Regulation of CRF mRNA in the Rat Extended Amygdala Following Chronic Cocaine: Sex Differences and Effect of Delta Opioid Receptor Agonism

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Abstract

Background: Cocaine withdrawal activates stress systems. Females are more vulnerable to relapse to cocaine use and more sensitive to withdrawal-induced negative affect. Delta opioid receptors modulate anxiety-like behavior during cocaine withdrawal in rats. This study measured the time course of gene regulation of one of the main stress peptides, corticotropin-releasing factor (CRF), and its type 1 receptor in male and female rats as well as the ability of the delta opioid receptor agonist SNC80 to normalize cocaine withdrawal-induced changes in CRF mRNA.

Methods: Rats were injected with cocaine or saline 3 times daily for 14 days. Brains were collected 30 minutes, 24 hours, 48 hours, 7 days, and 14 days following the last injection. The paraventricular nucleus of the hypothalamus, central amygdala, and bed nucleus of the stria terminalis were processed for quantitative reverse transcriptase PCR measurement of CRF and CRFR1 mRNA. Additional rats received SNC80 during early cocaine withdrawal, and CRF mRNA was measured in the central amygdala.

Results: CRF mRNA was elevated in the central amygdala at 24 hours and the paraventricular nucleus at 48 hours of cocaine withdrawal in males and females. Significant sex differences in cocaine-induced CRF upregulation were found in the bed nucleus of the stria terminalis at 30 minutes and 24 hours. SNC80 administration attenuated the increase in CRF mRNA in the central amygdala of female rats only.

Conclusions: CRF mRNA regulation during cocaine withdrawal is sex, time, and brain region dependent. Administration of a delta opioid receptor agonist during early withdrawal may ameliorate stress-related negative affect in females by abrogating the induction of CRF mRNA.

Key words: amygdala, bed nucleus of the stria terminalis, CRF, delta opioid receptor, sex differences

Introduction

Cocaine abuse and dependence are major public health problems with serious societal and economic consequences. Preventing cycles of relapse to drug use is the main goal of treatment for substance use disorders, yet there is currently no US Food and Drug Administration-approved pharmacological therapy for cocaine use disorder. Stress and withdrawal-induced negative affect greatly contribute to relapse vulnerability, particularly in females. Chronic cocaine use and withdrawal...
persistent activation of the hypothalamic-pituitary-adrenal (HPA) axis (Kuhn and Francis, 1997; Richter and Weiss, 1999; Koob, 2008) and involve the extended amygdala, which consists of the interconnected central amygdala (CeA), bed nucleus of the stria terminalis (BNST), and nucleus accumbens shell. Interaction between the HPA axis and extended amygdala stress systems is important in both the development of cocaine addiction and the withdrawal syndrome following cocaine cessation (Sarnyai et al., 1995; Richter and Weiss, 1999; Zorrilla et al., 2001; Maj et al., 2003) have been reported in these brain regions at different time points following chronic cocaine administration. The majority of these studies have investigated male rats. As female humans and rats progress from cocaine abuse to dependence more quickly (Griffin et al., 1989; Becker and Hu, 2008), they are more sensitive to the subjective effects of cocaine (Lucas et al., 1996; Sofuoglu et al., 1999; Back et al., 2005), and are more vulnerable to relapse (Erb et al., 1998; Hudson and Stamp, 2011), all stages of addiction involving stress systems, it is important to consider the underlying mechanisms responsible for these sex differences (Lynch et al., 2002; Lynch, 2018). Delta opioid receptors (DOR) play an important role in not only analgesia but also regulation of mood. DOR signaling is disrupted by chronic cocaine use, which may contribute to the negative effect experienced during cocaine withdrawal (Perrine et al., 2008). Indeed, cocaine withdrawal-induced anxiety is reduced by the selective DOR agonist SNC80 in both male and female rats (Perrine et al., 2008; Ambrose-Lanci et al., 2010). The molecular or cellular mechanisms underlying the ability of DOR agonists to reduce cocaine withdrawal-induced anxiety remain unknown. DOR and CRF are colocalized in the central and basolateral amygdala; DOR are found on dendrites of CRF-containing neurons and in axon terminals targeting CRF neurons (Reyes et al., 2017). This anatomical arrangement suggests that DOR and CRF systems interact to regulate stress-related responses, and this interaction may be implicated in negative affective states during cocaine withdrawal.

Building on previous work, this study aimed to provide a comprehensive time course analysis of gene regulation of CRF and its type 1 receptor in the CeA, PNV, and BNST of both male and female rats following chronic cocaine administration. Our preliminary studies did not find CRF or CRF-R1 mRNA regulation in the nucleus accumbens shell; therefore, this region was excluded from further study. An additional aim of this study was to investigate the ability of DOR agonist SNC80 to normalize cocaine withdrawal-induced increases in CRF gene expression in the CeA given its ability to reduce withdrawal-induced anxiety.

### Methods

#### Animals

Male and female Sprague Dawley rats (7 weeks old on arrival, Charles River Laboratories) were housed in pairs of the same sex in a room on a 12-hour-light/ dark cycle (lights on at 7:00 AM) for at least 1 week before experiments began. Rats had access to standard rat chow and water ad libitum. All procedures were in compliance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals and approved by Temple University’s Institutional Animal Care and Use Committee.

#### Drugs

Cocaine HCl (generously supplied by the National Institute on Drug Abuse drug supply program) was dissolved in 0.9% normal saline and injected i.p. in a volume of 1 mL/kg body weight. SNC80 ((+)-4-[[25,5R]-4-allyl-2,5-dimethyl-1-piperazinyl]-3-methoxybenzyl]-N,N-diethylbenzamide) (Tocris Bioscience, Minneapolis, MN) was dissolved in 2 µL of 1 M HCl per milligram SNC80, and sterile water was slowly added with vortexing to achieve a concentration of 10 mg/mL. SNC80 was injected s.c. in a volume of 1 mL/kg body weight. Rats were injected with saline or 15 mg/kg cocaine 3 times per day, 1 hour apart (i.e., binge pattern) starting at 9:30 AM for 14 days. An additional cohort of rats was injected with saline or cocaine for 14 days, followed by 10 mg/kg SNC80 or vehicle 8 hours and 20 hours (2 SNC80 injections total) following the last cocaine or saline injection. Brains were obtained 24 hours post last cocaine or saline injection.

#### Brain Tissue Collection

Rats were killed 30 minutes, 24 hours, 48 hours, 7 days, or 14 days following the last cocaine or saline injection by brief CO2 anesthesia followed by decapitation. Brains were removed, flash frozen in isopentane (−35°C to −40°C), and stored at −80°C. Frozen brains were sliced on a cryostat microscope, and 1-mm punches were used to dissect bilateral PNV, BNST, and CeA (supplementary Figure 1) (Paxinos and Watson, 2007) from 2 slices 300 µm thick. Tissue punches were placed in RNAlater-ICE (Invitrogen, Waltham, MA) overnight at −20°C before storage at −80°C.

#### Quantitative Reverse Transcriptase Polymerase Chain Reaction

Total RNA was isolated using a Quick-RNA Miniprep kit (Zymo Research, Irvine, CA), and RNA concentration was measured using a NanoDrop 2000 spectrophotometer. Samples were diluted to the same RNA concentration before cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA). Quantitative RT-PCR was performed...
using TaqMan Fast Advanced Master Mix and TaqMan Gene Expression Assays for CRF (Rn01462137_m1), CRFR1 (Rn00578611_m1), and the control 18S rRNA (Hs99999901_s1). Relative fold change was calculated using the ΔΔCt method (Livak and Schmittgen, 2001). To facilitate analysis of sex differences, quantitative fluorescent data were collected simultaneously from samples from 1 brain region at a single time point from both sexes. For time course data within individual sexes, mRNA levels in cocaine-injected rats were normalized to saline controls of the same sex at the same time point. To identify sex differences, mRNA levels in cocaine-injected males, cocaine-injected females, and saline-injected females were all normalized to male saline controls, which did not differ across the time course.

Statistics
Statistical analyses were performed with GraphPad Prism software using 2-way ANOVA (cocaine × time, cocaine × sex, or cocaine × SNC80), followed by Sidak post tests for planned comparisons between saline- and cocaine-injected rats at individual time points. *P < .05 was considered significant.

Results
Time Course of CRF Gene Expression in the CeA, PVN, and BNST
CRF mRNA was measured 30 minutes, 24 hours, 48 hours, 7 days, and 14 days following 14 days of binge-pattern cocaine or saline injections in male and female rats. In the CeA of female rats (Figure 1A), 2-way ANOVA indicated a significant interaction (F[4,38] = 3.240, P = .0221) between the main effects of cocaine (F[1,38] = 2.035, P = .1619) and time (F[4,38] = 2.020, P = .1112). Post hoc analysis revealed that CRF mRNA was elevated compared with saline-injected controls 24 hours following cocaine (3.05-fold, P = .0084) but not at other time points (supplementary Table 1). Similarly, 2-way ANOVA showed an increase in CRF mRNA in the CeA of cocaine-injected males vs saline-injected controls (Figure 1D) (interaction F[4,48] = 3.884, P = .0082; cocaine F[1,48] = 0.1017, P = .9181; time F[4,48] = 4.436, P = .0039), which was significant at 24 hours only (2.02-fold, post hoc P = .0255) (supplementary Table 1).

No significant differences in CRF mRNA levels between cocaine- and saline-injected males were found in the PVN at any time point (Figure 1E; supplementary Table 1). In the PVN of female rats (Figure 1B, 2-way ANOVA found a significant effect of time (F[4,48] = 3.039, P = .0287; cocaine F[1,38] = 3.784, P = .0592; interaction F[4,48] = 2.220, P = .0850), and post hoc analysis indicated that CRF mRNA was significantly elevated in cocaine-injected females compared with saline-injected controls (4.01-fold, post hoc P = .0081) 48 hours following the last injection (supplementary Table 1).

In the BNST of female rats (Figure 1C), 2-way ANOVA revealed a significant interaction (F[4,39] = 3.027, P = .0288) between time (F[4,39] = 2.624, P = .0493) and cocaine (F[1,39] = 2.274, P = .1396). Compared with saline-injected controls, CRF mRNA was significantly elevated (2.05-fold, post hoc P = .0105) 24 hours following the last cocaine injection (supplementary Table 1). In the BNST of male rats (Figure 1F), 2-way ANOVA indicated a significant interaction (F[4,46] = 15.58, P < .0001) as well as significant main effects of time (F[4,46] = 17.29, P < .0001) and cocaine (F[1,46] = 16.32, P = .0002). Compared with saline controls, CRF mRNA was elevated in the BNST of male rats 30 minutes post cocaine (6.01-fold, post hoc P < .0001) but not at other time points (supplementary Table 1).

Time Course of CRFR1 Gene Expression in CeA, PVN, and BNST
CRFR1 mRNA was measured 30 minutes, 24 hours, 48 hours, 7 days, and 14 days following 14 days of binge-pattern cocaine or saline injections in male and female rats. In the CeA of female rats (Figure 1A), 2-way ANOVA indicated a significant interaction (F[4,38] = 3.240, P = .0221) between the main effects of cocaine (F[1,38] = 2.035, P = .1619) and time (F[4,38] = 2.020, P = .1112). Post hoc analysis revealed that CRF mRNA was elevated compared with saline-injected controls 24 hours following cocaine (3.05-fold, P = .0084) but not at other time points (supplementary Table 1). Similarly, 2-way ANOVA showed an increase in CRF mRNA in the CeA of cocaine-injected males vs saline-injected controls (Figure 1D) (interaction F[4,48] = 3.884, P = .0082; cocaine F[1,48] = 0.1017, P = .9181; time F[4,48] = 4.436, P = .0039), which was significant at 24 hours only (2.02-fold, post hoc P = .0255) (supplementary Table 1).

No significant differences in CRF mRNA levels between cocaine- and saline-injected males were found in the PVN at any time point (Figure 1E; supplementary Table 1). In the PVN of female rats (Figure 1B, 2-way ANOVA found a significant effect of time (F[4,48] = 3.039, P = .0287; cocaine F[1,38] = 3.784, P = .0592; interaction F[4,48] = 2.220, P = .0850), and post hoc analysis indicated that CRF mRNA was significantly elevated in cocaine-injected females compared with saline-injected controls (4.01-fold, post hoc P = .0081) 48 hours following the last injection (supplementary Table 1).

In the BNST of female rats (Figure 1C), 2-way ANOVA revealed a significant interaction (F[4,39] = 3.027, P = .0288) between time (F[4,39] = 2.624, P = .0493) and cocaine (F[1,39] = 2.274, P = .1396). Compared with saline-injected controls, CRF mRNA was significantly elevated (2.05-fold, post hoc P = .0105) 24 hours following the last cocaine injection (supplementary Table 1). In the BNST of male rats (Figure 1F), 2-way ANOVA indicated a significant interaction (F[4,46] = 15.58, P < .0001) as well as significant main effects of time (F[4,46] = 17.29, P < .0001) and cocaine (F[1,46] = 16.32, P = .0002). Compared with saline controls, CRF mRNA was elevated in the BNST of male rats 30 minutes post cocaine (6.01-fold, post hoc P < .0001) but not at other time points (supplementary Table 1).

Figure 1. Withdrawal from chronic cocaine administration increases corticotropin-releasing factor (CRF) mRNA in a time- and brain region-specific manner in female (top) and male (bottom) rats. Fold changes in CRF mRNA compared with saline-injected controls of the same sex are shown for 5 time points after the last injection of saline or cocaine in the central amygdala (CeA; A, D), paraventricular nucleus (PVN; B, E), and bed nucleus of the stria terminalis (BNST; C, F). Post hoc tests: *P < .05, **P < .01, ****P < .0001. n = 5-7 rats/time point/group.
saline injections in both males and females. No significant differences (supplementary Table 1) were found in CRFR1 expression between cocaine- and saline-injected male (Figure 2D–F) or female (Figure 2A–C) rats in the CeA, PVN, or BNST.

**Sex Differences in CRF and CRFR1 Gene Regulation**

At the majority of time points and brain regions, males and females did not differ significantly when analyzed together (i.e., cocaine-injected males, cocaine-injected females, and saline-injected females were all normalized to male saline-injected controls); however, some sex differences did emerge. Thirty minutes following the last injection, 2-way ANOVA indicated a significant main effect of sex ($F[1,17] = 25.73, P < .0001$; cocaine $F[1,17] = 0.2262, P = .6404$; interaction $F[1,17] = 0.0271, P = .8712$), and post hoc tests showed that the CeA of both saline- and cocaine-injected females contained less CRF mRNA compared with male saline controls (0.317-fold, $P = .0073$ and 0.411-fold, $P = .0190$, respectively) (Figure 3A).

In the BNST 30 minutes following the last injection, 2-way ANOVA revealed a significant interaction between the main effects of sex and cocaine ($F[1,16] = 5.319, P = .0133$), but compared with male saline controls, post hoc tests showed the BNST contained significantly higher amounts of CRF mRNA in both saline- (6.92-fold, $P < .0001$) and cocaine-injected (8.33-fold, $P < .0001$) females (Figure 3B). Additionally, the BNST of both saline- and cocaine-injected female rats contained significantly more CRFR1 mRNA than that of saline- and cocaine-injected males 30 minutes following the last injection (Figure 3D) (2-way ANOVA, sex $F[1,16] = 24.88, P = .0001$; interaction $F[1,16] = 0.3297, P = .5738$; cocaine $F[1,16] = 0.1153, P = .7386$).

Another difference between sexes became apparent in the BNST 24 hours following the last injection (2-way ANOVA, sex $F[1,16] = 19.27, P = .0005$; interaction $F[1,16] = 6.059, P = .0256$; cocaine $F[1,16] = 4.235, P = .0563$). While CRF mRNA levels in saline- and cocaine-injected males did not differ (Figure 1F), CRF mRNA was higher in cocaine-injected females (1.61-fold) compared with saline-injected females (0.797-fold, $P = .0041$) (Figure 3C) when normalized to saline control males.

**Effect of SNC80 Administration on CRF mRNA Levels in the Central Amygdala and Bed Nucleus of the Stria Terminalis**

The effect of SNC80 on CRF mRNA levels in the CeA and BNST was assessed in male and female rats 24 hours following the last injection. In the CeA of females, 2-way ANOVA found a significant interaction ($F[1,23] = 7.189, P = .0133$) between the main effects of SNC80 ($F[1,23] = 7.042, P = .0142$) and cocaine ($F[1,23] = 0.2878, P = .5968$). Post hoc tests showed CRF mRNA levels in the CeA of cocaine-injected rats treated with SNC80 were significantly lower compared with cocaine-injected rats given vehicle ($P = .0058$; Figure 4A). In contrast to the findings in females, SNC80 administration did not regulate CRF mRNA levels in the CeA of male rats 24 hours following the last injection (Figure 4B; supplementary Table 1).

CRF mRNA in the BNST of female rats was higher 24 hours following chronic cocaine and normalized by SNC80 administration (Figure 4C); however, this did not reach statistical significance due to variability between animals. CRF mRNA levels in the BNST of males were not regulated by SNC80 administration (Figure 4D; supplementary Table 1).

**Discussion**

This study investigated the regulation of CRF and CRFR1 gene expression during early and protracted withdrawal from chronic cocaine administration. CRF mRNA was significantly higher in the CeA of both male and female rats injected with cocaine compared with those injected with saline 24 hours after the last injection.
injection. Regardless of treatment group, the CeA of female rats contained less CRF mRNA than males, and the BNST of females contained more CRFR1 mRNA than males when measured 30 minutes after the last injection. Additional sex differences in CRF regulation emerged in the BNST. Thirty minutes following the last injection, CRF mRNA levels in cocaine-injected males and both saline- and cocaine-injected females were higher than that of saline-injected male controls. Twenty-four hours later, CRF mRNA content returned to control levels in males and saline-injected females, while CRF mRNA remained elevated in cocaine-injected females.

The experiments described in this manuscript are based on an important foundation of previous work. Expanded by a thorough time-course analysis during withdrawal and by the addition of females treated in parallel to males, the present results support previous findings and extend those results in several ways. For example, 2 studies found an increase in CRF mRNA in the CeA of male rats 24 hours post cocaine (Maj et al., 2003; Erb et al., 2004), which is replicated here. In contrast, other studies have found an increase in CRF mRNA in the amygdala at 48 hours, not 24 hours, post cocaine (Zhou et al., 2003a; Rudoy et al., 2009). While the current study and others (Mantsch et al., 2007) show no significant differences in CRF mRNA in the PVN, another found a decrease in CRF mRNA in the hypothalamus 30 minutes following the last cocaine injection (Zhou et al., 1996). Congruent with the present study, previous work reported no differences in CRF mRNA in the BNST after 1, 3, or 10 days withdrawal (Erb et al., 2004). The reason(s) for discrepancies in CRF mRNA levels in the amygdala and hypothalamus are uncertain; however, differences may be due to the tissue included in the analysis. Studies that analyzed the whole amygdala found increases in CRF mRNA at 48 hours following chronic cocaine administration (Zhou et al., 2003a; Rudoy et al., 2009), while studies that selectively analyzed only the CeA agreed on an increase in CRF mRNA at 24 hours (Maj et al., 2003; Erb et al., 2004). It should be noted that other nuclei of the amygdala, such as the basolateral amygdala, also contain CRF and play a role in cocaine withdrawal-induced negative affect, which may have affected the study outcome. In similar fashion, results from studies investigating the whole hypothalamus (Zhou et al., 1996, 2003a, 2003b) differ from those that processed the PVN specifically (Mantsch et al., 2007; this study). Inter-animal variability in CRF mRNA expression levels in the PVN in the current study may also be a factor in the different outcomes. In terms of lack of changes in gene expression during long-term withdrawal, the present findings support several studies that showed no differences in CRF mRNA levels between rats given chronic saline or cocaine at longer withdrawal time points (i.e., 7, 10, or 14 days post cocaine) (Zhou et al., 1996, 2003a, 2003b; Erb et al., 2004). It should be noted that all of the above cited studies investigated male animals. To the authors’ knowledge, regulation of CRF mRNA during withdrawal from chronic cocaine has not been reported in females.

This study investigated the effect of the DOR agonist SNC80 on elevated CRF mRNA levels in the CeA during cocaine withdrawal. SNC80 administration significantly reduced elevated CRF mRNA levels in females but had no effect on CRF mRNA in males. This was unexpected given that DOR agonists can modulate anxiety-like behavior in both sexes (Perrine et al., 2006, 2008; Ambrose-Lanci et al., 2010; Randall-Thompson et al., 2010; van Rijn et al., 2010). Why SNC80 reduced CRF mRNA in females but not males is unclear. One possible explanation could be the timing of SNC80 administration. In previous behavioral studies, SNC80 was injected 30 to 60 minutes before anxiety testing. In the current study, SNC80 was injected 8 hours and 20 hours

**Figure 3.** Sex differences in regulation of corticotropin-releasing factor (CRF) and CRFR1 mRNAs during early withdrawal from chronic cocaine administration. Fold changes (compared with male saline controls) in CRF (A–C) and CRFR1 (D) mRNAs are shown in the central amygdala (CeA; A) and bed nucleus of the stria terminalis (BNST; B–D) of male and female rats either 30 minutes (A, B, D) or 24 hours (C) following the last injection. ANOVA main effect of sex: ### $P < .001$, #### $P < .0001$. Post hoc tests: **$P<.01$, ***$P<.001$, ****$P<.0001$, n=5–7 rats/group.
following the last cocaine or saline injection (i.e., 4 hours before brain collection). It is possible that the timing of CRF regulation by SNC80 differs between males and females. Sex differences in subcellular distribution of DORs have been documented in the hippocampus following stress (Mazid et al., 2016) and in the nucleus accumbens following chronic cocaine (Ambrose-Lanci et al., 2008). This may contribute to the difference in regulation of CRF by DOR agonists. It is likely that the effect of DOR agonists on CRF expression and, subsequently, downstream effects of CRF are modulated by a network of brain areas. Recent studies provide evidence of sex differences in stress-related brain circuits. Using cFos as a marker of neuronal activation, Bangasser and colleagues measured functional connectivity between stress-related brain regions and found sex differences in activated networks both in unstressed rats as well as in rats that received CRF by icv injection (Wiersielis et al., 2016; Salvatore et al., 2018). They further showed that behavioral responses to CRF, such as anxiety, are regulated by different brain area connections in males and females. Further studies investigating different SNC80 administration protocols or measuring CRF protein levels are needed to address the noted sex difference in CRF regulation by DOR agonists.

A growing body of literature highlights sex differences in the escalation of cocaine use, the subjective effects of cocaine, and susceptibility to relapse. One of the main triggers to relapse is stress, and sex differences in stress systems have emerged as a critical factor driving substance abuse and relapse in women. Cocaine-using women are more sensitive to CRF (Brady et al., 2009), and female rats show greater HPA axis activation in response to cocaine withdrawal (Kuhn and Francis, 1997; Fox et al., 2006). Female rats are also more sensitive to stress-induced reinstatement of cocaine seeking (Erb et al., 1998; Anker and Carroll, 2010; Buffalari et al., 2012). One possible explanation for this is the difference in CRF receptor trafficking and signaling. Sex differences in CRF mRNA (compared with saline + vehicle control) in the central amygdala (A, B) and bed nucleus of the stria terminalis (C, D) are shown for female (A, C) and male (B, D) rats injected with SNC80 or vehicle following chronic cocaine or saline administration. Brain tissue was obtained 24 hours following the last cocaine or saline injection. Post hoc tests: **P < .01. n = 6–8 rats/group.

While changes in CRF mRNA during withdrawal from chronic cocaine have been described in males, this study identifies important sex differences in CRF and CRF1 regulation in the CeA and particularly in the BNST. Perhaps these differences play a role in females’ increased vulnerability to cocaine withdrawal-induced negative affect and relapse. Since SNC80 mitigated cocaine withdrawal-induced increases in CRF mRNA in the CeA of females and mitigated withdrawal-induced anxiety (Perrine et al., 2008; Ambrose-Lanci et al., 2010), DOR agonists may be a valuable tool for managing withdrawal symptoms and preventing relapse, especially in females.

**Supplementary Materials**

Supplementary data are available at International Journal of Neuropsychopharmacology (IJNPPY) online.

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Statement of Interest

None.

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