Psychopharmacology (2015) 232:4469–4479
DOI 10.1007/s00213-015-4082-z

ORIGINAL INVESTIGATION

Increased mesocorticolimbic dopamine during acute and repeated social defeat stress: modulation by corticotropin releasing factor receptors in the ventral tegmental area

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Received: 17 July 2015 / Accepted: 3 September 2015 / Published online: 25 September 2015

Abstract
Rationale Stress activates a subset of dopamine neurons in the ventral tegmental area (VTA), increasing extracellular dopamine in the medial prefrontal cortex (mPFC) and nucleus accumbens shell (NAcSh). The stress neuropeptide corticotropin releasing factor (CRF) and its receptors (CRF-R1 and CRF-R2) are located within the VTA and directly and indirectly influence dopaminergic activity. However, it has yet to be shown in vivo whether VTA CRF receptor activation is necessary for acute and repeated stress-induced dopamine efflux.
Objective With intra-VTA CRF-R1 and CRF-R2 antagonism during social defeat, we assessed whether blockade of these receptors could prevent stress-induced dopamine increases in the mPFC and NAcSh using in vivo microdialysis.
Methods Rats were microinjected with a CRF-R1 or CRF-R2 antagonist into the VTA prior to social defeat stress on days 1, 4, 7, and 10. In vivo microdialysis for dopamine in the mPFC and NAcSh was performed during stress on days 1 and 10.
Results During the first social defeat, extracellular dopamine was significantly elevated in both the mPFC and NAcSh, and this increase in the NAcSh was blocked by intra-VTA CRF-R2, but not CRF-R1, antagonism. During the final social defeat, the dopaminergic increase was neither sensitized nor habituated in the mPFC and NAcSh, and intra-VTA CRF-R2, but not CRF-R1, antagonism prevented the dopamine increase in both brain regions.
Conclusion These findings show that CRF-R2 in the VTA is necessary for acute and repeated stress-induced dopamine efflux in the NAcSh, but is only recruited into mPFC-projecting dopamine neurons with repeated stress exposure.

Keywords Social defeat · Dopamine · Corticotropin releasing factor · CRF-R1 · CRF-R2 · Ventral tegmental area

Introduction

Persistent changes from repeated as opposed to acute stress exposure contribute to the etiology of many stress-related disorders, such as drug addiction (Sinha 2001), depression (Nestler et al. 2002), and several anxiety disorders (Heim and Nemeroff 2001). In the brain, stress can dynamically excite the mesocorticolimbic dopamine system, and repeated stress exposure causes enduring synaptic adaptations in dopamine neurons in the ventral tegmental area (VTA), which may be at the core of several stress-related psychiatric disorders (Polter and Kauer 2014). The present study explores the mechanisms by which acute and repeated stress may influence dopaminergic activity: we ask how the actions of the neuropeptide corticotropin releasing factor (CRF) on its receptors (CRF-R1 and CRF-R2) within the VTA modulate extracellular dopamine in VTA projection targets.

Various types of acute stressors rapidly and potently activate VTA dopamine neurons (Anstrom et al. 2009; Anstrom and Woodward 2005; Brischoux et al. 2009), resulting in extracellular dopamine increases in the medial prefrontal cortex (mPFC) and nucleus accumbens shell (NAcSh) (Abercrombie et al. 1989; Imperato et al. 1989, 1991). However, less is known about the effects of

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repeated stress on mesocorticolimbic dopamine activity. Depending on the intensity, duration, frequency, controllability, and predictability of the stressor, some have found that repeated stress causes a habituation (Imperato et al. 1992, 1993), sensitization (Jordan et al. 1994; Naef et al. 2013; Petty et al. 1997), or no change (Young 2004) in extracellular mPFC or NAcSh dopamine in response to the same stressor.

VTA dopamine activity can be modulated by several neuropeptides, including CRF, one key initiator of the central and peripheral stress response. In addition to stimulating the hypothalamic-pituitary-adrenal (HPA) axis stress response, CRF and its receptors are found in widespread extrahypothalamic regions, including the VTA (Swanson et al. 1983). CRF is released into the VTA during footshock (Wang et al. 2005) and social defeat (Holly et al. 2015, under review) stress. CRF-containing neurons form symmetric (inhibitory) and asymmetric (excitatory) synapses onto VTA dendrites, co-releasing GABA or glutamate, respectively; however, the synapses onto dopamine neurons are predominately glutamatergic (Tagliaferro and Morales 2008). Thus, CRF release into the VTA during stress is primed to excite VTA dopamine neurons projecting to the NAc and mPFC.

The VTA expresses both CRF-R1 and CRF-R2 (Ungless et al. 2003; Van Pett et al. 2000), but the synaptic location and function of these receptors has become the subject of considerable debate. Initial work showed that CRF increases VTA dopamine neuron firing rate through postsynaptic CRF-R1 activation (Korotkova et al. 2006; Wanat et al. 2008), while postsynaptic CRF-R2 activation induces transient potentiation of NMDA-mediated synaptic transmission (Hahn et al. 2009; Ungless et al. 2003) and enhancement of metabotropic glutamate receptors on VTA dopamine neurons (Fiorillo and Williams 1998). Other work, however, has demonstrated that both CRF-R1 and CRF-R2 are located presynaptically, as their selective activation alters AMPA- and not NMDA-mediated excitatory postsynaptic currents (EPSCs) (Manabe et al. 1993; Williams et al. 2014).

We have recently shown that pharmacological blockade of CRF-R1 or CRF-R2 within the VTA during intermittent social defeat stress can prevent the induction of later dopaminergic cross-sensitization to cocaine as well as escalated cocaine self-administration (Boyson et al. 2014). However, the influence of CRF in the VTA on dopaminergic efflux in VTA projection targets during social defeat remains to be characterized. The current study explores how VTA CRF receptor activation influences acute and repeated social stress-induced increases in extracellular mPFC and NAcSh dopamine. Rats were microinjected with CRF-R1 or CRF-R2 antagonists into the VTA prior to intermittent social defeat stress (days 1, 4, 7, and 10) and underwent in vivo microdialysis for dopamine in the mPFC and NAcSh during the first and last social defeat.

Methods

Subjects

Male Long-Evans rats (n=38, Charles River, Wilmington, MA) weighing 225–250 g at arrival were singly housed in custom-built acrylic chambers (30×20.5×24.5 cm) with wire mesh side panels. Stimulus "resident" rats were housed in a separate room in male-female pairs in large stainless steel cages (71×46×46 cm). All rats were provided food and water ad libitum, and the vivarium was maintained on a 12-h light/dark cycle (lights on at 20:00) under controlled temperature (21±1 °C) and humidity (30–40 %). All procedures were approved by the Tufts University Institutional Animal Care and Use Committee, following the guidelines set in the *Guide for Care and Use of Laboratory Animals* (National Research Council 2011).

Experimental design

Rats underwent intracranial surgery, after which they were exposed to episodic social defeat stress on days 1, 4, 7, and 10. Vehicle (artificial cerebrospinal fluid, aCSF), CRF-R1 antagonist (CP376395, CP, 500 ng/side), or CRF-R2 antagonist (Astressin2B, A2B, 1000 ng/side) was microinjected 10 min prior to the instigation phase of each social defeat, and in vivo microdialysis of both the mPFC and NAcSh performed concurrently with the defeats on days 1 and 10 (see Table 1 for the group sizes and Fig. 1 for the experimental design). Drug doses were chosen based on our prior dose-response work with these compounds (Boyson et al. 2014).

Intracranial surgery

After 1 week of habituation to the vivarium and at least 1 week before the first day of microdialysis, rats underwent intracranial surgery under ketamine (100 mg/kg, ip) and xylazine (6 mg/kg, ip) anesthesia. Bilateral microinjection cannulae (23 ga, 11 mm length, PlasticsOne, Roanoke, VA) were implanted at a 10° angle 5.2 mm posterior from bregma and 1.8 mm lateral from midline at a depth of 7.5 mm from the skull surface. Two unilateral microdialysis cannulae (8 mm length, Synaptech Inc, Marquette, MI) were also implanted, aimed at both the mPFC (+3.0 mm from bregma, +1.7 mm from midline, −4.0 mm from dura, 10° angle) and the NAcSh (+2.1 mm from bregma, +1.1 mm from midline, −5.8 mm from dura, 0° angle).

Microinjections

Drugs were microinjected into the VTA with an infusion pump (CMA 102, CMA Microdialysis, Chelmsford, MA) using 33 ga microinjectors protruding 1 mm beyond the guide cannulae

Table 1 Group sizes

Pretreatment	Day 1		Day 10		Included both days	
	mPFC	NAcSh	mPFC	NAcSh	mPFC	NAcSh
aCSF	7	9	5	5	3	4
CP	4	6	6	5	4	3
A2B	6	6	5	5	3	3

(PlasticsOne, Roanoke, VA). Drugs and vehicle were administered in a volume of 0.25 μl/side across 1 min, and injectors left in place for an additional 1 min after the infusion to allow adequate diffusion from the injection site and prevent backflow.

Social defeat stress

A modification of a previously described resident-intruder paradigm was used (Miczek 1979). Rats were exposed to four brief social defeat stress episodes, separated by approximately 72 h, on days 1, 4, 7, and 10. The defeats occurred in three phases: (1) instigation: the female resident was removed, and the experimental animal (intruder) in its home cage placed inside the resident's home cage for 10 min. This allowed for visual and olfactory instigation through the wire mesh panels, but prevented tactile contact. (2) Fight: The intruder's home cage was removed, and the experimental rat placed with the aggressive resident for 5 min. Attack latency and number of bites were recorded, and no statistical differences were observed between the treatment groups or microdialysis days and non-microdialysis days. (3) Threat: The experimental animal was then returned to its home cage inside the resident's cage for an additional 10 min, after which it was removed.

In vivo microdialysis

In vivo microdialysis for dopamine in the mPFC and NAcSh occurred on both days 1 and 10 of the social defeat protocol (Fig. 2). On the night before the microdialysis experiment, both microdialysis stylets were removed and replaced with microdialysis probes (2 mm active membrane, Synaptech Inc, Marquette, MI), which were perfused with aCSF (147 mmol/l NaCl, 2.7 mmol/l KCl, 1.2 mmol/l $CaCl_2$, 0.85 mmol/l $MgCl_2$) at a flow rate of 0.5 μl/min overnight. The flow rate was increased to 2.0 μl/min 2 h prior to sample collection the following day. Samples were collected by hand every 5 min into Eppendorf PCR tubes containing 4 μl antioxidant (20 mM phosphate buffer containing 25 mM EDTA-2Na and 0.5 mM ascorbic acid, pH 3.5), and tonic levels of dopamine were measured in five baseline samples. After baseline collection, animals were microinjected as described

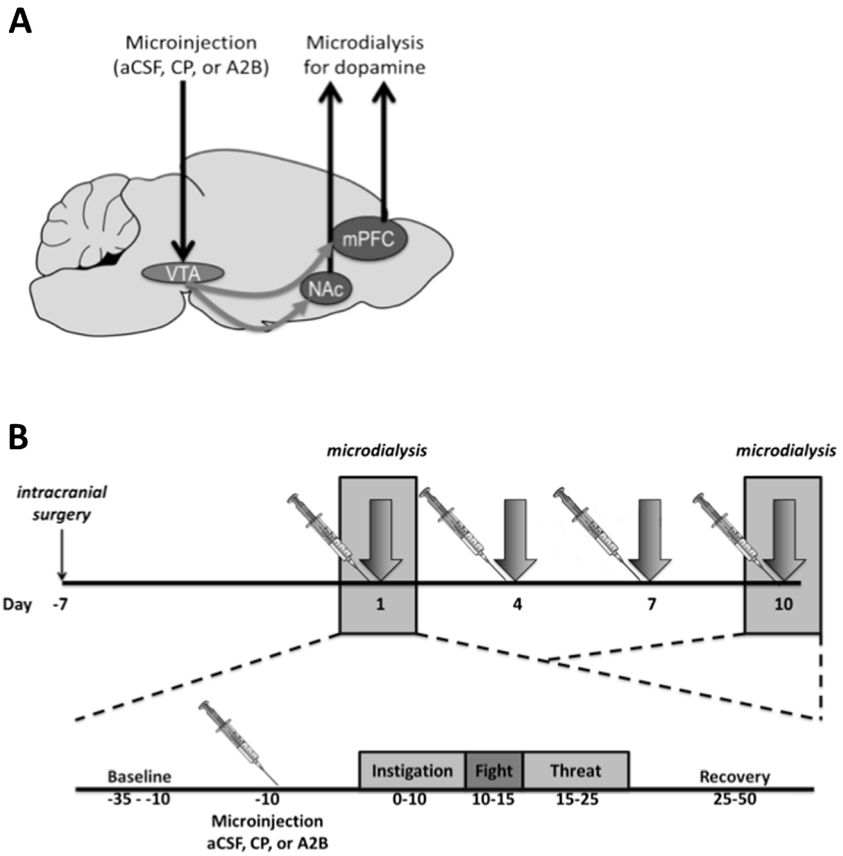

Fig. 1 Experimental design and timeline. **a** Rats were microinjected with vehicle (artificial cerebrospinal fluid, *aCSF*), CRF-R1 antagonist (CP376395, *CP*), or CRF-R2 antagonist (Astressin2B, *A2B*) into the ventral tegmental area (*VTA*) and microdialysis samples collected from the medial prefrontal cortex (*mPFC*) and nucleus accumbens (*NAc*). **b** Rats underwent intracranial surgery 7 days prior to the first social defeat. Social defeat stress (*gray arrows*) occurred on days 1, 4, 7, and 10, with intra-VTA microinjections (*syringes*) prior to each defeat. In vivo microdialysis was concurrently performed during the social defeat stress on days 1 and 10 (*gray boxes*), with sample times indicated on the timeline on the *bottom*

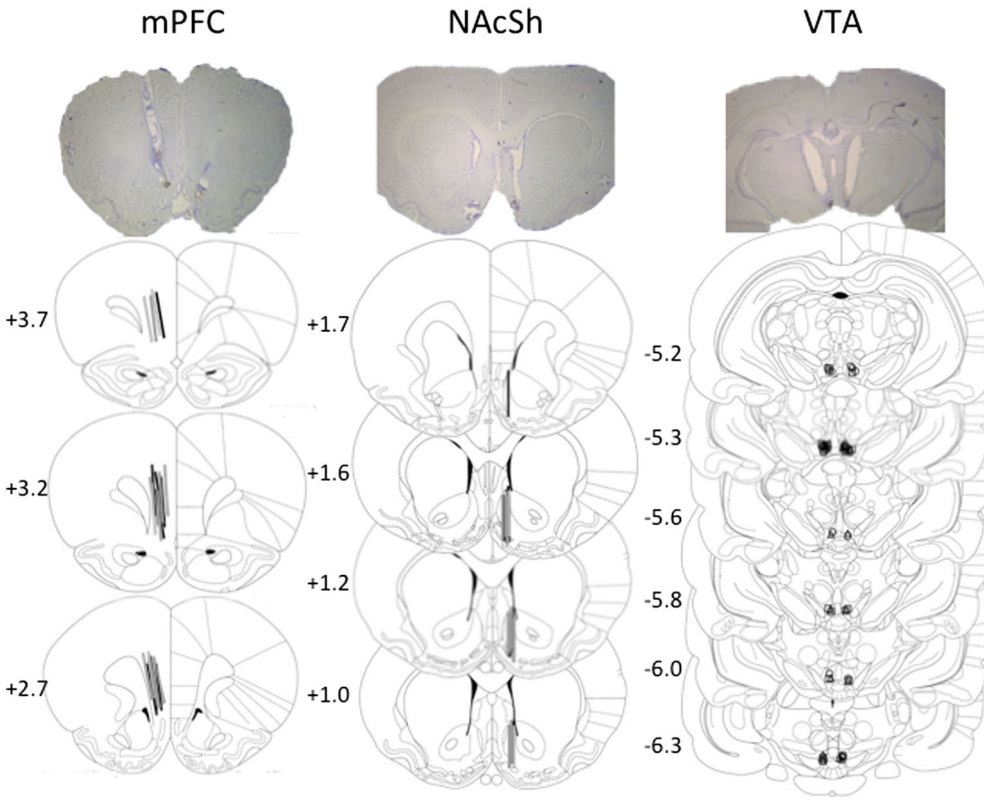

Fig. 2 Histology. (*Top*) Representative photopictomicrographs and (*bottom*) placements are depicted for the medial prefrontal cortex (*mPFC*, *left*, *black* = aCSF group, *light gray* = CP376395 group, *dark gray* = Astressin2B group), nucleus accumbens shell (*NAcSh*, *middle*, *black* = aCSF group, *light gray* = CP376395 group, *dark gray* = Astressin2B group), and ventral tegmental area (*VTA*, *right*, *circles* = aCSF group, *squares* = CP376395 group, *triangles* = Astressin2B group). Distributions of placements did not vary across treatment groups

above, and social defeat occurred in an adjacent resident rat home cage, with sample collection ongoing throughout the defeat. After the rat was removed from the threat phase, five additional samples were collected to evaluate the time course of dopaminergic changes after social stress termination. On days 4 and 7, microinjection and social defeat occurred in an identical manner, but microdialysis was not performed. Five rats were excluded from all analysis because their headmounts came off during the first defeat. On day 1, all samples from the mPFC (n=6 rats) and NAcSh (n=5 rats) were excluded due to complications during sampling (probes removed during defeat, tubings bitten by the resident, etc). On day 10, all samples from the mPFC (n=11 rats) and NAcSh (n=12 rats) were excluded either because their headmounts came off between the end of day 1 microdialysis and the end of the defeat on day 10 or due to complications during sampling as described above.

Dopamine was analyzed by HPLC as described previously (Boyson et al. 2014; Holly et al. 2012). Mobile phase (150 nM ammonium acetate, 50 mM citric acid, 27 μM EDTA, 10 % methanol, 1 % acetonitrile, pH adjusted to 4.6 by glacial acetic acid) was pumped by an LC10-AD pump (Shimadzu, Columbia, MD) at a flow rate of 0.200 ml/min. Samples were injected with a manual injector (Rheodyne 7725, IDEX Health and Science LLC, Rohnert Park, CA) with a 100-μl sample loop. Monoamines were separated by a cation-exchange column (CAPCELL PAK, 1.5 mm×250 mm, 5 μm ID, Shiseido, Tokyo, Japan) at 30 °C and quantified by electrochemical detection (DECADE II, Antec Leyden BV, Zoeteroude, Netherlands), and dopamine concentrations were calculated using a standard curve with known amounts of dopamine in a range of 1.975–18.75 pg.

Histology

At the culmination of the experiments, rats were anesthetized with pentobarbital (100 mg/kg, ip) and transcardially perfused with saline followed by 4 % paraformaldehyde. Brains were removed and placed in 4 % paraformaldehyde for at least 24 h prior to slicing into 55-μm sections for cresyl violet staining as described previously (Boyson et al. 2014; Holly et al. 2012). All rats had correct bilateral microinjection cannulae placement, and dopamine data were excluded from one or both regions in the event of missed mPFC (n=1) or NAcSh placement (n=3 in NAc core).

Statistical analysis

Statistical analysis was performed with SigmaPlot 11.0 (Systat Software Inc., San Jose, CA). Data were analyzed with two-way repeated measures analysis of variance (ANOVA), followed by post hoc analysis with Holm-Sidak corrections for multiple comparisons.

Results

Baseline concentrations of dopamine were variable (but not significantly different, Table 2) across the 2 years the study was performed due to varying HPLC column and cell conditions, so all dopamine values were analyzed and presented as percent change from the average of the five baseline samples. All rats showed less than 20 % variability in the five baseline samples. There was no effect of drug or vehicle microinjection, so all further analysis was performed comparing the last microinjection sample with the five samples during stress (two during the instigation phase, one during the fight, two during the threat phase). Furthermore, there was no significant effect of drug treatment or microdialysis day (day 1 or day 10) nor any interaction on baseline dopamine.

Acute stress phasically increases extracellular dopamine in the mPFC, independent of CRF-R1 or CRF-R2 in the VTA

Acute social defeat significantly increased extracellular dopamine in the mPFC (Fig. 3a, b). Two-way repeated measures ANOVA revealed a significant effect of sample ($F_{5, 70}$=7.031, p<0.001), with the aCSF-pretreated animals showing a significant elevation from baseline in all samples during stress with the exception of the first sample of the threat period (p<0.008). Thus, in naive rats with no social defeat experience, the extracellular dopamine in the mPFC immediately and significantly increased as the rats' home cages were moved inside the residents' home cages for instigation. This increased extracellular dopamine in the mPFC persisted and was sustained throughout the duration of instigation as well as the fight. On termination of the physical defeat encounter and beginning of the threat period, the extracellular dopamine in the mPFC initially returned to baseline, but rebounded for the second half of the threat period. Finally, the extracellular dopamine levels returned to baseline for the entire recovery period, after the rats were removed entirely from visual and olfactory contact with the resident.

During the first defeat, there was no effect of either CRF-R1 (Fig. 3a) or CRF-R2 (Fig. 3b) antagonism in the VTA on the stress-induced dopamine increase in the mPFC. There was no difference between baseline samples and samples immediately following the drug microinjections, indicating no direct effect of CRF antagonism on basal levels of dopamine, so analysis was performed between the last microinjection sample and the stress samples. Two-way repeated measures ANOVA revealed neither the main effect of drug pretreatment nor the pretreatment × sample interaction.

Table 2 Average baseline dopamine concentrations (pmol/12.5 μl± SEM)

Pretreatment	mPFC		NAcSh	
	Day 1	Day 10	Day 1	Day 10
aCSF	0.538±0.260	0.225±0.082	0.569±0.079	0.410±0.074
CP	0.220±0.004	9.142±0.067	0.403±0.010	0.212±0.085
A2B	0.192±0.163	0.228±0.004	0.321±0.065	0.355±0.176

Acute stress phasically increases extracellular dopamine in the NAcSh, mediated through CRF-R2, but not CRF-R1, in the VTA

Extracellular dopamine in the NAcSh was also significantly elevated above baseline during the first day of social defeat (Fig. 3c, d). A two-way repeated measures ANOVA revealed a significant main effect of the sample ($F_{5, 80}$=3.443, p=0.007), with aCSF-pretreated animals showing a significant elevation from baseline during the instigation and first portion of the threat period (p<0.026), but not during the fight or second threat period. The increase in extracellular dopamine in the NAcSh showed a slightly different time course than in the mPFC. Extracellular NAcSh dopamine immediately and significantly rose in response to the instigation period, but while extracellular dopamine levels were still elevated during the fight period, it was no longer statistically significant. In contrast with dopamine in the mPFC, extracellular dopamine in the NAcSh remained elevated during the initial half of the threat period following the fight, but returned to baseline for the second half of the threat period, and remained at baseline for the duration of sampling during the recovery period.

The stress-induced dopamine increase in the NAcSh during the first defeat requires intact CRF-R2 within the VTA (Fig. 3d). There was a main effect of drug pretreatment (two-way repeated measures ANOVA $F_{2, 16}$=3.856, p=0.043), although there was no pretreatment × sample interaction. The main effect of drug pretreatment was driven by rats given the CRF-R2 antagonist Astressin2B into the VTA, such that overall the NAcSh dopamine in the Astressin2B-pretreated group was less than that in the aCSF-pretreated controls (Holm-Sidak t=2.713, p=0.030), and dopamine levels were not significantly increased from baseline during or after stress. No effect of intra-VTA CRF-R1 antagonism was observed (Fig. 3c).

Repeated social defeat stress does not result in habituation or sensitization of the dopaminergic response in the mPFC and NAcSh

The dopaminergic response to stress in both the mPFC and NAcSh was not significantly different between day 1 and day 10 within the aCSF-pretreated controls, demonstrating neither habituation nor sensitization with repeated intermittent social defeat experience (Fig. 4). Additionally, the time course of

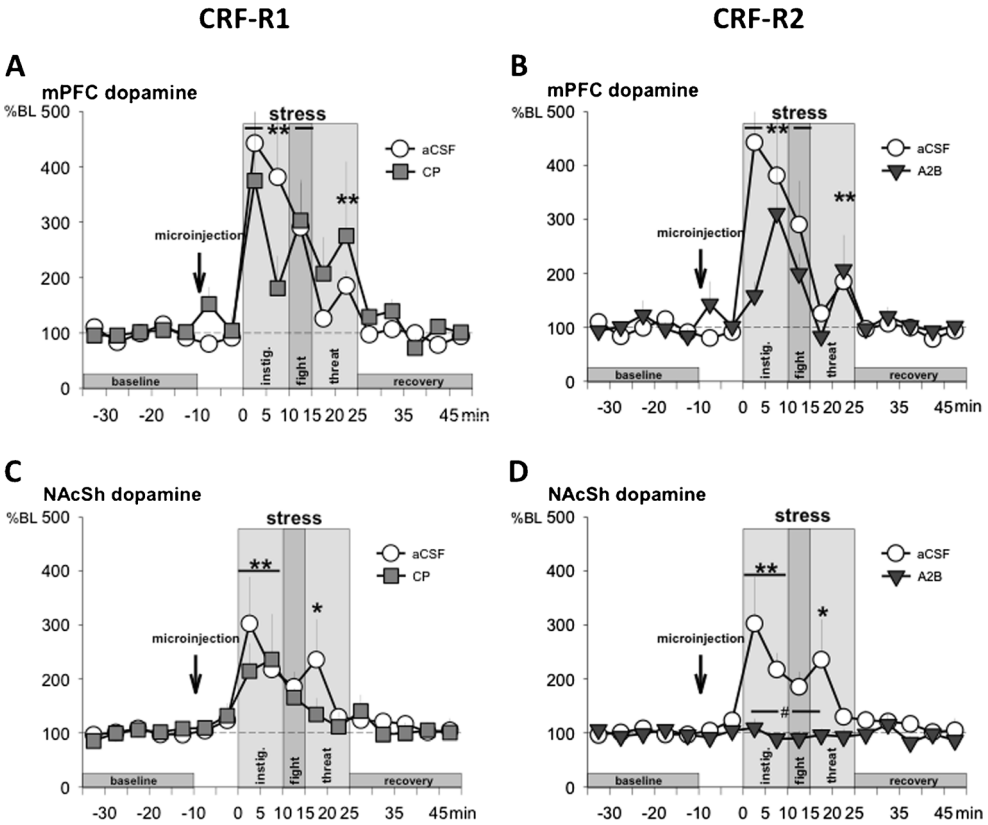

Fig. 3 Effect of intra-VTA CRF-R1 and CRF-R2 antagonism on acute stress-induced dopamine efflux in the mPFC and NAcSh. Extracellular dopamine, expressed as percent change from baseline (%BL), was measured in 5-min samples from the medial prefrontal cortex (*mPFC*; **a**, **b**) and nucleus accumbens shell (NAcSh; **c**, **d**) during the first day of social defeat stress. After five baseline samples, vehicle (artificial cerebrospinal fluid, *aCSF*, mPFC n=7, NAcSh n=8), CRF-R1 antagonist (CP376395, *CP*, mPFC n=4, **a**; NAcSh n=5, **c**), or CRF-R2 antagonist (Astressin2B, *A2B*, mPFC n=6, **b**; NAcSh n=6, **d**) was microinjected into the ventral tegmental area (VTA). Ten minutes later, rats underwent social defeat stress, consisting of instigation (*instig.*), *fight*, and *threat* periods, after which they were removed and dopamine measured an additional 25 min. *p<0.05, **p<0.01 versus baseline; #p<0.01 versus aCSF

changes in extracellular dopamine in both regions on the last day was closely similar to that of the first day.

In the mPFC, an a priori hypothesis-driven two-way repeated measures ANOVA revealed a main effect of sample ($F_{5, 10}$= 3.862, p=0.033), but no effect of microdialysis day nor an interaction. Extracellular dopamine was significantly elevated above baseline in both samples during the instigation period (p<0.010), but although elevated, was not significantly different from baseline during the fight period. Similar to day 1, extracellular dopamine in the mPFC decreased to baseline during the first half of the threat period, but the increase during the second half of the threat was no longer statistically significant.

In the NAcSh, an a priori hypothesis-driven two-way repeated measures ANOVA revealed a main effect of sample ($F_{5, 15}$=3.940, p=0.018), but no effect of microdialysis day nor an interaction. The time course of change from baseline in extracellular dopamine in the NAcSh on day 10 was statistically identical to day 1.

Intra-VTA antagonism of CRF-R2, but not CRF-R1, prevents the repeated stress-induced extracellular dopamine increase in the mPFC

In contrast to day 1, intra-VTA antagonism of CRF-R2 prevented the stress-induced increase in dopamine in the mPFC during stress, while CRF-R1 antagonism still had no effect (Fig. 5a, b). Again, there was no effect of microinjection compared to baseline, so further analysis was performed between the last microinjection sample and the five samples during stress. Two-way repeated measures ANOVA revealed significant main effects of sample ($F_{5, 65}$=4.986, p<0.001) and drug pretreatment ($F_{2, 13}$=4.773, p=0.028), with no interaction between sample and drug pretreatment. The drug pretreatment effect was driven by the CRF-R2 antagonist group, such that mPFC dopamine in rats given Astressin2B prior to defeat was significantly lower than that in aCSF-pretreated controls (Holm-Sidak t=2.888, p=0.025), and dopamine levels did not significantly deviate from baseline.

Intra-VTA antagonism of CRF-R2, but not CRF-R1, prevents the repeated stress-induced extracellular dopamine increase in the NAcSh

Similarly, intra-VTA antagonism of CRF-R2, but not CRF-R1, prevented the stress-induced dopamine increase in the NAcSh during stress (Fig. 5c, d). There were significant main effects of sample (two-way repeated measures ANOVA $F_{5, 55}$=2.888, p=0.022) and drug pretreatment ($F_{2, 11}$=5.211, p=0.026), with no interaction between sample and drug pretreatment. While rats pretreated with the CRF-R1 antagonist

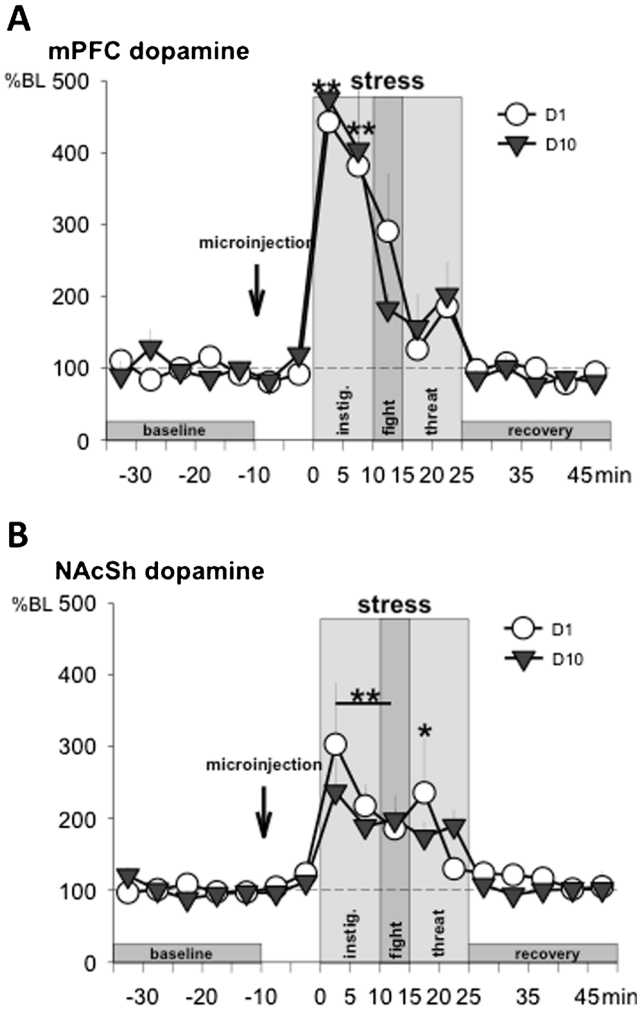

Fig. 4 Acute versus repeated social defeat stress-induced dopamine efflux in the mPFC and NAcSh. Extracellular dopamine, expressed as percent change from baseline (%BL), was measured in 5-min samples from the medial prefrontal cortex (*mPFC*, **a**) and nucleus accumbens shell (*NAcSh*, **b**) during the first (*D1*) and last (*D10*) day of social defeat stress. After five baseline samples, vehicle (artificial cerebrospinal fluid, aCSF) was microinjected into the ventral tegmental area (VTA). Ten minutes later, rats underwent social defeat stress, consisting of instigation (*instig.*), *fight*, and *threat* periods, after which they were removed and dopamine measured an additional 25 min. *$p<0.05$, **$p<0.01$ versus baseline; mPFC D1 $n=7$, D10 $n=5$; NAcSh D1 $n=9$, D10 $n=5$

did not differ from aCSF controls, those pretreated with the CRF-R2 antagonist did (Holm-Sidak $t=2.731$, $p=0.039$), and dopamine levels never significantly changed from baseline during stress.

Discussion

The current study replicates and extends past work, demonstrating that extracellular mesocorticolimbic dopamine is significantly increased during social defeat stress, with neither habituation nor sensitization in the dopaminergic response to repeated intermittent social defeat. We also demonstrate that CRF-R2, but not CRF-R1, in the VTA modulates stress-induced dopamine efflux in the mPFC and NAcSh.

CRF-dopamine interactions during acute stress

Consistent with previous findings using other stressors (Joseph et al. 2003), social defeat stress rapidly and potently increases extracellular dopamine within both the mPFC and NAcSh as measured by in vivo microdialysis. The greater percent change from baseline observed in this study compared with previous reports is likely due to the greater temporal resolution achieved in the current experiments. While previous experiments have used 10–30-min samples, the use of 5-min samples here resulted in a distinct, replicable time course in the dopaminergic changes during the stress exposure.

Although the experimental rats had never been exposed to social defeat, they exhibited immediate increases in extracellular dopamine in the mPFC and NAcSh as soon as they were placed in the aggressor's home cage. During the 10-min instigation phase, the resident rat bites and threatens the protective barrier, while the intruder often vocalizes audibly (personal observation). While it could be argued that the concurrent dopaminergic increase is due to novelty, Feenstra and Botterblom (1996) report that exposure to a novel environment only increases extracellular dopamine in the mPFC to 135 % baseline in 5.5-min samples, whereas the present assays reveal an increase of 443 % from baseline. Similarly, evidence for novel environment-induced changes in NAc dopamine is limited, with transient increases of no more than 120 % baseline using 10-min samples (Ladurelle et al. 1995). Furthermore, Tidey and Miczek (1996) found no extracellular dopamine increase in control rats placed in a resident's home cage without the resident present. Exposure to a novel social partner cannot account for such dramatic increases observed in the present study, as exposure to novel juveniles only results in approximately 120 % basal dopamine levels in both the mPFC and NAcSh (De Leonibus et al. 2006). As such, while novelty may contribute to the dopaminergic increase, the most likely interpretation of the observed dopamine increase in both the mPFC and NAcSh during the instigation and subsequent phases of social defeat is a stressful reaction to the aggressive display of the resident rat. Although indices of a stress response are not reported in the present study, social defeat stress has been previously demonstrated to induce a very large sympathetic and adrenocortical activation (Covington and Miczek 2005; Tornatzky and Miczek 1993).

The present results are also consistent with the findings from in vivo electrophysiology and fast-scan cyclic voltammetry during acute social defeat. Although a different procedure for social defeat stress was implemented, Anstrom et al.

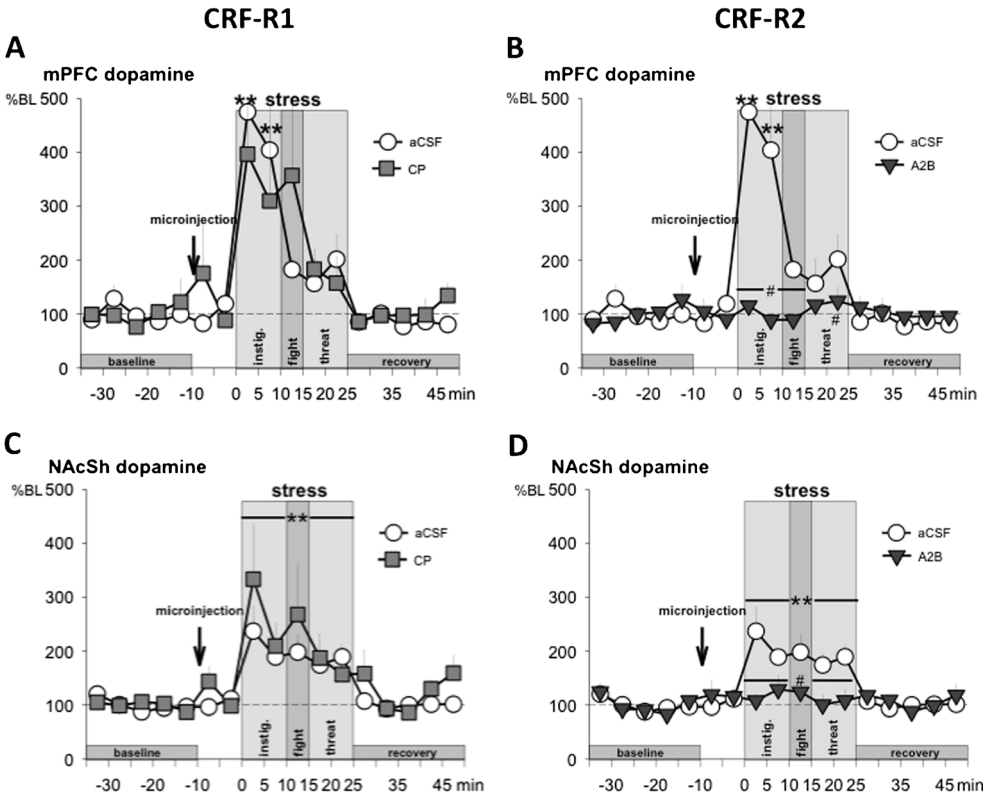

Fig. 5 Effect of intra-VTA CRF-R1 and CRF-R2 antagonism on repeated stress-induced dopamine efflux in the mPFC and NAcSh. Extracellular dopamine, expressed as percent change from baseline (%BL), was measured in 5-min samples from the medial prefrontal cortex (*mPFC*; **a**, **b**) and nucleus accumbens shell (*NAcSh*; **c**, **d**) during the last day of social defeat stress. After five baseline samples, vehicle (artificial cerebrospinal fluid, *aCSF*, mPFC n=5, NAcSh n=5), CRF-R1 antagonist (CP376395, *CP*, mPFC n=6, **a**; NAcSh n=4, **c**), or CRF-R2 antagonist (Astressin2B, *A2B*, mPFC n=5, **b**; NAcSh n=5, **d**) was microinjected into the ventral tegmental area (VTA). Ten minutes later, rats underwent social defeat stress, consisting of instigation (*instig.*), *fight*, and *threat* periods, after which they were removed and dopamine measured an additional 25 min. *p<0.05, **p<0.01 versus baseline; #p<0.01 versus aCSF

(2009) demonstrated an increase in VTA dopamine neuron burst firing as well as phasic increases in NAcSh dopamine during the 5-min social defeat encounter. Contrary to the present protocol, no threat period followed the social defeat, and increased burst firing and oxidation/reduction currents were measured after the experimental animals were returned to their home cage. One interpretation would assign negatively reinforcing effects to the termination of defeat, such that the increased burst firing and phasic dopamine increases in the nucleus accumbens are congruent with the current report of increased extracellular dopamine in the NAcSh following the social defeat. Alternatively, our observation that extracellular dopamine in the NAcSh when the experimental rats were returned to their home cage could be the result of reduced temporal resolution, as fast-scan cyclic voltammetry can capture phasic, subsecond dopaminergic increases.

Acute social defeat stress also increases extracellular CRF as measured by in vivo microdialysis (Holly et al. 2015, under review), and the present findings indicate that this phasic CRF release may act upon CRF-R2 to directly or indirectly increase extracellular dopamine in the NAcSh, but not mPFC. This effect on dopaminergic activity could be induced by synergistic action of CRF and CRF binding protein (CRF-BP) on CRF-R2 to potentiate NMDAR-mediated EPSCs on dopaminergic neurons (Ungless et al. 2003). However, without CRF-BP, CRF-R2 activation enhances mGluR function through a PKA pathway (Fiorillo and Williams 1998). Future work should clarify the role of CRF-BP in conjunction with CRF-R2 on NAcSh dopamine efflux during acute stress.

As there was no effect of CRF-R1 antagonism during acute stress, the current experiment demonstrates that CRF-R1 may have less of an impact on dopaminergic activity than previously thought. Prior electrophysiological work demonstrates that 500 nM–1 μM CRF enhances VTA dopamine neuron firing rate through CRF-R1 (Wanat et al. 2008). However, a dose-response work from both the central amygdala (Roberto et al. 2010) and VTA (Williams et al. 2014) finds that such high concentrations of CRF may not be physiologically relevant and produce different, sometimes opposite, effects from lower physiological CRF concentrations. Therefore, it is likely that while CRF-R1 is present in the VTA, it may not directly and immediately increase dopamine neuron firing rate during stress.

The present results find a more significant role of VTA CRF-R2 than CRF-R1 on acute stress-induced dopamine efflux in the NAcSh. Although the exact synaptic location of CRF-R2 remains unknown, the current work indicates that in naive animals, CRF-R2 could be expressed on NAcSh-projecting dopamine neurons, GABAergic interneurons modulating NAcSh-projecting dopamine neuron activity, the small

proportion of unidentified tertiary neurons within the VTA, or some combination of these possibilities. To date, electrophysiology studies examining the effects of CRF on VTA neuron activity have exclusively attempted to focus on dopamine neurons (Beckstead et al. 2009; Hahn et al. 2009; Korotkova et al. 2006; Ungless et al. 2003; Wanat et al. 2008; Williams et al. 2014), but only one has specifically attempted to investigate the effects of CRF on VTA GABA neurons (Korotkova et al. 2006). While CRF depolarizes VTA GABA interneurons in the presence of tetratotoxin and increases GABA firing rate, the specific roles of CRF-R1 and CRF-R2 were not investigated (Korotkova et al. 2006).

CRF-dopamine interactions during repeated stress

The extracellular mPFC and NAcSh dopamine response to social defeat stress was not altered with repeated experience (Fig. 4). Additionally, there was a shift in the role of CRF-R2 across repeated stress, such that CRF-R2 antagonism prevented the stress-induced increase in both mPFC and NAcSh dopamine during the final defeat, while CRF-R1 antagonism still had no effect (Fig. 5).

As observed during the first stress exposure, extracellular dopamine rapidly and substantially increased in the mPFC and NAcSh as soon as the instigation phase began. As these animals had encountered the same resident and same experimental procedures previously, novelty and social interaction are likely not playing significant roles in this dopaminergic increase. Additionally, although these defeats occurred at approximately the same time each day, no anticipatory rise in dopamine prior to the instigation phase was observed. This is in contrast to the anticipatory rise in NAc extracellular dopamine reported in the aggressive resident rats after repeated intruder confrontations (Ferrari et al. 2003), possibly due to the intermittency and uncontrollability of social defeat in the current experiment.

The time course and magnitude of extracellular dopamine changes during the social defeat procedure was also closely similar between the first and last days of social defeat stress, representing neither habituation nor sensitization. Few previous studies have evaluated the effects of repeated stress exposure on mPFC and NAcSh dopamine within the same animals. Of note, Imperato et al. (1992) demonstrated that repeated daily 120-min restraint stress resulted in a habituated extracellular dopamine response in the NAc when repeated for 6 days; however, a final restraint stress exposure after a 3-day break resulted in a return to the dopaminergic response to restraint observed on day 1. Brief footshock stress 24 h apart also does not show a habituated or sensitized response (Young 2004), while the very mild stress of daily 30-min tail pinch stress yields a sensitized dopamine response after 5 days (Naef et al. 2013). The unchanged dopaminergic response to intermittent social defeat stress may be due to the intermittency of stress exposure or the relative salience of the stressor. It should be considered that there may be interactive effects of repeated probe implantation and repeated social defeat stress in the present study. However, while morphological examinations were not performed in the present experiment, others have found minimal gliosis with no glial barrier formation with up to 30 probe implantations, no detrimental degeneration of dopaminergic fibers in the striatum, and no significant alterations in dopamine concentrations as measured by no-net-flux microdialysis (Georgieva et al. 1993; Lecca et al. 2006a, b, 2007a, b; Martin-Fardon et al. 1997), suggesting minimal effects of repeated probe insertion in the present study.

There was, however, a shift in the role of CRF-R2 from acute to repeated stress. During acute defeat, intra-VTA CRF-R2 antagonism only affected stress-induced dopamine efflux in the NAcSh, but by the last defeat, it prevented dopamine increases in both the NAcSh and mPFC. There are several possible explanations for this change. First, prior work demonstrates that an exposure to forced swim stress can cause the externalization of CRF-R2 in both the locus coeruleus and dorsal raphe nucleus (Bangasser and Valentino 2012; Wood et al. 2013). While receptor trafficking has not yet been examined in the VTA, it may be that CRF-R2 is normally expressed on NAcSh-projecting neurons and, after initial exposure to social defeat, becomes externalized on mPFC-projecting neurons as well. Second, it has recently been reported that the function of CRF-R2 can become reversed with stress exposure. After rats experience yohimbine stress-induced reinstatement to cocaine seeking, presynaptic CRF-R2 in the VTA shifts from stimulating to inhibiting GABA release onto VTA dopamine neurons (Williams et al. 2014). Thus, it may be that during acute defeat, CRF-R2 activation facilitates heterosynaptic regulation of mPFC-projecting VTA dopamine neurons, but after stress exposure, CRF-R2 activation removes the GABAergic brake on VTA dopamine neuronal activity. Finally, there may be a shift in which VTA dopamine neurons respond to repeated as opposed to acute stress exposure. Prior work identifying a subset of aversion-responsive VTA dopamine neurons only measured neurons in response to acute stress exposure (Brischoux et al. 2009). There may be another population of mPFC and/or NAcSh-projecting dopamine neurons that are only recruited after repeated stress exposure.

Conclusion

The present work demonstrates that dynamic interactions of CRF with CRF-R2 in the VTA to promote increases in extracellular dopamine in VTA projection targets during acute social defeat shift over the course of repeated intermittent social defeat. We have previously shown that activation of both CRF-R1 and CRF-R2 is necessary for the development of

neural cross-sensitization to cocaine and escalated cocaine self-administration during a 24-h "binge" (Boyson et al. 2014), pointing to a dissociation between stress-induced dopamine release and later addiction-related behaviors. Future work should clarify how stress-induced activation of VTA dopamine neurons is related to consequent maladaptations, such as increased vulnerability to drug use, depression, or other psychiatric disorders.

Acknowledgments This work was funded by NIDA grant DA031734 to KAM.

Conflict of interest All authors declare that they have no competing interests.

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