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Stress and Drug Dependence Differentially Modulate Norepinephrine Signaling in Animals with Varied HPA Axis Function

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Previous work has demonstrated the importance of genetic factors and stress-sensitive circuits in the development of affective disorders. Anxiety and numerous psychological disorders are comorbid with substance abuse, and noradrenergic signaling in the bed nucleus of the stria terminalis (BNST) is thought to be a source of this convergence. Here, we examined the effects of different stressors on behavior and norepinephrine dynamics in the BNST of rat strains known to differ in their HPA-axis function. We compared the effects of acute morphine dependence and social isolation in non-anxious Sprague Dawley (SD) rats, and a depression model, Wistar-Kyoto (WKY) rats. We found a shared phenotype in drug-dependent and singly housed SD rats, characterized by slowed norepinephrine clearance, decreased autoreceptor function, and elevated anxiety. WKY rats exhibited changes in anxiety and autoreceptor function only following morphine dependence. To ascertain the influence of LC inhibition on this plasticity, we administered the LC-terminal-selective toxin DSP-4 to SD and WKY rats. DSP-4-treated SD rats demonstrated a dependence-like phenotype, whereas WKY rats were unchanged. Overall, our findings suggest that individuals with varying stress susceptibilities have different noradrenergic signaling changes in response to stress. These changes may establish conditions that favor stress-induced reinstatement and increase the risk for addiction.
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INTRODUCTION

Researchers have demonstrated the importance of central noradrenergic activation in regulating behavioral and physiological responses to stress (Cecchi *et al*, 2002; Fendt *et al*, 2005). In part, this is because norepinephrine can engage the hypothalamic-pituitary-adrenal (HPA) axis. Through such actions, norepinephrine has been identified as an important neural substrate associated with the aversive components of morphine withdrawal (Delfs *et al*, 2000). Such evidence has given support to the view that the negative affect experienced during drug withdrawal is mediated in part by norepinephrine, and can contribute to the addiction cycle (Koob and Volkow, 2010). Interestingly, a number of psychological disorders and addictions are comorbid with stress and involve noradrenergic dysregulation (eg, post-traumatic stress disorder) (Hyman *et al*, 2006; Sinha, 2008). For example, exposing rats to intruder stress evokes an opiate-dependent like state and alters firing of norepinephrine neurons (Chaijale *et al*, 2013). Therefore, investigation of noradrenergic systems and their role in the initiation/termination of stress is important for understanding the pathophysiology of diseases that co-express with addiction.

The ventral bed nucleus of the stria terminalis (vBNST) is a major target of norepinephrine innervation within the brain (Kilts and Anderson, 1986). Here, forebrain, limbic, and brainstem inputs converge to relay information about both physical and psychological stressors. The BNST receives small noradrenergic input from the locus coeruleus (LC), and major noradrenergic innervation from the A1 and A2 (via the ventral noradrenergic bundle) cell groups, and projects to stress and reward centers (Forray and Gysling, 2004; Drolet, 2009). The BNST has neurons containing corticotropin-releasing factor (McElligott *et al*, 2010), excitatory and inhibitory projections to the paraventricular nucleus of the hypothalamus (Choi *et al*, 2007), and activates the HPA axis (Forray and Gysling, 2004). Norepinephrine is released in the BNST during restraint stress, oral administration of an aversive tastant, and during morphine withdrawal (Fuentealba *et al*, 2000; Pardon *et al*, 2002; Park *et al*, 2012). Thus, norepinephrine release in the BNST can integrate stressful and aversive stimuli to generate an appropriate physiological response.

Previously, we showed that two different rat strains, Sprague-Dawley and Lewis, markedly differed in the response of their noradrenergic system to morphine withdrawal (McElligott *et al*, 2013). Sprague-Dawley rats demonstrated profound plasticity of uptake and autoreceptor function, whereas control mechanisms were unchanged in Lewis rats.

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To better understand how genetic differences interact with drug withdrawal and stressors, we chose to compare the stress responses of Sprague-Dawley (SD) and Wistar-Kyoto (WKY) rats. WKY rats exhibit increased depressive phenotypes and HPA axis function relative to Sprague-Dawley rats (Cohen *et al*, 2006; Carr and Lucki, 2010), and under restraint-stress, extracellular norepinephrine varies between the two (Pardon *et al*, 2002). Here, we used fast-scan cyclic voltammetry to evaluate differences in norepinephrine overflow and regulation between SD and WKY rats in response to stress. We subjected animals to acute morphine-dependence, 2 weeks of social-isolation, and DSP-4 lesioning. In response to these stressors, we found robust neurochemical changes that differed between strains and corresponded with anxiety-like behavior.

METHODS

Animal Care

Experiments were performed in accordance with University of North Carolina at Chapel Hill (UNC) Institutional Animal Care and Use Committee's guidelines. Subjects were Sprague-Dawley and Wistar-Kyoto rats (males, 250–350 g on arrival from Charles River, Wilmington, MA) pair-housed in UNC animal facilities on a 12-hour day/light cycle. Animals were given *ad libitum* access to food and water, and their health was monitored daily during treatments. For social isolation, after 1-week of acclimation, subjects were randomly split into single or pair-housing for 2 weeks. Care was taken to reduce the number of animals used. For anesthetized voltammetry experiments, 108 Sprague-Dawley and 103 Wistar-Kyoto rats were used. A separate group of 32 Sprague-Dawley and 48 Wistar-Kyoto rats was used for anxiety measures on the Elevated Plus Maze (EPM).

Chemicals and Drugs

Drugs were purchased from Sigma-Aldrich (St Louis, MO), with the exception of α_{2C} antagonist JP-1302 dihydrochloride (Tocris Bioscience, Ellisville, MO), dissolved in sterile saline (0.9%), and used as received. JP-1302 and α_{2A} antagonist BRL-44408 maleate were administered to naïve animals in a range from 0.2 to 5 mg/kg i.p. to build a dose–response curve. Treated and control animals were given either 2 mg/kg BRL-44408 i.p. or 2 mg/kg α_{2A} agonist Guanfacine HCl i.p. to assay α_{2A} function. At the end of the experiment, animals were given 2 mg/kg dopamine D2 antagonist raclopride tartrate i.p., followed by 5 mg/kg α_2 antagonist idazoxan i.p. to validate signal per Park *et al*, 2009.

Measurement of Norepinephrine Release

Norepinephrine release in the vBNST was measured in anesthetized animals as described previously (Park *et al*, 2009) (Supplementary Methods).

Elevated Plus Maze

Anxiety-like behavior was measured as described previously (McElligott *et al*, 2013) (Supplementary Methods). Briefly, the number of entries and time spent in each section of the maze was measured over 5 min. Preference for the open arms was determined in each animal and was expressed as a ratio of open-arm time over closed-arm time.

Morphine Dependence

For 3 days, rats were administered 10 mg/kg morphine sulfate s.c. once daily, followed 4 h later by 1 mg/kg naloxone HCl to induce withdrawal. Somatic indices of withdrawal (eg, teeth chattering) were scored each day per Schulteis *et al*, 1999. Control animals received 3 days of 0.5 ml saline s.c., followed 4 h later by 1 mg/kg naloxone. On day 4, drug-free rats were assayed on the EPM or underwent surgery for norepinephrine measurements.

DSP-4 Lesioning

Wistar-Kyoto and Sprague-Dawley rats (150–200 g on arrival) were pair-housed, and administered two i.p., 50 mg/kg N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) injections in 1 ml/kg saline 3 days apart. Control animals were given two 1 ml/kg i.p. saline injections 3 days apart. Animals were allowed to recover for >10 days after the last injection.

Statistics

Results are presented as average values ± SEM. Two-way analysis of variance (ANOVA) with *post hoc* Bonferroni tests were used to determine statistical significance. An unpaired *t*-test was used to determine differences in anxiety between morphine-dependent and control WKY rats. Differences were considered significant when $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$.

RESULTS

First, we chose to extend prior work demonstrating strain-dependent differences in norepinephrine regulation during stress (Pardon *et al*, 2002; McElligott *et al*, 2013). We characterized vBNST norepinephrine synaptic function in anesthetized WKY rats with electrical stimulations of the ventral noradrenergic bundle, and measured subsequent norepinephrine overflow with fast-scan cyclic voltammetry (Park *et al*, 2009; Herr *et al*, 2012). This technique allows characterization of release and uptake of biogenic amines (John *et al*, 2006). We measured norepinephrine by placing the carbon-fiber microelectrode directly beneath the anterior commissure (electrode placement shown in Figure 1a, sample color plot encoding voltammetric recordings in Figure 1b). Release increased linearly with increasing stimulation duration in WKY rats (60 Hz stimulations, $r^2=0.99$, slope: 3.71 ± 0.24; Figure 1c) similar to our findings in SD rats (slope: 3.11 ± 0.21, McElligott *et al*, 2013). Despite known phenotypic variations (Carr and Lucki, 2010), we found no differences in norepinephrine clearance ($t_{1/2}$) between WKY and SD rats in a baseline state (1.6 ± 0.12 s *vs* 1.7 ± 0.10 s, $n=22$ and 26, respectively).

The adrenergic α_2 receptor serves as the inhibitory autoreceptor for norepinephrine, and two differentially regulated subtypes are present in the BNST (Scheinin *et al*, 1994). We assayed autoreceptor influence on release with α_2

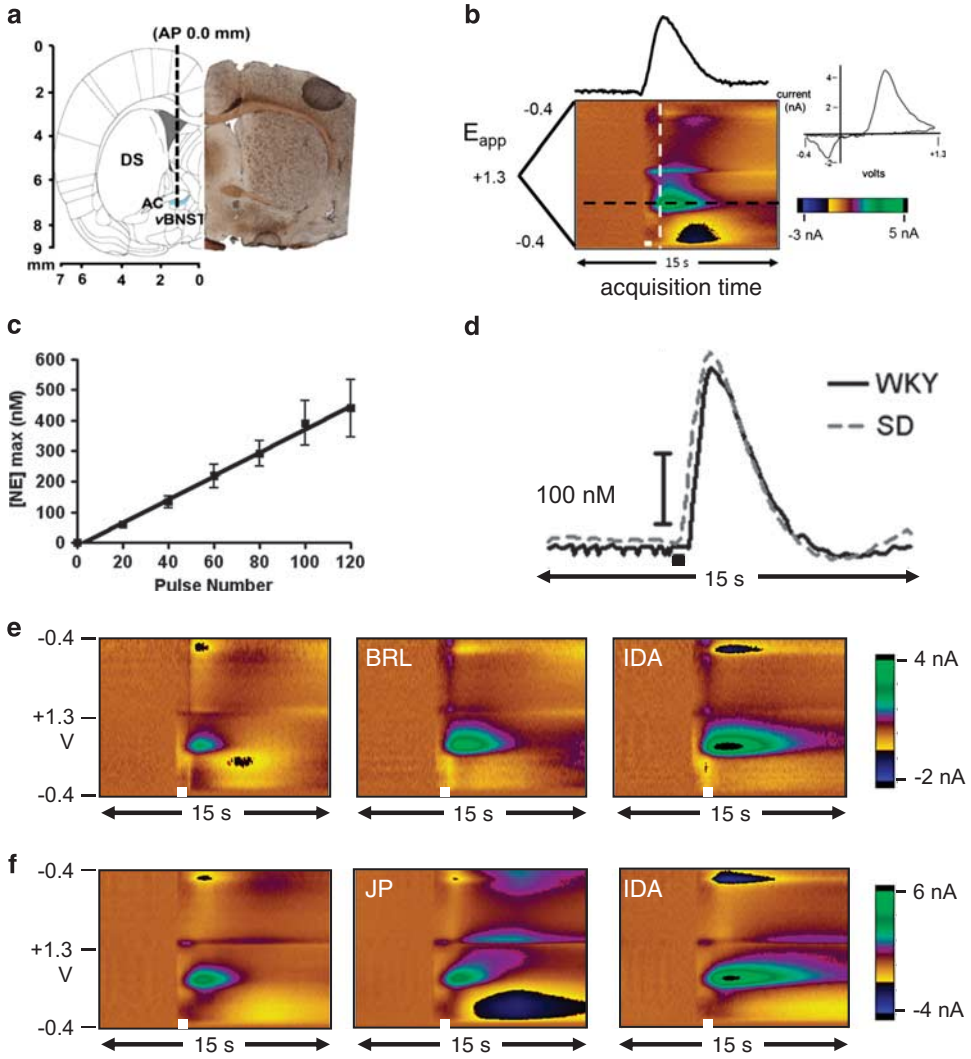

Figure 1 Fast-Scan Cyclic Voltammetry of norepinephrine in the ventral bed nucleus of the stria terminalis (vBNST). (a) Electrode tract (dashed line) and representative histology of carbon-fiber electrode placement in the vBNST (dashed circle; DS, dorsal striatum; AC, anterior commissure). (b) Norepinephrine measured in the vBNST after electrical stimulation (white bar). The cyclic voltammogram (current *vs* potential) is obtained from the white dashed line, and the concentration *vs* time trace from the black dashed line. (c) Input-output curve of $[NE]_{max}$ at 20, 40, 60, 80, and 120 stimulation pulses in WKY rats. (d) Representative concentration traces comparing norepinephrine release and uptake in SD and WKY rats. (e and f) Representative color plots of electrically evoked norepinephrine in the vBNST with α_{2A} (BRL), α_{2C} (JP), and non-selective α_2 (IDA) antagonists on board in WKY rats.

antagonist idazoxan in WKY and SD rats. After drug administration (5 mg/kg), the amplitude in evoked norepinephrine ($[NE]_{max}$) was increased similarly in both strains (WKY: 205 ± 10.3% *vs* SD: 216 ± 21.1%, $n = 6$ and 5, respectively). To examine α_2 subtype-specific effects, we employed α_{2A} and α_{2C} selective antagonists BRL-44408 and JP-1302, respectively. We found increased evoked norepinephrine in WKY rats following 2 mg/kg BRL-44408, but not 2 mg/kg JP-1302 (examples in Figure 1e and f, BRL: 140 ± 6.9% *vs* JP: 95 ± 4.5%, $n = 5$). Similar effects were found in SD rats: (BRL: 157 ± 15.4%. *vs* JP: 102 ± 7.3%, $n = 5$ and 6, respectively). The selected doses (2 mg/kg) approach saturation (Supplementary Figure 1) and are sufficient to produce robust behavioral effects *in vivo* (Sallinen *et al*, 2007). Thus, our findings indicate the α_{2A} subtype is the principle noradrenergic autoreceptor in the vBNST of both WKY and SD rats, and it exerts similar control over norepinephrine release in both strains.

Norepinephrine Dynamics were Differentially Altered in Morphine-Dependent SD and WKY Rats

We hypothesized that SD and WKY rats would not differ in their response to morphine-dependence, owing to their noradrenergic similarity in a baseline state. To compare noradrenergic plasticity, we established morphine-dependence in SD and WKY rats as before (Schulteis *et al*, 1999; McElligott *et al*, 2013). Briefly, rats received 10 mg/kg morphine followed 4 h later by 1 mg/kg naloxone once daily for 3 days. Consistent with our prior work, withdrawal was behaviorally evident in both strains by day 3 (Supplementary Figure 2). On day 4, rats were anesthetized and electrically evoked norepinephrine was recorded. First, we examined the effects on norepinephrine clearance and found clearance half-life showed a main effect of strain ($F = 5.8$, $P < 0.05$) and treatment ($F = 5.8$, $P < 0.05$). There was also a significant treatment × strain interaction (two-way ANOVA, $F = 10.35$,

$P<0.01$). In agreement with our previous work, morphine-dependent SD rats showed impaired uptake relative to controls (2.2 ± 0.16 s *vs* 1.5 ± 0.06 s, $n=12$ and 11, respectively, $P<0.001$). To our surprise, uptake was unaltered in WKY rats (1.5 ± 0.14 s *vs* 1.6 ± 0.10 s, $n=9$ and 10, respectively, $P>0.05$, Figure 2a). As before, the protocol we employed allows time for clearance of morphine and naloxone (Trescot *et al*, 2008), thus altered uptake in SD rats is a consequence of morphine withdrawal.

To assay the effects of morphine withdrawal on α_{2A} function in these two strains, we administered 2 mg/kg of selective antagonist BRL-44408, or agonist guanfacine (2 mg/kg, GFC) and determined the effects on release. The doses were selected based on dose–response analysis in Supplementary Figure 1 and 3. Response to autoreceptor drugs showed a main effect of morphine treatment (two-way ANOVA, BRL: $F=26$, $P<0.0005$; GFC: $F=18.2$, $P<0.005$) and strain (two-way ANOVA, GFC: $F=4.7$ $P<0.05$). *Post hoc* analysis revealed a significant decrease in response to BRL between morphine-dependent animals and their saline-naloxone controls (SD: $102 \pm 3.5\%$ for morphine withdrawal *vs* $167 \pm 9.1\%$ for control, $n=7$, respectively, $P<0.001$; WKY: $104 \pm 6.5\%$ for morphine withdrawal *vs* $147 \pm 6.4\%$ for control, $n=7$, respectively, $P<0.001$, Figure 2b). Additionally, the α_{2A} agonist, GFC, was less effective at inhibiting evoked norepinephrine release in both strains (SD: $87 \pm 3.6\%$ for morphine withdrawal *vs* $65 \pm 4.9\%$ for control, $n=7$, respectively, $P<0.05$; WKY: $102 \pm 8.9\%$ for morphine withdrawal *vs* $75 \pm 3.8\%$ for control, $n=7$, respectively, $P<0.01$, Figure 2c). Thus, following morphine withdrawal, α_{2A} function is desensitized in both SD and WKY rats.

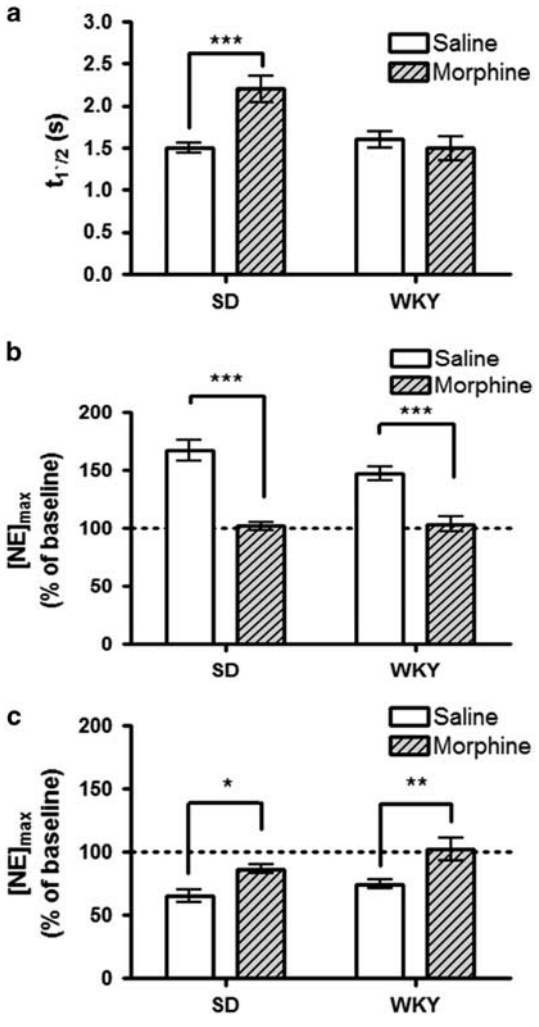

Figure 2 The effects of morphine dependence in Sprague-Dawley and Wistar-Kyoto rats. (a) Average norepinephrine clearance as measured by $t_{1/2}$. (b and c) Average evoked norepinephrine ($[NE]_{max}$) following administration of 2 mg/kg BRL-44408 (b), or guanfacine (GFC) (c) relative to pre-drug stimulated release. Data are presented as mean ± SEM. Bonferroni *post hoc* analysis: $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$.

WKY and SD Rats Exhibited Increased Anxiety-Like Behavior following Morphine-Dependence

We previously showed that morphine withdrawal increases anxiety-like behavior in SD rats (McElligott *et al*, 2013). To examine its impact on WKY rats, we assayed their behavior on the EPM. Withdrawal caused WKY rats to become more anxious, as they had reduced preference for the open arms compared with their saline-naloxone controls (unpaired *t*-test, Open/Closed time: 0.05 ± 0.03 *vs* 0.39 ± 0.12, $n=8$, respectively, $P<0.01$, Table 1). WKY rats treated with saline-naloxone did not demonstrate a change in open-arm preference relative to naïve animals (Table 2, pair-housed).

Social-Isolation Altered Norepinephrine Signaling in SD, but not WKY Rats

To further investigate differences between SD and WKY norepinephrine responses, we treated rats with chronic social isolation. This is a passive stressor that removes injection/handling stress, and is suggested to generate depression in rodents (Nestler and Hyman, 2010; Butler *et al*, 2014). Following 2 weeks of single-housing, rats were anesthetized and evoked norepinephrine was recorded. When comparing norepinephrine uptake between single (S) and pair (P)-housed animals, we found significantly slower uptake in SD-S rats compared with SD-P (2.3 ± 0.09 s *vs* 1.7 ± 0.10 s, $n=20$ and 26, respectively, $P<0.001$). However, WKY-S did not slow uptake relative to pair-housed controls (1.6 ± 0.06 s *vs* 1.6 ± 0.12 s, $n=21$ and 22, respectively, $P>0.05$, Figure 3a).

We next compared α_{2A} drug effects between single and pair-housed animals, and found a main effect of housing (two-way ANOVA, BRL: $F=24.12$, $P<0.0001$; GFC: $F=7.2$, $P<0.05$) and a housing × strain interaction (BRL: $F=8.2$, $P<0.01$; GFC: $F=5.4$, $P<0.05$). *Post hoc* analysis revealed that BRL was less effective in increasing norepinephrine in SD-S animals as compared with SD-P ($97 \pm 1.4\%$ *vs* $158 \pm 11.9\%$, $n=7$, respectively, $P<0.001$, Figure 3b). Similarly, the decrease in release following GFC was attenuated in single-housed animals ($91 \pm 3.2\%$ *vs* $64 \pm 7.9\%$, $n=7$, respectively, $P<0.01$, Figure 3c). WKY-S failed to decrease drug response when compared with WKY-P rats (BRL: $124 \pm 8.9\%$ *vs* $140 \pm 4.8\%$, $n=7$ respectively, $P>0.05$; GFC: $78 \pm 6.4\%$ *vs* $76 \pm 2.2\%$, $n=7$, respectively, $P>0.05$).

Social Isolation Induces Anxiety-Like Behavior in SD Rats

We assayed anxiety-like behavior in singly housed animals on the EPM. Enclosed arm time showed a main effect of

Table 1 Anxiety-like Behavior Following Morphine Withdrawal in WKY Rats

Morphine dependence	Ratio Open/Closed time	Total entries	Open time	Closed time
Wistar-Kyoto				
Saline-Naloxone ($n=8$)	0.39 ± 0.12	6 ± 1	22 ± 4 s	76 ± 14 s
Morphine-Naloxone ($n=8$)	0.05 ± 0.03*	6 ± 1	6 ± 3 s*	126 ± 24 s

Total number of entries and time spent in the open and enclosed arms of the elevated plus maze was evaluated. An animal's preference for open arms was expressed as a ratio of open-arm time over closed-arm time. Data are presented as mean ± SEM. Treated WKY were compared with their controls using an unpaired *t*-test. Starred values denote significance between the treated group and the control directly above it. *$P<0.05$.

Table 2 Anxiety-like Behavior Following Differing Stressors

	Ratio Open/Closed time	Total entries	Open time	Closed time
Social isolation				
Sprague-Dawley				
Pair-housed ($n=7$)	0.33 ± 0.09	14 ± 1	49 ± 11 s	163 ± 10 s
Single-housed ($n=8$)	0.12 ± 0.05*	12 ± 2	20 ± 7 s*	201 ± 12 s*
Wistar-Kyoto				
Pair-housed ($n=8$)	0.26 ± 0.06	9 ± 2	38 ± 8 s	156 ± 10 s
Single-housed ($n=8$)	0.23 ± 0.04	9 ± 1	32 ± 6 s	148 ± 9 s
Coerulean Lesioning				
Sprague-Dawley				
Saline ($n=8$)	0.26 ± 0.01	15 ± 2	47 ± 6 s	165 ± 8 s
DSP-4 lesioned ($n=8$)	0.12 ± 0.03*	6 ± 2*	25 ± 6 s*	207 ± 15 s*
Wistar-Kyoto				
Saline ($n=8$)	0.17 ± 0.05	6 ± 1	25 ± 8 s	162 ± 8 s
DSP-4 lesioned ($n=7$)	0.13 ± 0.03	7 ± 3	24 ± 4 s	193 ± 14 s

Total number of entries and time spent in the open and enclosed arms of the elevated plus maze was evaluated. An animal's preference for open arms was expressed as a ratio of open-arm time over closed-arm time. Data are presented as mean ± SEM. Treated animals were compared with their controls using a two-way ANOVA with Bonferroni *post hoc* analysis. Differences in anxiety following stress were determined for each strain by comparing treated animals with their respective controls. Starred values denote significance between the treated group and the control directly above it. *$P<0.05$.

strain (two-way ANOVA, $F=8.24$, $P<0.01$) and a housing × strain interaction ($F=4.89$, $P<0.05$). Open arm time showed a main effect of housing ($F=4.83$, $P<0.05$). Total number of entries showed a main effect of strain ($F=6.11$, $P<0.05$), and the reduced number of WKY entries agrees with previous studies (Cohen *et al*, 2006; Carr and Lucki, 2010). *Post hoc* analysis revealed a significant reduction in open arm preference in single-housed animals (Open time/Closed time: 0.33 ± 0.09 *vs* 0.12 ± 0.05, SD-S *vs* SD-P, $n=8$ and 7, respectively, $P<0.05$). WKY-S did not increase anxiety-like behavior relative to WKY-P rats (Table 2).

Coerulean Lesion Induced Noradrenergic Plasticity in SD Rats, but not WKY

As both morphine dependence and social stress can alter the firing rate of the LC (Chaijale *et al*, 2013; Van Bockstaele and Valentino, 2013), we wanted to mimic the effects of long-term LC inhibition on BNST norepinephrine and behavior. We lesioned coerulean norepinephrine terminals using the neurotoxin DSP-4, which reduces norepinephrine from LC but not medullary cells (Fritschy and Grzanna, 1989). The vBNST receives little LC input, and stimulation electrode placement in the ventral noradrenergic bundle targets axons primarily from A1/A2 cell groups. Not surprisingly, $[NE]_{max}$ was unchanged by DSP-4 treatment (SD: 219 ± 37 nM *vs* 322 ± 87 nM, $n=7$, and 6, respectively, $P>0.05$; WKY: 230 ± 23 nM *vs* 239 ± 19 nM, $n=7$ and 5, respectively, $P>0.05$). However, differences in synaptic function were found between vehicle and DSP-4-treated SD rats. Clearance half-life showed a strain × treatment interaction ($F=9.5$, $P<0.01$) and main effect of strain ($F=13.2$, $P<0.005$). DSP-4-treated SD rats had slower uptake than their controls (2.6 ± 0.27 s *vs*1.6 ± 0.15 s, $n=7$ and 8, respectively, $P<0.01$). WKY rats were unchanged (1.1 ± 0.23 s *vs* 1.5 ± 0.22 s, $n=5$ and 8, respectively, $P>0.05$, Figure 4a). Response to both BRL and GFC showed a strain × treatment interaction (two-way ANOVA, BRL: $F=6.7$, $P<0.05$; GFC: $F=4.5$, $P<0.05$), and main effect of treatment (BRL: $F=11.9$, $P<0.01$; GFC: $F=5.8$, $P<0.05$). *Post hoc* analysis revealed the response to BRL and GFC was blunted in DSP-4

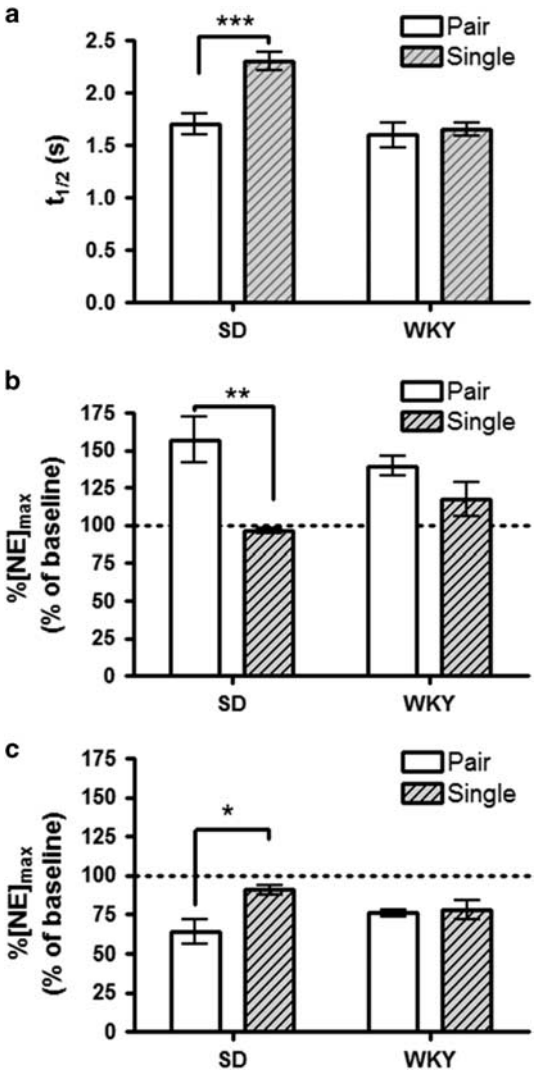

Figure 3 The effects of social isolation in Sprague-Dawley and Wistar-Kyoto rats. (a) Average norepinephrine clearance as measured by $t_{1/2}$. (b and c) Average evoked norepinephrine ($[NE]_{max}$) following administration of 2 mg/kg BRL-44408 (b), or guanfacine (GFC) (c) relative to pre-drug stimulated release. Data are presented as mean ± SEM. Bonferroni *post hoc* analysis: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$.

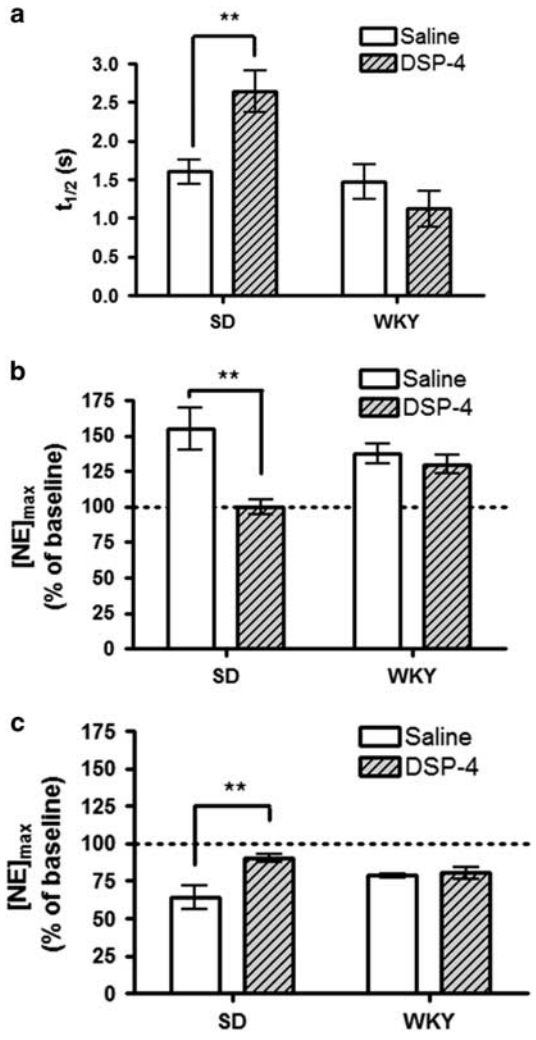

Figure 4 The effects of coerulean lesioning in Sprague-Dawley and Wistar-Kyoto rats. (a) Average norepinephrine clearance as measured by $t_{1/2}$. (b and c) Average evoked norepinephrine ($[NE]_{max}$) following administration of 2 mg/kg BRL-44408 (b) or guanfacine (GFC) (c) relative to pre-drug stimulated release. Data are presented as mean ± SEM. Bonferroni *post hoc* analysis: $^{**}P<0.01$.

treated SD rats (BRL: 100 ± 5.3% *vs* 155 ± 14.7%, $n=5$, respectively, $P<0.05$; GFC: 90 ± 2.8% *vs* 64 ± 7.9%, $n=5$ and 7, respectively, $P<0.01$) but not WKY rats (BRL: 130 ± 6.4% *vs* 138 ± 7.2% $n=5$, respectively, $P>0.05$; GFC: 80 ± 3.8% *vs* 78 ± 1.3% $n=5$, respectively, $P>0.05$, Figure 4c).

Coerulean Lesion Increased Anxiety-Like Behavior in SD Rats

The impact of DSP-4 lesioning on anxiety is dependent on dosage, recovery, and housing (Harro *et al*, 1995; Lapiz *et al*, 2001; Itoi *et al*, 2011). Our treatment increased anxiety-like behavior in SD rats, but not WKY rats as measured on the EPM. DSP-4-treated SD rats spent less time in the open arms, and more time in the enclosed arms (Open time/Closed time: 0.12 ± 0.03 *vs* 0.26 ± 0.01, $n=8$, DSP-4 *vs* saline, respectively, $P<0.05$. The number of entries was also reduced (6 ± 2 *vs* 15 ± 2, $n=8$, respectively, $P<0.05$). DSP-4-treated WKY rats did not show increased anxiety compared with controls (Table 2).

DISCUSSION

Extracellular neurotransmitter concentrations are balanced by release and uptake processes (Wightman *et al*, 1988). Norepinephrine release is controlled by both cell firing and autoreceptor modulation, and the norepinephrine transporter is the primary clearance mechanism (Xu *et al*, 2000) with metabolism operating on a slower time scale (Near *et al*, 1988). Previously, we showed Lewis rats have hindered uptake, blunted autoreceptor function (McElligott *et al*, 2013), and increased anxiety compared with SD rats. When

stressed with morphine withdrawal, Lewis rats showed no change in the regulation of BNST norepinephrine. This was in contrast to the dramatic alterations in norepinephrine clearance rate and autoreceptor function found in drug-dependent SD rats (McElligott *et al*, 2013). Here, we show that naïve SD and WKY rats are indistinguishable from each other with respect to evoked norepinephrine overflow, however, each strain diverges in their adaptations to stress. We found that following morphine withdrawal, WKY rats were similar to SD rats with increased anxiety and attenuated autoreceptor function, however, norepinephrine uptake rate in WKY rats was unaltered. Additionally, BNST norepinephrine control mechanisms were unchanged in socially isolated WKY rats, whereas SD rats challenged with social isolation became more anxious and exhibited exacerbated norepinephrine signaling. Following lesions of norepinephrine in the LC, SD rats showed reduced norepinephrine control and increased anxiety, surprisingly similar to acute morphine dependence, but WKY rats were unaffected. Overall, we find WKY rats respond only to select stressors and that anxiety correlates with the degree of regulation of extracellular norepinephrine in the BNST.

The α_2 receptors are the primary adrenergic autoreceptors, and they show subtype-specific desensitization and phosphorylation by G-protein receptor kinases (Jewell-Motz and Liggett, 1996). As two subtypes of α_2 receptors are expressed in the BNST (Scheinin *et al*, 1994), we paired fast-scan cyclic voltammetry measurements with receptor-subtype-specific pharmacology to assay control over norepinephrine release by each subtype. Knock-out mice were used to determine that α_{2A} acts as the principle autoreceptor (Trendelenburg *et al*, 2001). In agreement with this, we found the inhibition of α_{2A} increased norepinephrine overflow in the vBNST to a similar extent in both rat strains. Although blockade of α_{2C} receptors did not increase norepinephrine overflow, α_{2C} inhibition generated a large alkaline flux following stimulated norepinephrine release, seen in Figure 1f as blue current just below norepinephrine peak oxidation potential. The α_{2C} receptors in the vBNST may therefore be positioned to regulate blood flow/metabolism in the vBNST (Bucher *et al*, 2014).

WKY rats exhibit reduced locomotion in an open field, limited exploration in the EPM, low baseline startle, and limited stress responsivity as compared with SD rats (Pardon *et al*, 2002; Cohen *et al*, 2006). Correspondingly, increases in extracellular norepinephrine in the BNST, evaluated by microdialysis, were greater in SD during restraint stress than WKY rats (Pardon *et al*, 2002). Following the stresses of both social isolation and morphine withdrawal, we found in SD rats that uptake and autoreceptor regulation are downregulated. The decreased noradrenergic control in SD rats could generate the comparatively larger increase in extracellular norepinephrine found with microdialysis. In WKY rats, morphine withdrawal produced only attenuated autoreceptor function, whereas social stress had no effect on norepinephrine control mechanisms. These limited adaptations would result in the smaller alteration in the level of extracellular norepinephrine induced by stress (Pardon *et al*, 2002). Importantly, cellular activation in the BNST during stress is similar between the two strains (Ma and Morilak, 2004). Thus, the greater norepinephrine overflow others have measured in stressed SD likely reflect the changes in uptake rate and desensitized autoreceptors revealed in this work.

Non-specific organic cation transporters (OCTs) are expressed throughout the BNST (Gasser *et al*, 2009). The high-capacity, low-affinity OCT3 is thought to act as a secondary means of norepinephrine clearance, and is inhibited by physiological levels of corticosterone (Gasser *et al*, 2006). The extent to which BNST norepinephrine is taken up by OCTs *in vivo* is not currently known, however, NET knockout mice still demonstrate catecholamine clearance in brain slices (Xu *et al*, 2000) relative to dopamine transporter knockouts (Giros *et al*, 1996), indicating this may be a significant clearance mechanism for norepinephrine. Previously, we showed the altered norepinephrine clearance in SD rats following withdrawal was not due to decreased NET binding sites (McElligott *et al*, 2013). Instead, stress-induced corticosterone release may inhibit OCT3 and reduce clearance rate. WKY rats have elevated peak diurnal levels of corticosterone relative to SD rats (Rittenhouse *et al*, 2002), which may eliminate any OCT3 component of norepinephrine uptake because of chronic inhibition. In addition, WKY rats exhibit behavioral sensitivity to the NET inhibitor desipramine (Lopez-Rubalcava and Lucki, 2000), highlighting the importance of NET in WKY rats. The unaltered norepinephrine uptake in WKY rats corresponds with their low stress responsivity, and is likely the result of HPA axis dysfunction.

Social stressors promote drug self-administration and escalation, generate a long-lasting tolerance to opiate analgesia, and are as efficacious as physical stress at reinstating morphine place preference (Miczek *et al*, 2004; Ribeiro Do Couto *et al*, 2006; Butler *et al*, 2014). Exposure to stressful life events and HPA axis dysfunction have been implicated in the development of several psychiatric disorders that are comorbid with addiction, however, stress alone is not sufficient for their development (Faravelli *et al*, 2012). It has been suggested that stress can interact with genetic vulnerabilities in predisposed individuals to create the psychopathology. Valentino and coworkers(Chaijale *et al*, 2013) found stress activation of the endogenous opioid system sufficient to generate a cellular opiate dependence in SD rats. Here, we used chronic social isolation, a passive stressor suggested to induce depression and anxiety in rodents (Nestler and Hyman, 2010; Butler *et al*, 2014). Remarkably, after SD rats were socially isolated for 2 weeks, they resembled morphine-dependent SD rats, with increased anxiety and enhanced noradrenergic signaling in the vBNST. Moreover, following social isolation, SD rats anxiety and norepinephrine regulation resembled that of our previous work in Lewis rats (McElligott *et al*, 2013), a model of increased drug-intake and PTSD (Cohen *et al*, 2006; Sanchez-Cardoso *et al*, 2007; Picetti *et al*, 2012). In stark contrast, we found that WKY rats were unresponsive to social isolation, exhibiting no changes in anxiety-like behavior or norepinephrine signaling relative to their controls. These results support the idea that genetic differences can predispose individuals to psychological disorders or addictions, as their noradrenergic system may already resemble a drug-dependent or anxious state. Additionally, individuals with anxiety or depression may not be able to appropriately adapt to stress because of low responsivity of the noradrenergic system.

During stress, LC activity is tuned by a balance of CRF excitation, and endogenous opioid inhibition (Van Bockstaele and Valentino, 2013), and chronic stress can decrease LC discharge rates (Chaijale *et al*, 2013). The BNST receives a small input from the LC (Forray and Gysling, 2004), and its activation may be influenced by altered coerulean discharge rates following stress. Thus, we chose to mimic stress-induced LC inhibition by lesioning it with the potent and selective neurotoxin DSP-4. DSP-4 induces degradation of norepinephrine axons arising from the LC, while leaving brainstem norepinephrine innervation intact (Fritschy and Grzanna, 1989). The behavioral effects of DSP-4 treatment are variable (Harro *et al*, 1995; Lapiz *et al*, 2001; Itoi *et al*, 2011), and in our study, we found an anxiogenic effect in SD rats with no change in WKY rats relative to their respective controls. This LC inhibition also lowered the number of maze entries SD rats made to that of WKY. Surprisingly, the DSP-4 treatment produced a robust, dependence-like phenotype in the BNST norepinephrine regulation of SD rats. Such a response was not expected owing to the limited LC innervation of the BNST. These results may reflect feedback between the two main sources of central noradrenergic innervation through a common afferent (Van Bockstaele and Valentino, 2013) and should be a point of future study. Cross-talk between noradrenergic inputs is supported by work demonstrating the importance of both medullary and coerulean norepinephrine in mediating the aversiveness (Delfs *et al*, 2000) and somatic withdrawal signs of withdrawal (Maldonado, 1997). Importantly, LC activation during opiate withdrawal is partly a result of excitatory input from the nucleus paragigantocellularis, a structure that innervates the nucleus of the solitary tract (A2). Following DSP-4 treatment, we found no change in norepinephrine regulation in WKY rats, similar to WKY rats exposed to social isolation stress. However, WKY rats were responsive to morphine withdrawal, the aversiveness of which is due to medullary norepinephrine (Delfs *et al*, 2000). WKY rats overexpress kappa opioid receptors in the LC, leaving the LC in a chronically inhibited state (Carr and Lucki, 2010), a possible explanation for the low number of maze entries and lack of DSP-4 response. This persistent inhibition may attenuate any excitatory influence of CRF and partially explain the lack of noradrenergic facilitation to social isolation. Overall, these results suggest a plasticity of medullary inputs but not LC inputs in the BNST of WKY rats.

We have shown that certain stressors permit exacerbated BNST signaling that is accompanied by increased anxiety. Furthermore, we showed the signaling changes coincide with α_{2A} receptor function and are dependent on rat strain. Social isolation and persistent coerulean inhibition caused dramatic changes in the SD rat, and generated a morphine-dependence-like phenotype. WKY rats were unresponsive to both social isolation and DSP-4 treatment. Following morphine withdrawal, they demonstrated increased anxiety and an intermediate change in norepinephrine signaling: decreased α_{2A} function without a change in norepinephrine clearance. Taken together, this differential responsivity may reflect separate noradrenergic mechanisms for adaptation that depend on the stressor or its intensity. Moreover, our data support the idea that genetic factors contribute to stress response, which may in turn generate cellular conditions that favor drug use and future substance abuse issues.

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The authors declare no conflict of interest.

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