Role of mesolimbic endocannabinoid signaling in stress-driven cocaine use in rats

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Abstract

Stress is prevalent in the lives of those with substance use disorders (SUDs) and influences SUD outcomes. Understanding the neurobiological mechanisms through which stress promotes drug use is important for the development of effective SUD interventions. We have developed a model wherein exposure to a stressor, uncontrollable electric footshock, daily at the time of cocaine self-administration (SA) escalates intake in male rats. Here we test the hypothesis that stress-induced escalation of cocaine SA is the result of persistent recruitment of endocannabinoid signaling. Male Sprague-Dawley rats self-administered cocaine (0.5 mg/kg/inf, i.v.) during 2-h sessions comprised of four 30-min SA components separated by 5-min shock sequences or 5-min shock-free periods for 14 days. Footshock produced an escalation of cocaine SA that persisted following shock removal. Systemic administration of the cannabinoid receptor type 1 (CB1R) antagonist, AM251, attenuated cocaine intake only in rats with a history of stress. This effect was localized to the mesolimbic system, as intra-nucleus accumbens (NAc) shell and intra-ventral tegmental area (VTA) micro-infusions of AM251 also attenuated cocaine intake only in stress-escalated rats. Cocaine SA, regardless of stress history, increases CB1R binding site density in the VTA, but not NAc shell. Following extinction, cocaine-primed reinstatement (10 mg/kg, ip) was increased in rats with prior footshock during SA. AM251 attenuated reinstatement only in rats with a stress history. Altogether, these data suggest that repeated stress at the time of cocaine use recruits endocannabinoid signaling in mesolimbic regions to escalate intake and heighten relapse susceptibility.

Introduction

Despite intensive research efforts, substance use disorders (SUDs) remain among society's most significant public health challenges, and there is a pressing need for more effective interventions. While emphasis has recently been placed on opioids, cocaine use is a persistent problem. There is a strong link between stress and cocaine use. Co-morbidity between cocaine use disorder (CUD) and stress-related conditions [1], notably PTSD, is high [2]. Measures of cumulative lifetime stress positively correlate with CUD severity [3]. Life stress often precedes cocaine use [4-6] and can predict relapse [7; 8] and poor treatment outcomes [9]. Further, relapse susceptibility is greater in those with lower distress tolerance [10]. In a lab setting, personalized stress imagery can precipitate cocaine craving [11; 12] and the magnitude of craving and associated physiological responses predicts subsequent relapse [8; 13].

A key characteristic of SUDs is a progressive loss of control over drug intake which can be observed in rodents as escalating patterns of drug self-administration (SA) [14; 15]. We have demonstrated that delivery of electric footshock stress, daily at the time of SA, produces an emergent escalation of cocaine intake in male rats [16]. Shock-induced escalation of cocaine SA involves neuroadaptations that require elevated glucocorticoids [16], as does escalation of SA in rats provided daily long-access to the drug [17; 18]. Endocannabinoid signaling is glucocorticoid-regulated [19-24], is involved in buffering stress responses and is affected by chronic stress [25; 26]. Polymorphisms in the cannabinoid type 1 receptor (CB1R) gene, *cnr1*, are associated with CUD [27]. In rodents, CB1R antagonism has minimal effects on cocaine SA under conditions of stable/non-escalated intake [28-31]. However, in rats displaying escalated intake following repeated daily long-access SA, the CB1R antagonist, rimonabant, reduced breakpoints when tested for cocaine SA under a progressive ratio schedule that are not observed after daily short-access SA [31]. Altogether these findings suggest that endocannabinoid signaling via CB1Rs is regulated by stress, recruited under conditions resulting in escalated drug use, and promotes drug SA. Accordingly, we hypothesize that stressor-induced escalation of cocaine SA involves heightened CB1R signaling in brain regions implicated in cocaine use.

VTA (ventral tegmental area) dopamine neurons projecting to the nucleus accumbens (NAc) shell comprise a mesolimbic pathway that is critical for cocaine SA [32-35]. Dopaminergic signaling is regulated in both regions

by endocannabinoids [36-41]; and both cocaine and stress can influence VTA and NAc endocannabinoid signaling [31; 42-44]. Indeed, escalated cocaine intake observed under long-access SA conditions is associated with an upregulation of NAc shell CB1Rs and is selectively reduced by intra-NAc shell micro-infusions of rimonabant [31]. Here we test the hypothesis that repeated stress at the time of cocaine SA recruits VTA and/or NAc shell endocannabinoid signaling to escalate cocaine use. Further, we hypothesize that this recruitment of CB1R/endocannabinoid signaling is long-lasting and heightens susceptibility to later cocaine-primed reinstatement.

Materials and Methods

Additional methodological details are described in the supplemental methods.

Subjects: 155 Male Sprague-Dawley rats (60 days old; 275-300g at arrival; Envigo, Indianapolis, IN) were housed individually in a temperature- and humidity-controlled AAALAC-accredited animal facility on a 12:12 hr reverse light/dark cycle (0700-1900 lights off) with *ad libitum* food and water access. Procedures were conducted in the dark/active phase of the rat light cycle. Procedures were approved by the Institutional Animal Care and Use Committee at Marquette University and conducted in compliance with NIH Guidelines.

Surgery: All rats received intravenous catheters for drug SA. Some rats also received intra-NAc shell or intra-VTA guide cannula immediately following the catheter surgery. Guide cannula were implanted 0.5 mm above the target brain region. Rats recovered for a minimum of one week before the commencement of SA training.

Cocaine SA: Rats were trained to self-administer cocaine (0.5 mg/kg/0.2 ml infusion over 5 sec) by lever pressing under a fixed ratio 4 schedule during daily sessions as described [16] (Fig 1A). The program consisted of four 30-min SA blocks wherein the levers were extended, the active lever light was on, and the houselight was off. During the infusion, the active lever light was extinguished for a 10-sec time-out period during which presses were recorded but not reinforced. Separating the four 30-min SA blocks (and preceding the first block) were 5-min drug-free periods during which the houselight was on, the levers were retracted, and the active lever light was off.

Experiment 1: Electric footshock stress-induced escalation of cocaine intake

Rats were separated into two groups following establishment of baseline SA (<10% change in infusions over 3 days) (Fig. 1A). The “no shock” group continued to self-administer cocaine for 14 days. The “shock” group received intermittent electric footshock stress (3 x 0.5mA, 500 msec duration, 30-sec average inter-shock interval) during the four 5-min drug-free components over the 14-day SA period. To test whether stress-induced escalation of cocaine intake persisted in the absence of the stressor, the shock component was removed after the 14-day SA period and the rats were allowed to self-administer cocaine for at least 5 days under “no shock” conditions. Control rats continued to self-administer under the same parameters during this time.

Experiment 2: Effect of systemic CB1R antagonism on stress-escalated cocaine intake

Rats received CB1R antagonist (AM251) injections prior to SA. Rats self-administered cocaine for 14 days after which the shock components were removed for at least one day prior to testing with AM251. During testing, rats received injections of AM251 (1 mg/kg, i.p.) or vehicle (1:1:18 ethanol: Cremaphor: saline) 30 min before the SA session (Fig. 1E).

Experiment 3: Effect of CB1R antagonism in the NAc shell and VTA on stress-escalated cocaine intake

Following 14 days of SA under no shock/shock conditions, rats received bilateral intra-NAc shell or intra-VTA AM251 (1, 3 µg/0.3 µL/side) or vehicle (100% DMSO) micro-infusions were tested for SA under shock-free conditions (15-min pretreatment; see Experiment 2). Each rat received AM251 and vehicle in counter-balanced order. After the experiment, rats were euthanized, and brains were collected for cannula placement verification. Only rats with confirmed placements were included for analyses.

Experiment 4: CB1R binding changes in the NAc shell and VTA following stress-induced escalation of cocaine intake

To examine CB1R binding, a binding assay was performed in postmortem tissue. Rats self-administered under no shock/shock conditions (see Experiment 1). Saline control groups were added to isolate changes in binding resulting from cocaine SA or repeated shock alone. Saline access was provided under parameters otherwise identical to those for cocaine. Rats were euthanized, brains were collected and frozen 24h after the last session. NAc shell and VTA were isolated from frozen brains and CB1R binding determined (Supplemental Methods).

Experiment 5: Cocaine-primed reinstatement following stress-induced escalation of cocaine intake; requirement for CB1R

Following 14 days of SA, rats underwent extinction training wherein cocaine was replaced with saline. Once extinction criteria were met (<15 lever presses/2h), reinstatement testing was conducted during which rats received cocaine injections (0, 2.5, 5, 10 mg/kg, i.p.) immediately prior to the test session. Each rat received all doses in counter-balanced sequence. Rats met extinction criteria between consecutive reinstatement tests. To test the role of CB1R signaling in enhanced cocaine-induced reinstatement, rats underwent SA and extinction/reinstatement. On reinstatement test day, rats received AM251 (1 mg/kg, i.p.) or vehicle followed by a cocaine (10 mg/kg, i.p.) or saline injection 30 min later, immediately followed by reinstatement testing. The sequence of AM251/vehicle tests was counter-balanced.

Statistical analysis: Statistical analyses were conducted using 2-way or 3-way repeated measures ANOVA, followed by Dunnett's or Holm-Sidak-corrected post hoc tests when appropriate.

Results

For all experiments, relevant statistical analyses are reported in the main text and full statistical analyses are reported in supplemental tables (Tables S1-4).

Experiment 1: Electric footshock stress-induced escalation of cocaine intake

Study design is shown in Fig 1a. Rats receiving footshock during cocaine SA, but not no-shock controls, showed significant escalation of cocaine SA (Fig. 1b-e). Two-way ANOVA revealed a significant stress x SA day interaction for cocaine infusions (Fig. 1B; $F_{(14,700)}$=4.719, p<.0001) and active (Fig. 1C; $F_{(14, 700)}$=2.561, p<.01), but not inactive, lever presses (Fig. 1C; $F_{(14, 700)}$=1.539, p=.09). There were no significant differences in baseline SA between the groups; and shocked but not control rats showed significantly increased intake (p<.01) and active lever pressing (p<.01) on each SA day compared to baseline. Additionally, rats in the shock group had significantly greater intake relative to controls on SA days 5-14 (p<.05) as well as greater cumulative cocaine intake (Fig. S1A; $t_{(50)}$=3.18, p<.01) and area under the curve (AUC; Fig. S1B; $t_{(50)}$=5.14, p<.0001). Intake across the four SA blocks each day was consistent in both groups (Fig. 1D-E); there were no SA day x block interactions (No shock: $F_{(42,966)}$=1.14, p=0.25; Shock: $F_{(42,1134)}$=1.34, p=.07). To determine whether escalated intake persists in the absence of shock, rats in the shock group were allowed to continue SA under

shock-free conditions for at least 5 days (Fig. 1F). These rats displayed escalated SA during this persistence phase (P1-5), comparable to when shock was present (SA 14). Two-way ANOVA revealed no significant SA day x stress interaction ($F_{(5,130)}$=0.22, p=0.95) but an overall stress effect ($F_{(1,26)}$=7.43, p<.05).

Experiment 2: Effect of systemic CB1R antagonism on stress-escalated cocaine intake

To examine endocannabinoid involvement in stress-escalated SA, the effects of AM251 were determined. Rats received AM251 (1 mg/kg, i.p.) or vehicle injections 30 min prior to SA. Two-way ANOVA revealed a significant AM251 x stress interaction ($F_{(1,14)}$=18.56, p<.001); AM251 significantly attenuated intake in the shock (p<.001) but not control group.

Experiment 3: Effect of CB1R antagonism in the NAc shell and VTA on stress-escalated cocaine intake

In this study, rats received micro-infusions of AM251 into NAc shell or VTA following 14 days of cocaine SA under stress or non-stress conditions (Fig 2A). As expected, SA was established in both groups and two-way ANOVA revealed a significant overall effect of stress ($F_{(1,33)}$=12.09, p<.0001; Fig 2B). Rats in the shock group had significantly greater cumulative cocaine intake (Fig. S2C; $t_{(33)}$=3.58, p<.01) and AUC (Fig. S2D; $t_{(33)}$=2.47, p<.05) compared to controls. To test for involvement of NAc shell CB1R signaling, rats received intra-NAc shell micro-infusions of AM251 (1, 3 µg/side) or vehicle 15 min before SA (Fig. 2C). Three-way ANOVA revealed a significant drug x stress x dose interaction ($F_{(1,28)}$=7.59, p<.05). AM251 did not attenuate SA in non-shocked controls at either dose (p<.05). However, both AM251 doses attenuated SA in shocked rats (1 µg: p<.01; 3 µg: p<.01). Separate rats received intra-VTA AM251 micro-infusions (1, 3 µg/side) or vehicle (Fig. 2E). While there was no effect of AM251 dose, there was an overall AM251 effect ($F_{(1,23)}$=22.11, p<.001) and a significant AM251 x stress interaction ($F_{(2,23)}$=4.31, p<.05). High dose AM251 (3 µg) attenuated SA in controls (p<.05), but both AM251 doses attenuated SA in shocked rats (1 µg: p=.05; 3 µg: p<.05).

Experiment 4: CB1R binding changes in the NAc shell and VTA following stress-induced escalation of cocaine intake

CB1R binding parameters were determined in membranes harvested from VTA and NAc shell 24 hrs after the last SA day in shocked and control rats. Separate groups were included that had saline access for SA with or without shock. SA data from rats used for this study are shown in Fig. 3A. Two-way ANOVA with SA condition as a between-subject factor and day as a within-subject factor revealed a significant SA condition x day

interaction ($F_{(42,700)}$=1.58, p<.05). For cocaine SA, rats in the shock group had significantly greater cumulative cocaine intake (Fig. S3C; $t_{(25)}$=2.56, p<.05) compared to controls. For AUC, a 2-way ANOVA revealed a significant SA condition x shock interaction (Fig. S3D; $F_{(1,45)}$=4.52, p<.05); AUC in the cocaine SA/shock group was significantly increased compared to every other group (p<.05). In the NAc shell, all data sets ($F_{(6,263)}$=1.38, p=0.22) were fit by the same curve, indicating that the binding parameters are not significantly different among the treatment groups (Fig. 3C). By contrast, in the VTA (Fig. 3E), one curve could not adequately fit all data sets ($F_{(6,233)}$=2.93, p<.01), and B_{max} and K_D values were determined from the mean binding data for each group (Table S5). K_D values did not differ across groups, and there was high overall variance in the calculated B_{max} values, likely because binding site density in the VTA is very low. As an additional approach, we compared specific binding at the highest ^{3}H-CP 55,940 concentration in each sample using two-way ANOVA. There was a significant overall effect of SA condition ($F_{(1,41)}$=74.49, p<.01), with any history of cocaine SA producing higher amounts of ^{3}H-CP55940 binding (Fig. 3F).The individual binding values at the highest ^{3}H-CP 55,940 concentration in the NAc shell are shown in Fig. 3D.

Experiment 5: Cocaine-primed reinstatement following stress-induced escalation of cocaine intake; requirement for CB1R

To determine if stress during cocaine SA produces long-lasting changes that influence reinstatement, rats underwent extinction followed by cocaine-primed reinstatement testing (Fig. 4A). Two-way repeated measures ANOVA revealed an overall effect of extinction day ($F_{(4,128)}$=29.37, p<.001) but no stress x extinction interaction ($F_{(4,128)}$=0.66, p=0.62), indicating no differences in extinction between groups (Fig. 4B). Rats in the shock group took longer to reach extinction criterion (6.00 ± 0.80 days) than controls (4.25 ±0.28 days), but this difference did not reach significance (p=.059). Rats were tested for reinstatement using three cocaine priming doses (2.5, 5, 10 mg/kg, i.p.). Three-way ANOVA revealed a significant cocaine dose x stress x day interaction ($F_{(3,60)}$=6.80, p<.001). While both groups showed significant reinstatement compared to extinction (p<.05, Fig. 4C), rats in the shock group showed greater reinstatement at each cocaine dose tested compared to controls (p<.05).

To examine the contribution of CB1R signaling to enhanced reinstatement susceptibility in shocked rats, we assessed the effects of AM251 (1 mg/kg, i.p.) on cocaine-primed reinstatement (10 mg/kg, i.p.). Three-way ANOVA revealed a significant AM251 x stress x day interaction (Fig. 4D; $F_{(1,15)}$=19.64, p<.001). While

reinstatement was observed in both groups ($p<.001$), it was significantly increased in shocked rats ($p<.01$). Moreover, only shocked rats showed a significant attenuation of cocaine-induced reinstatement by AM251 ($p<.001$).

Discussion

We previously reported that chronic footshock at the time of daily cocaine SA escalates drug intake but not food-reinforced responding in male rats [16]. Here we extend these findings by demonstrating that escalated cocaine SA persists after chronic stress is terminated and is associated with heightened cocaine-primed reinstatement, despite insignificant differences in extinction compared to non-shocked controls. Thus, our data suggest that the combination of stress and cocaine SA produces unique and lasting neuroadaptations that promote drug use and likely exacerbate SUD severity.

Stress-escalated cocaine SA involves recruitment of CB1R signaling. Post-stress cocaine SA is selectively reduced by the CB1R antagonist, AM251, thus restoring SA to levels observed in non-shocked rats. This observation is consistent with findings that another CB1R antagonist, rimonabant, produces pronounced reductions in breakpoints for cocaine when tested under a progressive ratio schedule only in rats that display escalated intake following repeated daily long-access SA [31]. By contrast, CB1R antagonism has minimal effects on cocaine SA under conditions of stable/non-escalated intake [28; 29; 31; 45], although it has been reported that SA is attenuated in CB1R knockout mice [46] but see [47; 48].

Brain endocannabinoid/CB1R signaling is activated by and plays a critical role in adaptation to stress. In addition to buffering the physiological stress response, endocannabinoids promote active coping strategies. In the context of SUDs, such strategies may manifest as heightened drug seeking behavior. Indeed, CB1Rs are expressed throughout the mesocorticolimbic system, including the VTA and NAc shell – regions implicated in stress adaptation and drug SA/seeking [49-52] and key sites for CB1R-dependent reward. Self-administration of THC directly into these regions is prevented by CB1R antagonism, and site-specific THC micro-infusions into these regions produces CB1R-dependent conditioned place preference [53].

Here we provide evidence that CB1R signaling in the NAc shell and VTA is recruited by footshock stress at the time of cocaine SA and contributes to escalating use patterns. Intra-VTA or intra-NAc shell AM251 micro-infusions selectively attenuated SA in rats that received shock during SA and restored SA to levels observed in non-shocked controls. By contrast, intra-NAc shell AM251 failed to alter SA in non-shocked rats,

consistent with findings that intra-NAc shell AM251 reduces SA in rats displaying escalated intake following repeated long-access to cocaine (6-h daily) without affecting SA in short-access rats (1-h daily) [31]. Intra-VTA AM251 did have modest effects on SA in control rats at the highest dose tested; which is consistent with reports that VTA CB1R antagonism attenuates cocaine-induced CPP in mice [54; 55] and cocaine-enhanced motivation assessed in rats self-administering food pellets under a progressive ratio schedule of reinforcement [56].

To determine how endocannabinoid signaling is altered in rats displaying stress-escalated SA, we assessed CB1R binding parameters in membranes prepared from dissected VTA and NAc shell [57-60]. Neither cocaine SA alone, repeated footshock alone, nor the combination, altered NAc shell CB1R binding. However, cocaine SA increased VTA CB1R binding assessed using a single point analysis regardless of whether rats previously received shock. These findings were unexpected, as previous work found increased CB1R binding in the NAc shell but not VTA following cocaine SA [61]. Considering the large differences in SA observed between shocked rats, which displayed robust escalation, and non-shocked controls, which did not escalate at all, the comparable increase in VTA CB1R binding in both groups was surprising and implies that endocannabinoid production/release, clearance/catabolism, and/or CB1R signaling may be differentially altered in the two groups.

Based on the established role for dopamine in cocaine SA, we hypothesize that stress-escalated SA potentiates CB1R-mediated activation of dopaminergic signaling via effects in both the VTA and NAc shell. In support of this notion, cocaine mobilizes endocannabinoids in the VTA [43] and NAc shell [29] and CB1R agonists activate mesolimbic neurons [62] and promote NAc dopamine release [63-65]. Moreover, CB1R antagonism attenuates the NAc dopamine response to cocaine [38] and the dopamine response to ip cocaine is blunted in CB1R knockout mice [66].

Given that CB1R signaling is inhibitory to neurotransmitter release, we hypothesize that stress-escalated SA increases activation of CB1R on GABAergic terminals, resulting in disinhibition of mesolimbic neurons. CB1R activation can reduce both inhibitory [67] and excitatory [36] drive onto VTA dopamine neurons. Notably, cocaine exposure promotes CB1R-dependent long-term depression at inhibitory synapses (iLTD) [55] while suppressing CB1R-mediated LTD at excitatory synapses [68] in the VTA, changes consistent with enhanced excitatory drive onto mesolimbic dopamine neurons. Likewise, in the NAc, CB1R activation

attenuates both inhibitory [69] and excitatory [70] synaptic transmission and mediates LTD at excitatory synapses [71], including those on parvalbumin+ GABAergic interneurons [72]. As in the VTA, cocaine has been reported to reduce CB1R-dependent LTD in the NAc [73]. While we hypothesize that the recruitment of CB1R signaling promotes escalation and reinstatement through enhanced attenuation of inhibitory transmission in the VTA and NAc, it is possible that alterations in CB1R regulation of excitatory synaptic transmission are also involved.

Despite no differences in extinction responding between groups, rats that received shock during SA displayed heightened cocaine-primed reinstatement compared to non-shocked controls. These findings are consistent with reports of persistent increases in cocaine-primed reinstatement following stress in rats [74-78], although none of those studies applied stress during a period of ongoing cocaine SA. Prior reports have suggested that the magnitude of drug-primed reinstatement increases according to the amount of previously self-administered cocaine [15; 79; 80]. However, regression analyses failed to demonstrate a relationship between the magnitude of reinstatement and various indicators of earlier SA, such as cumulative intake and area under the curve, suggesting that heightened reinstatement was not solely attributable to increased intake in shocked rats (Fig. S2).

As was the case with SA, cocaine-primed reinstatement was reduced by AM-251, but only in rats that received shock during earlier SA. Moreover, AM251 restored cocaine-primed reinstatement to levels observed in controls, suggesting that the heightened susceptibility to drug-primed cocaine seeking was the result of persistent recruitment of endocannabinoid signaling. Prior studies examining the effects of CB1R antagonism on cocaine-primed reinstatement produced mixed results. De Vries et al [28] (iv rimonabant), Xi et al [81] (ip AM251), and Adamczyk et al [45] (ip AM251) reported that CB1R antagonism attenuates cocaine-primed reinstatement, while we [82] (ip AM251) and Kupferschmidt [83] (icv AM251) found no effect on cocaine-primed reinstatement in rats and Ward et al [84] (ip rimonabant) found no effects in mice. Reasons for the discrepancies among rat studies are unclear but may include differences in rat strains/vendors, housing conditions, experimental designs, and treatment parameters. Nonetheless, the findings suggest that the impact of stress during SA on endocannabinoid signaling is long-lasting and may influence relapse susceptibility.

We previously reported that shock-escalated cocaine SA requires elevated glucocorticoids [4]. Moreover, increases in glucocorticoids at the time of SA contribute to both escalated intake and long-term

heightened susceptibility to cocaine-primed and stressor-induced reinstatement [17; 18]. Endocannabinoids mediate many of the neurobiological and physiological effects of glucocorticoids [56] and our previous studies implicated CB1R/endocannabinoid signaling in the acute effects of corticosterone on cocaine seeking [24]. Thus, we hypothesize that combination of stress and cocaine produces additive or synergistic increases in CB1R activation, which contributes to an escalation of cocaine SA.

Several procedural/design considerations warrant discussion. First, shock-escalated SA persisted for at least five days after the shock was removed from the sessions, which, along with the observation of heightened reinstatement, provides evidence of long-lasting neuroadaptations involving endocannabinoid signaling that promote cocaine seeking. Second, we previously reported that shock failed to escalate food-reinforced behavior (45 mg sucrose pellet [16]), indicating some specificity for cocaine SA. Third, we previously found that the effects of shock require delivery within the drug context and/or at the time of SA; shock escalation was not observed when shock was applied within the SA chambers 4 h after completion of the daily SA sessions or in a different chamber [16]. Fourth, rates of shock-induced escalation varied slightly across cohorts. In some cohorts, escalation was observed within the first session, while in others, it took several days to emerge. The reason for this is unclear. Fifth, escalated SA was observed in each of the four 30-min components during the session and was not confined to the first component of each session when “drug loading” typically occurs. Finally, the present study was conducted using only male rats. Prior work has demonstrated that footshock potentiates the reinstatement of cocaine-induced reinstatement following SA in male but not female rats [85]. Sex differences in endocannabinoid signaling have been reported [86; 87] and extend to endocannabinoid-related effects of chronic stress [88] and cocaine [89]. Considering reports that stress-reactivity differs between cocaine-dependent men and women [90], future studies examining sex differences in CB1R-dependent stress-induced escalation of SA are needed.

These findings add to the literature demonstrating that adverse life events influence the severity of SUDs. While we found that chronic stress during an ongoing period of SA escalated intake, Miczek and colleagues have demonstrated that a prior history of social defeat intensifies later cocaine SA as observed by more prolonged binge-like behavior when rats are provided unlimited daily access to cocaine [91; 92]. Along with earlier findings [31] these results suggest that the recruitment of mesolimbic endocannabinoid signaling promotes escalating patterns of cocaine use and that heightened CB1R signaling may contribute to CUD. The

latter assertion is supported by studies demonstrating that polymorphisms related to the *CN1R* gene, are associated with CUD [93-96] but see [97]. Moreover, the findings indicate that CB1R-targeting medications (e.g., negative allosteric modulators [98; 99] are promising interventions for CUD, particularly for those whose cocaine use is stress related.

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Author Contributions

JRMc, CJH, and JRM designed the project and experiments. JRMc and JRM supervised all of the experiments. JRMc, CPW, and DMS conducted the surgeries and JRMc ran the behavioral experiments with assistance from CPW, DMS, RS, and JCM. CPW and RS completed the histology. JRMc ran the behavior and collected tissue for the CB1R binding assay and CJH supervised and LAK completed the binding assays and analysis of the binding data. JRMc analyzed and graphed all data. JRMc and JRM wrote the first draft of the manuscript with editing and reviewing by CJH. Funding was acquired by JRMc, CJH, and JRM.

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Competing Interests

JRMc, CPW, DMS, JCM, LAK, and RS declare no potential competing interest. CJH is a member of the scientific board of directors of Phytecs, Inc; has equity in Formulate Biosciences, Inc and has received consultation fees from Pharmavite, LLC. JRM is a co-founder of and has equity in Promentis Pharmaceuticals, Inc.

References

1. Kosten TR and Kleber HD. 1988. Differential diagnosis of psychiatric comorbidity in substance abusers. J Subst Abuse Treat 5: 201-206.

2. Back S, Dansky BS, Coffey SF, Saladin ME, Sonne S, Brady KT. 2000. Cocaine Dependence with and without Posttraumatic Stress Disorder: A Comparison of Substance Use, Trauma History and Psychiatric Comorbidity. The American Journal on Addictions 9: 51-62.

3. Mahoney JJ, Newton TF, Omar Y, Ross EL, Garza RDL. 2013. The relationship between lifetime stress and addiction severity in cocaine-dependent participants. European Neuropsychopharmacology 23: 351-357.

4. Wallace BC. 1989. Psychological and environmental determinants of relapse in crack cocaine smokers. J Subst Abuse Treat 6: 95-106.

5. Preston KL and Epstein DH. 2011. Stress in the daily lives of cocaine and heroin users: relationship to mood, craving, relapse triggers, and cocaine use. Psychopharmacology (Berl) 218: 29-37.

6. Panlilio LV, Stull SW, Bertz JW, Burgess-Hull A, Lanza ST, Curtis BL *et al*. 2021. Beyond abstinence and relapse II: momentary relationships between stress, craving, and lapse within clusters of patients with similar patterns of drug use. Psychopharmacology (Berl) 238: 1513-1529.

7. Waldrop AE, Back SE, Brady KT, Upadhyaya HP, McRae AL, Saladin ME. 2007. Daily stressor sensitivity, abuse effects, and cocaine use in cocaine dependence. Addict Behav 32: 3015-3025.

8. Back SE, Hartwell K, DeSantis SM, Saladin M, McRae-Clark A, Price KL *et al*. 2010. Reactivity to laboratory stress provocation predicts relapse to cocaine. Drug Alcohol Depend 106: 21-27.

9. Panlilio LV, Stull SW, Kowalczyk WJ, Phillips KA, Schroeder JR, Bertz JW *et al*. 2019. Stress, craving and mood as predictors of early dropout from opioid agonist therapy. Drug Alcohol Depend 202: 200-208.

10. Banducci AN, Bujarski SJ, Bonn-Miller M, Patel A, Connolly KM. 2016. The impact of intolerance of emotional distress and uncertainty on veterans with co-occurring PTSD and substance use disorders. J Anxiety Disord 41: 73-81.

11. Sinha R, Catapano D, O'Malley S. 1999. Stress-induced craving and stress response in cocaine dependent individuals. Psychopharmacology (Berl) 142: 343-351.

12. Sinha R, Fuse T, Aubin L-, O'Malley SS. 2000. Psychological stress, drug-related cues and cocaine craving. Psychopharmacology (Berl) 152: 140-148.

13. Sinha R, Garcia M, Paliwal P, Kreek MJ, Rounsaville BJ. 2006. Stress-Induced Cocaine Craving and Hypothalamic-Pituitary-Adrenal Responses Are Predictive of Cocaine Relapse Outcomes. Arch Gen Psychiatry 63: 324-331.

14. Ahmed SH and Koob GF. 1998. Transition from Moderate to Excessive Drug Intake: Change in Hedonic Set Point. Science 282: 298-300.

15. Mantsch JR, Yuferov V, Mathieu-Kia A, Ho A, Kreek MJ. 2004. Effects of extended access to high versus low cocaine doses on self-administration, cocaine-induced reinstatement and brain mRNA levels in rats. Psychopharmacology (Berl) 175: 26-36.

16. Mantsch JR and Katz ES. 2007. Elevation of glucocorticoids is necessary but not sufficient for the escalation of cocaine self-administration by chronic electric footshock stress in rats. Neuropsychopharmacology 32: 367-376.

17. Mantsch JR, Baker DA, Serge JP, Hoks MA, Francis DM, Katz ES. 2008. Surgical adrenalectomy with diurnal corticosterone replacement slows escalation and prevents the augmentation of cocaine-induced reinstatement in rats self-administering cocaine under long-access conditions. Neuropsychopharmacology 33: 814-826.

18. Graf EN, Hoks MA, Baumgardner J, Sierra J, Vranjkovic O, Bohr C *et al*. 2011. Adrenal activity during repeated long-access cocaine self-administration is required for later CRF-Induced and CRF-dependent stressor-induced reinstatement in rats. Neuropsychopharmacology 36: 1444-1454.

19. Di S, Malcher-Lopes R, Halmos KC, Tasker JG. 2003. Nongenomic Glucocorticoid Inhibition via Endocannabinoid Release in the Hypothalamus: A Fast Feedback Mechanism. The Journal of Neuroscience 23: 4850-4857.

20. Hill MN, Ho W-V, Meier SE, Gorzalka BB, Hillard CJ. 2005. Chronic corticosterone treatment increases the endocannabinoid 2-arachidonylglycerol in the rat amygdala. Eur J Pharmacol 528: 99-102.

21. Campolongo P, Roozendaal B, Trezza V, Hauer D, Schelling G, McGaugh JL *et al*. 2009. Endocannabinoids in the rat basolateral amygdala enhance memory consolidation and enable glucocorticoid modulation of memory. Proceedings of the National Academy of Sciences 106: 4888-4893.

22. Hill MN, Karatsoreos IN, Hillard CJ, McEwen BS. 2010. Rapid elevations in limbic endocannabinoid content by glucocorticoid hormones in vivo. Psychoneuroendocrinology 35: 1333-1338.

23. Hill MN, McLaughlin RJ, Pan B, Fitzgerald ML, Roberts CJ, Lee, T T - Y. *et al*. 2011. Recruitment of Prefrontal Cortical Endocannabinoid Signaling by Glucocorticoids Contributes to Termination of the Stress Response. Journal of Neuroscience 31: 10506-10515.

24. McReynolds JR, Doncheck EM, Li Y, Vranjkovic O, Graf EN, Ogasawara D *et al*. 2018. Stress Promotes Drug Seeking Through Glucocorticoid-Dependent Endocannabinoid Mobilization in the Prelimbic Cortex. Biol Psychiatry 84: 85-94.

25. Morena M, Patel S, Bains JS, Hill MN. 2016. Neurobiological Interactions Between Stress and the Endocannabinoid System. Neuropsychopharmacology 41: 80-102.

26. deRoon-Cassini T, Stollenwerk TM, Beatka M, Hillard CJ. 2020. Meet Your Stress Management Professionals: The Endocannabinoids. Trends Mol Med 26: 953-968.

27. López-Moreno JA, Echeverry-Alzate V, Bühler K. 2012. The genetic basis of the endocannabinoid system and drug addiction in humans. Journal of Psychopharmacology 26: 133-143.

28. Vries TJD, Shaham Y, Homberg JR, Crombag H, Schuurman K, Dieben J *et al*. 2001. A cannabinoid mechanism in relapse to cocaine seeking. Nat Med 7: 1151-1154.

29. Caillé S, Alvarez-Jaimes L, Polis I, Stouffer DG, Parsons LH. 2007. Specific alterations of extracellular endocannabinoid levels in the nucleus accumbens by ethanol, heroin, and cocaine self-administration. J Neurosci 27: 3695-3702.

30. Adamczyk P, McCreary AC, Przegalinski E, Mierzejewski P, Bienkowski P, Filip M. 2008. The effects of fatty acid amide hydrolase inhibitors on maintenance of cocaine and food self-administration and on reinstatement of cocaine-seeking and food-taking behavior in rats. Journal of physiology and pharmacology : an official journal of the Polish Physiological Society PMID - 19826190 60: 119-125.

31. Orio L, Edwards S, George O, Parsons LH, Koob GF. 2009. A role for the endocannabinoid system in the increased motivation for cocaine in extended-access conditions. J Neurosci 29: 4846-4857.

32. Roberts DCS and Koob GF. 1982. Disruption of cocaine self-administration following 6-hydroxydopamine lesions of the ventral tegmental area in rats. Pharmacology Biochemistry and Behavior 17: 901-904.

33. Caine SB, Heinrichs SC, Coffin VL, Koob GF. 1995. Effects of the dopamine D-1 antagonist SCH 23390 microinjected into the accumbens, amygdala or striatum on cocaine self-administration in the rat. Brain Res 692: 47-56.

34. Carlezon WA, Devine DP, Wise RA. 1995. Habit-forming actions of nomifensine in nucleus accumbens. Psychopharmacology (Berl) 122: 194-197.

35. Pontieri FE, Tanda G, Chiara GD. 1995. Intravenous cocaine, morphine, and amphetamine preferentially increase extracellular dopamine in the "shell" as compared with the "core" of the rat nucleus accumbens. Proceedings of the National Academy of Sciences 92: 12304-12308.

36. Melis M, Pistis M, Perra S, Muntoni AL, Pillolla G, Gessa GL. 2004. Endocannabinoids mediate presynaptic inhibition of glutamatergic transmission in rat ventral tegmental area dopamine neurons through activation of CB1 receptors. J Neurosci 24: 53-62.

37. Riegel AC and Lupica CR. 2004. Independent presynaptic and postsynaptic mechanisms regulate endocannabinoid signaling at multiple synapses in the ventral tegmental area. J Neurosci 24: 11070-11078.

38. Cheer JF, Wassum KM, Sombers LA, Heien ML, Ariansen JL, Aragona BJ *et al*. 2007. Phasic dopamine release evoked by abused substances requires cannabinoid receptor activation. J Neurosci 27: 791-795.

39. Pan B, Hillard CJ, Liu QS. 2008. D2 dopamine receptor activation facilitates endocannabinoid-mediated long-term synaptic depression of GABAergic synaptic transmission in midbrain dopamine neurons via cAMP-protein kinase A signaling. J Neurosci 28: 14018-14030.

40. Oleson EB, Beckert MV, Morra JT, Lansink CS, Cachope R, Abdullah RA *et al*. 2012. Endocannabinoids Shape Accumbal Encoding of Cue-Motivated Behavior via CB1 Receptor Activation in the Ventral Tegmentum. Neuron 73: 360-373.

41. Mateo Y, Johnson KA, Covey DP, Atwood BK, Wang H, Zhang S *et al*. 2017. Endocannabinoid Actions on Cortical Terminals Orchestrate Local Modulation of Dopamine Release in the Nucleus Accumbens. Neuron 96: 1112-1126.e5.

42. Wang W, Sun D, Pan B, Roberts CJ, Sun X, Hillard CJ *et al*. 2010. Deficiency in Endocannabinoid Signaling in the Nucleus Accumbens Induced by Chronic Unpredictable Stress. Neuropsychopharmacology 35: 2249-2261.

43. Wang H, Treadway T, Covey DP, Cheer JF, Lupica CR. 2015. Cocaine-Induced Endocannabinoid Mobilization in the Ventral Tegmental Area. Cell Rep 12: 1997-2008.

44. Tung L, Lu G, Lee Y, Yu L, Lee H, Leishman E *et al*. 2016. Orexins contribute to restraint stress-induced cocaine relapse by endocannabinoid-mediated disinhibition of dopaminergic neurons. Nature Communications 7: 12199.

45. Adamczyk P, Miszkiel J, McCreary AC, Filip M, Papp M, Przegaliński E. 2012. The effects of cannabinoid CB1, CB2 and vanilloid TRPV1 receptor antagonists on cocaine addictive behavior in rats. Brain Res 1444: 45-54.

46. Soria G, Mendizábal V, Touriño C, Robledo P, Ledent C, Parmentier M *et al*. 2005. Lack of CB1 cannabinoid receptor impairs cocaine self-administration. Neuropsychopharmacology 30: 1670-1680.

47. Cossu G, Ledent C, Fattore L, Imperato A, Böhme GA, Parmentier M *et al*. 2001. Cannabinoid CB1 receptor knockout mice fail to self-administer morphine but not other drugs of abuse. Behav Brain Res 118: 61-65.

48. Martín-García E, Bourgoin L, Cathala A, Kasanetz F, Mondesir M, Gutiérrez-Rodriguez A *et al*. 2016. Differential Control of Cocaine Self-Administration by GABAergic and Glutamatergic CB1 Cannabinoid Receptors. Neuropsychopharmacology 41: 2192-2205.

49. Tsou K, Brown S, Sañudo-Peña MC, Mackie K, Walker JM. 1998. Immunohistochemical distribution of cannabinoid CB1 receptors in the rat central nervous system. Neuroscience 83: 393-411.

50. Pickel VM, Chan J, Kash TL, Rodríguez JJ, MacKie K. 2004. Compartment-specific localization of cannabinoid 1 (CB1) and mu-opioid receptors in rat nucleus accumbens. Neuroscience 127: 101-112.

51. Pickel VM, Chan J, Kearn CS, Mackie K. 2006. Targeting dopamine D2 and cannabinoid-1 (CB1) receptors in rat nucleus accumbens. J Comp Neurol 495: 299-313.

52. Winters BD, Krüger JM, Huang X, Gallaher ZR, Ishikawa M, Czaja K *et al*. 2012. Cannabinoid receptor 1-expressing neurons in the nucleus accumbens. Proc Natl Acad Sci U S A 109: 2717.

53. Zangen A, Solinas M, Ikemoto S, Goldberg SR, Wise RA. 2006. Two brain sites for cannabinoid reward. J Neurosci 26: 4901-4907.

54. Pan B, Zhong P, Sun D, Liu QS. 2011. Extracellular signal-regulated kinase signaling in the ventral tegmental area mediates cocaine-induced synaptic plasticity and rewarding effects. J Neurosci 31: 11244-11255.

55. Tong J, Liu X, Vickstrom C, Li Y, Yu L, Lu Y *et al*. 2017. The Epac-Phospholipase Cε Pathway Regulates Endocannabinoid Signaling and Cocaine-Induced Disinhibition of Ventral Tegmental Area Dopamine Neurons. J Neurosci 37: 3030-3044.

56. Engi SA, Beebe EJ, Ayvazian VM, Cruz FC, Cheer JF, Wenzel JM *et al*. 2021. Cocaine-induced increases in motivation require 2-arachidonoylglycerol mobilization and CB1 receptor activation in the ventral tegmental area. Neuropharmacology 193: 108625.

57. Hillard CJ, Edgemond WS, Campbell WB. 1995. Characterization of Ligand Binding to the Cannabinoid Receptor of Rat Brain Membranes Using a Novel Method: Application to Anandamide. Journal of neurochemistry 64: 677-683.

58. Hill MN, Patel S, Carrier EJ, Rademacher DJ, Ormerod BK, Hillard CJ *et al*. 2005. Downregulation of Endocannabinoid Signaling in the Hippocampus Following Chronic Unpredictable Stress. Neuropsychopharmacology 30: 508-515.

59. Hill MN, Carrier EJ, Ho W-V, Shi L, Patel S, Gorzalka BB *et al*. 2008. Prolonged glucocorticoid treatment decreases cannabinoid CB1 receptor density in the hippocampus. Hippocampus 18: 221-226.

60. McLaughlin RJ, Hill MN, Dang SS, Wainwright SR, Galea LAM, Hillard CJ *et al*. 2013. Upregulation of CB1 receptor binding in the ventromedial prefrontal cortex promotes proactive stress-coping strategies following chronic stress exposure. Behavioural brain research 237: 333-337.

61. Adamczyk P, Faron-Górecka A, Kuśmider M, Dziedzicka-Wasylewska M, Papp M, Filip M. 2012. Long-lasting increase in [3H]CP55,940 binding to CB1 receptors following cocaine self-administration and its withdrawal in rats. Brain Res 1451: 34-43.

62. Gessa GL, Melis M, Muntoni AL, Diana M. 1998. Cannabinoids activate mesolimbic dopamine neurons by an action on cannabinoid CB1 receptors. Eur J Pharmacol 341: 39-44.

63. Cheer JF, Wassum KM, Heien ML, Phillips PE, Wightman RM. 2004. Cannabinoids enhance subsecond dopamine release in the nucleus accumbens of awake rats. J Neurosci 24: 4393-4400.

64. Solinas M, Justinova Z, Goldberg SR, Tanda G. 2006. Anandamide administration alone and after inhibition of fatty acid amide hydrolase (FAAH) increases dopamine levels in the nucleus accumbens shell in rats. J Neurochem 98: 408-419.

65. Hernandez G, Oleson EB, Gentry RN, Abbas Z, Bernstein DL, Arvanitogiannis A *et al*. 2014. Endocannabinoids promote cocaine-induced impulsivity and its rapid dopaminergic correlates. Biol Psychiatry 75: 487-498.

66. Li X, Hoffman AF, Peng XQ, Lupica CR, Gardner EL, Xi ZX. 2009. Attenuation of basal and cocaine-enhanced locomotion and nucleus accumbens dopamine in cannabinoid CB1-receptor-knockout mice. Psychopharmacology (Berl) 204: 1-11.

67. Szabo B, Siemes S, Wallmichrath I. 2002. Inhibition of GABAergic neurotransmission in the ventral tegmental area by cannabinoids. Eur J Neurosci 15: 2057-2061.

68. Wang R, Hausknecht KA, Gancarz-Kausch A, Oubraim S, Shen RY, Haj-Dahmane S. 2020. Cocaine self-administration abolishes endocannabinoid-mediated long-term depression of glutamatergic synapses in the ventral tegmental area. Eur J Neurosci 52: 4517-4524.

69. Manzoni OJ and Bockaert J. 2001. Cannabinoids inhibit GABAergic synaptic transmission in mice nucleus accumbens. Eur J Pharmacol 412: 3.

70. Robbe D, Alonso G, Duchamp F, Bockaert J, Manzoni OJ. 2001. Localization and mechanisms of action of cannabinoid receptors at the glutamatergic synapses of the mouse nucleus accumbens. J Neurosci 21: 109-116.

71. Robbe D, Kopf M, Remaury A, Bockaert J, Manzoni OJ. 2002. Endogenous cannabinoids mediate long-term synaptic depression in the nucleus accumbens. Proc Natl Acad Sci U S A 99: 8384-8388.

72. Manz KM, Ghose D, Turner BD, Taylor A, Becker J, Grueter CA *et al*. 2020. Calcium-Permeable AMPA Receptors Promote Endocannabinoid Signaling at Parvalbumin Interneuron Synapses in the Nucleus Accumbens Core. Cell Rep 32: 107971.

73. Fourgeaud L, Mato S, Bouchet D, Hémar A, Worley PF, Manzoni OJ. 2004. A single in vivo exposure to cocaine abolishes endocannabinoid-mediated long-term depression in the nucleus accumbens. J Neurosci 24: 6939-6945.

74. Conrad KL, McCutcheon JE, Cotterly LM, Ford KA, Beales M, Marinelli M. 2010. Persistent increases in cocaine-seeking behavior after acute exposure to cold swim stress. Biol Psychiatry 68: 303-305.

75. Ball KT, Stone E, Best O, Collins T, Edson H, Hagan E *et al*. 2018. Chronic restraint stress during withdrawal increases vulnerability to drug priming-induced cocaine seeking via a dopamine D1-like receptor-mediated mechanism. Drug Alcohol Depend 187: 327-334.

76. Glynn RM, Rosenkranz JA, Wolf ME, Caccamise A, Shroff F, Smith AB *et al*. 2018. Repeated restraint stress exposure during early withdrawal accelerates incubation of cue-induced cocaine craving. Addict Biol 23: 80-89.

77. Schwendt M, Shallcross J, Hadad NA, Namba MD, Hiller H, Wu L *et al*. 2018. A novel rat model of comorbid PTSD and addiction reveals intersections between stress susceptibility and enhanced cocaine seeking with a role for mGlu5 receptors. Transl Psychiatry 8: 209.

78. Garcia-Keller C, Smiley C, Monforton C, Melton S, Kalivas PW, Gass J. 2020. N-Acetylcysteine treatment during acute stress prevents stress-induced augmentation of addictive drug use and relapse. Addict Biol 25: e12798.

79. Sutton MA, Karanian DA, Self DW. 2000. Factors that determine a propensity for cocaine-seeking behavior during abstinence in rats. Neuropsychopharmacology 22: 626-641.

80. Baker DA, Tran-Nguyen T, Fuchs RA, Neisewander JL. 2001. Influence of individual differences and chronic fluoxetine treatment on cocaine-seeking behavior in rats. Psychopharmacology (Berl) 155: 18-26.

81. Xi ZX, Gilbert JG, Peng XQ, Pak AC, Li X, Gardner EL. 2006. Cannabinoid CB1 receptor antagonist AM251 inhibits cocaine-primed relapse in rats: role of glutamate in the nucleus accumbens. J Neurosci 26: 8531-8536.

82. McReynolds JR, Doncheck EM, Vranjkovic O, Ganzman GS, Baker DA, Hillard CJ *et al*. 2016. CB1 receptor antagonism blocks stress-potentiated reinstatement of cocaine seeking in rats. Psychopharmacology (Berl) 233: 99-109.

83. Kupferschmidt DA, Klas PG, Erb S. 2012. Cannabinoid CB1 receptors mediate the effects of corticotropin-releasing factor on the reinstatement of cocaine seeking and expression of cocaine-induced behavioural sensitization. Br J Pharmacol 167: 196-206.

84. Ward SJ, Rosenberg M, Dykstra LA, Walker EA. 2009. The CB1 antagonist rimonabant (SR141716) blocks cue-induced reinstatement of cocaine seeking and other context and extinction phenomena predictive of relapse. Drug Alcohol Depend 105: 248-255.

85. Doncheck EM, Liddiard GT, Konrath CD, Liu X, Yu L, Urbanik LA *et al*. 2020. Sex, stress, and prefrontal cortex: influence of biological sex on stress-promoted cocaine seeking. Neuropsychopharmacology 45: 1974-1985.

86. Craft RM, Marusich JA, Wiley JL. 2013. Sex differences in cannabinoid pharmacology: a reflection of differences in the endocannabinoid system? Life Sci 92: 476-481.

87. Ney LJ, Matthews A, Bruno R, Felmingham KL. 2018. Modulation of the endocannabinoid system by sex hormones: Implications for posttraumatic stress disorder. Neurosci Biobehav Rev 94: 302-320.

88. Reich CG, Taylor ME, McCarthy MM. 2009. Differential effects of chronic unpredictable stress on hippocampal CB1 receptors in male and female rats. Behav Brain Res 203: 264-269.

89. Peterson BM, Martinez LA, Meisel RL, Mermelstein PG. 2016. Estradiol impacts the endocannabinoid system in female rats to influence behavioral and structural responses to cocaine. Neuropharmacology 110: 118-124.

90. Back SE, Brady KT, Jackson JL, Salstrom S, Zinzow H. 2005. Gender differences in stress reactivity among cocaine-dependent individuals. Psychopharmacology (Berl) 180: 169-176.

91. E. 3C, H. and Miczek KA. 2001. Repeated social-defeat stress, cocaine or morphine. Effects on behavioral sensitization and intravenous cocaine self-administration "binges". Psychopharmacology (Berl) 158: 388-398.

92. Quadros IM and Miczek KA. 2009. Two modes of intense cocaine bingeing: increased persistence after social defeat stress and increased rate of intake due to extended access conditions in rats. Psychopharmacology (Berl) 206: 109-120.

93. Comings DE, Muhleman D, Gade R, Johnson P, Verde R, Saucier G *et al*. 1997. Cannabinoid receptor gene (CNR1): association with i.v. drug use. Mol Psychiatry 2: 161-168.

94. Ballon N, Leroy S, Roy C, Bourdel MC, Charles-Nicolas A, Krebs MO *et al*. 2006. (AAT)n repeat in the cannabinoid receptor gene (CNR1): association with cocaine addiction in an African-Caribbean population. Pharmacogenomics J 6: 126-130.

95. Zuo L, Kranzler HR, Luo X, Yang BZ, Weiss R, Brady K *et al*. 2009. Interaction between two independent CNR1 variants increases risk for cocaine dependence in European Americans: a replication study in family-based sample and population-based sample. Neuropsychopharmacology 34: 1504-1513.

96. Clarke TK, Bloch PJ, Ambrose-Lanci L, Ferraro TN, Berrettini WH, Kampman KM *et al*. 2013. Further evidence for association of polymorphisms in the CNR1 gene with cocaine addiction: confirmation in an independent sample and meta-analysis. Addict Biol 18: 702-708.

97. Herman AI, Kranzler HR, Cubells JF, Gelernter J, Covault J. 2006. Association study of the CNR1 gene exon 3 alternative promoter region polymorphisms and substance dependence. Am J Med Genet B Neuropsychiatr Genet 141b: 499-503.

98. Jing L, Qiu Y, Zhang Y, Li JX. 2014. Effects of the cannabinoid CB_1 receptor allosteric modulator ORG 27569 on reinstatement of cocaine- and methamphetamine-seeking behavior in rats. Drug Alcohol Depend 143: 251-256.

99. Nguyen T, Gamage TF, Finlay DB, Decker AM, Langston TL, Barrus D *et al*. 2022. Development of 3-(4-Chlorophenyl)-1-(phenethyl)urea Analogues as Allosteric Modulators of the Cannabinoid Type-1 Receptor: RTICBM-189 is Brain Penetrant and Attenuates Reinstatement of Cocaine-Seeking Behavior. J Med Chem 65: 257-270.

Figure Legends

Figure 1. Chronic electronic footshock administered daily at the time of self-administration induces a persistent escalation of cocaine intake. A) Schematic of modified short-access paradigm and experimental timeline wherein 2-hr daily self-administration was separated into 4 30-min self-administration blocks (levers extended, drug cue light on, house light off) separated by 4 5-min drug-free periods (levers retracted, drug cue light off, house light on). Following stable self-administration on FR4 schedule of reinforcement, animals in the stress group received intermittent electric footshock stress during the drug-free period daily for 14 days. Under this paradigm footshock (shock) stress administered daily at the time of self-administration induced a significant escalation of cocaine intake as assessed by an increase in infusions (B) and active lever presses (C) across days while no shock rats showed fairly stable responding. Daily infusions of cocaine were similar across all 4 self-administration blocks within each day in both the no shock (D) and shock (E) groups (no shock: n=20; shock n=17; **p<.01, *p<.05, compared to baseline). F) Animals in the stress group continue to show elevated cocaine intake when the shock stressor was removed (P1-P5; No Shock, n=13; Shock, n= 15; *p<.05, compared to No Shock). G) Systemic cannabinoid receptor type 1 (CB1R) antagonism with AM251 (0, 1 mg/kg, i.p.) given 30 min prior to self-administration significantly attenuated cocaine intake in animals with a history of shock (n=7) while having no effect in rats with a history of no shock (n=9; ***p<.001 compared to Vehicle). Data are presented as mean ± SEM.

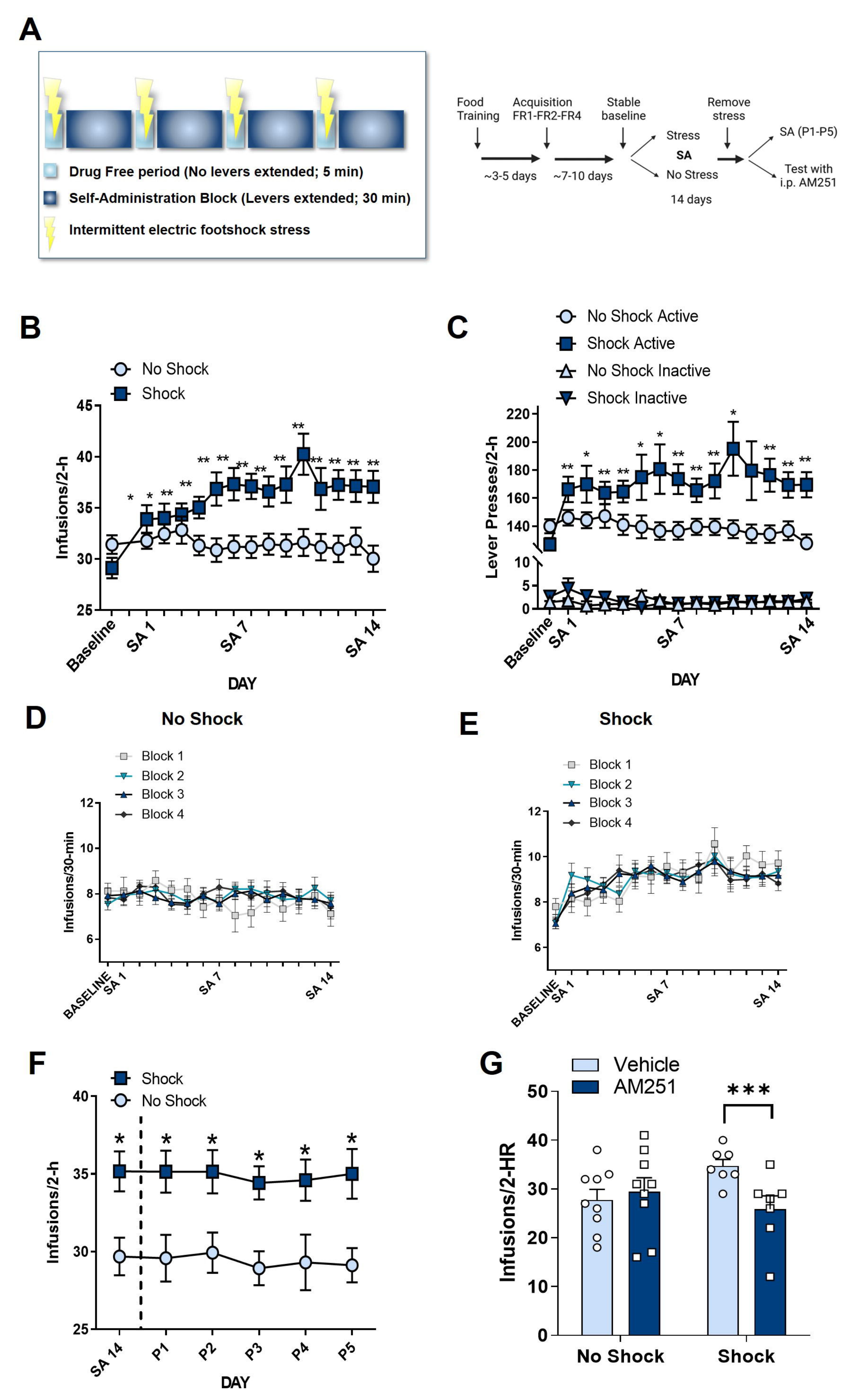
Figure 2. Cannabinoid receptor type 1 antagonism in the nucleus accumbens shell and ventral tegmental area attenuate cocaine intake in rats that underwent stress-induced escalation of cocaine intake. A) Experimental timeline. B) Self-administration data for all animals that received intra-cranial CB1R antagonism. Repeated daily footshock stress induces a significant escalation of cocaine intake (No shock, n=16; Shock, n=19; *p<.05 compared to baseline). C) Intra-nucleus accumbens (NAc) shell administration of the CB1R antagonist AM251 (0, 1, 3 µg/side) attenuates cocaine intake only in rats with a history of shock (n=8/dose) but has no effect on cocaine intake in rats with a history of no shock (n=7-9/dose; **p<.01 compared to No Shock-Vehicle, ##p<.01 compared to Vehicle). D) Injection needle tips of all rats included in the intra-NAc shell experiment. Coordinates are listed from Bregma. E) Intra-ventral tegmental area (VTA) administration of AM251 (0, 1, 3 µg/side) attenuates cocaine intake in rats with a history of shock (n=7-8/dose) at both doses of AM251 but only

attenuates cocaine intake in rats with a history of no shock (n=6-7/dose) at the 3 µg dose (*p<.05 compared to No Shock-Vehicle, #p<.05 compared to Vehicle). F) Injection needle tips of all rats included in the intra-VTA experiment. Coordinates are listed from Bregma. Data are presented as mean ± SEM.

Figure 3. Cocaine self-administration increases cannabinoid receptor type 1 binding in the ventral tegmental area but not nucleus accumbens shell. A) Self-administration data for all animals that were used for the CB1R binding experiment. Repeated daily footshock stress induces a significant escalation of cocaine intake (No Shock, n=14; Shock, n=13) but has no effect on saline self-administration (No Shock, n=14; Shock, n=13). B) Experimental timeline and schematic of dissection boundaries for the NAc shell (left) and VTA (right). C) CB1R binding in the NAc shell is similar across all four self-administration conditions as assessed by the full binding curve from the radiolabeled ligand CB1R binding assay. The binding at the highest concentration of CP 55, 940 is represented on the right and there are no differences between groups. E) In the VTA, cocaine self-administration increases CB1R binding. The full binding curves are represented on the left and binding at the highest dose of CP 55,940 is higher in rats that underwent cocaine self-administration but there is no effect of stress on CB1R binding (**p<.01 compared to Saline SA at the 2.0 nM concentration of CP 55,940). Data are presented as mean ± SEM.

Figure 4. A history of stress-induced escalation of cocaine intake results in increased cocaine-induced reinstatement that is dependent upon CB1R activation. A) Experimental timeline. There is no difference in the number of days to reach extinction criterion between animals with a history of no shock (n=16) or shock (n=18). B) Animals with a history of shock show similar rates of extinction compared to rats with a history of no shock. C) Rats with a history of stress at the time of self-administration (n=12) show increased cocaine-induced reinstatement at multiple doses (2.5, 5, 10 mg/kg, i.p.) compared to rats with a history of no stress at the time of self-administration (n=10; #p<.05, ##p<.01, compared to saline; *p<.05, **p<.01, compared to No Shock). D) Systemic administration of AM251 (0, 1 mg/kg, i.p.) 30 min prior to a cocaine injection (10 mg/kg, i.p.) attenuates cocaine-induced reinstatement only in rats with a history of stress at the time of cocaine self-administration (n=8). The same treatment has no effect on cocaine-induced reinstatement in rats with no

history of stress at the time of cocaine self-administration (n=9; *p<.05, ***p<.001, compared to extinction; @@p<.01, compared to No Stress SA-Vehicle; ###p<.001, compared to Vehicle). Data are presented as mean ± SEM.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Drug Free period (No levers extended; 5 min)
Self-Administration Block (Levers extended; 30 min)
Intermittent electric footshock stress
Food Training
Acquisition FR1-FR2-FR4
Stable baseline
Remove stress
Stress
SA
No Stress
14 days
~3-5 days
~7-10 days
SA (P1-P5)
Test with i.p. AM251
B
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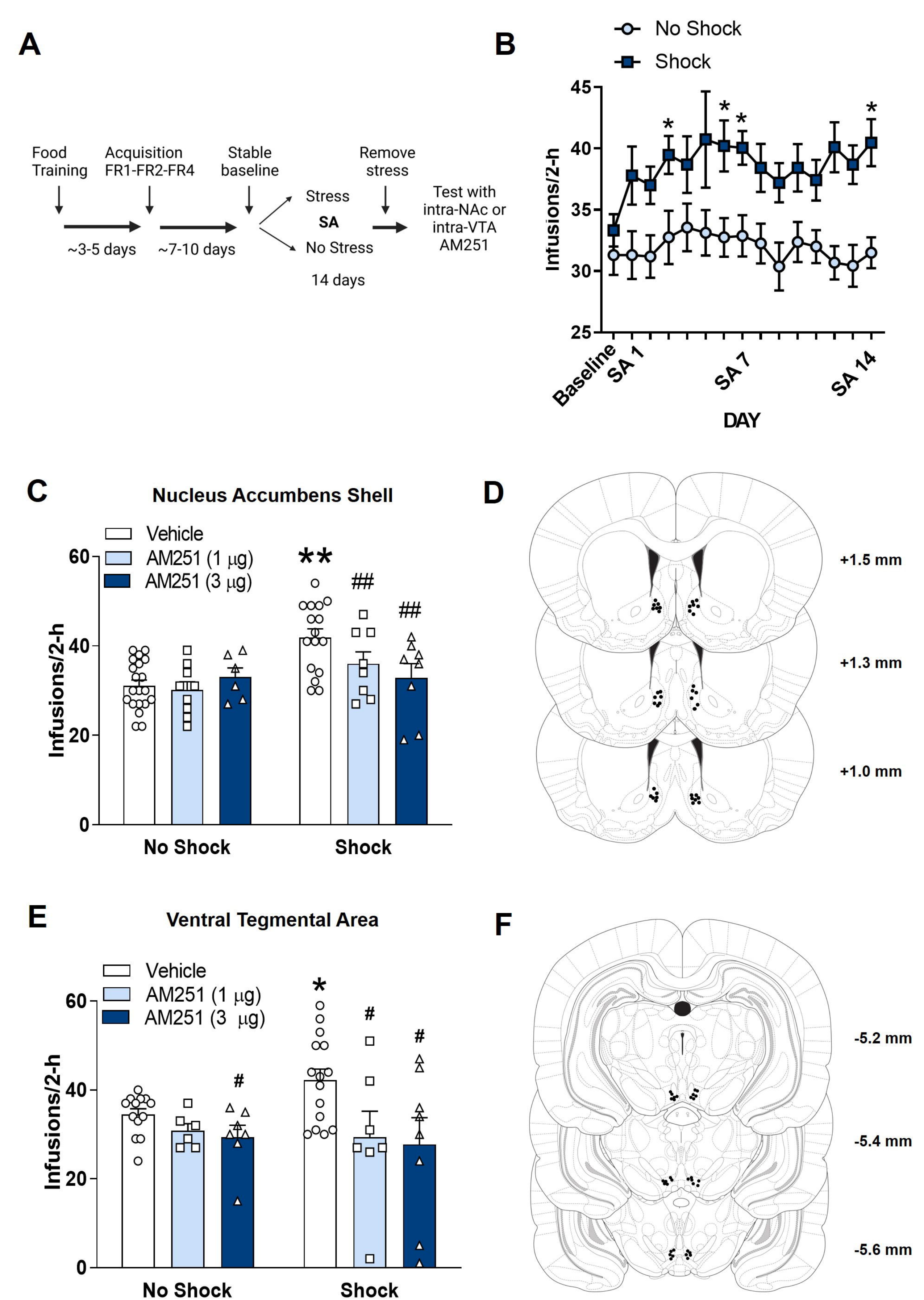
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Food Training
Acquisition FR1-FR2-FR4
Stable baseline
Remove stress
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Test with intra-NAc or intra-VTA AM251
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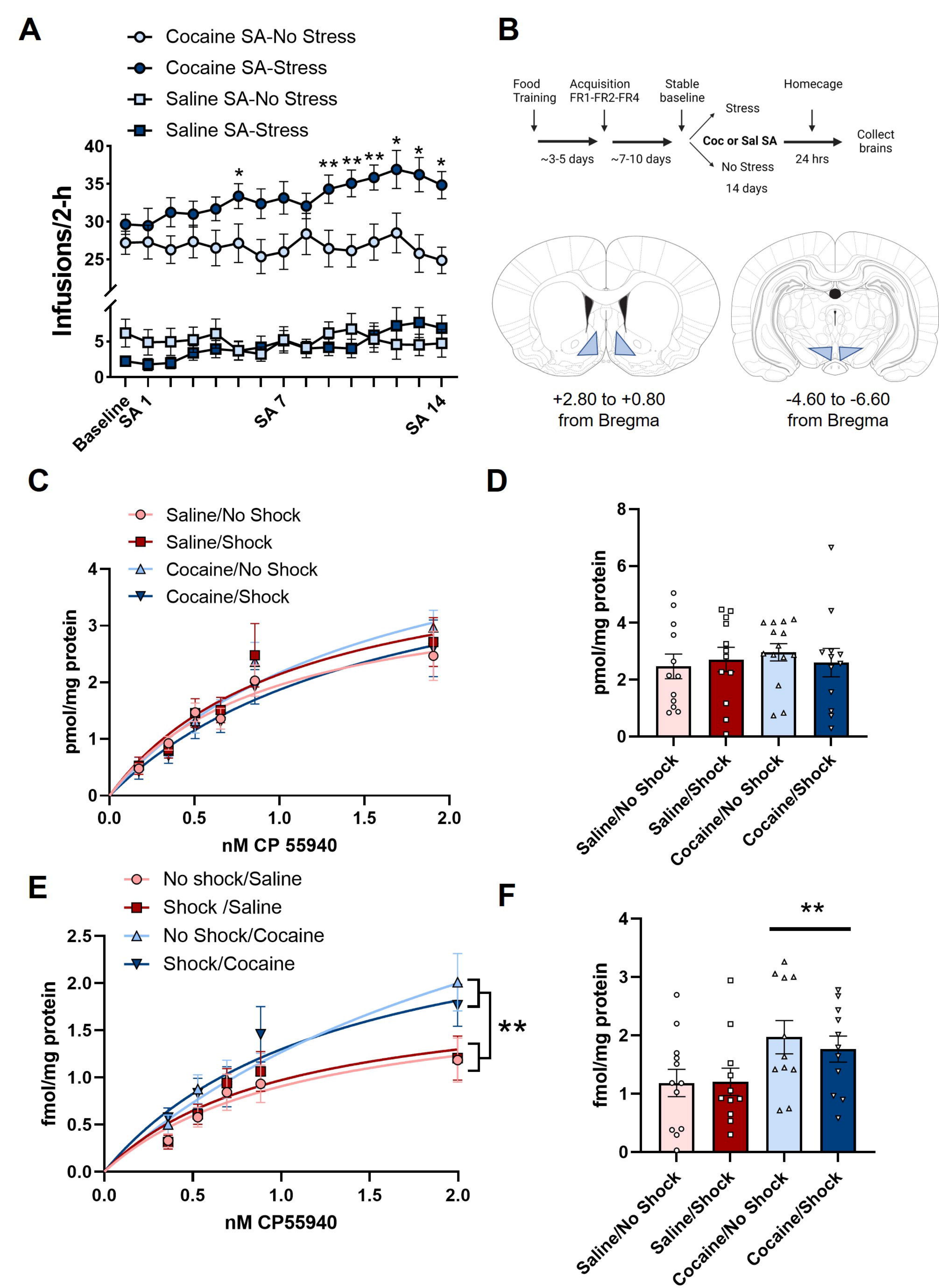
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Cocaine SA-Stress
Saline SA-No Stress
Saline SA-Stress
Infusions/2-h
Baseline
SA 1
SA 7
SA 14
B
Food Training
Acquisition FR1-FR2-FR4
Stable baseline
Homecage
Stress
Coc or Sal SA
No Stress
14 days
Collect brains
~3-5 days
~7-10 days
24 hrs
+2.80 to +0.80 from Bregma
-4.60 to -6.60 from Bregma
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Saline/No Shock
Saline/Shock
Cocaine/No Shock
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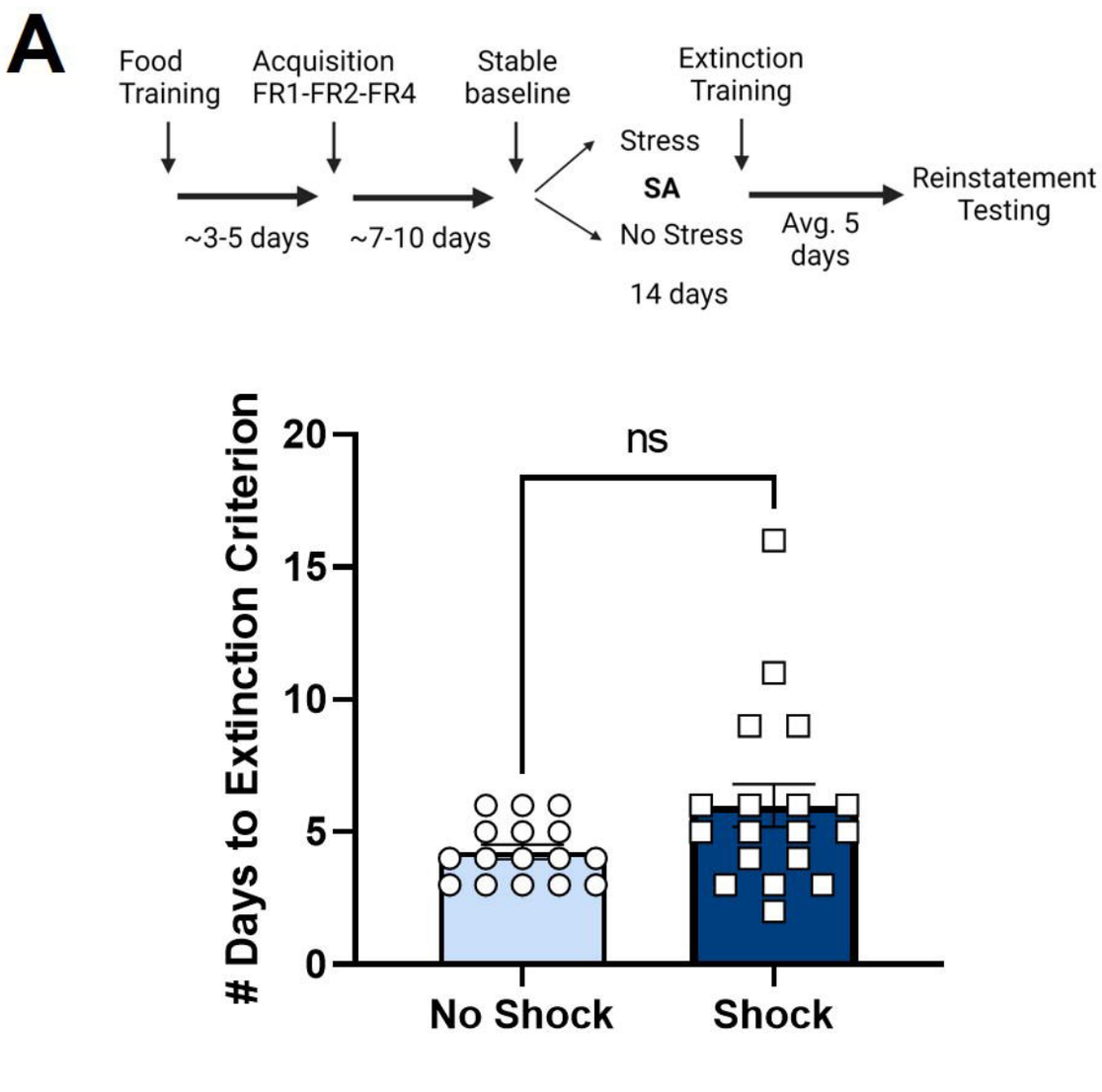
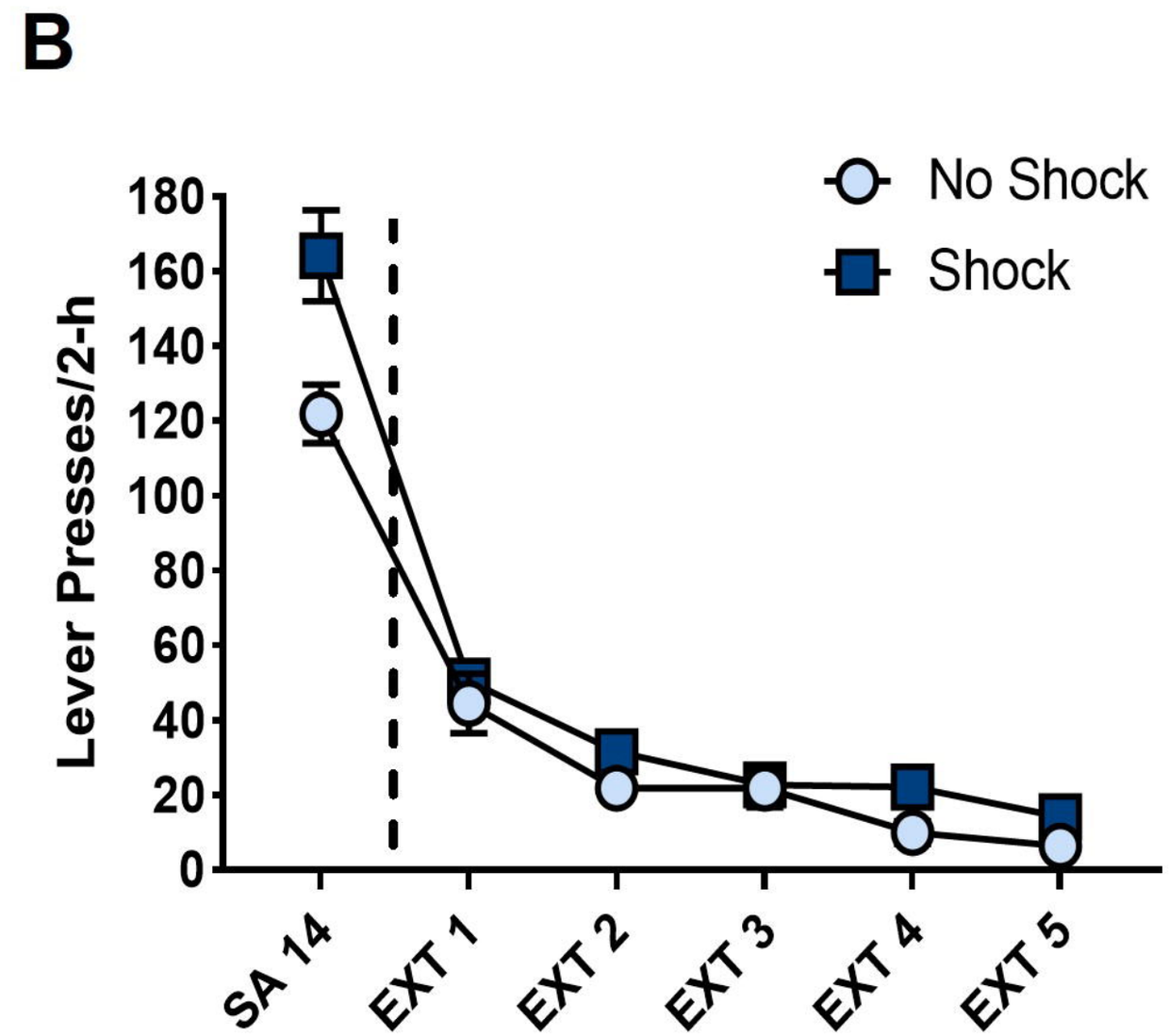
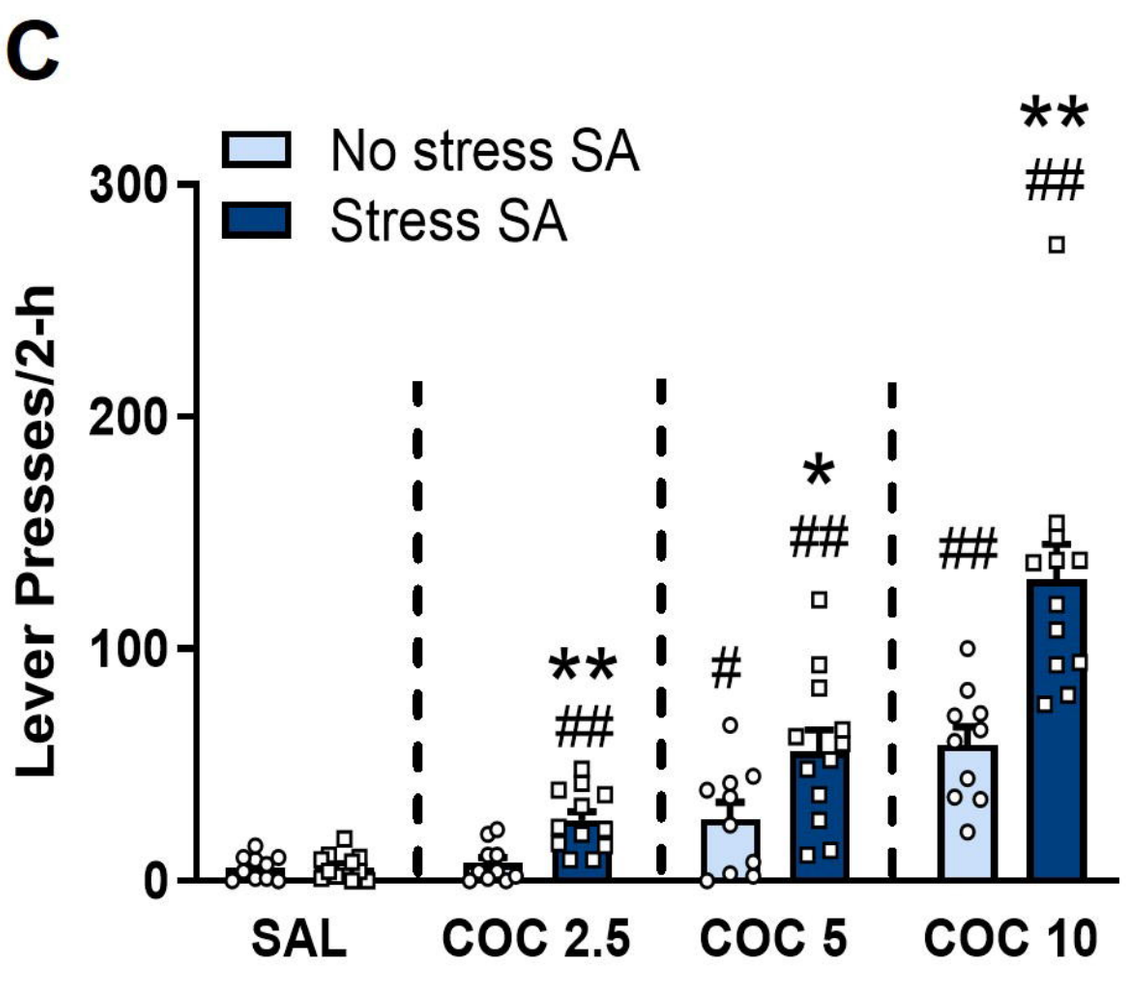
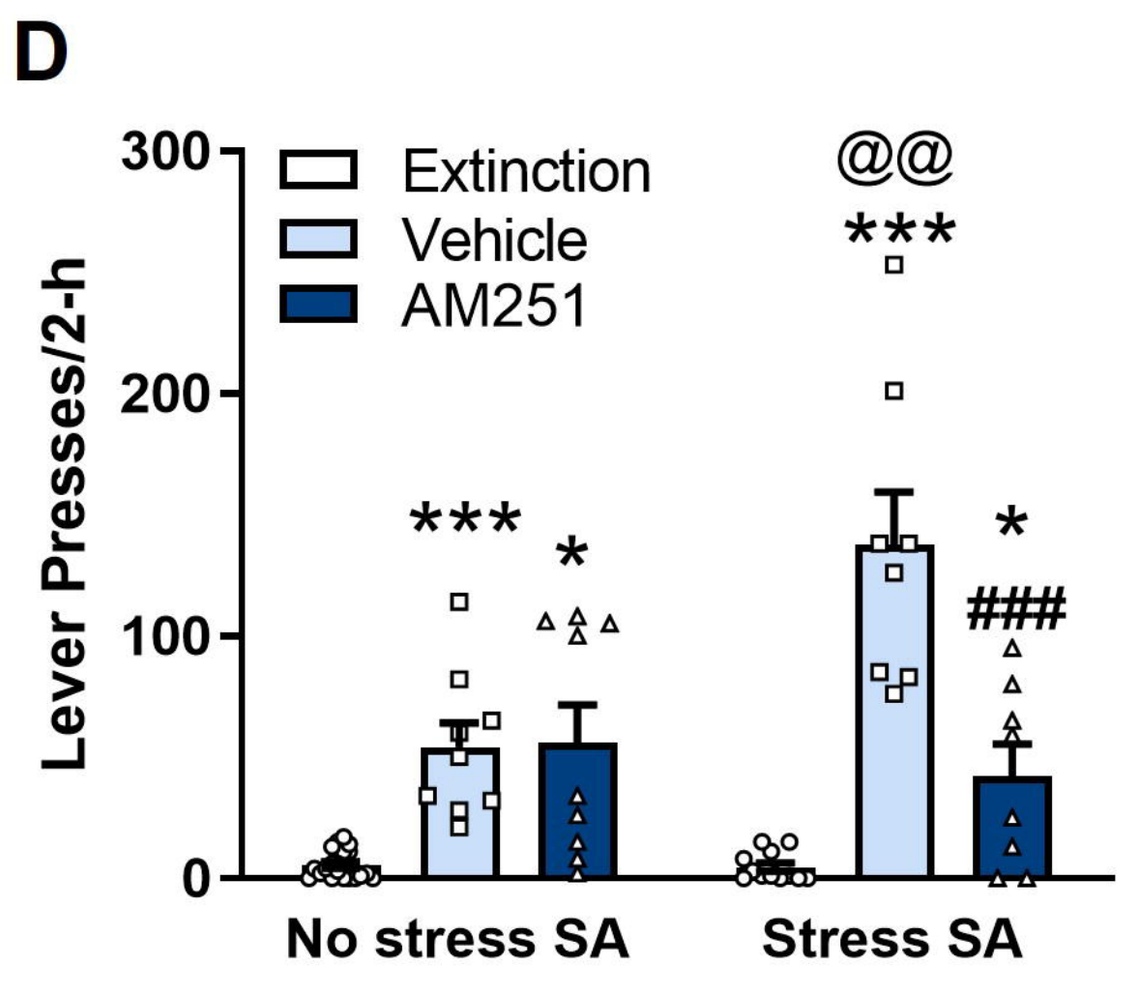
nM CP 55940
D
pmol/mg protein
Saline/No Shock
Saline/Shock
Cocaine/No Shock
Cocaine/Shock
E
No shock/Saline
Shock /Saline
No Shock/Cocaine
Shock/Cocaine
fmol/mg protein
nM CP55940
F
fmol/mg protein
Saline/No Shock
Saline/Shock
Cocaine/No Shock
Cocaine/Shock


A
Food Training
Acquisition FR1-FR2-FR4
Stable baseline
Extinction Training
Stress
SA
No Stress
14 days
Reinstatement Testing
~3-5 days
~7-10 days
Avg. 5 days
Days to Extinction Criterion
ns
No Shock
Shock


B
No Shock
Shock
Lever Presses/2-h
SA 14
EXT 1
EXT 2
EXT 3
EXT 4
EXT 5


C
No stress SA
Stress SA
Lever Presses/2-h
SAL
COC 2.5
COC 5
COC 10


D
Extinction
Vehicle
AM251
Lever Presses/2-h
No stress SA
Stress SA