdaloid nucleus and intraamygdaloid division of the bed nucleus of stria terminalis (Plates 25 through 30; Paxinos and Watson, 1997).

For quantification every fourth coronal (120 μm apart) section was taken through the antero-posterior extent of the PGi and CNA. The number of c-fos, FG, ENK or CRF, dually labeled cells (FG+c-fos) and triple labeled cells (FG, c-fos and ENK or CRF) were counted in 12 PGi-containing sections and 6 CNA-containing sections. Counts of c-fos in the LC were determined from 6 sections from each rat. The total number of labeled (FG, c-fos, Enk or CRF, FG/c-fos and FG/c-fos/ENK or FG/c-fos/CRF) cells from all sections quantified was determined for each rat as well as the percentage of FG-fos labeled neurons that were triple labeled and the group means were compared by a one-way ANOVA with Tukey post-hoc tests for comparisons between individual groups (JMP 9.0.0, SAS Institute).

Results

Repeated stress and retrograde labeling. Repeatedly stressed rats clustered into two populations based on either a relatively short (SL) or long (LL) latency to assume the subordinate defeat posture, consistent with other reports (Wood et al., 2010). The range of latencies for the SL rats was 183-219 s with a mean latency of 196±12 s (n=3).

The range of latencies for LL rats was 679-764 s with a mean latency of 730±18 s (n=4) and this was significantly different than the mean latency of SL rats (p=0.005).

Of 14 rats (7 control, 7 stress) that were injected with FG into the LC 11 (5 control, 3 stress LL rats and 3 stress SL rats) had optimally placed injections that targeted the region of LC. Figure 1Aii shows a representative brightfield photomicrograph depicting an FG injection site into the LC. FG injections into the LC yielded consistent retrograde labeling of perikarya in both the PGi and the CNA in all cases examined (Fig. 1B,C). The mean number of retrogradely labeled neurons after repeated social stress was not different between experimental groups in either the PGi ($F(2,8)=2.034$, $p=0.169$) or CNA ($F(2,8)=1.511$, $p=0.278$; Table 1).

Repeated Social stress-induced activation of LC-projecting ENK neurons in the PGi. Figure 2 shows examples of FG, c-fos and ENK immunolabeling in the PGi. A clear distinction in PGi neuronal activation was observed between rats with different coping strategies after repeated exposure to social stress. More PGi neurons were activated in LL rats compared to control and SL rats as evidenced by the total number of c-fos profiles ($F(2,8)=19$, $p=0.0009$; Table 1). Moreover, a greater number of PGi cells that project to the LC were activated in LL rats compared to control and SL rats (FG+c-fos; $F(2,8)=52$, $p<0.0001$; Table 1). Interestingly, ENK-labeled neurons were also more abundant in LL rats compared to controls, but not SL rats ($F(2,8)=7.59$, $p=0.014$; Table 1). Importantly, there was evidence of greater ENK-drive to the LC in LL rats compared to SL and control rats as indicated by a greater percentage of

retrogradely labeled-c-fos expressing neurons that also were ENK immunoreactive (F(2,8)=14, p=0.002; Table 1).

Repeated Social stress-induced activation of LC-projecting CRF neurons in the CNA. Figure 3 shows examples of FG, c-fos and CRF immunolabeling in the CNA. In direct contrast to the patterns of activation within the PGi, more CNA neurons overall were activated in SL rats compared to LL or control rats based on the number of c-fos profiles (F(2,8)=33, p=0.0001; Table 1) and more CNA LC-afferents were activated in SL rats compared to other groups (FG+c-fos; F(2,8)=40, p<0.0001; Table 1). Notably, CRF-immunolabeled neurons were more numerous in the CNA of SL rats compared to all other groups (F(2,8)=20, p=0.0007; Table 1). Moreover, SL rats showed greater activation of CRF-LC projecting neurons as indicated by the percentage of CRF-immunolabeled LC-projecting neurons exhibiting c-fos immunolabeling compared to both controls and LL rats (F(2,8)=43, p<0.0001; Table 1). There was a tendency for more triple labeled neurons in LL rats compared to controls (p=0.052, Tukey's HSD).

Acute Social Stress-induced activation of LC-projecting ENK neurons in the PGi and CRF neurons in the CNA. Consistent with previous reports that the LL phenotype emerges with repeated exposure to social stress (Wood et al., 2010), a group of rats that were exposed to a single session of resident-intruder stress all had short latencies to defeat ranging between 112-300 s with a mean of 205±40 s (n=4). As for repeatedly stressed rats, a single exposure to stress did not affect retrograde labeling from the LC and the numbers of FG labeled neurons were similar in stressed and matched controls

in both the PGi ($F(1,6)=2.445$, $p=0.169$) and CNA ($F(1,6)=1.206$, $p=0.314$; Table 2). The single stress exposure increased c-fos labeling compared to control manipulation in both the PGi ($F(1,6)=14$, $p=0.0009$) and CNA ($F(1,6)=86$, $p<0.0001$) (Table 2). Acute social stress was associated with increased ENK-immunolabeled cells in the PGi ($F(1,6)=11.5$, $p=0.015$) and CRF-immunolabeled cells in the CNA ($F(1,6)=54.5$, $p=0.0003$) compared to control manipulation (Table 2). Importantly, rats acutely exposed to the social stress displayed evidence of both greater ENK and CRF drive to the LC from the PGi and CNA respectively, when compared to controls. The percentage of LC-projecting PGi neurons and CNA neurons that express c-fos and were also ENK- or CRF-immunoreactive, respectively was greater in stressed compared to control rats ($F(1,6)=71$, $p<0.0001$ and $F(1,6)=52$, $p<0.0001$, respectively; Table 2).

Acute and repeated social stress and LC activation. To determine whether differences in stress-induced activation of LC afferents have consequences for LC activity, c-fos immunoreactive profiles were quantified in this region in the different groups. Figure 4 shows examples of c-fos immunoreactive profiles in representative LC sections of rats exposed to a single or repeated resident-intruder stress or their matched controls. The number of c-fos-immunoreactive profiles in the LC was greater in acute stressed compared to control rats ($F(1,6)=71$, $p=0.0002$; Fig. 4). Notably, after 5 repeated exposures c-fos immunoreactive profiles in the LC were elevated above controls in SL, but not LL rats ($F(2,9)=4.92$, $p=0.036$; Fig. 4).

Statistical Table

	Data structure	Test Type	Power
a. Table 1-PGi	Normal Distribution	ANOVA/Tukey	0.99
b. Table 1-CNA	Normal Distribution	ANOVA/Tukey	0.99
c. Table 2-PGi	Normal Distribution	ANOVA/Tukey	0.82
d. Table 2-CNA	Normal Distribution	ANOVA/Tukey	1.0

Discussion

Electrophysiological studies demonstrated that during acute stress LC neurons are co-regulated in an opposing manner by CRF-mediated excitation and opioid-mediated inhibition, with CRF excitation predominating (Curtis et al., 2001; Curtis et al., 2012). The present neuroanatomical evidence that CNA-CRF and PGI-ENK afferents to the LC are both engaged during acute resident-intruder stress and that the net effect is LC activation is consistent with the electrophysiological findings. Importantly, the current study demonstrated that with repeated social stress, the establishment of different coping strategies is associated with strategy-specific changes in the circuitry that regulates the brain norepinephrine system. Specifically, in rats with a tendency to resist subordination, the inhibitory ENK drive to the LC was maintained while activation of CNA-CRF LC afferents was lost and LC activation was no longer apparent. In direct contrast, rats that show a propensity towards subordination exhibited the opposite pattern, with a prominent activation of excitatory CNA-CRF afferents and LC activation, but a loss of PGI-ENK drive. The results are consistent with the differential stress-induced internalization of μ-opioid receptors (MOR) in LC neurons of LL rats and CRF1 receptors in LC neurons of SL rats (Chaijale et al., 2013). Together, they suggest that repeated social stress engages distinct circuits that have opposing influences to regulate activity of the LC-NE system in rats with different coping strategies. This differential circuit activation may determine the coping strategy and/or the pathological consequences of the stress.

Technical Considerations. Interpretations of the current findings must take into account certain caveats of retrograde tract tracing and immunolabeling of neuropeptides. The extent and localization of the FG injection cannot be identical across rats. Nonetheless, monitoring electrophysiological characteristics during micropipette placement greatly optimizes the degree of accuracy of injections. The use of iontophoresis through small diameter micropipettes limits diffusion and reduces the degree of axonal damage and uptake by fibers of passage. Cases that are analyzed are conservatively limited to those in which the injection fills the LC and dorsolateral peri-LC where LC dendrites extend. Only cases with retrograde labeling in the major LC afferents, the nucleus PGi and CNA are used. Confirming the consistency of retrograde labeling, there were no differences between groups in the number of retrogradely labeled neurons in the PGI or CNA. Importantly, this was also true when comparing acute and repeatedly stressed rats even though FG was present for a shorter duration in acutely stressed rats (4 days) compared to repeatedly stress rats (8 days). Another caveat is that greater visualization of the neuropeptides could be obtained with colchicine, which was not used in the present study because it affects c-fos expression as well as retrograde labeling (Gorenstien et al., 1985; Monti-Graziadei and Berkely, 1991). Because of this, the percentage of triple labeled neurons could be underestimated in this study. Given that CRF and ENK neurons were readily visible in all analyzed cases, the lack of colchicine should not have a bearing on interpretations. Finally, c-fos profiles were observed in control rats although their number was substantially less compared to acutely stressed rats or repeatedly stressed SL rats. This was not unexpected as the controls were significantly manipulated. To mimic the

handling of the stressed rats, controls were placed into a novel cage, picked up at 15 min and put behind a wire mesh barrier that limited their access to the entire cage. This procedure assured that any differences between control and stressed groups were entirely due to the presence of the resident.

Stress-induced co-regulation of LC activity. Activation of LC neurons by acute stressors is an important central limb of the stress response. Converging lines of evidence implicate CRF as a neurotransmitter that mediates this response. CRF-immunoreactive axon terminals synapse with tyrosine hydroxylase immunoreactive dendrites in the nucleus LC and peri-LC (Van Bockstaele et al., 1996; Van Bockstaele et al., 1998). CRF increases LC neuronal discharge rates *in vivo* and in slice preparations *in vitro* (Curtis et al., 1997; Jedema and Grace, 2004). Importantly, local administration of CRF antagonists prevent LC activation by certain stressors (Valentino et al., 1991; Lechner et al., 1997; Kawahara et al., 2000). LC neurons are also regulated by endogenous opioids during acute stress. ENK axon terminals synapse with LC dendrites (Van Bockstaele et al., 1995). MOR agonists potently inhibit LC neurons *in vivo* and in slice preparations *in vitro* (Williams and North, 1984; Nestler et al., 1994). MOR regulation of LC activity is engaged during acute stress, as indicated by a naloxone-sensitive LC neuronal inhibition that is unmasked when stressed rats are administered a CRF antagonist (Curtis et al., 2001; Curtis et al., 2012). Notably, there is little co-localization of CRF and ENK in axon terminals in the LC region and convergence of CRF and ENK axon terminals onto the same LC dendrites is more prominent (Tjoumakaris et al., 2003). Additionally, evidence suggests that these derive

453 from distinct sources, with ENK deriving primarily from the PGi and CRF from the CNA
454 (Drolet et al., 1992; Van Bockstaele et al., 1998; Tjoumakaris et al., 2003). Although the
455 net effect of acute stress on LC neuronal discharge rate is CRF-mediated excitation,
456 eliminating the excitatory CRF influence with an antagonist reveals a naloxone-
457 sensitive, opioid-mediated inhibition (Curtis et al., 2001; Curtis et al., 2012). The
458 engagement of opioid afferents to the LC during acute stress serves to maintain LC
459 activation in an optimal range and to facilitate a return to baseline discharge when the
460 stressor is terminated. (Curtis et al., 2001; Curtis et al., 2012). The present
461 neuroanatomical findings in rats exposed to a single resident-intruder stress were
462 consistent with the electrophysiological results by providing evidence for activation of
463 both ENK and CRF afferents to the LC and overall LC activation as indicated by c-fos
464 expression.
465
466 *Role of coping strategy*. Rats initially exposed to resident-intruder stress assume
467 defeat with short latencies (Wood et al., 2010) (the present study). With repeated
468 exposure, two subpopulations emerge that are characterized by a propensity vs.
469 resistance to assume the subordinate defeat posture (Wood et al., 2010). Interestingly,
470 it is the LL population that emerges and becomes significantly different by the fourth
471 exposure (Wood et al., 2010). The two populations are also distinguished by the
472 consequences of stress. For example, SL rats show evidence for hypothalamic-
473 pituitary-adrenal axis dysfunction, anhedonia, immobility in the forced swim test and
474 decreased heart rate variability (Wood et al., 2010; Wood et al., 2012; Wood et al.,
475 2015). As these are characteristics that are seen in major depression, the SL and LL

476 phenotypes have been interpreted to represent stress-vulnerable and stress-resilient
477 populations, respectively. This may be a simplistic interpretation as both coping
478 strategies may be associated with distinct pathologies and at the same time have
479 evolutionary adaptive purpose. Nonetheless, it is important to identify the
480 neurobiological basis and neurocircuitry underlying the different coping strategies.

481 A comparison of CRF1 and MOR cellular localization following a bout of repeated
482 resident-intruder stress suggested that ENK and CRF were differentially engaged in rats
483 with different coping strategies to repeated social stress (Chaijale et al., 2013). Thus,
484 MOR was internalized in LC dendrites selectively in LL rats, whereas CRF1 was
485 selectively internalized in SL rats. As receptor internalization is a consequence of
486 agonist binding, these findings suggest that ENK is preferentially released to interact
487 with MOR in LL rats and CRF released to interact with CRF1 in SL rats.

488 The present findings using functional neuroanatomy support the interpretations of
489 the electron microscopic results (Fig. 5). In LL rats PGi-ENK afferents to the LC were
490 activated after the fifth social stress and this was not apparent in SL rats. Rather, in SL
491 rats, CNA-CRF inputs to the LC were activated. The net effect was selective activation
492 of LC neurons in SL rats. Although the neuroanatomical results cannot be analyzed
493 across days in the same rats, an examination of the results across days suggests that
494 as the LL coping style emerges, the ENK drive is maintained, while the CRF drive is
495 diminished in LL rats resulting in a loss of LC activation. In SL rats, the CRF drive is
496 maintained and the ENK drive is lost so that LC activation remains. Taken with findings
497 that administering a CRF antagonist prior to each resident-intruder stress biases the
498 coping style towards LL, the results suggest that CRF promotes the SL coping strategy

499 (Wood et al., 2012). Notably, in all cases in which triple labeled neurons increased in
500 the PGI or CNA, the number of neurons expressing either ENK or CRF was also greater
501 in the respective group, suggesting that the repeated stress may be affecting
502 neurotransmitter expression in LC afferents that are activated during the stress.
503
504 *Functional and Clinical Implications*. Given that CRF1 and MOR activation
505 regulate the LC in opposing manners, the differential engagement of CRF and ENK
506 afferents in rats with different coping styles has important implications. One question is
507 whether there is a causal link between afferent regulation of the LC and coping strategy.
508 The finding that systemic administration of a CRF antagonist before each exposure to
509 resident-intruder stress shifts the coping strategy phenotype towards LL, supports
510 causality between CRF and the SL strategy (Wood et al., 2012). It is also possible that
511 coping strategy determines the afferent regulation that is engaged. Alternatively, the
512 circuitry may be designed such that the afferents engaged to modulate LC activity and
513 the circuitry determining the coping strategy are regulated in parallel by an upstream
514 circuit component.
515 Afferent regulation of LC activity would be predicted to impact the behavioral
516 consequences of stress. The “depressive-like” behavioral phenotype of SL rats is
517 attenuated in rats pretreated with a CRF antagonist and this treatment biases the
518 coping strategy towards LL (Wood et al., 2012). It is tempting to speculate that this is in
519 part a result of CRF actions at the level of the LC. Notably, rats with intracranial
520 implants for LC recordings exhibit an LL phenotype response to repeated social stress
521 and show electrophysiological evidence for opioid regulation of the LC (Chaijale et al.,

2013). In these rats naloxone produces an activation of LC neurons that resembles that seen during opioid withdrawal. These findings have suggested that repeated social stress produces a state of mild opioid dependence in rats with the LL coping strategy that could predispose to substance abuse. Together, the present results suggest that different stress coping strategies are associated with distinct circuitry that can regulate the LC-norepinephrine system in opposing manners and result in pathology that is unique to the specific coping phenotype.

Figure legends

Figure 1. Fluorogold injection and retrograde labeling. **Ai-Ci.** Schematic diagrams adapted from the Rat Brain Atlas (Paxinos and Watson, 1998) showing the antero-posterior levels of the representative injection site (A) and retrograde labeling (B-C). **Aii.** Brightfield photomicrograph showing a representative Fluorogold (FG) injection within the rat locus coeruleus (LC). **Bii-Cii.** Brightfield photomicrographs of representative retrograde labeling in the nucleus paragigantocellularis (PGi; panel Bii at approximately plate 67 (Paxinos and Watson, 1997) and central nucleus of the amygdala (CNA; panel Cii at approximately plate 26 (Paxinos and Watson, 1997) following FG injection into the LC. The arrows indicate immunoperoxidase labeled cells. Scale bars = 50 μm (A) and 25 μm (B,C).

Figure 2. Activation of locus coeruleus-projecting enkephalin neurons in the nucleus paragigantocellularis (PGi). **A.** Scatterplot showing the percentage of FG-c-fos-labeled neurons that were also immunolabeled for ENK for individual control, short latency and long latency rats. Lines through the points indicate the group mean. **B.** Representative immunofluorescence photomicrograph from a long latency rat showing c-fos profiles (blue), fluorogold labeling (green), ENK-immunoreactivity (red) and triple labeled neurons (yellow). Arrows point to single labeled neurons and arrowheads point to triple labeled neurons. Scale bar = 10 μm. **C.** High magnification photomicrographs illustrating activated LC-projecting ENK neurons in the PGi of a long latency rat. FG, c-fos and ENK panels show single labeling for the retrograde tracer, FG (green), c-fos immunoreactivity (blue) and ENK immunoreactivity (red), respectively. The merged

image shows all labels. Thin arrows point to the same triple labeled neuron in all images. Scale bar = 10 µm.

Figure 3. Activation of locus coeruleus-projecting corticotropin-releasing factor neurons in the central nucleus of the amygdala (CNA). **A.** Scatterplot showing the percentage of FG-c-fos-labeled neurons that were also immunolabeled for CRF for individual control, short latency and long latency rats. Lines through the points indicate the group mean. **B.** Representative immunofluorescence photomicrograph from a short latency rat showing c-fos profiles (blue), fluorogold labeling (green), CRF-immunoreactivity (red) and triple labeled neurons (yellow). Arrows point to single labeled neurons and arrowheads point to triple labeled neurons. Scale bar = 10 µm. **C.** High magnification photomicrographs illustrating activated LC-projecting CRF neurons in the CNA of a short latency rat. FG, c-fos and CRF panels show single labeling for the retrograde tracer, FG (green), c-fos immunoreactivity (blue) and CRF immunoreactivity (red), respectively. The merged image shows all labels. Thin arrows point to the same triple labeled neuron in all images. Scale bar = 10 µm.

Figure 4: Activation of LC neurons by a single or repeated exposure to resident-intruder stress. **Ai, Aii**. Representative sections from rats exposed to a single control manipulation (Ai) and a single resident-intruder stress (Aii). **Aiii**. Scatterplot showing the number of c-fos profiles in the LC after a single stress or control manipulation for individual control and stressed rats. Lines through the points indicate the group mean. **Bi, Bii**. Representative sections from a control rat exposed to 5 repeated manipulations

576 (Bi) and an SL rat exposed to 5 repeated resident-intruder exposures (Bii). **Biii**.
577 Scatterplot showing the number of c-fos profiles in the LC after the fifth stress or control
578 manipulation for individual control, SL and LL rats. Lines through the points indicate the
579 group mean.
580
581 **Figure 5.** Schematic depicting distinct engagement of CRF and ENK afferents to the LC
582 and adaptations in receptors in LC neurons depending on social stress history and
583 coping strategy. Acute social stress engages CRF inputs to the LC from the CNA and
584 ENK inputs from the PGi to the LC. The emergence of different coping strategies with
585 repeated social stress is associated with distinct biases towards regulation by one
586 afferent. In rats that resist defeat (LL rats) PGi-ENK afferents to the LC remain engaged
587 resulting in MOR internalization and upregulation in this phenotype. The CRF influence
588 is decreased in LL rats. In contrast, in rats exhibiting a subordinate coping style (SL),
589 amygdalar-CRF afferents are engaged and CRF1 becomes internalized in LC dendrites.
590 The PGi-ENK influence is diminished in these rats. The selective engagement of
591 afferents with opposing effects that are related to coping styles may be a basis for
592 individual differences in the pathological consequences of social stress.
593
594 **Visual Abstract.** See Abstract
595

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706

707

Table 1

Repeated Social Stress						
PGi						
	c-fos	FG	ENK	FG+c-fos	%FG+c-fos	%FG+c-fos expressing ENK
Control	31 ± 3	129 ± 2	234 ± 7	22 ± 1	17 ± 1	9 ± 2
SL	31 ± 1	120 ± 4	256 ± 12	21 ± 1	18 ± 1	11 ± 3
LL	55 ± 4*, #	120 ± 4	286 ± 5*	40 ± 1*, #	33 ± 1*, #	**41 ± 9****, ##
CNA						
	c-fos	FG	CRF	FG+c-fos	%FG+c-fos	%FG+c-fos expressing CRF
Control	75 ± 4	240 ± 6	130 ± 5	17 ± 2	7 ± 1	12 ± 1
SL	160 ± 6*, ŧ	252 ± 10	212 ± 10*, ŧ	56 ± 4*, ŧ	22 ± 1*, ŧ	**57 ± 5*****, ŧ ŧ
LL	102 ± 12	212 ± 28	127 ± 16	27 ± 3	13 ± 1*	26 ± 5

***p<0.001, **p<0.01, *p<0.05 post hoc comparisons to control values; ## p<0.01, # p<0.05 post hoc comparisons to SL values, ŧ ŧ p<0.01, ŧ p<0.05 post hoc comparisons to LL values

Table 2

Acute Social Stress						
PGi						
	c-fos	FG	ENK	FG+c-fos	%FG+c-fos	%FG+c-fos expressing ENK
Control	37 ± 4	137 ± 5	224 ± 8	18 ± 3	13 ± 2	15 ± 4
Defeat	57 ± 4**	155 ± 12	255 ± 6*	12 ± 2	15 ± 2	**52 ± 2*** **
CNA						
	c-fos	FG	CRF	FG+c-fos	%FG+c-fos	%FG+c-fos expressing CRF
Control	88 ± 4	220 ± 6	142 ± 7	20 ± 4	9 ± 2	19 ± 2
Defeat	180 ± 9***	237 ± 12	221 ± 9***	57 ± 2***	24 ± 2***	**70 ± 7*** **

***p<0.001, **p<0.01, *p<0.05 one-way ANOVA comparisons to control values