Corticotropin releasing factor and dopamine interactions in a heterogeneous ventral tegmental area: How can aversive experiences heighten cocaine self-administration?

A dissertation

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ABSTRACT

Stress rapidly and potently excites certain dopamine neurons in the ventral tegmental area (VTA), promoting phasic dopamine increases in the medial prefrontal cortex (mPFC) and nucleus accumbens shell (NAcSh). Repeated stressful episodes have been linked to addiction both clinically and preclinically, so stress-induced activation of this mesocorticolimbic dopamine system may trigger long-term changes leading to later heightened vulnerability to addiction. One possible mediator of this VTA dopamine activation is the stress neuropeptide corticotropin releasing factor (CRF), but the interactions of CRF and VTA dopamine neurons during stress have received only limited attention.

This dissertation explores interactions of CRF and dopamine neurons in the VTA during acute stress (Aim 1) and repeated stress (Aim 2), as well as how these interactions can affect later reward-related behaviors and neurochemistry (Aim 3). Specifically, this work demonstrates that extracellular dopamine is increased in both the mPFC and NAcSh during acute social defeat, which is paralleled by increased extracellular CRF within the posterior, but not anterior VTA. Intra-VTA antagonism of CRF-R2, but not CRF-R1, prevents acute social defeat stress-induced dopamine efflux in the NAcSh, but not mPFC. Repeated stress results in increased tonic CRF within the VTA, and a shift to a phasic response in the anterior, but not posterior, VTA during stress. Additionally, the non-habituated dopaminergic increases in both the mPFC

and NAcSh during repeated defeat are prevented with intra-VTA CRF-R2 antagonism. However, intra-VTA antagonism of both CRF-R1 and CRF-R2 prior to social defeat prevents the induction of neural cross-sensitization to cocaine as well as escalated cocaine self-administration during a 24 h “binge”. Finally, this work shows that VTA CRF and its receptors play a long-lasting role in cocaine seeking, such that increased extracellular CRF tone within the VTA of previously stressed animals drives augmented context-induced reinstatement to cocaine seeking after abstinence, an effect which can be prevented with intra-VTA antagonism of both CRF-R1 and CRF-R2 prior to reinstatement testing. Together, these data establish a fundamental role of CRF and its receptors in the VTA in mediating dopaminergic function during stress, promoting long lasting neural adaptations of mesocorticolimbic circuitry traditionally associated with reward.

ACKNOWLEDGEMENTS

In writing the acknowledgements section, I had no idea where to start. So I will just start at the beginning. To this day, the best talk I have ever attended was on a sweltering hot day in Rome in the late summer of 2009. To this day, I also think that is the only talk I have ever really taken notes at. I still have them. I pulled them out as inspiration and motivation to push me through the flurried writing of this dissertation, the final struggle. I smiled when I pulled them out, and not just because I found 50 euro. Klaus Miczek's name is excitedly circled in the program, and my notes are filled with exclamation points (although admittedly, those who know me realize that is just a characteristic writing style of mine). The European Behavioral Pharmacology Society Biennial meeting is by far my favorite meeting; it's small, and there are only two concurrent sessions at a time. Luckily Klaus gave the plenary lecture, as I apparently wanted nothing to do with any addiction related sessions during that meeting and would have never seen Klaus speak otherwise. I remember talking to my mom that evening, saying I figured out who I wanted to work with in graduate school. I remember talking to my quasi-mentor Joe Porter, who encouraged me to go introduce myself, which I did not. But clearly I eventually met Klaus, and was sitting in his lab a year later. So, first and foremost, I am eternally grateful to have heard Klaus speak that day.

The journey to where I am now has simultaneously flown by and seemed never ending. I would be nowhere without the phenomenal

mentorship I have been so blessed with on this trek. My undergraduate academic advisor, Dr. Bradley Olson, deserves significant credit for telling me I was far too good to research in his unproductive social psychology lab, and for dragging me into the office of Dr. Adam Prus, where I got back in touch with science. Dr. Prus quickly helped me realize that my personality was unsuitable for a career in clinical psychology, and got me excited about how the brain drives behavior. Of course, I am also very grateful to Dr. Prus' own Ph.D. advisor, Dr. Joe Porter, who served as an excellent, unwavering, supportive grandmentor and has been a source of strength and encouragement throughout the ups and downs.

My experience at Pfizer shaped me substantially as well. My boss Dr. Rouba Kozak was not a boss, she was and is a true mentor. The others kindled the fire, but Rouba set my passion for science and psychopharmacology ablaze with her relentless criticism, demand for perfection, and adamant convictions on how science should be conducted. She made me love *in vivo* microdialysis in behaving animals, but loathe it in passive or anesthetized procedures. She started getting me to think like a scientist.

But it was Klaus, Joe DeBold, and Akiko Shimamoto who flipped the switch. Akiko fueled my passion for neurochemistry, inspiring me to take graduate analytical chemistry courses, and has been a constant source of scientific, career, and emotional support. Klaus challenged me in every possible way, to the point I thought I wouldn't make it. But due to his

unsurpassed mentorship, I have transitioned from someone who tackles technically challenging experiments to a true scientist, who critically explores questions from a hypothesis-driven angle. And last, but most certainly not least, Joe provided stoic balance, incredible insight and alternative ideas, and possibly most importantly didn't let me quit when I tearfully tried.

I also would not have made it this far without the-now-Dr. Chris Boyson, who was a rock for my first four years, balancing out my fieriness and acting as a sounding board for hypotheses. The soon-to-be-Dr. Lara Hwa also provided immense emotional support and is an absolute inspiration to me. I probably would have electrocuted myself or set Bacon Hall on fire without Tom Sopko, so credit is absolutely due there as well. More importantly, Tom was an unwavering source of support, as well as constantly available outlet for my sassiness or procrastination, to which I am immeasurably grateful. Young lab siblings Xiao Han and Emily Newman also made my time at Bacon immensely more enjoyable, as did the eternal optimism of joyful postdocs Dr. Lucas Albrechet-Souza and Dr. Kyle Gobrogge.

My friends and family also gave me colossal strength and encouragement throughout the last five years. Dr. Amber LaCrosse has always been a genuine inspiration for me, but also the world's best cheerleader. Rachel Parkin and Michael Malia were always there with a cocktail, listening ears, and delightfully snarky comments. Brian Sullivan

has provided ample distractions in this final push, and made what has been one of the most stressful phases of my life a little less so. He has always been there with a hug and somehow motivational sports reference ("Look, if the Red Sox can come from 0-3 to win the ALCS in 2004, you can write your dissertation!"). I couldn't have done this without the support of my mom either, reminding me that the pressure was probably completely self-imposed ("Remember second grade?"), that eating and sleeping are important, that Dr. Miczek is definitely a wonderful man and I need to stop being a basket case, but most importantly, that I can do it and no matter what I make her and the rest of my family proud.

Finally, I would like to dedicate this dissertation to my biological mother. As Hans Selye so eloquently said, "*Every stress leaves an indelible scar.*" A scar is a truly appropriate analogy. Our most severe stresses are permanent, they are visible, and they make us who we are. It is the scars of my first 6+ years, and the loss of my first mother, that made Klaus' talk at EBPS enrapture me. It is those scars that drive me to research the neurobiology and neuroadaptations induced by repeated stress.

TABLE OF CONTENTS

FIGURES

Every stress leaves an indelible scar.

–Hans Selye, 1936

I. INTRODUCTION

1. Introduction

Both stressful and rewarding stimuli rapidly and potently excite dopamine neurons in the ventral tegmental area (VTA). Furthermore, these seemingly opposite experiences interact with each other at both a neural and behavioral level. A history of exposure to stressful stimuli or situations is strongly associated with later addictive behavior, with both clinical and preclinical work demonstrating stress plays a powerful role in the initiation, escalation, and relapse to drug abuse (Shaham, Erb, & Stewart, 2000; Sinha, 2007, 2009). However, the converse is also true—a history of cocaine self-administration results in greater susceptibility to chronic social stress in mice (Covington et al., 2011).

As stress and reward are interacting at a behavioral level, it is logical that they interact at a basic neural level as well. Indeed, it appears that intersecting as opposed to parallel neural circuitry may be driving these two distinct experiences of aversion and reward, both mediated by the mesocorticolimbic dopamine system. Classic evidence has established a clear function of mesocorticolimbic dopamine in rewarding and reinforcing processes, and a key role of mesocorticolimbic dopamine in the response to acute and repeated stress is becoming increasingly apparent.

Unfortunately, with this rapidly growing body of research on the role of dopamine in the effects of stressful and aversive stimuli, the nature, schedule, and intensity of stressors is often overlooked. This may stem from a lack of consensus on the definition of stress. When Selye popularized the term stress as a biomedical construct in 1936, he proposed that stress was any demand on the body that resulted in adaptation, but that all stressors resulted in identical non-specific physiological responses (Selye, 1936).

However, over the last 80 years, it has become quite evident that while there may be some non-specific responses, different stressors can result in distinct, specific responses. Mason (1971) first questioned Selye's hypothesis of non-specific responses, noting that stressors could increase, decrease, or not change HPA axis activity. Chrousos and Gold (1992) expanded upon this, defining stress as a state that resulted from a threat to homeostasis, yielding behavioral and physiological adaptations that could be specific to the stressor or non-specific when the threat to homeostasis reaches a homeostatic threshold. McEwen (1998) applied the concept of allostasis as an active adaptive process to maintain stability through change. For the purposes of this review, we choose to define stress as a threat to homeostasis, either physical or perceived, which results in *specific* allostatic compensatory responses.

Prior work from this and other laboratories has unambiguously demonstrated that different schedules, intensities, or modalities of stressor

presentation can result in dramatically different behavioral and physiological responses. For example, intermittent and chronic social defeat stress engender opposite effects on subsequent cocaine-stimulated dopamine increases in the nucleus accumbens shell (NAcSh) as well as cocaine self-administration (Miczek, Nikulina, Shimamoto, & Covington, 2011). But how does stressor specificity interact with later reward-related behaviors? And how is mesocorticolimbic dopamine poised to play a key interactive role between the seemingly opposite experiences of reward and aversion?

First, the structure, connectivity, and function of VTA dopamine neurons will be reviewed, followed by evidence for VTA dopamine neuron activation and adaptation in response to both acute and repeated stress, with careful attention paid to the nature, schedule, and intensity of the stressor. Finally, afferent control of VTA dopamine neuron firing will be reviewed, with a focus on the role of corticotropin releasing factor (CRF). The structure, connectivity, and function of VTA dopamine neurons with specific regard to reward-related behaviors have been thoroughly reviewed previously (Ikemoto, 2007), so that will be summarized here only briefly.

This dissertation investigates the interactions between CRF and dopamine neurons in the VTA during acute and repeated stress, and how they may play a role in subsequent cocaine taking and seeking. Ultimately, I propose that driven in part by CRF, VTA dopamine neurons

rapidly fire in response to both reward and aversion, increasing extracellular dopamine in the mPFC and NAcSh, and certain intensities and schedules of stress can induce neuroadaptations within these neurons to result in intensified responses to later aversive and rewarding stimulation.

2. Heterogeneity in structure and function of VTA dopamine neurons

Prior to the development of current labeling techniques, the VTA was not considered a separate structure from the cell bodies of the substantia nigra (SN). The first classical description of the VTA was made with Golgi and Nissl preparations by Tsai (1925a, 1925b), who concluded that the cell-free space overlying the sulcus lateralis mesencephali, along with smaller cell size and close relationship to the tracti mammillo- and olfacto-tegmentalis, warranted a separation from the SN (Oades & Halliday, 1987). Later studies validated Tsai's initial hypothesis that this area indeed has a discrete population of dopaminergic cells serving a distinct function from SN dopaminergic neurons, leading other researchers to initially term the region the ventral tegmental area of Tsai.

As thoroughly reviewed by Oades (1987), dopamine cells have been isolated in many animals, including fish (Lefranc, L'Hermite, & Tusques, 1969), birds (Fuxe & Ljunggren, 1965), rats (Carlsson, Dahlstroem, Fuxe, & Lindqvist, 1965), and other mammals (Fuxe & Owman, 1965), but the VTA as a structure appears to be evolutionarily

conserved only in higher order vertebrates. Lower vertebrates do not show a defined VTA, with the 'peripeduncular area' containing both dopamine and serotonin cells (Dube & Parent, 1982), and broader development of the VTA observed in only a few teleosts and reptiles (Oades & Halliday, 1987). However, there is a high degree of similarity between the VTA of mammals, including opossum (Crutcher & Humbertson, 1978), rat (Lindvall & Bjorklund, 1974; Phillipson, 1979a, 1979b, 1979c), rabbit (Blessing, Chalmers, & Howe, 1978), dog (Shimada, Ishikawa, & Tanaka, 1976), cat (Pin, Jones, & Jouvet, 1968; Poitras & Parent, 1978; Taber, 1961), non-human primate (Felten, Laties, & Carpenter, 1974; Garver & Sladek, 1975; Hubbard & Di Carlo, 1974; Jacobowitz & MacLean, 1978; Tanaka, Ishikawa, & Shimada, 1982), and human (Bogerts, 1981; Bogerts, Hantsch, & Herzer, 1983; Nobin & Bjorklund, 1973; Olson, Nystrom, & Seiger, 1973). Further, the number of dopamine neurons increases with phylogenetic order, such that Balb/C mice have an estimated 25,000 dopamine neurons, albino rats 40,000, and a 33 year old man 450,000 (German, Schlusselberg, & Woodward, 1983).

Regardless of homology between higher-order species, researchers have struggled to clearly define the boundaries and function of the VTA. As reviewed below, the VTA is a heterogeneous structure in regards to cytoarchitecture, neurochemical and electrophysiological profiles, and afferent/efferent connections, so it is not surprising that there

is evidence that VTA dopamine neurons may serve multiple functions, such as reward and aversion.

2.1 Heterogeneity in dopaminergic cytoarchitecture.

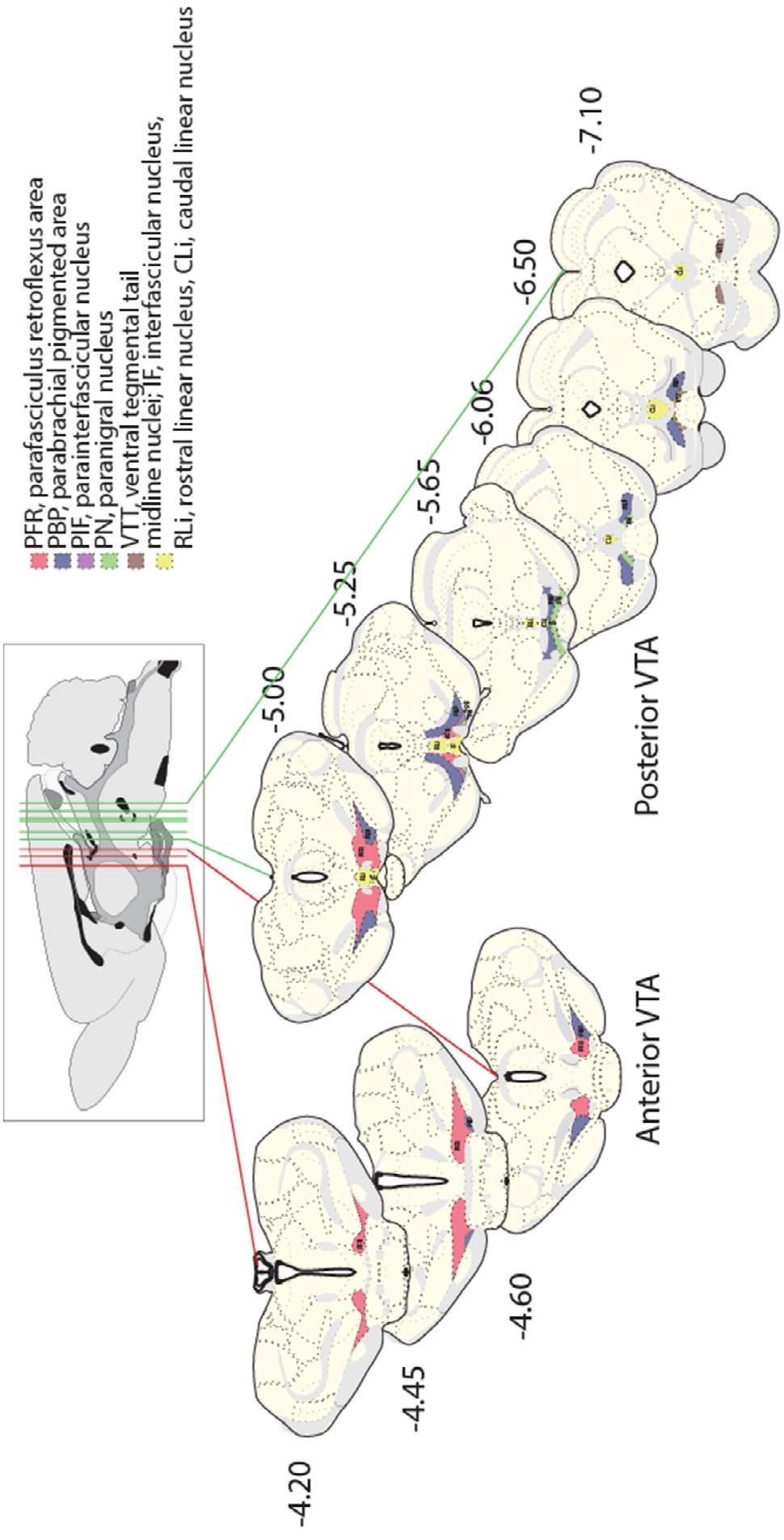
The VTA is characterized by considerable heterogeneity in dopaminergic cell cytoarchitecture. The VTA is comprised of four major zones or subnuclei (Figure 1, see also Ikemoto, 2007). The parafasciculus retroflexus area and ventral tegmental tail (VTT) contain few dopaminergic cell bodies, while the paranigral nucleus (PN) and parabrachial pigmented area (PBP) are rich in dopaminergic neurons. Some researchers include a fifth subregion, the dopamine-rich parainterfasicular nucleus (PIF), located between the PBP and PN in the posterior VTA. Additionally, the adjacent regions of the caudal linear nucleus (CLi), interfasicular nucleus (IF), and rostral linear nucleus of the raphe (RLi) are often considered VTA subregions (Oades & Halliday, 1987; L. W. Swanson, 1982). However, even within these subregions, the dopaminergic cell body characteristics are still not homogeneous (for thorough review, see Ikemoto, 2007).

As described by Ikemoto (2007), the parafasciculus retroflexus area contains a low density of small- to medium-sized dopaminergic cell bodies, which show light to moderate immunoreactivity for tyrosine hydroxylase (TH, the rate limiting enzyme in dopamine synthesis, used as a marker of dopaminergic cells) and are continuous with dopaminergic cell bodies in the posterior hypothalamic area. The densest TH-positive staining is found in the middle two-thirds of the VTA, divided into the PN,

PIF, and PBP. Within the PN, TH-positive cell bodies are oriented mediolaterally, tilting towards the IF. The dopamine cell bodies are

Figure 1. Subregional distinctions in the VTA.

Coronal sections are arranged from anterior (-4.20) to posterior (-7.10) from bregma. The division between anterior and posterior VTA is drawn between the interpeduncular nucleus and the interpeduncular fossa. PFR (red): parafasciculus retroflexus area; PBP (blue): parabrachial pigmented area; PIF (purple): parainterfascicular nucleus; PN (green): paranigral nucleus; VTT (brown): ventral tegmental tail; midline nuclei (yellow): IF, interfascicular nucleus, RLi, rostral linear nucleus, CLi, caudal linear nucleus.


PFR, parafasciculus retroflexus area
PBP, parabrachial pigmented area
PIF, parainterfascicular nucleus
PN, paranigral nucleus
VTT, ventral tegmental tail
midline nuclei; IF, interfascicular nucleus,
RLi, rostral linear nucleus, CLi, caudal linear nucleus
-4.20
-4.45
-4.60
-5.00
-5.25
-5.65
-6.06
-6.50
-7.10
Anterior VTA
Posterior VTA

relatively homogenous within the PN, medium in size, and medium to darkly stained. The PBP, on the other hand, is heterogeneous in cytoarchitecture, and the borders have not been consistently defined within the rat or mouse. The PBP contains both large and medium cell bodies, with no unified orientation. Within the anterior VTA, the PBP contains large, intensely stained cell bodies, which are continuous with the anterior SN pars compacta. In the posterior VTA, the PBP is just dorsolateral to the PN, and contains cell bodies and fibers that form a net-like structure. Finally, the VTT has a low density of dopaminergic cell bodies, which are small and moderately stained. The midline nuclei, which are often considered part of the VTA, are also rich in TH-positive cell bodies. Most notably, the IF contains the densest population of dopaminergic cell bodies in the ventral midbrain. The CLi also contains a dense population of relatively homogenous dopaminergic cell bodies, which are medium in size and medium-dark in TH staining.

It is clear from this and other existing immunohistochemical data that a great deal of cytoarchitectonic heterogeneity exists not only within the VTA, but also within specific subregions of the VTA. Not only has this led to a difficulty in establishing clear boundaries of the VTA and its subregions, but this heterogeneity in structure points to further heterogeneity in neurochemical and electrophysiological profiles, as well as overall function.

2.2 Heterogeneity in neurochemical profile.

VTA neurons also differ in terms of their neurotransmitter profile. VTA neurons have typically been classified as principal (primarily dopaminergic), secondary (GABAergic), or tertiary (other) on the basis of immunohistochemistry for TH, as well as electrophysiological and pharmacological properties (Cameron, Wessendorf, & Williams, 1997; Grace & Onn, 1989; Johnson & North, 1992). Tertiary neurons are hyperpolarized by opioids and serotonin, and while one-third of these have been identified as atypical-dopaminergic, the neurochemical profile of the remaining two-thirds has yet to be clearly characterized (Cameron et al., 1997; Lammel, Lim, & Malenka, 2014). In sum, the VTA is comprised of approximately 65% dopaminergic neurons, 35% GABAergic neurons, and less than 3% glutamatergic neurons which do not express TH (Nair-Roberts et al., 2008; Sesack & Grace, 2010). However, it should be noted that some VTA dopamine neurons projecting to the NAc can also co-release glutamate (Hnasko, Hjelmstad, Fields, & Edwards, 2012; Stuber, Hnasko, Britt, Edwards, & Bonci, 2010), adding to further neurochemical heterogeneity within the region.

2.3 Heterogeneity in electrophysiological profile.

Despite this immense anatomical and neurochemical heterogeneity in the VTA, it has until recently been common practice of virtually all *in vivo* electrophysiological studies, particularly those evaluating reward and drug related synaptic adaptations, to consider VTA dopamine neurons as

a single homogenous population (reviewed in Lammel et al., 2014; Ungless & Grace, 2012). As *in vivo* electrophysiological measurements do not allow for the direct confirmation of the neurochemical identity of the neurons being recorded, neurons are putatively characterized based on standard classification criteria. Conventionally, putative dopamine neurons have been identified electrophysiologically based on the following: broad action potentials, low-frequency pacemaker activity, D2 agonist-induced hyperpolarization, and/or the presence of large *Ih* (hyperpolarizing inward) currents generated by hyperpolarization-activated cyclic nucleotide-regulated cation channels, or HCN channels (Kitai, Shepard, Callaway, & Scroggs, 1999; Ungless & Grace, 2012).

However, it has somewhat recently been established that these conventional criteria are not necessarily reliable (as reviewed extensively in Ungless & Grace, 2012). Briefly, some researchers have shown that the presence of large *Ih* currents within the VTA can be observed in non-dopaminergic neurons (Margolis, Lock, Hjelmstad, & Fields, 2006; Margolis, Mitchell, Ishikawa, Hjelmstad, & Fields, 2008; Zhang, Placzek, & Dani, 2010). Furthermore, some verified VTA dopamine neurons are not responsive to dopamine bath application (Bannon & Roth, 1983; Lammel et al., 2008) and others have very small or negligible *Ih* currents (Brischoux, Chakraborty, Brierley, & Ungless, 2009; Ford, Mark, & Williams, 2006; Hnasko et al., 2012; Jones & Kauer, 1999; Lammel et al., 2008; Lammel, Ion, Roeper, & Malenka, 2011; Margolis et al., 2006;

Zhang et al., 2010). As such, it appears that the VTA dopamine neuron heterogeneity extends to electrophysiological profiles as well. Unfortunately, this electrophysiological heterogeneity has been lost in many earlier studies, confounding previous conclusions drawn about VTA dopamine neuron function.

Not surprisingly, it appears that these electrophysiologically distinct dopamine neurons are located within discrete anatomical subregions of the VTA. Most *in vivo* electrophysiological studies have used these conventional classification methods described above to identify putative dopamine neurons, and as such have primarily focused on dorsal portions of the VTA, specifically within a region medial to the medial terminal nucleus of the accessory optical tract (Lammel et al., 2014; Ungless, Argilli, & Bonci, 2010; Zhang et al., 2010), where putative dopamine neurons fit these conventional criteria. Thus, the studies focusing on the function of these specific, “conventional” dopamine neurons in this small portion of the VTA may not be applicable to “non-conventional” dopamine neurons in other subregions of the VTA. These other regions of the VTA, such as the ventromedial posterior VTA consisting of the PN and PBP, have been largely ignored as many of the DA neurons do not conform to established conventional criteria such as large *Ih* (Lammel et al., 2008). Therefore, it has been proposed that while the correlation between *Ih* and dopamine phenotype may be high in the commonly targeted dorsolateral region of the VTA (specifically the anterior PBP), other subregions such as

the PN and posterior PBP, which have been largely ignored, contain dopamine neurons with a distinct electrophysiological profile (Borgland, Ungless, & Bonci, 2010; Lammel et al., 2008; Lammel et al., 2014). The distinction in electrophysiological profile among these anatomical regions is crucial, as these regions have been implicated in vastly different behavioral functions (discussed in Section 2.6).

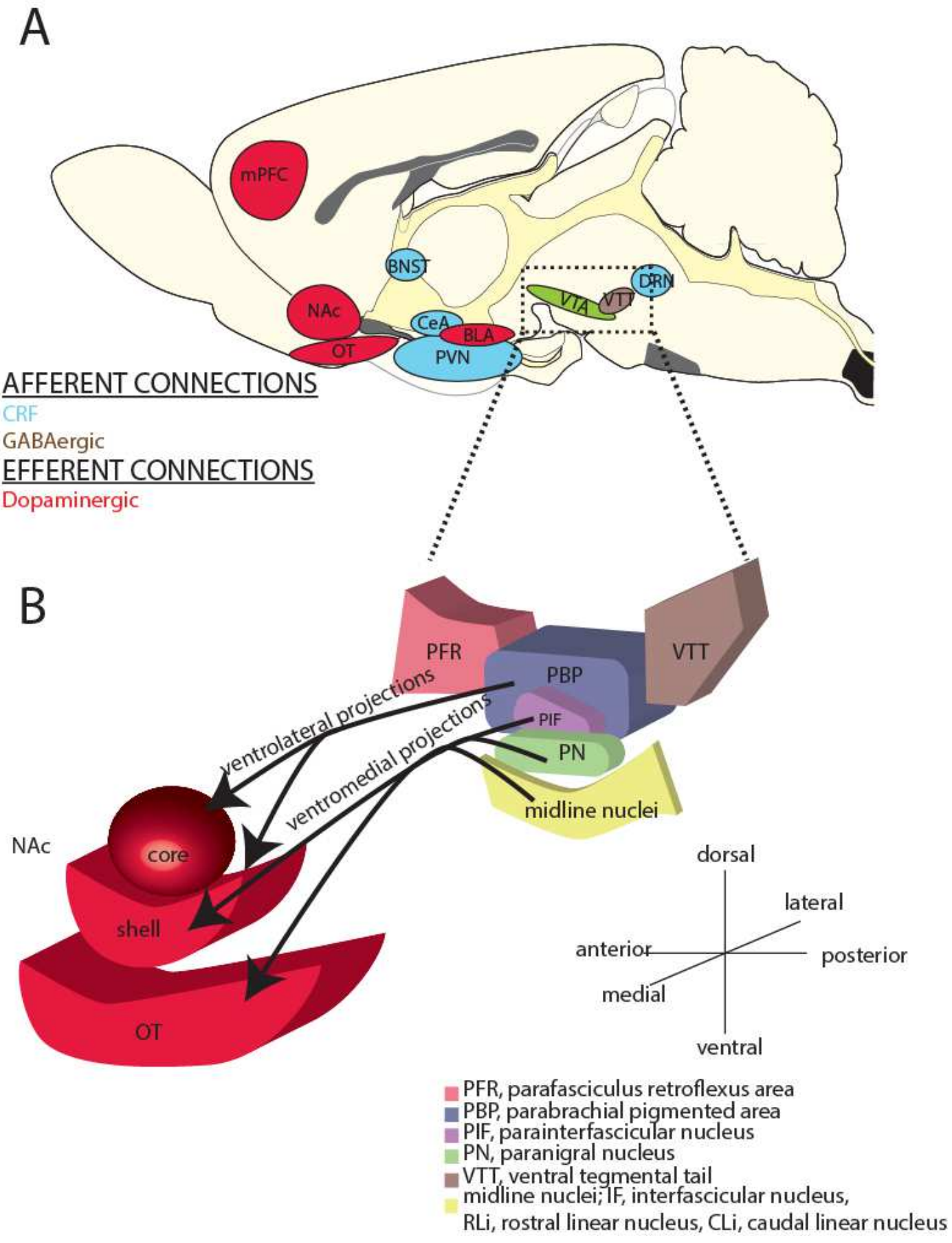
2.4 Heterogeneity in efferent connections.

This discrepancy is also critical because recent studies have shown a clear distinction within VTA subregions in terms of projection targets, which in turn have important behavioral and functional implications. VTA dopamine neurons project to many different brain regions, and appear to do so with clear mediolateral topography in a non-overlapping fashion at an approximate 45° angle to the midline (See Figure 2, Albanese & Minciacchi, 1983; Fallon, 1981; Ikemoto, 2007). While few have closely examined the heterogeneity in efferent and afferent connections within VTA subregions, the most intensively mapped connections have been between VTA subregions and the striatum. Most notably for the purposes of this dissertation, dopamine-rich cell bodies in the ventromedially-located PN and dorsoposteromedial portions of the PBP selectively project to the medial nucleus accumbens (NAc) shell, medial prefrontal cortex (mPFC), medial olfactory tubercle (OT), and basolateral amygdala (BLA, Figure 2B), regions heavily implicated in

Figure 2. Afferent and efferent connections of the VTA.

A. The ventral tegmental area (VTA, green) sends dopaminergic projections (red) to the medial prefrontal cortex (mPFC), nucleus accumbens (NAc) shell and core, olfactory tubercle (OT), and basolateral amygdala (BLA). The VTA is enervated by CRF projecting neurons (blue) from the central amygdala (CeA), bed nucleus of the stria terminalis (BNST), paraventricular nucleus of the hypothalamus (PVN), and dorsal raphe nucleus (DRN).

B. Striatal efferents project along a mediolateral trajectory to the NAc and OT, such that laterally located parabrachial pigmented area (PBP, blue) dopamine neurons send projections to the lateral NAc core and shell, while the medially located paraintrafascicular nucleus (PIF, purple), paranigral nucleus (PN, green), and midline nuclei (yellow) project to the medial NAc shell and medial OT.


A
mPFC
BNST
NAc
CeA
BLA
OT
PVN
VTA
VTT
DRN
AFFERENT CONNECTIONS
CRF
GABAergic
EFFERENT CONNECTIONS
Dopaminergic
B
PFR
PBP
VTT
PIF
PN
midline nuclei
ventrolateral projections
ventromedial projections
NAc
core
shell
OT
dorsal
lateral
anterior
posterior
medial
ventral
PFR, parafasciculus retroflexus area
PBP, parabrachial pigmented area
PIF, parainterfascicular nucleus
PN, paranigral nucleus
VTT, ventral tegmental tail
midline nuclei; IF, interfascicular nucleus,
RLi, rostral linear nucleus, CLi, caudal linear nucleus

reward and reward processing (although these regions certainly mediate other functions as well)(Ikemoto, 2007).

Following the mediolateral projection topography, lateral PBP dopamine neurons send heavy projections to the ventrolateral striatum, which has been less heavily implicated in reward-related functions (Ikemoto, 2007). The PFR and VTT do not contain dense dopamine cell bodies, but the sparse dopamine cell bodies of the PFR selectively project to the nucleus of the diagonal band. Midline nuclei are also distinct in terms of projection targets, with IF dopamine neurons projecting selectively to the dorsomedial NAc shell, and RLi to the diagonal band and pallidal zone of the OT (Ikemoto, 2007). Future work should evaluate VTA heterogeneity in non-striatal dopamine projection sites, such as the mPFC and BLA.

2.5 Heterogeneity in afferent connections.

Accordingly, there may also be sub-regional differences in afferent connectivity to the VTA, although this has not yet been thoroughly investigated. VTA dopamine neurons receive innervation from widespread regions throughout the brain. The direct monosynaptic inputs to midbrain dopamine neurons have been thoroughly mapped recently (Watabe-Uchida, Zhu, Ogawa, Vamanrao, & Uchida, 2012). By using a Cre/loxP gene expression system with rabies-virus-based transsynaptic retrograde tracing, Watabe-Uchida and colleagues (2012) mapped direct monosynaptic inputs to genetically defined midbrain dopamine neurons.

VTA dopamine neurons receive limited input from the cortex (primarily the prefrontal cortex), hippocampal, and thalamic regions, with more numerous synaptic contacts made from the ventral striatum, pallidum, lateral hypothalamus, and midbrain. Notably, VTA dopaminergic neurons receive the most innervation from the ventral striatum, particularly the NAc, while the densest innervation originates from the dorsal raphe nucleus (DRN). NAc cells projecting to VTA dopamine neurons form extremely dense patches within the NAc. Moreover, these neurons are morphologically distinct from NAc GABAergic medium spiny neurons, indicating distinct heterogeneity within the ventral striatum. Future circuit tracing experiments should investigate whether this heterogeneity extends to the VTA, with differences in afferent connectivity within the various subregions. A more detailed examination of how these afferent connections can influence dopamine function during stress is covered in Section 5.

2.6 Heterogeneity in VTA dopamine neuron function.

The aforementioned heterogeneity in anatomy, neurochemistry and electrophysiological profile, and connectivity point to diversity in the overall behavioral functions mediated by VTA dopamine neurons. As many of the dopaminergic projection targets of the VTA have been heavily implicated in reward, there has been considerable attention paid to VTA dopamine neurons in these processes. Both natural rewards (Berridge, 1996) and

drugs of abuse (Di Chiara & Imperato, 1988) stimulate release of dopamine from VTA neurons projecting to the NAc, leading to a well-accepted hypothesis that this connection at least partially drives reward-related functions.

However, recent evidence has also shown heterogeneity within these projection targets in terms of reward-related function. Cocaine infused directly into the medial NAc shell produces significantly greater changes in locomotion compared to cocaine infused directly into the lateral NAc shell (Ikemoto, 2002, 2007), while rats will selectively self-administer cocaine and amphetamine into the medial but not lateral NAc shell (Ikemoto, 2002; Ikemoto & Donahue, 2005), with similar differences observed between the medial and lateral OT (Ikemoto, 2002; Ikemoto & Donahue, 2005). Accordingly, it is not surprising that the posteromedial VTA, centered around the PN and PBP, has been shown to also play a stronger role in reward processes than the anterior and lateral portions of the VTA (Rodd-Henricks, McKinzie, Li, Murphy, & McBride, 2002; Sellings & Clarke, 2003; Sellings, McQuade, & Clarke, 2006). Specifically, cocaine, nicotine, opiates, ethanol, and cannabinoids are all selectively self-administered into the posterior but not anterior VTA (Ikemoto, Qin, & Liu, 2006; Ikemoto & Wise, 2002; Rodd et al., 2005; Rodd-Henricks, McKinzie, Crile, Murphy, & McBride, 2000; Zangen, Solinas, Ikemoto, Goldberg, & Wise, 2006).

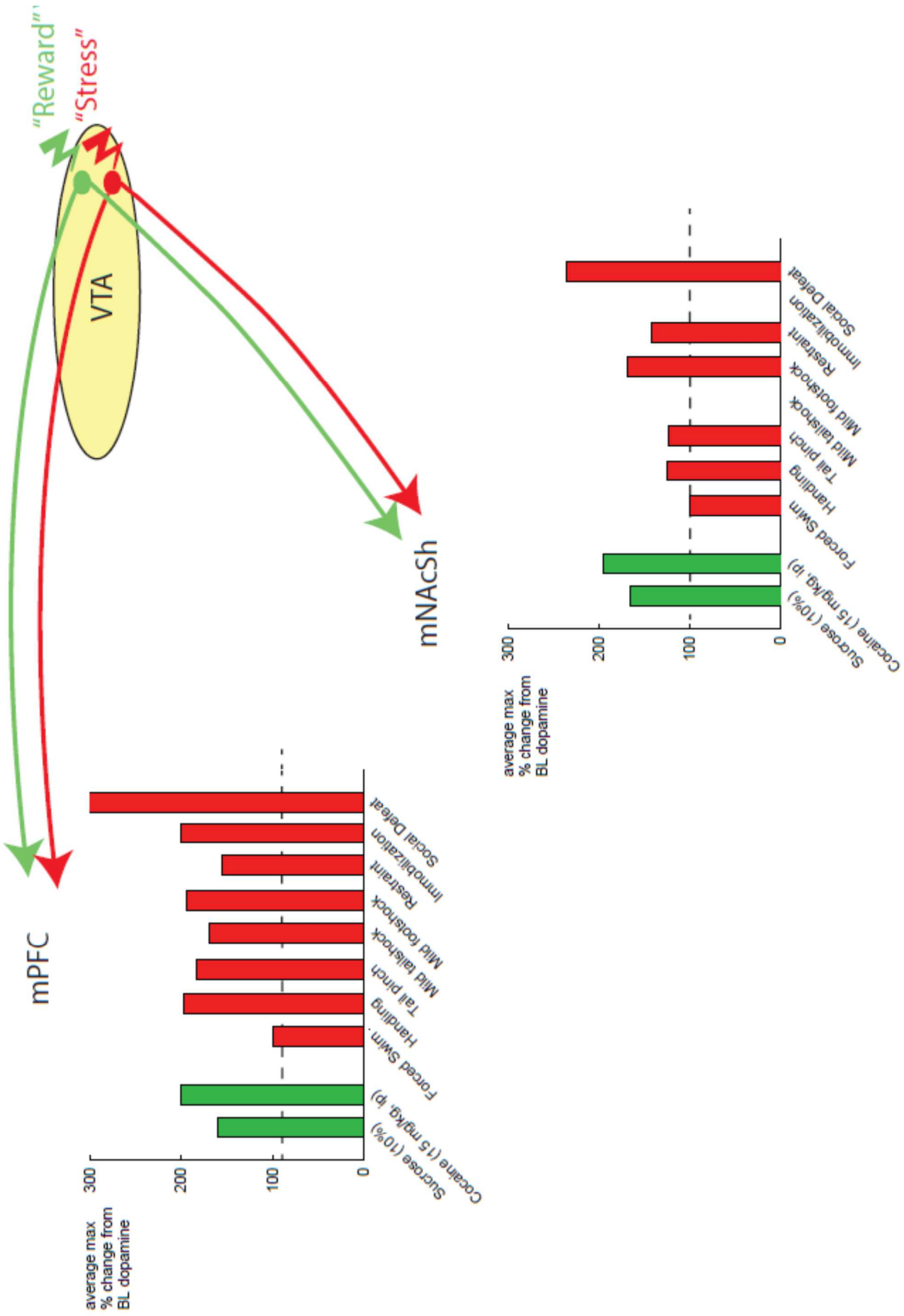
This mesolimbic dopamine circuitry stemming from the VTA and projecting to the ventral striatum and medial prefrontal cortex (Figure 2A) thus plays a fundamental role in reward. This is paralleled by subsequent reports that rewards, as well as their predictive cues, can elicit strong phasic firing within the DA cell bodies of the VTA (Schultz, 1998). These pioneering findings have lead to a prominent hypothesis that this system primarily serves to mediate reward, hedonia, and related energizing processes (but see Salamone & Correa, 2012). Given this overwhelming evidence for a key role of VTA dopamine neurons in reward, it was initially surprising and controversial to many reward and addiction researchers that VTA dopamine also could be involved in stress and other aversive events.

3. Effects of acute stress on VTA dopamine neuron activity

Electrophysiological studies have shown that aversive stimuli inhibit putative VTA dopamine neuron firing (e.g. Mantz, Thierry, & Glowinski, 1989; Mirenowicz & Schultz, 1996; Schultz & Romo, 1987; Ungless, Magill, & Bolam, 2004). However, microdialysis studies examining extracellular dopamine and its metabolites collected over minutes and hours have found a robust dopaminergic increase during stress in VTA projection targets, particularly the mPFC and NAcSh. Various stressors such as restraint, footshock, tail pinch/shock, social threat, and others potently increase extracellular dopamine in the NAc and mPFC (Section 3.1, see Tables 1-9, Figure 3). Across these studies, the nature and

Figure 4. Reward and stress activate VTA dopamine neurons, increasing extracellular dopamine in the mPFC and NAcSh.

Both rewarding and stressful stimuli induce dopaminergic increases in ventral tegmental area (VTA) projection targets, namely the medial prefrontal cortex (mPFC) and nucleus accumbens shell (NAcSh), to a similar degree. Average maximal percent change from baseline dopamine is representative of papers presented in Tables 1-9.


"Reward"
"Stress"
VTA
mPFC
mNAcSh
average max
% change from
BL dopamine
300
200
100
0
Sucrose (10%)
Cocaine (15 mg/kg, ip)
Forced Swim
Handling
Tail pinch
Mild tailshock
Mild footshock
Restraint
Immobilization
Social Defeat

degree of the dopaminergic increase varies according to stressor and intensity. Recent electrophysiological studies have also found a discrete subset of VTA dopamine neurons that increase firing in response to aversive stimulation, providing an explanation for the observed microdialysis results (Section 3.2). Additional studies have found evidence for long-lasting neuroadaptive changes on VTA dopamine neurons after a single stress exposure, which also point to VTA dopamine neuron activation during stress, indicating that acute stress can alter VTA dopamine neuron responsivity to future stressors or drugs of abuse (Section 3.3).

3.1 Microdialysis evidence.

Early postmortem studies demonstrated altered dopamine and dopamine metabolite concentrations in brains of rodents following stress (Deutch et al., 1991; Deutch, Tam, & Roth, 1985; Dunn & File, 1983; Fadda et al., 1978; Kramarcy, Delanoy, & Dunn, 1984). Upon the advancement of the microdialysis technique to monitor extracellular monamine levels in awake, freely moving animals in the late 1980s, researchers began to more directly assess dopamine in response to a variety of stressors.

Imperato and colleagues (1989) were the first to use microdialysis to demonstrate a clear increase in extracellular dopamine in response to restraint stress. She and others have found that restraint stress reliably increases extracellular dopamine in the rat NAc (Table 1) and mPFC

(Table 2) to a roughly equivalent degree (average maximal percent change from baseline from Tables 1 and 2 142.5% and 155% for the NAc and mPFC, respectively).

Studies examining extracellular dopamine in the mPFC in response to immobilization have found a slightly greater response (average maximal percent change from baseline 200%, Table 2), indicating there may be a difference in severity between these two similar stressors. This may be explained by a confound within the results of the experiments utilizing restraint stress. All but two studies (Garrido et al., 2013; Mokler, Torres, Galler, & Morgane, 2007) were conducted in the light phase of the light-dark cycle. Restraint stress during the light phase (inactive period) results in significantly reduced body weight gain and development of stomach ulcers, whereas no such effects are produced by restraint during the active dark phase (Koolhaas et al., 2011; Pare & Glavin, 1986; Rybkin et al., 1997). Wild Norway rat spends the resting (light) phase hiding in narrow burrow systems (Koolhaas et al., 2011), so restraint may be a less potent stressor during this phase. Rather than the physical compression used in restraint stress, immobilization involves restricting paw movement in a more spread-out manner, and as such may serve as a more powerful stressor during the inactive light phase.

While some studies have demonstrated that dopamine levels in both the NAc and mPFC remain elevated for the duration of restraint (Cuadra, Zurita, Gioino, & Molina, 2001; Cuadra, Zurita, Lacerra, &

Molina, 1999; Garrido et al., 2013; Jackson & Moghaddam, 2004; Mokler et al., 2007), when restraint is prolonged (>60 min), dopamine levels return to baseline within 70-120 min (Imperato, Angelucci, Casolini, Zocchi, & Puglisi-Allegra, 1992; Imperato, Cabib, & Puglisi-Allegra, 1993; Imperato, Puglisi-Allegra, Casolini, & Angelucci, 1991; Imperato et al., 1989; Imperato et al., 1990; Puglisi-Allegra, Imperato, Angelucci, & Cabib, 1991). Thus, there appears to be a habituation of the dopamine response upon extended stressor presentation. However, as restraint is the only stressor examined in microdialysis studies to date that is amenable to such prolonged presentation, it is not clear if this habituation would extend to other types of stress.

Mild footshock has also been shown to potently increase dopamine in both the NAc (Table 3) and mPFC (Table 4) to comparable degrees (average maximal percent change from baseline 169.22% and 194% for NAc and mPFC, respectively). Notably, all reports of microdialysis during footshock stress have used less than 0.55mA intensity, generally considered to be mild. Future work could examine the relationship between footshock intensity and extracellular dopamine in the NAc and mPFC. Mild to moderate tailshock (1.0mA) also produces significant increases in extracellular dopamine in the mPFC (Table 7, average maximal percent change from baseline 169%). Like footshock, varying intensities of tailshock have not been examined, and could be the focus of future work.

Microdialysis has also been used to examine the dopamine response to acute tail pinch (Tables 5 and 6). Unlike restraint and footshock, it appears that tail pinch stress may differentially increase extracellular dopamine in the NAc and mPFC, with greater dopamine increases observed in the mPFC (average maximal percent change from baseline 124% and 184% for NAc and mPFC, respectively). As none of the studies examined the dopamine response in both the NAc and mPFC, it is possible that there are differences in intensity of tail pinch pressure between labs. However, it may also be the case that the very mild stress of tail pinch is not sufficient to activate VTA dopamine neurons, and the cortical dopamine response is due to another function, such as novelty.

Likewise, handling, often considered a very mild stressor, has differential effects on extracellular dopamine in the NAc and mPFC (average maximal percent change from baseline 126% and 197% for NAc and mPFC, respectively, Tables 8 and 9). Duration of handling stress does not appear to reliably affect extracellular dopamine concentrations, but there may be a strain difference in reactivity to handling, as the greatest changes in mPFC dopamine were observed in Wistar as opposed to Sprague Dawley rats.

However, not all stressors examined have produced increases in extracellular NAc and mPFC dopamine. Acute forced swim stress, often thought to be a much milder stressor than footshock and restraint stress (Jordan, Kramer, Zukas, & Petty, 1994), does not alter extracellular

dopamine in the NAc or the mPFC (Azzi et al., 1998; Jordan et al., 1994). Likewise, the similarly mild stressor of airpuff to the face or low-dose cytokine (IL-8) injection does not alter extracellular dopamine in either brain region, although these stressors work synergistically to increase dopamine when administered concurrently (Merali, Lacosta, & Anisman, 1997).

One potentially important distinction is that all the above-mentioned stressors involve some sort of direct physical tactile contact/stimulation of the animal. However, stressors that do not involve direct contact with the animals' body can also elicit strong increases in extracellular dopamine in both the NAc and mPFC. The "psychological" stress of observing 9 other rats receiving severe (3.0mA) footshocks elicits a significant increase in extracellular dopamine in the NAc shell, but not core (Y. L. Wu, Yoshida, Emoto, & Tanaka, 1999), one of the only studies to examine the difference in responsivity to stress between these subregions of the NAc. Additionally, presentation of a predator (fox) odor produces a gradual increase to 205% baseline levels in extracellular dopamine in the mPFC (W. R. Wu, Li, & Sorg, 2003).

It is possible that these findings showing stress-induced elevations in extracellular NAc and mPFC dopamine are in line with a hypothesis that VTA dopamine neurons primarily subserve reward-related functions as opposed to stress-related functions. The removal of a stressor or aversive stimulus is negative reinforcement, and can strengthen subsequent

associated behaviors (Thorndike, Columbia University. Teachers College. Institute of Psychological Research., & Carnegie Corporation of New York., 1932). Considerable behavioral evidence has demonstrated that the termination of a stressor or aversive stimulus can serve as a reward (e.g. Navratilova et al., 2012; Tanimoto, Heisenberg, & Gerber, 2004). Thus, it could be expected that rather than stress activating these dopaminergic neurons, it is actually the offset of stress that excites VTA DA neurons, resulting in the observed extracellular dopamine increases in VTA projection sites. Indeed, approximately half of the VTA dopamine neurons inhibited by footshock also show excitation at the termination of the aversive stimulation (Brischoux et al., 2009).

Some microdialysis studies have found some support for this explanation. Although restraint stress produces a sustained elevation in extracellular dopamine in both the NAc and mPFC (Tables 1 and 2), when restraint or immobilization is prolonged until dopamine levels return to baseline, most have shown that there is a strong, rapid increase in extracellular dopamine levels again upon release (Imperato et al., 1992; Imperato et al., 1991; Jackson & Moghaddam, 2004; Lillrank, Lipska, Kolachana, & Weinberger, 1999; Pozzi, Acconcia, Ceglia, Invernizzi, & Samanin, 2002; Puglisi-Allegra et al., 1991; C. J. Swanson, Perry, & Schoepp, 2004). However, it is difficult to evaluate a dopamine response to the termination of a stressor, as most other types of stress studied (i.e. footshock, tail pinch, etc.) are much shorter in duration, rarely spanning

greater than two microdialysis samples, and do not show a return to baseline prior to the termination of the stressor. Therefore, it cannot be concluded that any significant increases after termination of the stressor are due to negative reinforcement as opposed to carryover from the aversive experience. Regardless, this hypothesis that the dopamine increase is due to negative reinforcement as opposed to stress itself cannot explain the sustained dopaminergic increases observed in microdialysis studies where the stressors or aversive stimuli outlast the sampling time.

Thus, while the temporal resolution and correlational nature of these microdialysis experiments could not conclusively prove that VTA dopamine neurons are excited by stress as opposed to the removal of a stressor, the magnitude and duration of dopaminergic increases in these target areas indicates a likely effect on VTA firing in response to stress. Overall, as summarized in Figure 3, most stressors elicit an increase in extracellular dopamine in VTA projection targets, with the most potent stressors eliciting the greatest changes from baseline, to a degree comparable to natural and drug rewards. However, with milder stressors, there appears to be a greater increase in the mPFC dopamine compared with NAc dopamine, indicating a possible lower threshold of stimulation or alternative function for mPFC projecting dopamine neurons.

3.2 Electrophysiological evidence.

Initially, these extracellular increases in dopamine concentration in VTA projection targets were difficult to reconcile, as most studies had shown a suppression of VTA dopamine neuron neuronal firing during stress or aversive stimulus presentation (Guarraci & Kapp, 1999; Mantz et al., 1989; Mirenowicz & Schultz, 1996; Schultz & Romo, 1987; Ungless et al., 2004). However, recent evidence has demonstrated that there is in fact a subset of dopamine neurons within the VTA that are rapidly and potently excited by stress and aversive stimuli. Single unit recordings in awake rats showed that both firing rate and burst firing are increased in putative VTA dopamine neurons during restraint stress (Anstrom & Woodward, 2005), while multiunit recording showed similar increases in burst firing, but not firing rate, during social defeat stress (Anstrom, Miczek, & Budygin, 2009). Burst firing is thought to play an important functional role in dopamine release, as these bursts of firing frequency may overwhelm the dopamine transporter, resulting in supralinear increases in extracellular dopamine concentration (Gonon, 1988).

As described in section 2.3, most *in vivo* electrophysiological studies have focused on the dorsolateral VTA dopamine neurons using classic criteria, particularly large *Ih* current. When examining ventromedial VTA dopamine neurons, which were characterized by smaller *Ih* currents, Brischoux and colleagues (2009) found there was a subset of neurons rapidly and strongly excited by stress. Similarly, others have found increased activity in these “non-conventional” VTA dopamine neurons in

response to aversive stimulus presentation (Cohen, Haesler, Vong, Lowell, & Uchida, 2012; Zweifel et al., 2011). It is highly likely that a subpopulation of dopamine neurons responsive to aversive or stressful stimuli have been missed in many other studies due to sampling bias and mischaracterization of VTA dopamine neurons (Brischoux et al., 2009; Ungless et al., 2010; Ungless & Grace, 2012). In light of this growing evidence, it has been proposed that there are at least two subpopulations of VTA dopamine neurons: one group encoding reward-prediction error that is suppressed by aversive stimulation, and a second group, with atypical *Ih* and high baseline burst firing, that is phasically stimulated by stressors and aversive stimuli (Ungless et al., 2010). Future work will undoubtedly further characterize this small subset of dopamine neurons excited by stress, and, as with the microdialysis studies, should span a range of stressor intensities and modalities.

3.3 Evidence for neuroadaptations on VTA dopamine neurons.

Exposure to a single acute stressor can also promote long-lasting neuroplastic changes in VTA dopamine neurons in a manner similar to exposure to drugs of abuse (Dong et al., 2004; Graziane, Polter, Briand, Pierce, & Kauer, 2013; Niehaus, Murali, & Kauer, 2010; Saal, Dong, Bonci, & Malenka, 2003). Acute stress exposure induces long-term potentiation (LTP) at glutamatergic synapses onto VTA dopamine neurons, while blocking the formation of LTP at GABAergic synapses onto VTA dopamine neurons (Graziane et al., 2013; Niehaus et al., 2010).

During induction of LTP at glutamatergic synapses, new AMPA receptors are inserted, increasing the AMPA/NMDA ratio and increasing later excitability of the postsynaptic neuron (Malinow & Malenka, 2002). This alteration in the AMPA/NMDA ratio enhances calcium permeability and changes calcium dynamics in the synapse, such that sub-threshold stimulation can induce robust long-term potentiation (Polter & Kauer, 2014). Acute exposure to stress increases this ratio of AMPA to NMDA receptors on excitatory synapses onto VTA dopamine neurons (Dong et al., 2004; Graziane et al., 2013; Saal et al., 2003).

However, consistent with the theme of importance of VTA heterogeneity, distinct regional differences in AMPA/NMDA ratio alterations have been observed after acute stress exposure. Injection of formalin into the hindpaw results in a significant increase in the AMPA/NMDA ratio in medial VTA dopamine neurons projecting to the mPFC, whereas VTA dopamine neurons projecting to the NAc shell do not exhibit such alterations (Lammel et al., 2011). These increases in AMPA/NMDA ratio are present within 2 hours of stress, and have been observed for at least 24 hours (Daftary, Panksepp, Dong, & Saal, 2009). Furthermore, intra-VTA antagonism of both AMPA and NMDA receptors prevents tail pinch-induced dopamine efflux in the mPFC, although the NAc has not been examined (Butts & Phillips, 2013).

There has also been recent evidence that acute exposure to stress can block the induction of LTP at $GABA_A$ synapses onto VTA dopamine

neurons (Graziane et al., 2013; Niehaus et al., 2010). VTA dopamine neurons are relatively depolarized at baseline, and thus typically at or very close to the threshold for firing action potentials (Graziane et al., 2013; Johnson & North, 1992). This loss of LTP at inhibitory synapses onto VTA dopamine neurons may represent the removal of a brake on the system, which combined with the induction of LTP in excitatory synapses can lead to increased responsivity of VTA dopamine neurons to future activation, whether by additional stressors or rewards such as drugs of abuse.

4. Effects of repeated stress on VTA dopamine neuron activity

Activation of VTA dopamine neurons during acute stress exposure and subsequent neuroadaptations may result in altered VTA dopamine response to later activation. The effects of repeated or chronic stress on VTA dopamine neurons have remained largely unstudied by electrophysiological measures, as electrophysiological evidence for a subset of VTA dopamine neuron activation by stress has only recently emerged. However, a few microdialysis studies indicate that repeated exposure to stress might indeed alter dopaminergic release in VTA projection targets, particularly the NAc and mPFC. Repeated stress exposure can affect both tonic (basal levels) and phasic (release in response to stimulation) dopamine in the NAc and mPFC.

Of note, the schedule, intensity, and nature of stressors or aversive stimuli again have differential effects on extracellular NAc and mPFC dopamine, related to altered VTA dopamine neuron activity. For example,

repeated intermittent exposure to social defeat stress increases dopaminergic tone in the NAc (Miczek et al., 2011), while chronic social defeat reduces overall dopaminergic tone in both males and females (Miczek et al., 2011; Shimamoto, Debold, Holly, & Miczek, 2011). Chronic, inescapable restraint stress, a relatively severe stressor, also decreases dopamine tone in the NAc (Mangiavacchi et al., 2001), while other animal models used to study depression such as chronic cold stress and chronic mild stress have no effect on basal dopaminergic tone in the NAc, striatum, or mPFC (Di Chiara, Loddo, & Tanda, 1999; Gresch, Sved, Zigmond, & Finlay, 1994). Both chronic restraint and repeated social defeat stress increase both spontaneous and burst firing in VTA dopamine neurons in both *in vivo* and *ex vivo* studies (Anstrom et al., 2009; Anstrom & Woodward, 2005; Cao et al., 2010; Krishnan et al., 2007).

The importance of this alteration in VTA dopamine activity is clearly demonstrated by the fact that such increases in activity are only present in mice that exhibit behavioral signs of susceptibility to chronic social defeat stress, as opposed to those seemingly resilient to equivalent amounts of stress (Cao et al., 2010; Feder, Nestler, & Charney, 2009; Krishnan et al., 2007). Furthermore, these effects are relatively long lasting, as they are still observed 3 weeks after termination of stress (Razzoli, Andreoli, Michielin, Quarta, & Sokal, 2011).

In addition to altered tonic dopamine in VTA projection targets, the phasic dopamine response in the NAc and mPFC to subsequent stressors

is also altered. While daily restraint stress for 6 consecutive days results in a habituation of the extracellular dopamine response in the NAc across time, when restraint is again repeated after 72 h, the extracellular dopamine phasic response in the NAc is equivalent to the response on the first day (Imperato et al., 1992; Imperato et al., 1993). Repeated footshock stress (Young, 2004) and intermittent social defeat stress (Holly and Miczek, unpublished data), on the other hand, do not show such habituation in the phasic extracellular NAc dopamine response, while a sensitized response is observed after repeated tail pinch stress (Naef, Gratton, & Walker, 2013) or forced swim stress (Jordan et al., 1994; Petty, Jordan, Kramer, Zukas, & Wu, 1997).

In addition to altered responses to repeated presentations of the same stressor, a history of repeated stress can also alter the subsequent phasic extracellular dopamine response to a different stressor. Prior history of chronic variable stress, considered by some to be an animal model of some cardinal symptoms of depression, results in a significantly greater phasic extracellular dopamine response in the mPFC as a result of restraint stress in comparison to previously non-stressed rats (Cuadra et al., 2001; Cuadra et al., 1999), as well as the phasic extracellular dopamine response in both the NAc and mPFC to later tail pinch stress (Di Chiara et al., 1999). Continuous chronic cold exposure, another model of repeated stress shown to elicit anhedonic-like responses in rodents, also produces greater mPFC phasic extracellular dopamine responses to

tail pinch (Finlay, Zigmond, & Abercrombie, 1995) and tail shock (Gresch et al., 1994; Murphy, Sved, & Finlay, 2003). A similar sensitized effect of extracellular NAc dopamine is observed in animals with a history of isolation rearing in response to footshock stress (Fulford & Marsden, 1998), and history of prior social defeat stress results in greater NAc and mPFC response to social threat compared to previously non-stressed controls (Tidey & Miczek, 1996, 1997; Watt et al., 2014). Furthermore, when repeated footshock stress is paired with a conditioned stimulus, an augmented response is observed in both the NAc and mPFC, with the CS alone significantly elevating extracellular dopamine above baseline (Feenstra, Vogel, Botterblom, Joosten, & de Bruin, 2001; Young, 2004; Young, Joseph, & Gray, 1993).

Overall, while the effects of repeated stress on VTA dopamine neuron activity and related tonic and phasic dopamine levels in the NAc and mPFC have received only limited attention, current evidence points to a clear effect of repeated stress on subsequent tonic dopamine activity as well as subsequent response to both identical and different stressors. As with the effects of acute stress, the nature, intensity, and schedule of repeated stress may produce different effects, such that mild or intermittent stressors may potentiate basal VTA dopamine neuron activity and more severe or chronic stressors may reduce basal VTA dopamine activity, but the response to later stressors of a different nature is generally cross-sensitized.

5. Modulation of VTA dopamine neuron activity during stress and aversion by corticotropin releasing factor

How some VTA dopamine neurons become activated during stress and aversive experiences may be mediated by afferent connections. As described in Section 2.5, the VTA receives input from a wide range of brain regions. Of these diverse areas, several have been heavily implicated in stress and aversion, and as such their connectivity with VTA dopamine neurons is receiving rapidly growing attention.

One potential mediator of stress-induced changes in VTA dopamine neuron activity is the neuropeptide corticotropin releasing factor (CRF). Due to their key function in the initiation of physiological stress response, CRF and its receptors (CRF-R1 and CRF-R2) may be playing an important role in the behavioral and neural interactions of stress and reward. CRF is a neuropeptide most known for stimulating the hypothalamic-pituitary-adrenal axis response to stress (Sarnyai, Shaham, & Heinrichs, 2001). However, CRF also has widespread receptors and cell bodies throughout extrahypothalamic regions of the brain (L. W. Swanson, Sawchenko, Rivier, & Vale, 1983). While CRF actions within the amygdala and associated structures have received the most attention for their roles in stress and drug abuse (Koob & Le Moal, 2005; Koob & Volkow, 2010), CRF receptors within the VTA also directly influence the mesolimbic DA system.

The source of CRF into the VTA is quickly becoming the subject of considerable debate. Preliminary anatomical tracing work revealed CRF

input to the VTA stemmed from the paraventricular nucleus of the hypothalamus (PVN), the oval nucleus of the bed nucleus of the stria terminalis (BNSTov), the central nucleus of the amygdala (CeA, See Figure 2, Rodaros, Caruana, Amir, & Stewart, 2007; Tagliaferro & Morales, 2008). However, these studies were *a priori* hypothesis driven, such that the authors only investigated regions they already believed to innervate the VTA, and may have missed other important inputs. Recent work tracing monosynaptic inputs to the ventral posteromedial VTA shows CRF innervation exclusively from the dorsal raphe nucleus (DRN) and anterior VTA (Gobrogge et al., unpublished, Figure 4).

Importantly, extracellular CRF is increased in the VTA during footshock stress as a result of neuronal release as opposed to increased circulation (B. Wang et al., 2005). 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 almost entirely glutamatergic (Tagliaferro & Morales, 2008). Thus, CRF release into the VTA during stress is primed to excite VTA dopamine neurons projecting to the NAc and mPFC. Moreover, recent evidence indicates that CRF-containing neurons primarily target ventral posteromedial VTA dopamine neurons (Gobrogge et al., unpublished, Figure 5), where dopamine neurons excited by stress have been identified (Brischoux et al., 2009; Ungless et al., 2010).

Figure 4. DRN-CRH neurons synapse onto dopamine cells in the paranigral nucleus of the VTA.

An AAV-flex tracer (500nl) was bilaterally injected into the dorsal raphe nucleus (DRN) and allowed to spread for 1 month, after which mice were perfused, brains removed, and tissue sectioned at 40 μm. Tissue was stained for tyrosine hydroxylase (TH, blue, a marker of dopaminergic cells) and postsynaptic density 95 (PSD95, green, a marker of synaptic connection). Large white arrows pointing to white color co-localization in "MERGE" panel indicate putative synaptic contact between AAV-CRH neurons/processes with TH immunoreactive cells in the paranigral nucleus of the posterior ventral tegmental area (VTA). Smaller white arrow heads pointing to magenta color co-localization demonstrate close apposition, but not putative synapses due to lack of PSD95 co-localization, between DRN-AAV-CRH neurons/processes with TH immunoreactive cells in the paranigral nucleus of the posterior VTA. Courtesy of Kyle Gobrogge, unpublished.

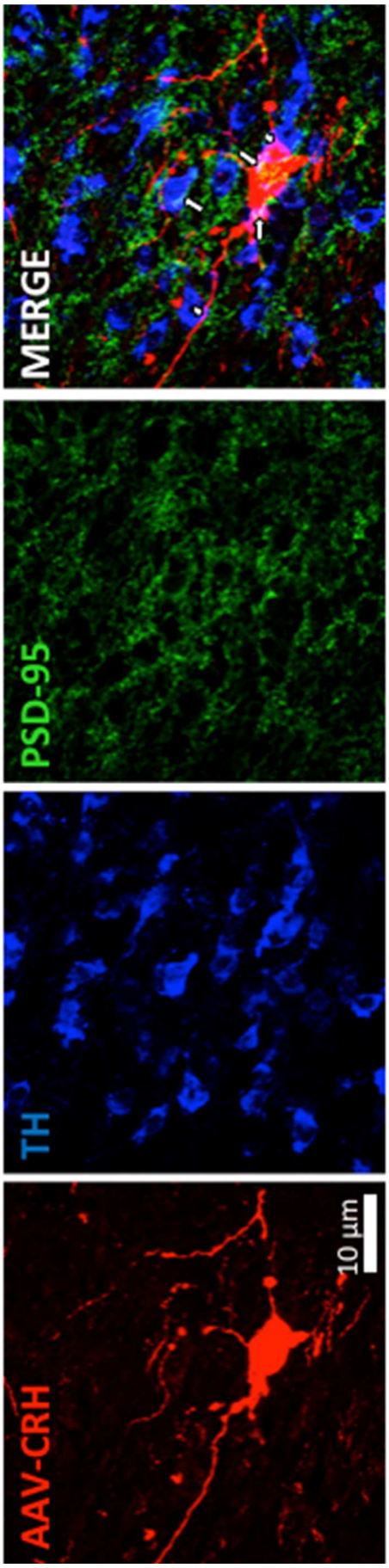
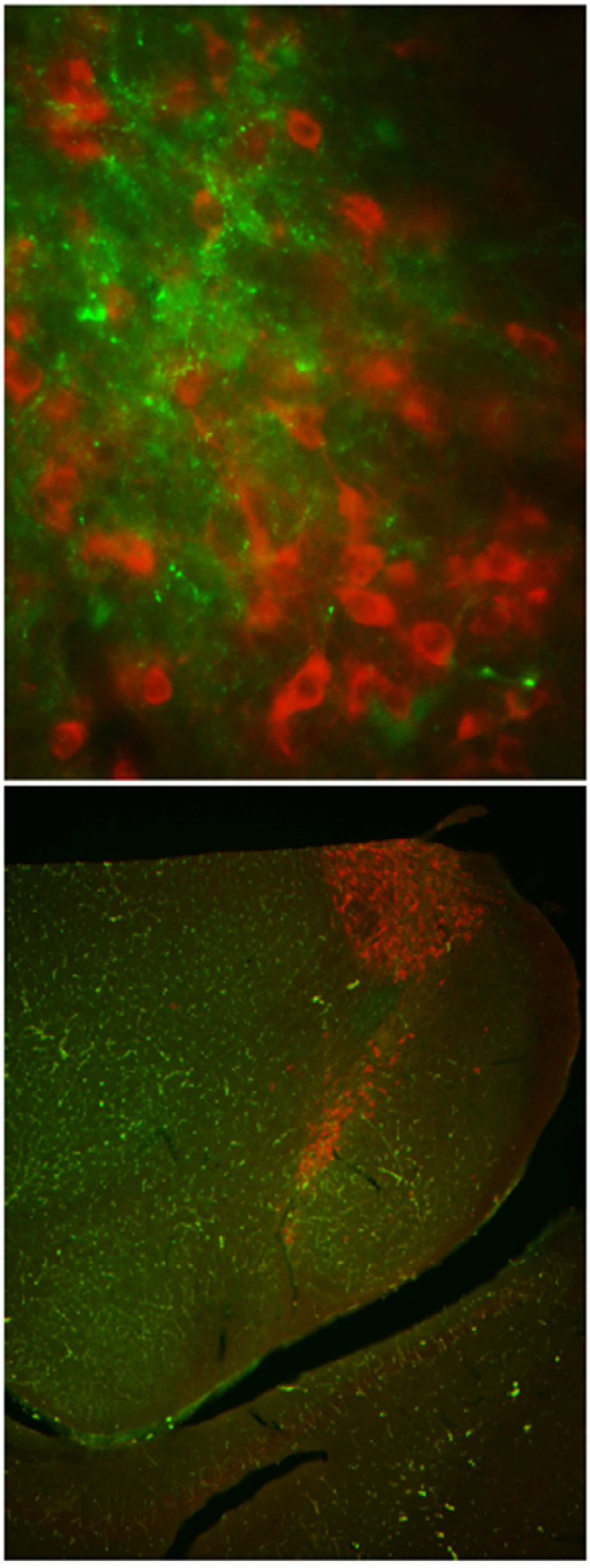

MERGE
PSD-95
TH
AAV-CRH
10 µm

Figure 5. Co-localization of CRF+ fibers and TH+ neurons in the ventral posteromedial paranigral nucleus (PN) of the VTA.

Coronal sections of the ventral tegmental area (VTA) were stained for immunohistochemistry of corticotropin releasing factor (CRF, green) and tyrosine hydroxylase (TH, red). Left figure shows a merged 20x magnification of a coronal section encompassing the VTA, stained bright red. The right panel shows a 400X magnification of the posteromedial paranigral nucleus of the VTA. Courtesy of Kyle Gobrogge, unpublished.



The VTA expresses both CRF-R1 and CRF-R2, although specific sub-regional examination of the distribution of these receptors has yet to be reported (Ungless et al., 2003; Van Pett et al., 2000). Additionally, CRF binding protein (CRF-BP) is present in approximately 25% of VTA dopamine neurons in naïve animals, primarily within the PBP (H. L. Wang & Morales, 2008). However, the synaptic location as well as function of these receptors has been the subject of considerable debate.

Initial work indicated that CRF exerted its action on VTA dopamine neurons postsynaptically, resulting in potentiation of NMDA-mediated excitatory postsynaptic currents (EPSCs, Ungless et al., 2003). Subsequently, it was demonstrated that activation of postsynaptic CRF-R1 on VTA dopamine neurons rapidly increases action potential firing rate (Korotkova, Brown, Sergeeva, Ponomarenko, & Haas, 2006; Wanat, Hopf, Stuber, Phillips, & Bonci, 2008). It appears that this effect is at least partially mediated through protein-kinase-C-dependent enhancement of *Ih* (Wanat et al., 2008). VTA CRF-R1 activation has also been shown to facilitate slow dopamine D_2- and $GABA_B$-receptor mediated synaptic transmission, although this enhancement is reduced with repeated stress or psychostimulant exposure (Beckstead et al., 2009).

Activation of postsynaptic CRF-R2 on VTA dopamine neurons, on the other hand, induces transient potentiation of NMDA mediated synaptic transmission (Hahn, Hopf, & Bonci, 2009; Ungless et al., 2003). This effect is similarly driven by activation of the protein kinase C pathway, but

requires the presence of CRF-BP (Ungless et al., 2003). Therefore, regardless of CRF-R2 distribution, only the small subset of PBP dopamine neurons expressing CRF-BP (~25%) would exhibit this effect. Activation of VTA CRF-R2 can also enhance the function of metabotropic glutamate receptors (mGluRs) on VTA dopamine neurons via activation of a protein kinase A pathway (Fiorillo & Williams, 1998). Notably, CRF-BP prevents this effect. This evidence suggests CRF may be at least partially mediating some of the immediate and long lasting effects of stress on VTA dopamine neurons, however more careful examination of anatomical subregions and dopaminergic cell type is required.

Other work, however, has found conflicting results, demonstrating that both CRF-R1 and CRF-R2 are located presynaptically, as their selective activation alters AMPA- and not NMDA- mediated EPSCs (Manabe, Wyllie, Perkel, & Nicoll, 1993; Williams, Buchta, & Riegel, 2014). In the naïve rat, presynaptic CRF-R1 and CRF-R2 heterosynaptically regulate VTA dopamine function, such that CRF-R1 activation facilitates glutamate release onto VTA dopamine neurons and CRF-R2 activation facilitates GABAergic release at the same multi-part synapse (Williams et al., 2014). This same study also found that a history of cocaine self-administration reversed the function of CRF-R2, such that it no longer facilitated a GABAergic brake on VTA dopamine function, and instead facilitated additional glutamatergic release during yohimbine stress-induced reinstatement to previously extinguished cocaine seeking. As the

effects of acute or repeated stress have not been evaluated in regards to CRF receptor function within the VTA, it will be crucial for future studies to investigate whether similar neuroadaptations occur in dopamine-CRF interactions in the VTA after stress exposure.

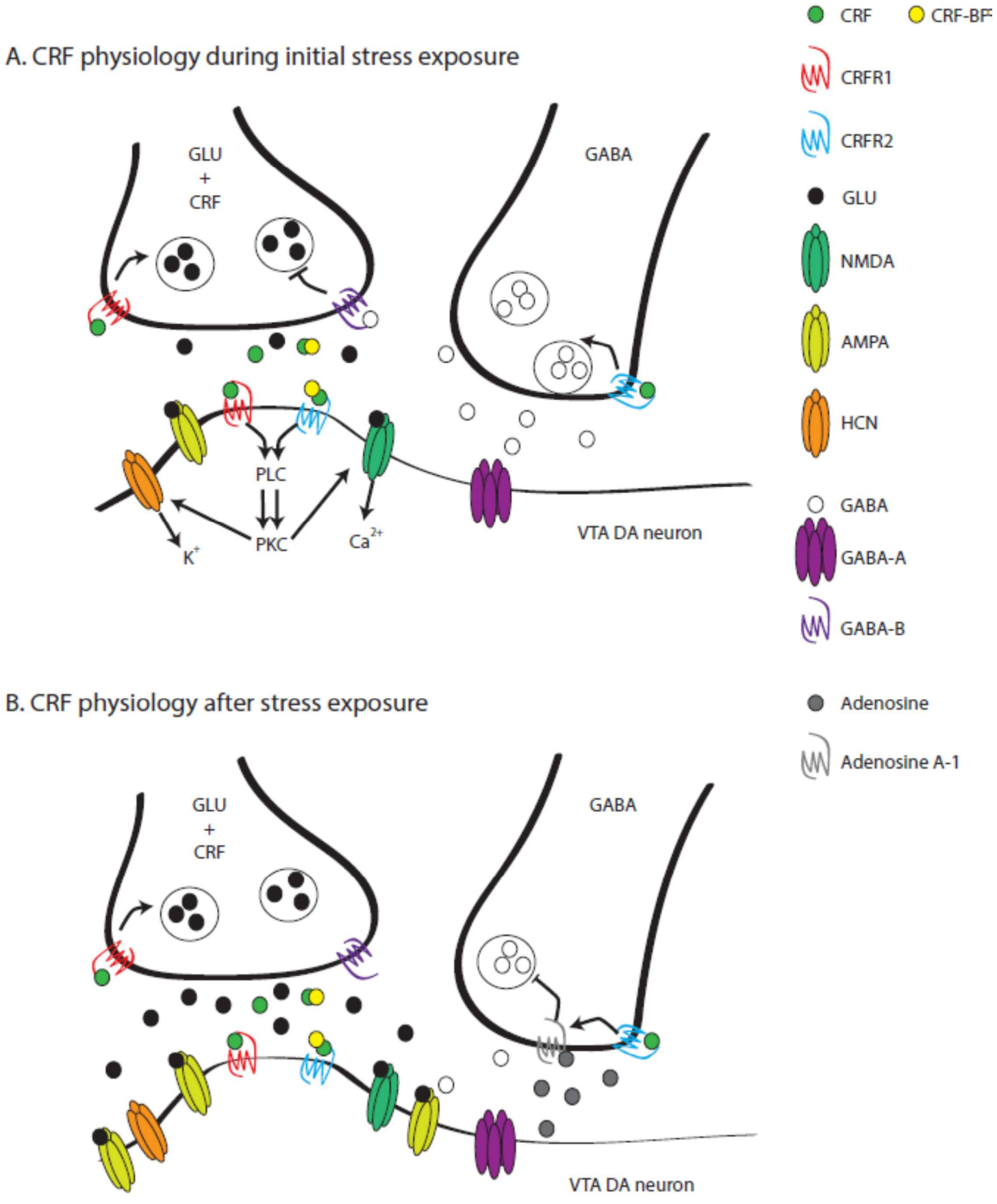
Although more work examining the specific effects of stress on CRF and CRF-R function within the VTA are needed, recent evidence suggests CRF and its receptors within the VTA are necessary during stress exposure for the development of later stress-induced maladaptive dopamine-dependent behavioral effects. Both systemic and intra-VTA antagonism of CRF-R1 during social defeat stress can prevent the development of later behavioral cross-sensitization to cocaine, as well as escalated cocaine self-administration during a 24 h “binge” (Boyson, Miguel, Quadros, Debold, & Miczek, 2011).

Thus, CRF signaling from glutamatergic neurons synapsing onto VTA dopamine neurons, as well from inhibitory neurons synapsing onto VTA GABA interneurons, plays a role in regulating VTA dopamine neuron function. When CRF is co-released with glutamate in the VTA during stress (B. Wang et al., 2005), it activates both CRF-R1 and CRF-R2, which may excite VTA dopamine neurons both directly through facilitation of AMPA- and/or NMDA-mediated EPSCs as well as indirectly, through the removal of the GABAergic interneuron brake on VTA dopamine neurons (Figure 6). During acute stress exposure, this VTA dopamine neuron activation (Section 3.2) leads to increased extracellular dopamine

Figure 6. Hypothesized CRF physiology before and after stress.

A. Under normal conditions, presynaptic CRF-R1 (red) potentiates glutamate (GLU, black) release onto ventral tegmental area (VTA) dopamine (DA) neurons. CRF-R2 (blue) heterosynaptically regulates glutamatergic input via potentiation of GABA (white) release, activating postsynaptic GABA-A and presynaptic GABA-B receptors (purple) on GLU terminals. During acute stress, postsynaptic CRF-R1 activates the phospholipase C (PLC) pathway, increasing K+ permeability through hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, increasing VTA DA neuronal firing rate. Postsynaptic CRF-R2 activation also activates the PLC-PKC pathway, enhancing N-methyl-D-aspartate (NMDA, green) permeability. Ultimately, during stress, the VTA DA neuron becomes more receptive to glutamatergic signaling.

B. After stress, presynaptic CRF-R2 function reverses, such that (through the adenosine A-1 receptor, gray) GABAergic regulation of the VTA DA neuron is inhibited, resulting in increased glutamatergic release into the synapse. Repeated stress also increases AMPA/NMDA ratio on the postsynaptic VTA DA neuron, such that it is now hyperexcitable.


A. CRF physiology during initial stress exposure
GLU
+
CRF
GABA
PLC
PKC
K+
Ca2+
VTA DA neuron
CRF
CRF-BP
CRFR1
CRFR2
GLU
NMDA
AMPA
HCN
GABA
GABA-A
GABA-B
B. CRF physiology after stress exposure
GLU
+
CRF
GABA
VTA DA neuron
Adenosine
Adenosine A-1

in VTA projection targets (Section 3.1), and in leads to the induction of glutamatergic LTP and prevention of GABAergic LTP within the VTA (Section 3.3), altering later VTA dopamine neuron activity both at rest and in response to additional stressors (Section 4).

6. Conclusions

Two critical themes regarding the role of VTA dopamine neurons in response to stress have emerged: (i) VTA heterogeneity matters, and (ii) the nature of the stressor matters. Recent research demonstrates that there may be at least two distinct types of VTA dopamine neurons mediating different behavioral functions, particularly reward and aversion. Anatomical, neurochemical, and electrophysiological data have all pointed to a subset of dopamine neurons in the ventral posteromedial VTA that have previously been ignored and are rapidly and potently excited by stress. Future research should be driven to determining the specific afferent and efferent connections of this particular subtype, as well as the distribution of CRF receptors within the region.

The nature of stressors and aversive stimuli are also crucial to the interpretation of both microdialysis and electrophysiology results. A general tendency within past research has been to extrapolate findings with one type of stressor to a general response to all types stress. However, as reviewed here, it is clear that the nature, intensity, and schedule of repeated stress can have vastly different effects on dopamine release in VTA projection targets. Notably, the comparatively mild,

inescapable stress of chronic cold induces a pronounced reduction in VTA dopamine neuron activity, whereas the more severe inescapable stress of acute restraint can increase this neuronal activity (Moore, Rose, & Grace, 2001; Valenti, Gill, & Grace, 2012). Of note, chronic stressors generally used as animal models of depression, such as chronic cold exposure or chronic mild stress, generally tend to blunt subsequent tonic dopaminergic activity, while more severe acute or intermittent stressors, such as those typically associated with anxiety or heightened vulnerability to subsequent addictive-like behaviors, tend to augment tonic dopaminergic activity. However, even within each so-called class of stressors, different stimuli can still promote profoundly different effects on not only tonic and phasic dopamine, but also behavior. This may be due, in part, to differential release of CRF by various stressors. Corticosterone is differentially released with distinct types of stressors (Koolhaas et al., 2011), so it is likely the same may be true of CRF.

Ultimately, understanding how stress and reward work at a neural level may lead to more promising therapies for addiction. Mesocorticolimbic dopamine plays a fundamental role in both stress and reward, but how these systems interplay is still poorly understood. The connections between CRF and dopamine neurons within the VTA may be a fundamental mediator of stress-induced escalations in maladaptive reward-related behavior, particularly addiction. Thus, this dissertation explores interactions of CRF and VTA dopamine neurons during acute

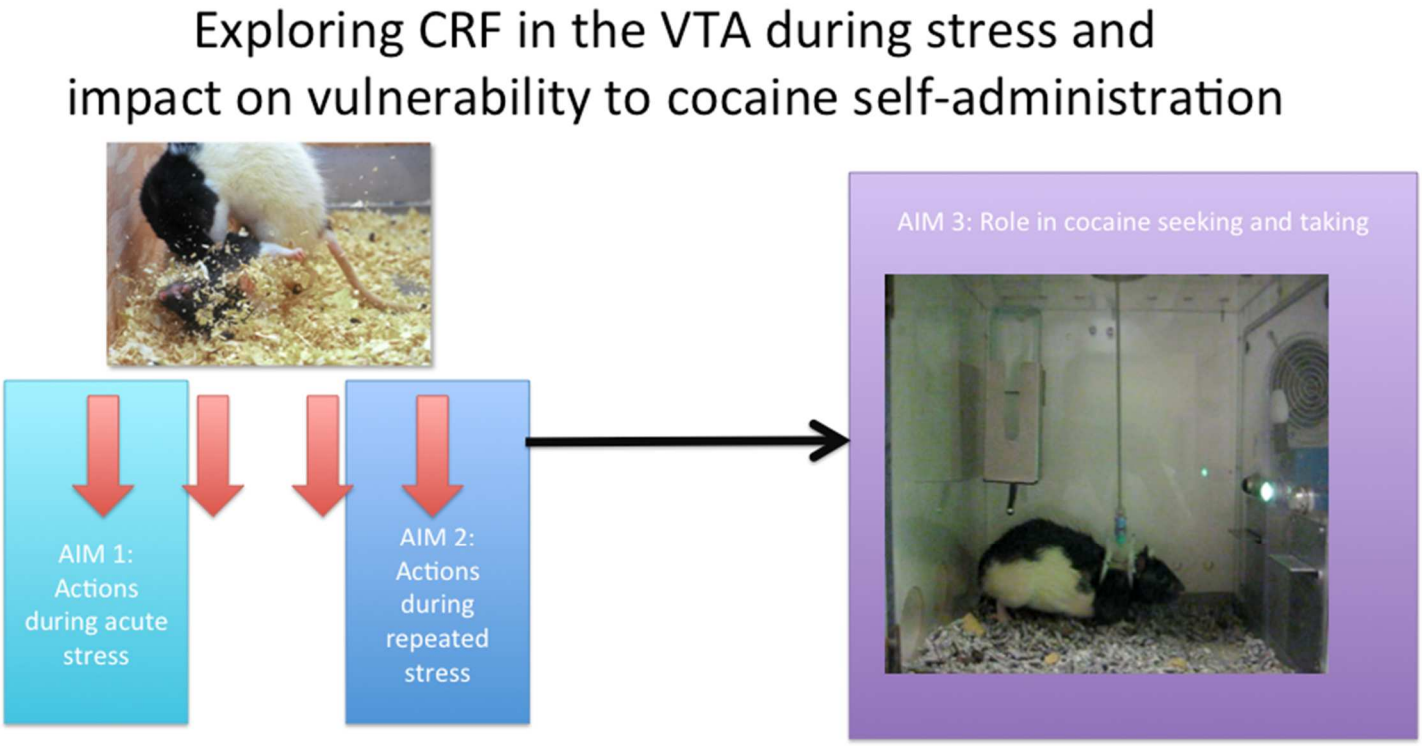
and repeated intermittent social defeat stress, how these interactions may change across the course of repeated intermittent stress, and ultimately influence subsequent behavioral and neural responses to cocaine.

First, this work examines how CRF and dopamine neurons in the VTA interact during acute social defeat stress through *in vivo* microdialysis of CRF within the VTA or dopamine within the mPFC and NAcSh following intra-VTA CRF receptor antagonism. Second, this work examines whether these interactions change with repeated stress exposure through *in vivo* microdialysis of the same animals on day 10 of the intermittent social defeat stress paradigm. Next, how these interactions during social defeat stress affect subsequent neural cross-sensitization to cocaine as well as escalated cocaine self-administration are investigated. Finally, the role of VTA CRF receptors are explored in the long term effects of social defeat stress.

II. AIMS AND EXPERIMENTAL DESIGN

This dissertation explores the interactions of CRF and dopamine neurons in the VTA during acute stress (Aim 1) and repeated stress (Aim 2), as well as how these interactions can affect subsequent reward related behaviors and neurochemistry (Aim 3, see Figure 7). Specifically, this work shows that dopamine is increased in the mPFC and NAcSh during both acute and repeated social defeat stress (Experiment 1), which is paralleled by increases in extracellular CRF in the VTA (Experiment 2).

Figure 7. Dissertation Aims.


Exploring CRF in the VTA during stress and impact on vulnerability to cocaine self-administration
AIM 1: Actions during acute stress
AIM 2: Actions during repeated stress
AIM 3: Role in cocaine seeking and taking

Moreover, antagonism of VTA CRF receptors prior to stress can attenuate the dopaminergic response to both acute and repeated social defeat, which will prevent the induction of neural cross-sensitization to cocaine (Experiment 3A) and escalated cocaine self-administration during a 24 h “binge” (Experiment 3B). Finally, intermittent social defeat stress causes long-lasting increased tonic CRF levels in the VTA (Experiment 4A), and antagonism of VTA CRF receptors can reverse heightened context-induced reinstatement in previously stressed rats (Experiment 4B). Together, these data establish a crucial role of CRF receptors in the VTA mediating dopaminergic function during stress and promoting long-lasting neural adaptations of the mesocorticolimbic circuitry traditionally associated with reward.

Aim 1: Investigating the interactions of CRF and dopamine neurons in the VTA during acute stress

The first aim is to characterize the interactions of CRF and its receptors within the VTA and how this may modulate subsequent dopamine release in VTA projection targets (mPFC and NAcSh). First, this work demonstrates that dopamine is phasically increased in VTA projection targets (mPFC and NAcSh) during acute social defeat stress (Experiment 1A), and that these phasic increases are due to similar phasic increases in extracellular CRF (Experiment 2A) and its actions on its receptors in the VTA (Experiment 1A). This aim is achieved by *in vivo*

microdialysis of either dopamine in the mPFC and NAcSh (Experiment 1) or CRF in the VTA (Experiment 2) on the first day of social defeat stress.

Nearly all *in vivo* microdialysis studies have shown that acute stress increases extracellular dopamine in both the mPFC and NAcSh (see Tables 1-9). Therefore, it was expected that the first day of social defeat stress would similarly increase extracellular dopamine in the mPFC and NAcSh.

The influence of CRF in the VTA on this dopaminergic increase has yet to be studied. In the only study to examine CRF in the VTA by microdialysis, Wang and colleagues (2005) found that footshock stress during reinstatement to cocaine seeking significantly increases VTA CRF in both cocaine experienced and cocaine naïve rats. However, the authors began reinstatement procedures immediately after footshock stress, so time course of CRF increases due to stress alone could not be evaluated. Therefore, it was expected that CRF will similarly be increased in the VTA of naïve rats undergoing their first social defeat.

Later, Wang and colleagues (2007) found that VTA dopamine was increased during footshock-induced reinstatement, and that this increase could be blocked by administration of a CRF-R2, but not CRF-R1, antagonist into the VTA. Although this experiment was conducted in cocaine-experienced rats and measured dopamine in the VTA rather than its projection targets, it is the only study to date to directly measure the impact of CRF receptor antagonism on stress-induced efflux of dopamine.

Therefore, although electrophysiological data has pointed to a role of CRF-R1 activation in VTA dopamine neuronal firing *ex vivo* (Wanat et al., 2008), it was hypothesized that the dopaminergic increases in the mPFC and NAcSh during social defeat stress could be blocked by intra-VTA antagonism of CRF-R2 but not CRF-R1.

Aim 2: Investigating the interactions of CRF and dopamine neurons in the VTA during repeated stress

The second aim of this dissertation is to determine whether these phasic dopaminergic increases during social defeat stress are habituated, sensitized, or unchanged by repeated intermittent social defeat (Experiment 1B), and whether these changes are similarly reflected in extracellular CRF in the VTA (Experiment 2B) or prevented by blockade of VTA CRF receptors (Experiment 1B). This aim is achieved by performing *in vivo* microdialysis on day 10 of the intermittent social defeat protocol in the same animals from Aim 1 experiments.

As discussed in Section 4 of the introduction, repeated stress can have different effects on phasic dopamine depending on the nature, schedule, and intensity of the stressor. While extracellular dopamine becomes habituated with daily restraint stress, after a 72 h break, the dopaminergic response to restraint is equivalent to the first day (Imperato et al., 1992). Additionally, the dopaminergic response to repeated mild footshock stress is not habituated after daily presentation (Young, 2004). Therefore, it is expected that due to the intermittent schedule of social

defeat stress in the present experiment, there will not be a change in the percent change from baseline dopamine between days 1 and 10 in either the mPFC or NAcSh.

The role of VTA CRF in the dopaminergic response to repeated stress has never been studied. It was expected that the increase in extracellular CRF during social defeat on day 10 will be similar to that of day 1, mirroring the anticipated results from dopamine microdialysis. Additionally, as expected in the acute stress experiment, it was expected that CRF-R2, but not CRF-R1, antagonism in the VTA would blunt the mPFC and NAcSh dopaminergic response to stress. In addition to the prior rationale based on the ineffectiveness of CRF-R1 antagonists in attenuating the dopaminergic response to footshock stress (B. Wang et al., 2007), work from the Valentino laboratory has demonstrated trafficking of CRF-R2 to the cellular membrane in the locus coeruleus and dorsal raphe nucleus after acute stress, while CRFR1 becomes internalized (Reyes, Valentino, & Van Bockstaele, 2008; Waselus, Nazzaro, Valentino, & Van Bockstaele, 2009; Wood et al., 2013). Thus, if a similar effect is occurring in the VTA, CRF-R1 will become internalized throughout the course of intermittent social defeat stress, such that CRF-R1 is not present on the membrane on the 10th day of the protocol, rendering antagonism ineffective.

Aim 3: Investigating long-term effects of CRF-dopamine interactions in the VTA during and after repeated stress on cocaine taking and seeking

Finally, the third aim is to investigate whether these interactions between CRF and dopamine neurons in the VTA are relevant for the induction of neural cross-sensitization to cocaine (Experiment 3A) and escalated cocaine self-administration during a 24 h “binge” (Experiment 3B). This was investigated through intra-VTA antagonism of CRF-R1 or CRF-R2 prior to each social defat, and testing for cross-sensitization to cocaine 10 d later with *in vivo* microdialysis of dopamine in the NAcSh, or through intravenous cocaine self-administration. Additionally, the role of VTA CRF (Experiment 4A) and its receptors (Experiment 4B) on the expression of context-induced reinstatement to cocaine seeking was assessed. In this experiment, as opposed to antagonizing VTA CRF receptors during stress, they will be antagonized prior to reinstatement, after the induction of any stress-induced neuroadaptations.

Previous studies from this laboratory have shown that both systemic and intra-VTA antagonism of CRF-R1 prior to social defeat stress can prevent the induction of behavioral sensitization as well as escalated cocaine self-administration during a 24 h “binge”, pointing to a key role of CRF-R1 during social defeat stress on engendering long lasting neuroadaptations leading to heightened vulnerability to several cocaine-related measures (Boyson et al., 2011). However, the CRF-R1 antagonist (CP-154526) used in that study had solubility issues, so we

sought to replicate these findings with a newer, more water-soluble compound. As such, it was expected that the CRF-R1 antagonist (CP-376395) used in the current experiment would also prevent the induction of behavioral sensitization and escalated cocaine self-administration. Furthermore, the role of CRF-R2 in the VTA has yet to be evaluated, but it was expected that intra-VTA CRF-R2 antagonism would additionally prevent these long-lasting effects of intermittent social defeat due to its expected prevention of stress-induced dopamine efflux in the mPFC and NAcSh.

Dopaminergic cross-sensitization to cocaine is thought to be a possible mechanism underlying later escalated cocaine self-administration (Thomas, Kalivas, & Shaham, 2008). As such, the role of VTA CRF receptors on preventing stress-induced cross-sensitization to cocaine was investigated. Similar to the expected results in cocaine self-administration, it was expected that intra-VTA antagonism of both CRF-R1 and CRF-R2 during social defeat stress would prevent the induction of dopaminergic cross-sensitization to cocaine.

Finally, it was hypothesized that intermittent social defeat stress-induced neuroadaptations in the VTA can not only affect cocaine taking, but also cocaine seeking. A translational model of cocaine abuse was first developed, whereby rats were exposed to handling or intermittent social defeat stress, allowed to freely self-administer cocaine for 10 d, after which they were subjected to 15 d forced abstinence, and lastly tested for

context-induced reinstatement. Previously stressed rats showed significantly more cocaine seeking than non-stressed rats. It was hypothesized that CRF-dopamine neuroadaptations in the VTA due to intermittent social defeat stress have long lasting effects and play a role in this heightened context-induced reinstatement. Accordingly, intra-VTA antagonism of both CRF-R1 and CRF-R2 was expected to attenuate cocaine seeking in the previously stressed rats.

III. METHODS

Experimental Design

Experiments 1 and 2 assess CRF-dopamine interactions in the VTA during acute (Experiments 1A, 2A) and repeated (Experiments 1B, 2B) stress using *in vivo* microdialysis during social defeat. Animals were exposed to intermittent social defeat stress on days 1, 4, 7, and 10, with concurrent microdialysis during the defeats on day 1 and day 10. Animals were tested for either extracellular dopamine in the mPFC and NAcSh after intra-VTA microinjection of aCSF, CRF-R1 antagonist or CRF-R2 antagonist (Experiment 1) or extracellular CRF in the VTA (Experiment 2).

The impact of these CRF-dopamine interactions and neuroadaptations during repeated social defeat stress on subsequent addiction-related endpoints is examined in Experiments 3 and 4. In Experiments 3A and 3B, animals were microinjected with aCSF, CRF-R1 antagonist, or CRF-R2 antagonist prior to each social defeat, and later

evaluated for neural cross-sensitization to cocaine using *in vivo* microdialysis (Experiment 3A) or escalated cocaine self-administration in a 24h "binge" (Experiment 3B). Separate rats underwent intermittent defeat or handling and cocaine self-administration followed by forced abstinence, after which they were tested for context-induced reinstatement to cocaine seeking (Experiment 4). These rats were either assessed for extracellular CRF using *in vivo* microdialysis during reinstatement (Experiment 4A) or microinjected with aCSF, CRFR1 antagonist, or CRFR2 antagonist into the VTA prior to reinstatement (Experiment 4B). Group sizes for each experiment are shown in Tables 11-14.

General Methods

Subjects

Male Long Evans rats (Charles River, Wilmington, MA) weighing 225-250g on arrival were individually housed in custom built acrylic chambers (30 x 20.5 x 24.5 cm) lined with Cellu-Dri™ pellet bedding (Shepherd Specialty Papers, Kalamazoo, MI). Stimulus "resident" rats were housed in male-female pairs in a separate room in large stainless steel cags (71 x 46 x 46 cm) as described previously (Miczek, 1979). All rats were housed in a vivarium with controlled temperature (21±1°C) on a 12hr light/dark cycle (lights on 2000-0800h). All procedures were approved by the Tufts University Institutional Animal Care and Use Committee following the guidelines set forth in the *Guide for Care and Use of Laboratory Animals* (National Research Council, 2011).

Social defeat stress

A modification of a previously described resident-intruder paradigm (Boyson et al., 2014; Tornatzky & Miczek, 1995) was used. Rats were subjected to 4 social defeats, separated by approximately 72 h, on days 1, 4, 7, and 10. The female resident is removed prior to each defeat, which consists of three phases: 1) Instigation: The experimental animal was placed in a wire mesh enclosure inside the resident's home cage for 10 min. This allowed for visual and olfactory instigation and threat, but prevented tactile contact. 2) Defeat: The protective enclosure was removed, and the experimental rat placed with the aggressive resident until the experimental rat was held in supine for 6s, was bitten 10 times, or 5 min had elapsed from the first attack bite. In the microdialysis experiments (Experiments 1 and 2), all defeats were 5 min to account for dialysate sample times. Attack latency and number of bites were recorded. No statistical difference in attack bites, latency, or duration was observed between the experiments. 3) Threat: The experimental rat was then returned to the wire mesh protective cage inside the resident's home cage for an additional 10 min, after which it was returned to its home cage.

Microinjections

Drugs were microinjected into the VTA with an infusion pump (CMA 102, CMA Microdialysis, Chelmsford, MA) using 33 gauge microinjectors protruding 1 mm beyond the guide cannulae (PlasticsOne, Roanoke, VA). All drugs and vehicle were administered in a volume of 0.25 µl/side across

1 min. Injectors were kept in place for 1 additional min following the microinjection to allow for adequate diffusion from injection site and prevent backflow. Microinjections occurred 10 min prior to the instigation phase of social defeat stress (Experiments 1 and 3) or context-induced reinstatement (Experiment 4B).

Histology

At the termination of experiments, rats were anesthetized with sodium pentobarbital (100 mg/kg, ip) and transcardially perfused with 0.9% saline and 4% paraformaldehyde. Brains were removed and placed in paraformaldehyde overnight, after which they were sliced into 50 μm sections and mounted onto microscope slides. Sections were stained with cresyl violet and coverslipped as described previously (Boyson et al., 2014; Holly, Shimamoto, Debold, & Miczek, 2012). Slides were examined under light microscopy for verification of microdialysis probe and microinjection cannula placement.

Experiment 1: Microdialysis during defeat for dopamine in the mPFC and NAcSh after CRFR1 or CRFR2 antagonism

Objective and experimental design

Experiment 1 first seeks to demonstrate that acute (Aim 1, Experiment 1A) and repeated (Aim 2, Experiment 1B) social defeat stress results in increased extracellular dopamine in the mPFC and NAcSh. Additionally, Experiment 1 evaluates whether CRF actions on its receptors in the VTA mediate the dopaminergic response to acute and repeated social defeat

(Aim 2). In this experiment, rats underwent intracranial surgery, after which they were exposed to intermittent social defeat stress on days 1, 4, 7, and 10 as described above. Vehicle (aCSF), CRF-R1 antagonist (CP376395, 500 ng/side), or CRF-R2 antagonist (Astressin2B, 1000 ng/side) was microinjected 10 min prior to the instigation phase of each social defeat, and microdialysis occurred concurrently with the defeats on days 1 and 10 (see Figure 8 for experimental design). Drug doses were chosen based on prior studies (Blacktop et al., 2011; Boyson et al., 2014).

Intracranial surgery

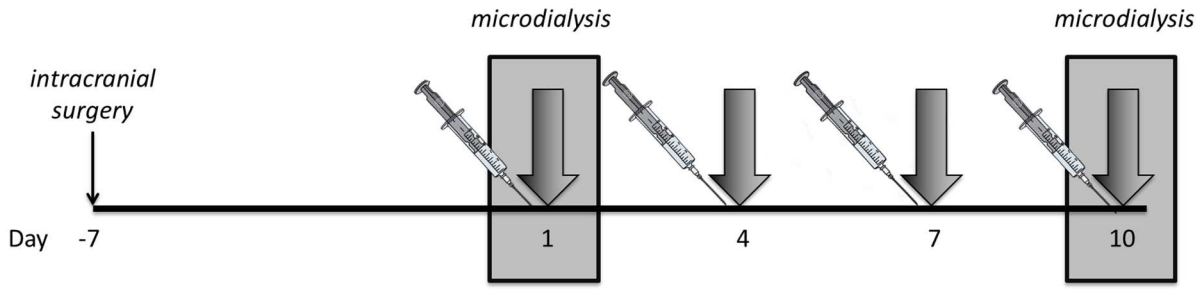
After one week of habituation to the vivarium and at least one week before the first day of microdialysis, rats underwent intracranial surgery under ketamine (100 mg/kg, ip) and xylazine (6 mg/kg, ip) anesthesia. Bilateral microinjection cannulae (23 ga, 11 mm length, PlasticsOne, Roanoke, VA) were implanted at a 10° angle 5.2 mm posterior from bregma and 1.8 mm lateral from the midline at a depth of 7.5 mm from the skull surface. 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, at 10° angle) and the NAcSh (+2.1 mm from bregma, +1.1 mm from midline, -5.8 mm from dura, at 0° angle).

In vivo microdialysis

In vivo microdialysis occurred on both days 1 and 10 of the social defeat protocol (Figure 7). On the night before the microdialysis experiment, the

Figure 8. Timeline for Experiment 1.

Animals were implanted with bilateral microinjection cannulae aimed at the ventral tegmental area (VTA) and unilateral microdialysis cannulae aimed at the medial prefrontal cortex (mPFC) and nucleus accumbens shell (NAcSh) one week prior to experimental manipulation. As indicated by the syringes, rats were microinjected with vehicle (aCSF), CRF-R1 antagonist (CP376395, 500 ng/side), or CRF-R2 antagonist (Astressin2B, 1000 ng/side) 10 min prior to the instigation phase of social defeat stress (indicated by arrows) on days 1, 4, 7, and 10. *In vivo* microdialysis was conducted concurrently on days 1 and 10 (indicated by gray boxes), with samples analyzed for dopamine in both the mPFC and NAcSh before, during, and after social defeat stress.


microdialysis
microdialysis
intracranial
surgery
Day
-7
1
4
7
10

microdialysis guide cannulae were removed and replaced with microdialysis probes (2 mm active membrane, Synaptech Inc, Marquette, MI), which were perfused with artificial cerebrospinal fluid (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 next 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-2-Na 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 above, and social defeat occurred in an adjacent resident rat's home cage, with sample collection ongoing throughout the defeat. After the rat was removed from the threat phase, an additional five samples were collected to evaluate the time course of dopaminergic changes. On days 4 and 7, social defeats occurred in an identical manner, but microdialysis was not performed.

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 a 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 x 250 mm, 5 µm ID, Shiseido, Tokyo, Japan) at 30°C and quantified by electrochemical detection (DECADE II, Antec Leyden BV, Zoeteroude, Netherlands). Dopamine concentrations were calculated using a standard curve with known amounts of monoamines in a range of 1.975-18.75 pg.

Experiment 2: Microdialysis during defeat for CRF in the VTA

Objective and experimental design

Experiment 2 directly measures extracellular CRF during acute (Aim 1, Experiment 2A) and repeated (Aim 2, Experiment 2B) social defeat stress. Similar to experiment 1, rats were exposed to intermittent social defeat stress, with concurrent microdialysis on days 1 and 10 (Figure 9).

Intracranial surgery

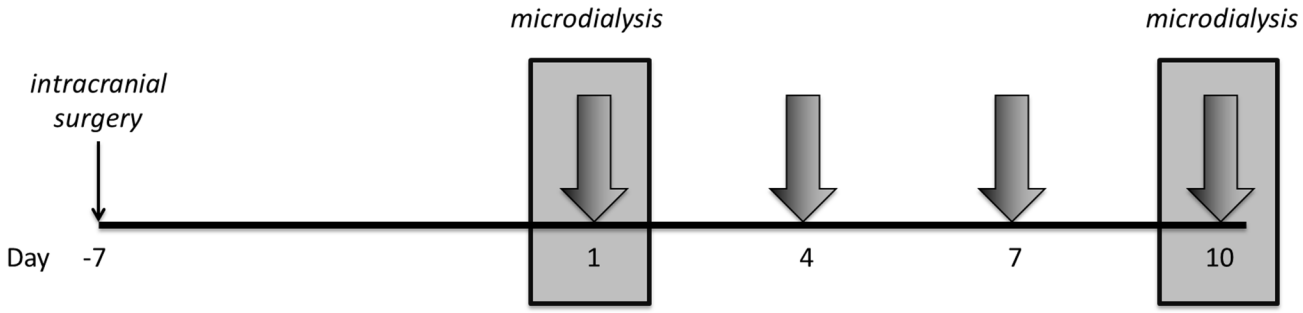
After one week of habituation to the vivarium and at least one week before the first day of microdialysis, rats underwent intracranial surgery under ketamine (100 mg/kg, ip) and xylazine (6 mg/kg, ip) anesthesia. A unilateral microdialysis cannula (8 mm length, Synaptech Inc, Marquette, MI) was implanted at a 10° angle 5.2 or 5.4 mm posterior from bregma and 1.8 mm lateral from the midline at a depth of 7.5 mm from the skull surface.

In vivo microdialysis

In vivo microdialysis occurred on both days 1 and 10 of the social defeat protocol as described above. On the night before the microdialysis experiment, the microdialysis guide cannula was removed and replaced

Figure 9. Timeline for Experiment 2.

Animals were implanted with unilateral microdialysis cannulae aimed at the ventral tegmental area (VTA) at least one week prior to experimental manipulation. Rats underwent intermittent social defeat stress on days 1, 4, 7, and 10 (indicated by arrows) with concurrent *in vivo* microdialysis for corticotropin releasing factor (CRF) in the VTA on days 1 and 10 (indicated by gray boxes).


microdialysis
microdialysis
intracranial
surgery
Day
-7
1
4
7
10

with a microdialysis probe (2 mm active membrane, Synaptech Inc, Marquette, MI), which was perfused with aCSF at a flow rate of 0.5 µl/min overnight. The following morning, aCSF was replaced with aCSF containing 0.2% bovine serum albumin (BSA) and the flow rate increased to 2.0 µl/min two hours prior to sample collection. Samples were collected by hand into Eppendorf Protein LoBind tubes every 12.5 min, and tonic levels of CRF were measured in five baseline samples. After baseline collection, social defeat occurred in an adjacent resident rat's home cage, with sample collection ongoing throughout the defeat. After the rat was removed from the threat phase, an additional five samples were collected to evaluate the time course of CRF changes. On days 4 and 7, social defeats occurred in an identical manner, but microdialysis was not performed. CRF was quantified by a commercially available enzyme immunoassay (Peninsula Laboratories, San Carlos, CA).

Experiment 3: Role of VTA CRF receptors during stress on the induction of dopaminergic cross-sensitization to cocaine and escalated cocaine self-administration

Objective and experimental design

Experiment 3 (Aim 3) evaluates whether activation of CRF receptors in the VTA during social defeat stress mediates previously reported dopaminergic cross-sensitization to cocaine (Experiment 3A) as well as escalated cocaine self-administration during a 24 h "binge" (Experiment 3B). To meet this objective, rats were microinjected with vehicle (aCSF),

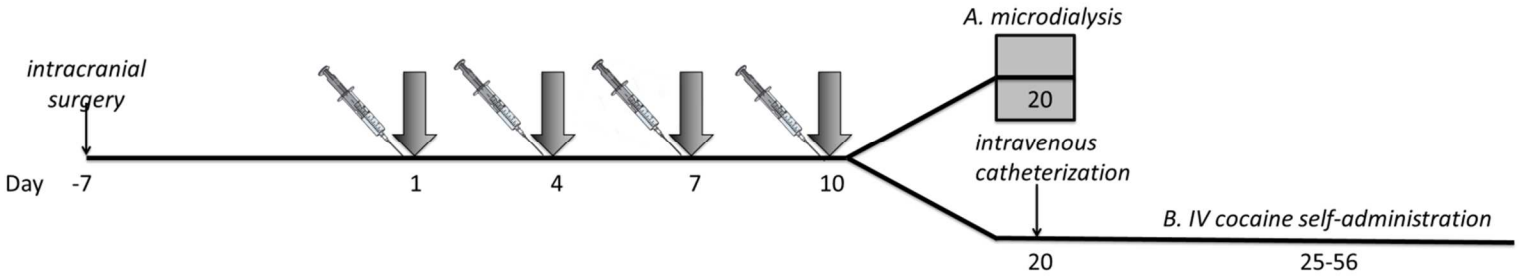
CRFR1 antagonist (CP376395, 50 or 500 ng/side), or CRFR2 antagonist (Astressin2B, 100 or 1000 ng/side) 10 min prior to the instigation phase of each defeat. The higher dose of each drug was chosen based on previous *in vivo* work (Blacktop et al., 2011; Henry, Vale, & Markou, 2006), with lower doses chosen to examine dose-responsivity. Ten days later, rats were either tested for dopaminergic cross-sensitization to cocaine by *in vivo* microdialysis of dopamine in the NAcSh in response to acute cocaine (Experiment 3A) or implanted with an indwelling catheter in the right jugular vein for subsequent intravenous cocaine self-administration, where they were ultimately exposed to a 24 h “binge” (Experiment 3B, Figure 10).

Intracranial surgery

After one week of habituation to the vivarium, 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 the midline at a depth of 7.5mm from the skull surface. In Experiment 3A, a unilateral microdialysis cannula (8 mm length, Synaptech Inc, Marquette, MI) was also implanted, aimed at the NAcSh (+2.1 mm from bregma, +1.1 mm from midline, -5.8 mm from dura, at 0° angle).

Figure 10. Timeline for Experiment 3.

Rats were microinjected (indicated by syringes) with vehicle (aCSF), CRF-R1 antagonist (CP376395, 500 or 50 ng/side), or CRF-R2 antagonist (Astressin2B, 1000 or 100 ng/side) 10 min prior to the instigation phase of social defeat stress (indicated by arrows) on days 1, 4, 7, and 10. Rats were then tested for dopaminergic cross-sensitization to cocaine by *in vivo* microdialysis on day 20 (Experiment 3A), or cocaine self-administration behavior, culminating in a 24 hour “binge” (Experiment 3B).


A. microdialysis
intracranial surgery
20
intravenous catheterization
Day -7
1
4
7
10
B. IV cocaine self-administration
20
25-56

Experiment 3A: In vivo microdialysis

On the night before the microdialysis experiment, the microdialysis guide cannula was removed and replaced with a microdialysis probe (2 mm active membrane, Synaptech Inc, Marquette, MI), which was perfused overnight with aCSF at a flow rate of 0.5 µl/min. The flow rate was increased to 1.5 µl/min 30 min prior to sample collection the next day. Samples were collected every 10 min by an automated refrigerated fraction collector (CMA 142, CMA Microdialysis, Chelmsford, MA) into collection vials containing 5 µl antioxidant (20 mM phosphate buffer containing 25 mM EDTA-2-Na and 0.5 mM ascorbic acid, pH 3.5) to prevent oxidation and degradation of dopamine. Tonic levels of dopamine were assessed in five baseline samples, followed by i.p. injections of saline (at 55 min) and cocaine (10 mg/kg, at 75 min). Samples were collected for an additional 115 min after cocaine injection to assess the time course of dopaminergic changes. Samples were analyzed for dopamine by HPLC as described in Experiment 1.

Experiment 3B: Intravenous cocaine self-administration culminating in 24 h "binge"

Intravenous catheterization. A separate group of rats were implanted with a catheter (SILASTIC silicon tubing, ID 0.63 mm, OD 1.17 mm, Dow Corning, Midland, MI) in the right jugular vein under ketamine (100 mg/kg, ip) and xylazine (6 mg/kg, ip) anesthesia. The catheter was subcutaneously passed over the shoulder, where it exited from a small

incision at the base of the neck and was affixed to a pedestal mounted inside a harness (SAI Infusion Technologies, Lake Villa, IL). Rats were then allowed to recover for at least 5 d prior to being moved from their home cage to permanent housing in intravenous cocaine self-administration chambers. To ensure catheter patency, catheters were flushed daily with 0.2 ml saline and 0.2 ml heparinized saline (20 IU/ml) and 0.17 ml pulses of saline were delivered every 30 min when self-administration sessions were not running. If patency was questioned, propofol (10 mg/ml) was injected to test the catheter.

Acquisition/Maintenance. Rats were allowed to freely self-administer cocaine (0.75 mg/kg/infusion) without priming or autoshaping during daily self-administration sessions, which were terminated after 15 infusions or 5 h access. Sessions were signaled by a stimulus light, and two retractable levers were extended from one wall of the home cage. Pressing the left active lever resulted in cocaine infusion paired with a cue light, followed by a 30 second time out, during which stimulus and cue lights were off. During the time out, pressing the right inactive lever was recorded, but neither punished nor reinforced. Rats were initially trained on a fixed ratio (FR) 1 schedule of reinforcement, where every lever press resulted in a cocaine infusion. After two consecutive days of 15 infusions at FR1, the schedule was gradually increased to FR5, where every fifth lever press resulted in a cocaine infusion. If rats did not meet the acquisition criteria of 15 infusions within the first 2 d of cocaine self-

administration, they were shaped with female urine or Fruit Loops on the active lever. Due to this shaping procedure, differences in acquisition across treatment groups could not be assessed. Rats were maintained on the FR5 schedule of reinforcement for 5 days to ensure stable and consistent responding across all groups. Sessions then alternated between FR5 maintenance sessions and progressive ratio sessions for 6 days (3 sessions each).

Progressive ratio. During the progressive ratio (PR) sessions, rats must respond with an increasing number of lever presses to attain an infusion of cocaine (0.3 mg/kg/infusion). The PR schedule of reinforcement, as adapted from Richardson and Roberts (1996), was as follows: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178. Sessions were terminated after 60 min without cocaine infusion, and the dependent variable was the “break point”, or total number of lever presses before the rat ceased responding.

24 h “binge”. After the last progressive ratio session, rats were given one more day of FR maintenance, and the following day given unlimited access to cocaine (0.3 mg/kg/infusion) on an FR5 schedule for 24 h. Total infusions and “binge” duration (time of last infusion) served as dependent variables. After completion of the “binge”, catheter patency was checked by injection of propofol.

Experiment 4: Role of VTA CRF and its receptors after stress on context-induced reinstatement after forced abstinence

Objective and experimental design

Experiment 4 (Aim 3) investigates the role of VTA CRF and its receptors in context-induced reinstatement after forced abstinence. Compared to non-stressed control rats, previously stressed rats show greater context-induced reinstatement following forced abstinence, a translational model of cocaine seeking and relapse. This experiment first investigates whether extracellular CRF is differentially increased in the VTA during reinstatement in previously stressed as opposed to control rats by using *in vivo* microdialysis for CRF (Experiment 4A). Separately, the role of VTA CRF receptors in context-induced reinstatement was assessed by intra-VTA microinjection of aCSF, CRFR1 antagonist (CP376395, 500 ng/side) or CRFR2 antagonist (Astressin2B, 1000 ng/side) 20 min prior to reinstatement (Experiment 4B, Figure 11).

Intravenous cocaine self-administration

Rats underwent intermittent social defeat stress, followed by intravenous catheterization surgery, as described in Experiment 3B, on day 11. After 5 days recovery, rats began cocaine self-administration. As in Experiment 3B, sessions were signaled by a stimulus light and both levers extended. Pressing the left active lever resulted in intravenous infusion of cocaine (0.75 mg/kg/infusion) paired with a cue light and a 30 second time out, during which pressing the right inactive lever, while

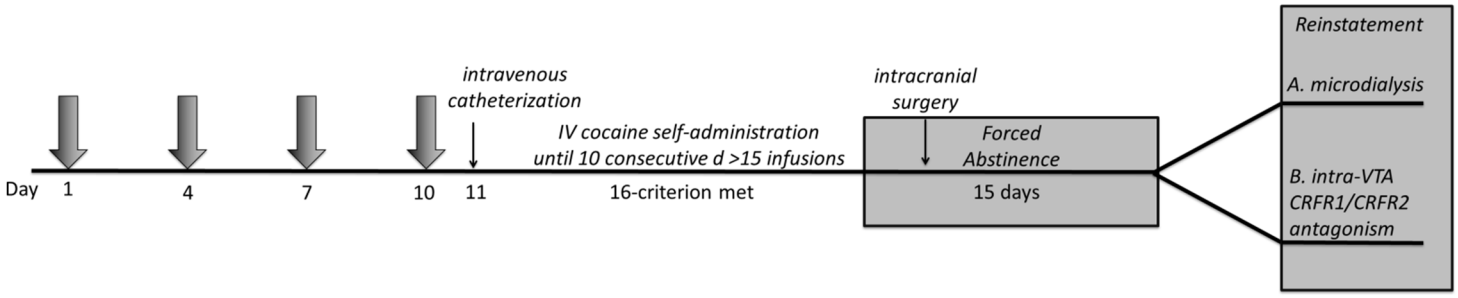
recorded, resulted in neither reinforcement nor punishment. Rats were initially trained on an FR1 schedule of reinforcement, and sessions terminated after 30 infusions or 3 hours. Behavioral shaping, consisting of placing female urine or Fruit Loops on the active lever, began on day 2 to accelerate and normalize acquisition between the two groups. After 2 consecutive days of greater than 15 infusions, the schedule of reinforcement was gradually increased to FR3, where the rats were maintained until they reached a criterion of a total of 10 consecutive days of greater than 15 infusions. After the criterion was met, rats were moved from the self-administration room into a separate vivarium room for 15±2 days of forced abstinence.

Intracranial surgery

Approximately 7 days into abstinence, rats underwent intracranial surgery under ketamine (100 mg/kg, ip) and xylazine (6 mg/kg, ip) anesthesia. Rats were implanted with either unilateral microdialysis guide cannulae (8 mm length, Synaptech Inc, Marquette, MI, Experiment 4A) or bilateral microinjection cannluae (23 ga, 11 mm length, PlasticsOne, Roanoke, VA, Experiment 4B) at a 10° angle 5.2 mm posterior from bregma and 1.8 mm lateral from the midline at a depth of 7.5 mm from the skull surface. Animals were allowed to recover for at least 7 days prior to context-induced reinstatement testing.

Figure 11. Timeline for Experiment 4.

Rats were intermittently defeated or handled on days 1, 4, 7, and 10 (indicated by arrows), after which they were catheterized for intravenous cocaine self-administration. Cocaine self-administration was conducted in daily sessions until rats reached the criterion of 10 consecutive days of greater than 15 infusions. After this criterion was met, rats were moved into a temporary housing room for 15 days of forced abstinence. Rats were then tested for context-induced reinstatement to cocaine seeking on the 15th day of abstinence, with either concurrent *in vivo* microdialysis for corticotropin releasing factor (CRF) in the ventral tegmental area (VTA, Experiment 4A), or intra-VTA antagonism of CRF-R1 or CRF-R2 (Experiment 4B).


Reinstatement
A. microdialysis
B. intra-VTA
CRFR1/CRFR2
antagonism
intravenous
catheterization
intracranial
surgery
IV cocaine self-administration
until 10 consecutive d >15 infusions
Forced
Abstinence
Day
1
4
7
10
11
16-criterion met
15 days

Context-Induced Reinstatement

For reinstatement, rats were brought from their temporary housing room to their previous cocaine self-administration chamber, which was not cleaned during abstinence to retain all odor contextual cues. When the 3 hour session began, the two levers were extended and the stimulus light illuminated, however pressing the active and inactive lever was recorded, but did not result in any reinforcement or previously paired cue light or timeout period.

Experiment 4A: In vivo microdialysis for CRF in the VTA during context-induced reinstatement

The night before reinstatement, rats were prepared for *in vivo* microdialysis in their temporary housing room. As with other microdialysis experiments, guide cannulae were removed and replaced with a microdialysis probe (2 mm active membrane, Synaptech Inc, Marquette, MI), which was perfused with aCSF at a flow rate of 0.5 µl/min overnight. On the morning of reinstatement, the aCSF is replaced with aCSF containing 0.2% BSA and the flow rate increased to 2.0 µl/min 2 h prior to sample collection. Samples were collected manually every 10 min into Protein LoBind Eppendorf tubes. After 5 baseline samples, rats were quickly brought to their self-administration chamber for context-induced reinstatement. Samples continued to be collected throughout the 3 hour reinstatement session.

Experiment 4B: Intra-VTA antagonism of CRFR1 and CRFR2 during context-induced reinstatement

A separate group of rats were microinjected in their temporary housing room with vehicle (aCSF), CRFR1 antagonist (CP376395, 500 ng/side), or CRFR2 antagonist (Astressin2B, 1000 ng/side) 20 min prior to reinstatement. Rats were then brought to their self-administration chamber for the context-induced reinstatement session as described above.

IV. RESULTS

Aim 1: Investigating the interactions of CRF and dopamine neurons in the VTA during acute stress

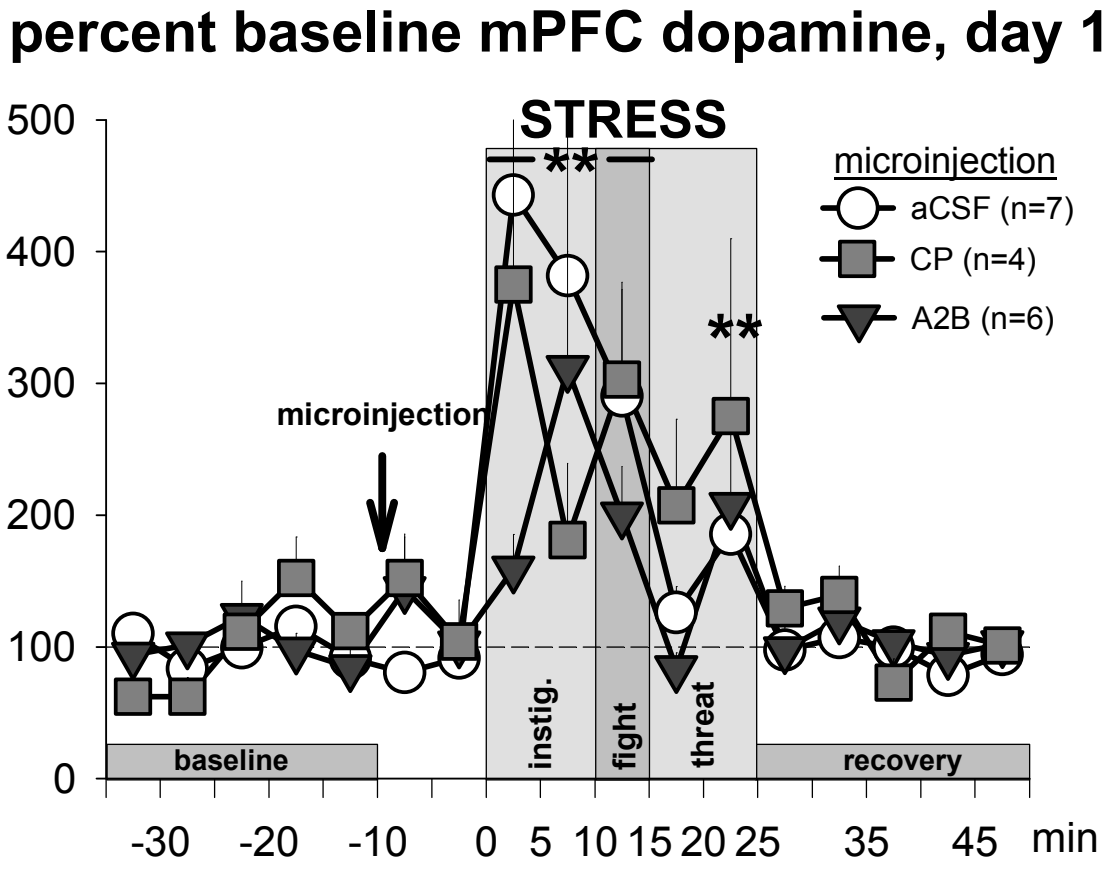
Acute stress phasically increases extracellular dopamine in both the mPFC and NAcSh.

During the first day of social defeat, extracellular dopamine in the mPFC was significantly increased above baseline (Figure 12). As baseline concentrations of dopamine were variable across the two years the study was performed due to varying HPLC column and cell conditions, all dopamine values were analyzed as percent change from average baseline. Additionally, there was no effect of aCSF injection, so all further analysis was performed comparing the last microinjection sample with the five samples during stress (two during instigation phase, one during fight phase, and two during threat phase).

Two-way repeated measures ANOVA revealed a significant effect of sample ($F_{5,70}$=7.031, $p<0.001$), with the aCSF pre-treated animals

Figure 12. Extracellular dopamine in the mPFC during acute social defeat.

On the first day of social defeat stress, dopamine in the medial prefrontal cortex (mPFC) is rapidly increased in animals pretreated with aCSF (white circles) into the ventral tegmental area (VTA). This effect is not attenuated by intra-VTA antagonism of CRF-R1 with 500ng CP376395 (CP, gray squares), or CRF-R2 with 1000ng Astressin2B (A2B, dark gray triangles). Data are represented as group means±SEM of percent change from individual baseline concentrations. **=$p<0.01$ vs. last sample before instigation.


percent baseline mPFC dopamine, day 1
STRESS
**
**
microinjection
aCSF (n=7)
CP (n=4)
A2B (n=6)
microinjection
500
400
300
200
100
0
baseline
instig.
fight
threat
recovery
-30
-20
-10
0
5
10
15
20
25
35
45
min

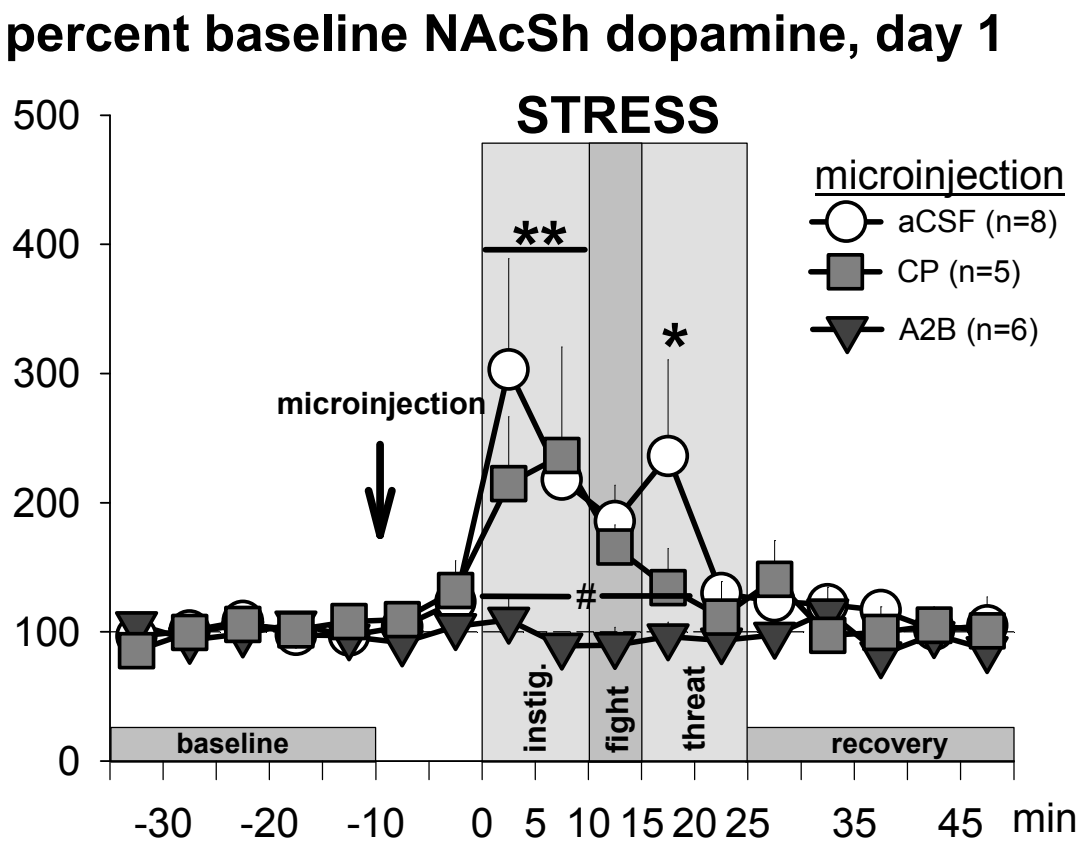
showing significant differences from baseline during all samples during stress with the exception of the first sample in the threat period ($p<0.008$).

Thus, in these naïve 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 during the fight. Upon termination of the physical defeat encounter and return to the home cage inside the resident's cage for 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.

Similarly, extracellular dopamine in the NAcSh was significantly elevated above baseline during the first day of social defeat (Figure 13). A two-way repeated measures ANOVA revealed a significant main effect of sample ($F_{5,80}=3.443$, $p=0.007$), with aCSF pre-treated animals showing 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. Thus, 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

Figure 13. Extracellular dopamine in the NAcSh during acute social defeat

On the first day of social defeat stress, dopamine in the nucleus accumbens shell (NAcSh) is rapidly increased in animals pretreated with aCSF (white circles) into the ventral tegmental area (VTA). This effect is not attenuated by intra-VTA antagonism of CRF-R1 with 500ng CP376395 (CP, gray squares), but is prevented with intra-VTA antagonism of CRF-R2 with 1000ng Astressin2B (A2B, dark gray triangles). Data are represented as group means±SEM of percent change from individual baseline concentrations. *=$p<0.05$, **=$p<0.01$ vs. last sample before instigation; #=$p<0.05$ vs. aCSF.


percent baseline NAcSh dopamine, day 1
STRESS
microinjection
aCSF (n=8)
CP (n=5)
A2B (n=6)
**
*
#
microinjection
500
400
300
200
100
0
instig.
fight
threat
baseline
recovery
-30
-20
-10
0
5
10
15
20
25
35
45
min

period, but while extracellular dopamine levels were still elevated during the fight period, it was no longer significant. In contrast with what was observed in the mPFC, extracellular dopamine in the NAcSh remained elevated during the initial half of the threat period following the fight, but statistically returned to baseline for the second half of the threat period, and remained at baseline for the duration of sampling during the recovery period.

CRF-R2 antagonism prevents the acute stress-induced dopamine efflux in the NAcSh, but not mPFC.

During the first defeat, there was no effect of either CRFR1 or CRFR2 antagonism in the VTA on the stress-induced dopamine increase in the mPFC (Figure 12). 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 five stress samples. Two-way repeated measures ANOVA revealed neither main effect of drug pretreatment nor pretreatment x sample interaction.

In contrast, the stress-induced dopamine increase in the NAcSh during the first defeat requires intact CRF-R2 within the VTA (Figure 13). 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 x 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 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.

Acute stress phasically increases extracellular CRF in the posterior, but not anterior, VTA

Regardless of probe placement within the VTA, baseline concentrations of CRF did not vary between groups on day 1 (mean±SEM for control-anterior VTA (aVTA)= 0.325±0.043 nM; control-posterior VTA (pVTA)= 0.293±0.012 nM; stress-aVTA=0.294±0.038 nM; stress-pVTA=0.216±0.063 nM).

Probe placement within the VTA had a significant effect on the stress-induced efflux of CRF on day 1, so rats were split based on placement within the anterior VTA (aVTA) and posterior VTA (pVTA) with -5.00 mm from bregma as the division line, based upon the emergence of the PBP and PN regions of the VTA (Ikemoto, 2007). Two-way repeated measures ANOVA on nM concentrations of CRF revealed a non-significant trend to an interaction between probe placement and sample ($F_{7,49}$=2.104, p=0.061), while similar analysis of the percent baseline CRF revealed both a significant interaction between probe placement and sample ($F_{7,49}$=3.363, p=0.005), as well as main effect of sample ($F_{7,49}$=2.585, p=0.024). As there were no significant main effects or

interactions in the nM concentration data, likely due to variability from multiple EIA runs, post hoc analyses were conducted on the percent change from baseline data, which was considerably less variable.

While there was no effect of stress on CRF in the aVTA, CRF was significantly elevated above baseline in the pVTA during the first stress sample and first two recovery samples, with levels significantly above those of the aVTA during the first stress ($p=0.003$) and second recovery ($p=0.019$), but not first recovery ($p=0.068$), samples (Figure 14). Analysis of area under the curve revealed a non-significant trend for greater CRF efflux in the pVTA compared to the aVTA (Student's $t=1.728$, $p=0.06$, inset of Figure 14).

Aim 2: Investigating the interactions of CRF and dopamine neurons in the VTA during repeated stress

Intra-VTA antagonism of CRF-R2, but not CRF-R1, prevents the repeated stress-induced extracellular dopamine increase in both 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 no habituation or sensitization with repeated intermittent social defeat experience (Figures 15 and 16 for mPFC and NAcSh, respectively). Additionally, the time course of changes in extracellular dopamine in both regions on the last day was closely similar to that of the first day. In the mPFC, extracellular dopamine was significantly elevated above baseline in both samples during the

Figure 14. Extracellular CRF in the VTA during acute social defeat.

On the first day of social defeat, corticotropin releasing factor (CRF) is phasically increased in the posterior portion of the ventral tegmental area (pVTA, dark gray), while unchanged in the anterior VTA (aVTA, white). Data are represented as group means±SEM of percent change from individual baseline CRF concentration across 12.5 min samples. Inset depicts area under the curve (AUC) after the initiation of social defeat in the aVTA (white) and pVTA (dark gray). **=p<0.01 vs. last sample before defeat; ##=p>0.01 vs. pVTA.

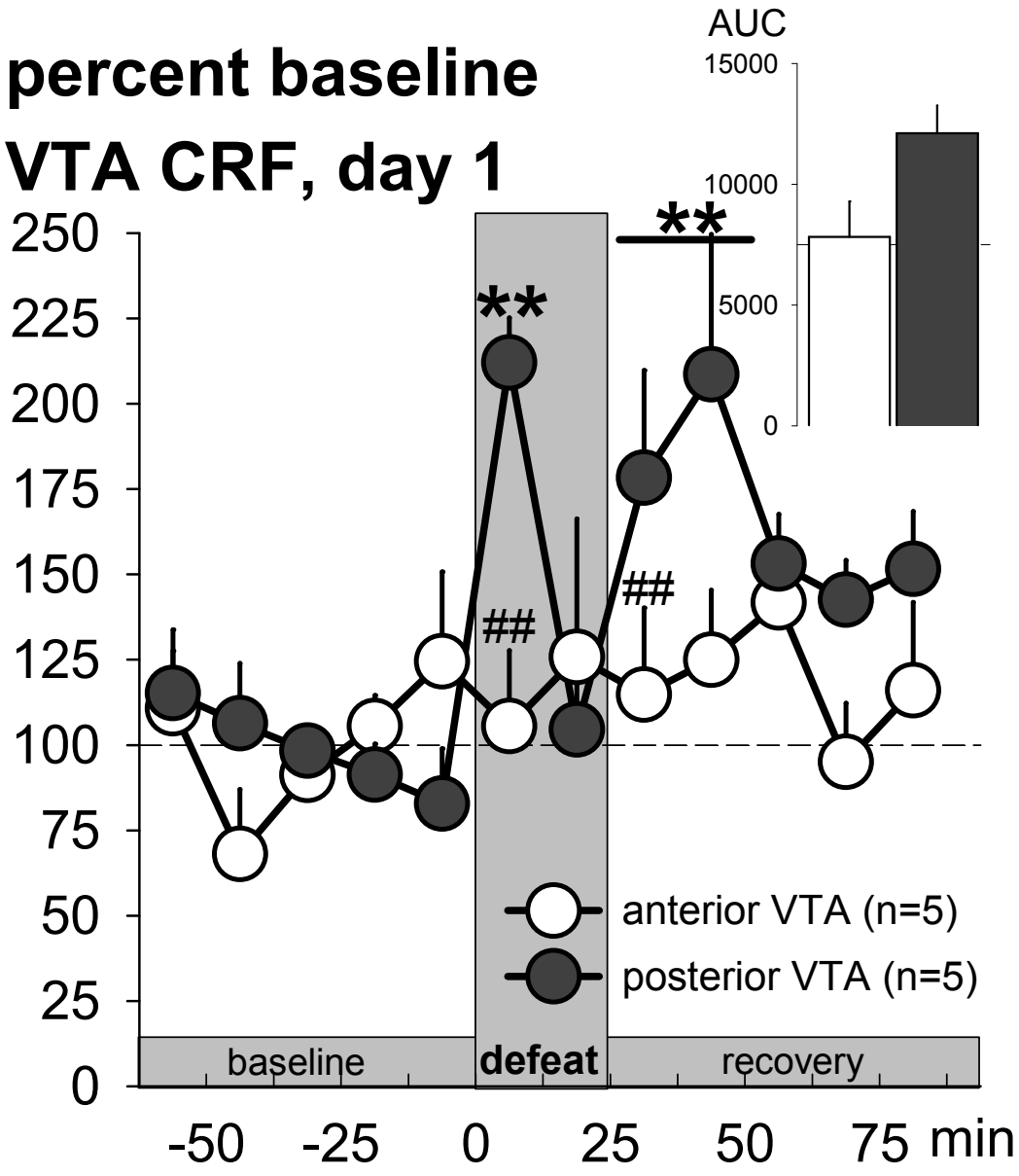

percent baseline
VTA CRF, day 1
AUC
15000
10000
5000
0
250
225
200
175
150
125
100
75
50
25
0
**
**
##
##
anterior VTA (n=5)
posterior VTA (n=5)
baseline
defeat
recovery
-50
-25
0
25
50
75
min

Figure 15. Extracellular dopamine in the mPFC during acute and repeated social defeat in vehicle controls.

A similar time course and magnitude of increased extracellular dopamine in the medial prefrontal cortex (mPFC) is observed during acute (D1, white circles) and repeated (D10, dark gray triangles) social defeat within the aCSF-pretreated group. Data are represented as group means±SEM of percent change from individual baseline concentrations. **=$p<0.01$ vs last sample before instigation phase.

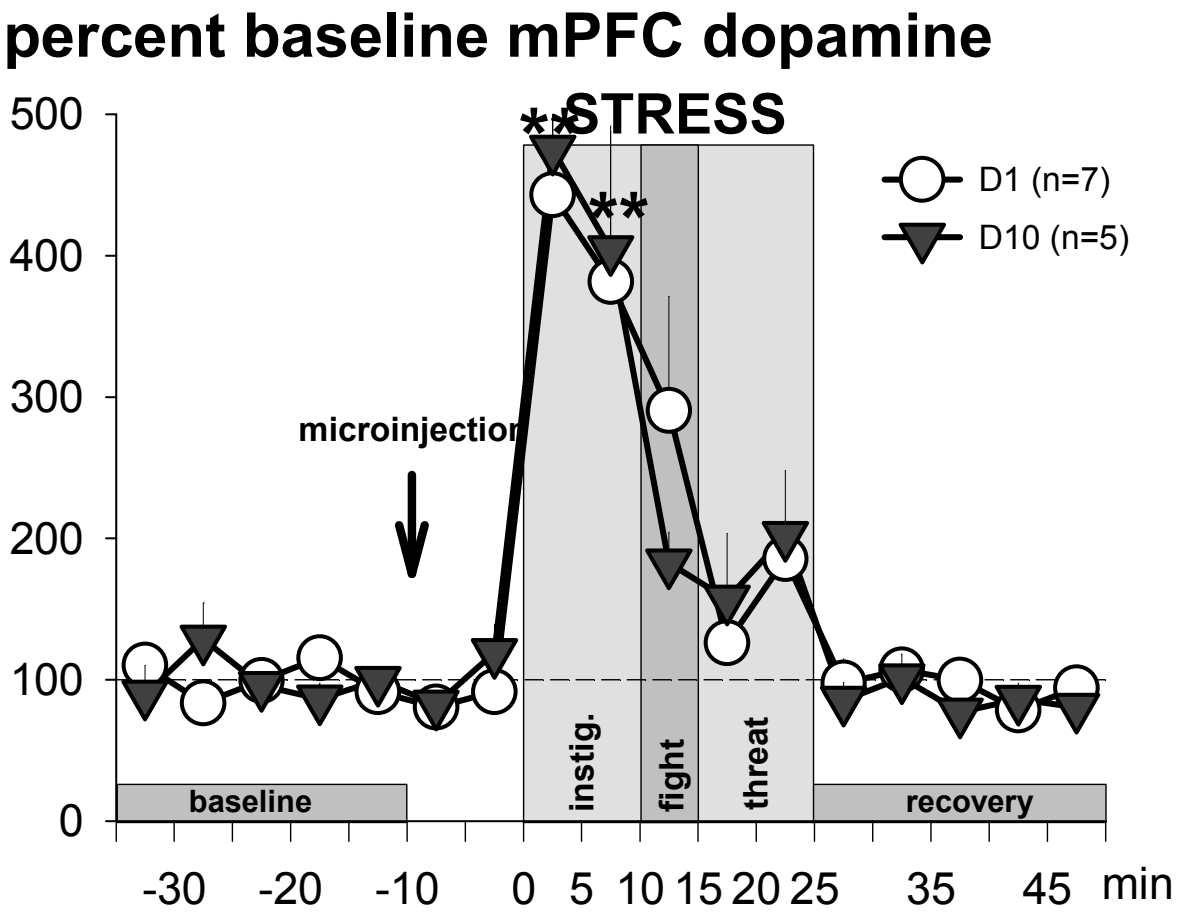
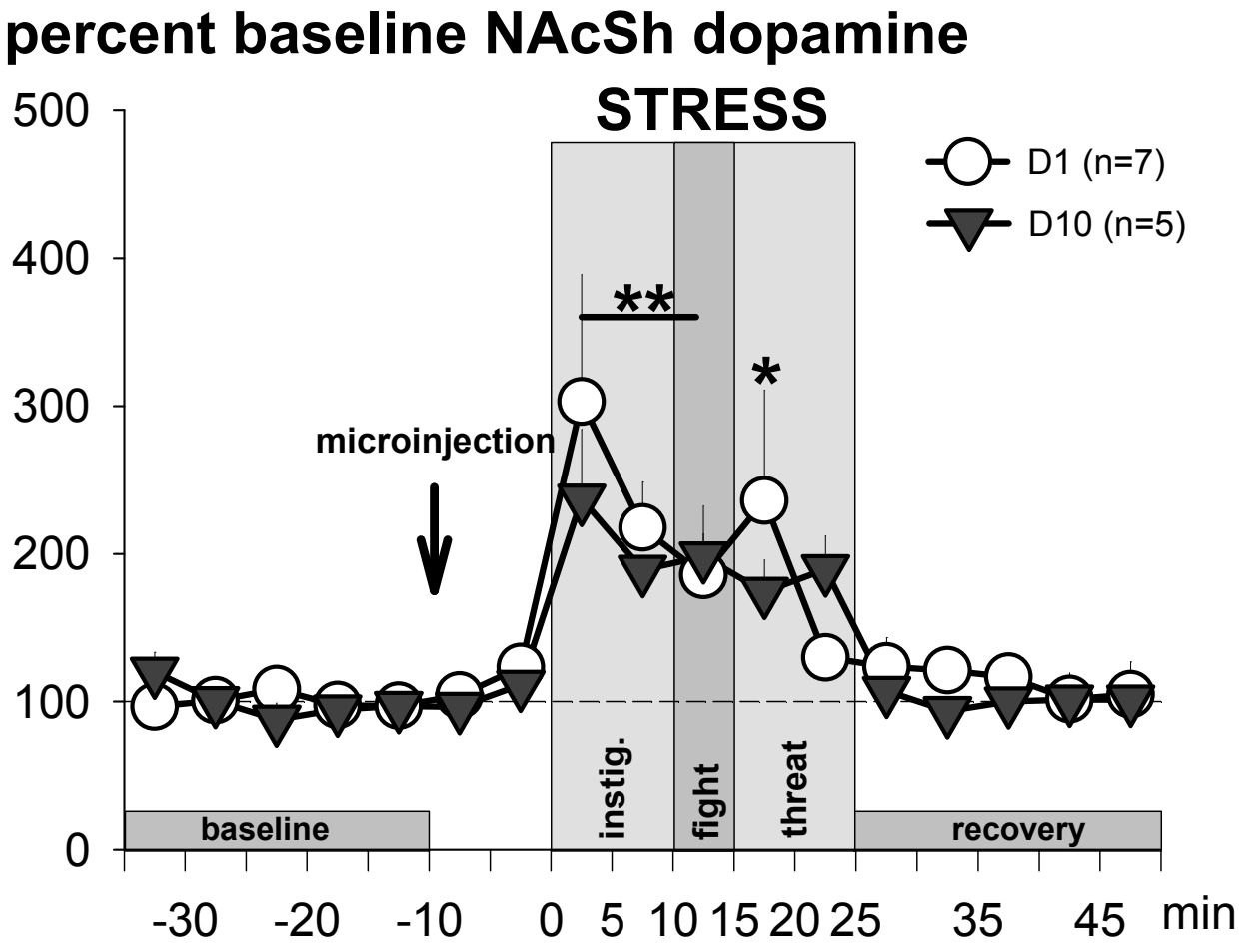

percent baseline mPFC dopamine
**STRESS
**
**
D1 (n=7)
D10 (n=5)
microinjection
500
400
300
200
100
0
baseline
instig.
fight
threat
recovery
-30
-20
-10
0
5
10
15
20
25
35
45
min

Figure 16. Extracellular dopamine in the NAcSh during acute and repeated social defeat in vehicle controls.

A similar time course and magnitude of increased extracellular dopamine in the nucleus accumbens shell (NAcSh) is observed during acute (D1, white circles) and repeated (D10, dark gray triangles) social defeat within the aCSF-pretreated group. Data are represented as group means±SEM of percent change from individual baseline concentrations. *=$p<0.05$, **=$p<0.01$ vs last sample before instigation phase.


percent baseline NAcSh dopamine
STRESS
D1 (n=7)
D10 (n=5)
500
400
300
200
100
0
**
*
microinjection
instig.
fight
threat
baseline
recovery
-30
-20
-10
0
5
10
15
20
25
35
45
min

instigation period ($p<0.010$), but now, 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 threat was no longer statistically significant. The time course of change from baseline in extracellular dopamine in the NAcSh on day 10 was statistically identical to that of day 1.

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 (Figure 17). 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 were significantly lower than aCSF pretreated controls (Holm-Sidak $t=2.888$, $p=0.025$), and dopamine levels did not significantly deviate from baseline.

Similarly, intra-VTA antagonism of CRF-R2, but not CRF-R1, was able to prevent the stress-induced dopamine increase in the NAcSh during stress (Figure 18). There were significant main effects of sample (two-way

Figure 17. Extracellular dopamine in the mPFC during repeated social defeat

On the last day of social defeat stress, dopamine in the medial prefrontal cortex (mPFC) is rapidly increased in animals pretreated with aCSF (white circles) into the ventral tegmental area (VTA). This effect is not attenuated by intra-VTA antagonism of CRF-R1 with 500ng CP376395 (CP, gray squares), but is prevented with pretreatment of CRF-R2 with 1000ng Astressin2B (A2B, dark gray triangles) prior to each defeat. Data are represented as group means±SEM of percent change from individual baseline concentrations. **=$p<0.01$ vs. last sample before instigation; #=$p<0.05$ vs. aCSF.

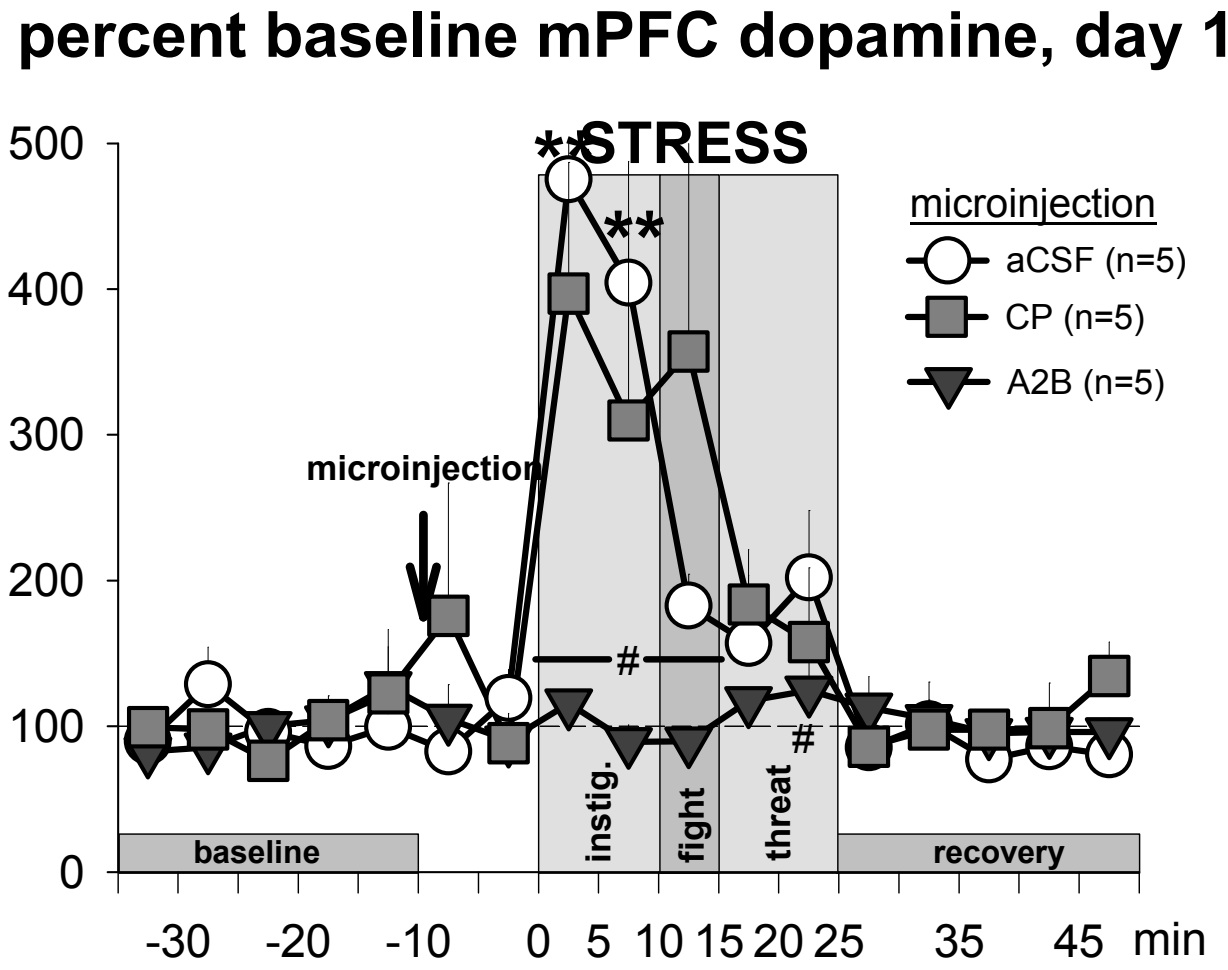
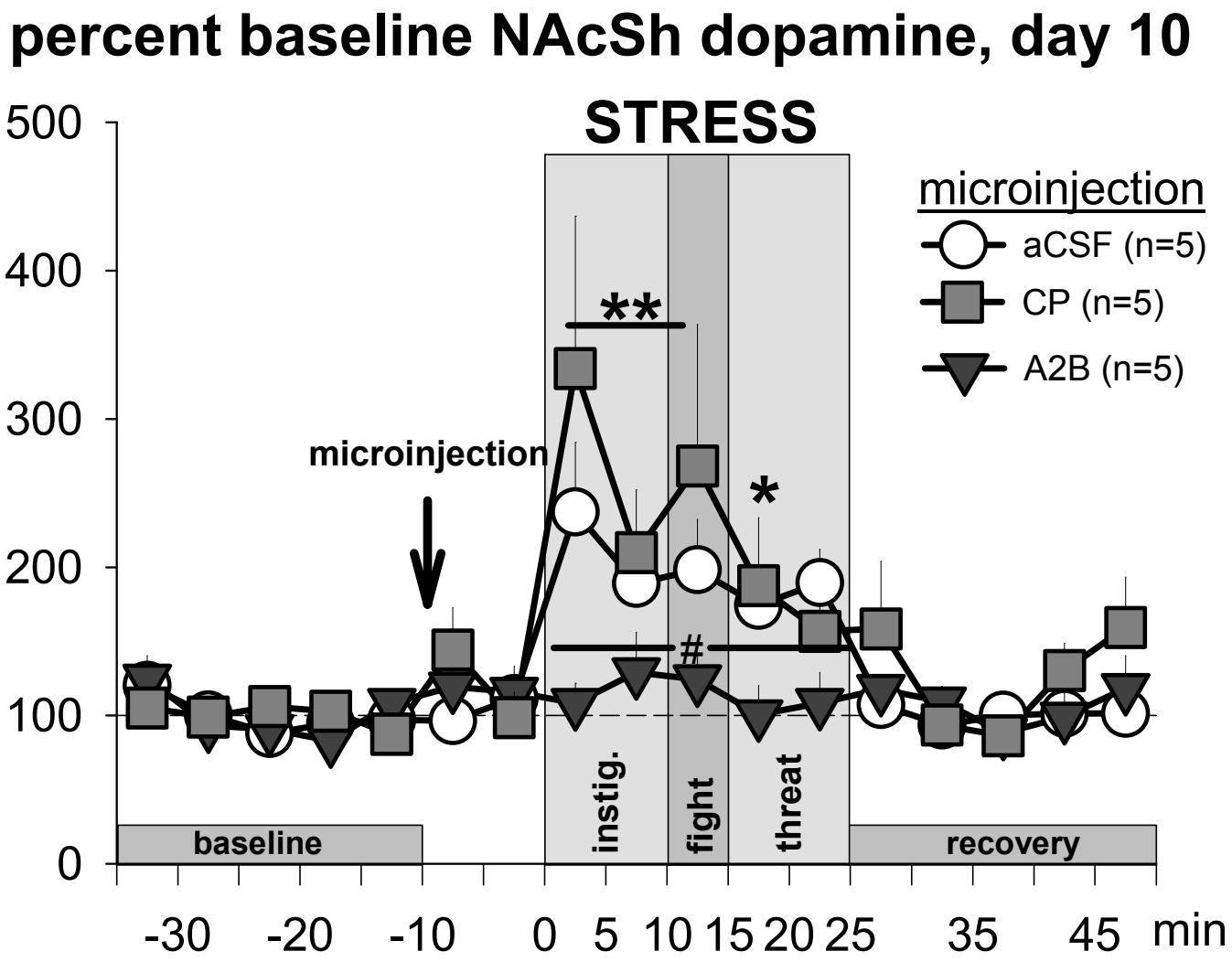

percent baseline mPFC dopamine, day 10
**STRESS
**
microinjection
aCSF (n=5)
CP (n=5)
A2B (n=5)
#
#
microinjection
baseline
instig.
fight
threat
recovery
500
400
300
200
100
0
-30
-20
-10
0
5
10
15
20
25
35
45
min

Figure 18. Extracellular dopamine in the NAcSh during repeated social defeat

On the last day of social defeat stress, dopamine in the nucleus accumbens shell (NAcSh) is rapidly increased in animals pretreated with aCSF (white circles) into the ventral tegmental area (VTA). This effect is not attenuated by intra-VTA antagonism of CRF-R1 with 500ng CP376395 (CP, gray squares), but is prevented with pretreatment of CRF-R2 with 1000ng Astressin2B (A2B, dark gray triangles) prior to each defeat. Data are represented as group means±SEM of percent change from individual baseline concentrations. **=$p<0.01$ vs. last sample before instigation; #=$p<0.05$ vs. aCSF.


percent baseline NAcSh dopamine, day 10
STRESS
microinjection
aCSF (n=5)
CP (n=5)
A2B (n=5)
microinjection
**
*
#
baseline
instig.
fight
threat
recovery
500
400
300
200
100
0
-30
-20
-10
0
5
10
15
20
25
35
45
min

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 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.

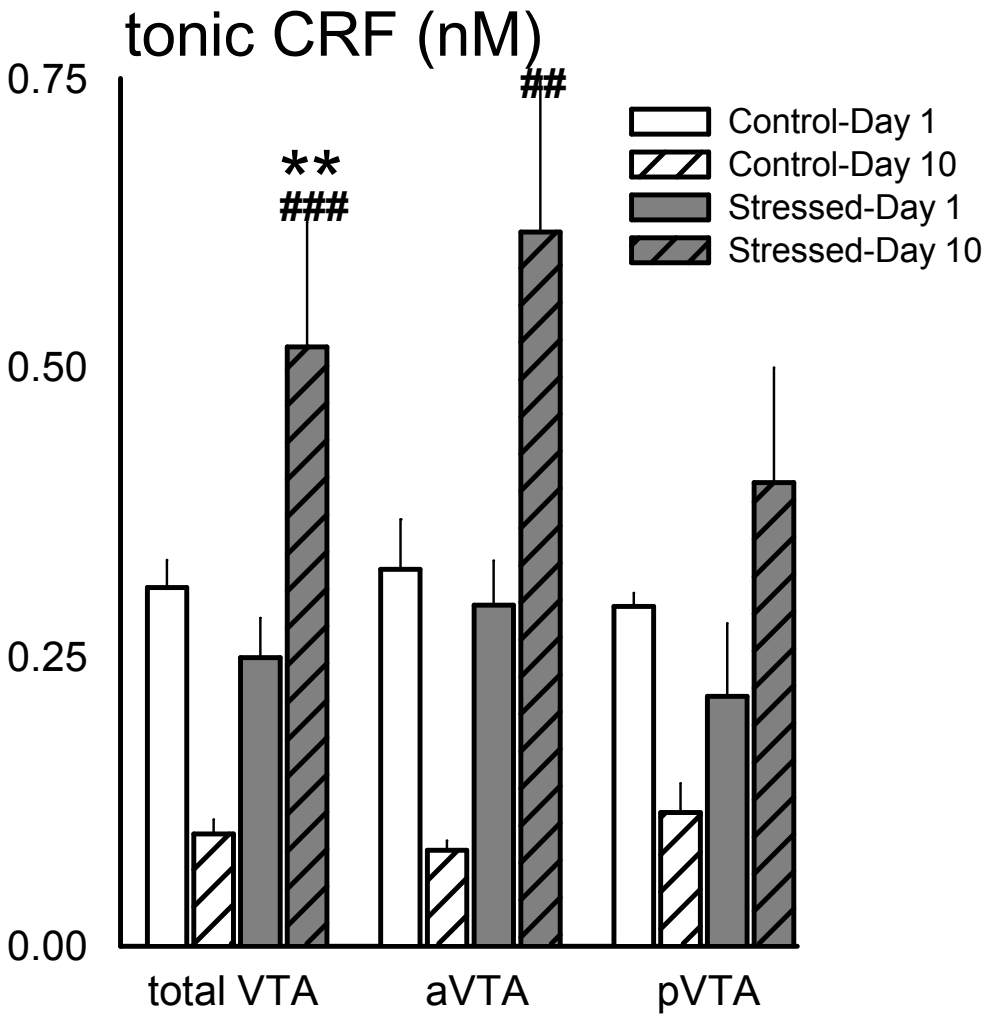
Repeated stress increases tonic CRF in the VTA

On the last day of intermittent social defeat stress, tonic levels of CRF were increased in stressed rats regardless of probe placement within the VTA, while they were decreased within non-stressed controls (Figure 19; mean±SEM nM concentration control-aVTA= 0.083±0.008 nM; control-pVTA= 0.115±0.025 nM; stressed-aVTA=0.617±0.192 nM; stressed-pVTA=0.321±0.126 nM).

Two way repeated measures ANOVA of nM baseline concentrations revealed a significant main effect of stress ($F_{1,15}$=4.906, p=0.043) and an interaction of stress condition and day ($F_{1,15}$=13.247, p=0.002), but no main effect of day. Within the stressed group, there was a significant increase in tonic CRF from day 1 to day 10 (Holm-Sidak t=3.182, p=0.006), but with the current sample size the decrease in tonic CRF observed within non-stressed controls did not reach statistical significance (Holm-Sidak t=2.083, p=0.055). There was, however, a significant effect of stress group on day 10, with tonic CRF levels

Figure 19. Tonic extracellular CRF in the VTA

Mean±SEM nanomolar (nM) corticotropin releasing factor (CRF) concentrations in the ventral tegmental area (VTA) for five baseline samples are shown for non-stressed control (white) and stressed (gray) animals on days 1 (solid) and 10 (striped) within the total VTA (left), anterior VTA (aVTA, middle), and posterior VTA (pVTA, right). **=$p<0.01$ vs. day 1; ##=$p<0.01$, ###=$p<0.001$ vs control.


tonic CRF (nM)
0.75
0.50
0.25
0.00
**
###
##
Control-Day 1
Control-Day 10
Stressed-Day 1
Stressed-Day 10
total VTA
aVTA
pVTA

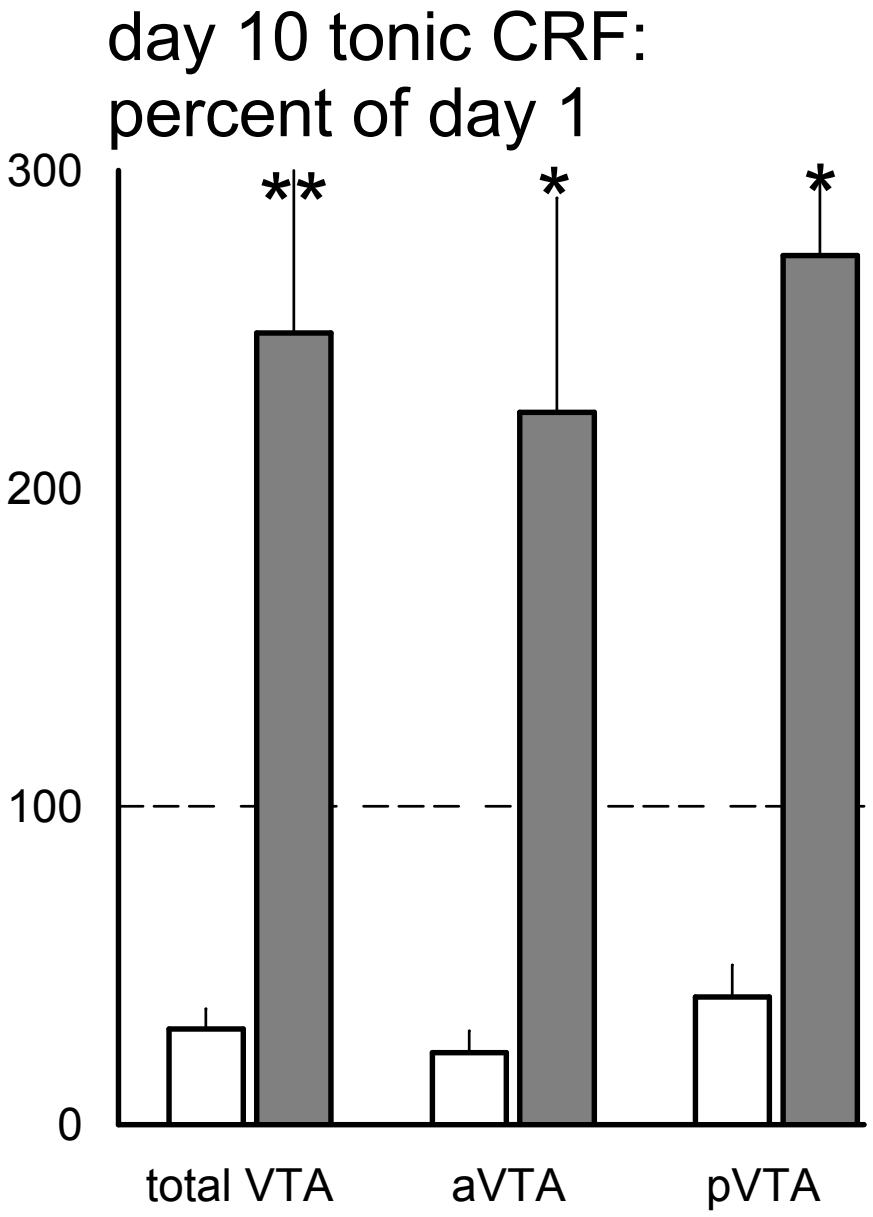
substantially greater within stressed animals than those of non-stressed controls (Holm Sidak t=3.617, p<0.001).

Separate analysis of VTA subregions revealed the same statistical effects and trends. Within the anterior VTA, there was a significant interaction between stress group and day ($F_{1,7}$=6.825, p=0.035), a nonsignificant trend towards an overall effect of group ($F_{1,7}$=4.661, p=0.068), and no overall effect of day. Due to the low sample size, the only significant post hoc comparison was a significantly elevated CRF tone in stressed compared to non-stressed rats on day 10 (Holm-Sidak t=3.357, p=0.005). Low sample size within the posterior VTA group resulted in no significant main effects or interactions.

Due to variability in baseline CRF concentration, CRF concentration on day 10 was also analyzed as a percentage of day 1 concentration within each rat. In this manner, the change in CRF concentration from day 1 to day 10 was readily apparent, with stressed rats exhibiting an increase in CRF tone compared to day 1 and non-stressed rats a decrease (Figure 20). Two way ANOVA revealed a significant main effect of stress ($F_{1,13}$=9.451, p=0.009) with no effects of VTA subregion or stress x subregion interaction. There was a significant effect of stress group within the posterior VTA (Holm-Sidak t=2.424, p=0.031), but the effect of stress failed to reach significance within the anterior VTA group (Holm-Sidak t=1.905, p=0,079), likely due to low sample size and greater variability.

Figure 20. Tonic extracellular CRF in the VTA on day 10 expressed as percentage of day 1.

Tonic concentrations of corticotropin releasing factor (CRF) in the ventral tegmental area (VTA) are increased from day 1 to day 10 within the stressed group (gray), while decreased in the non-stressed group (white), regardless of probe placement in the anterior VTA (aVTA, middle) or posterior VTA (pVTA, right). Data are expressed as the mean±SEM for each group of ratio of the average baseline concentration of CRF on day 10 to day 1 of individual rats, multiplied by 100. *=$p<0.05$, **=$p<0.01$ vs non-stressed controls.


day 10 tonic CRF:
percent of day 1
300
200
100
0
**
*
*
total VTA
aVTA
pVTA

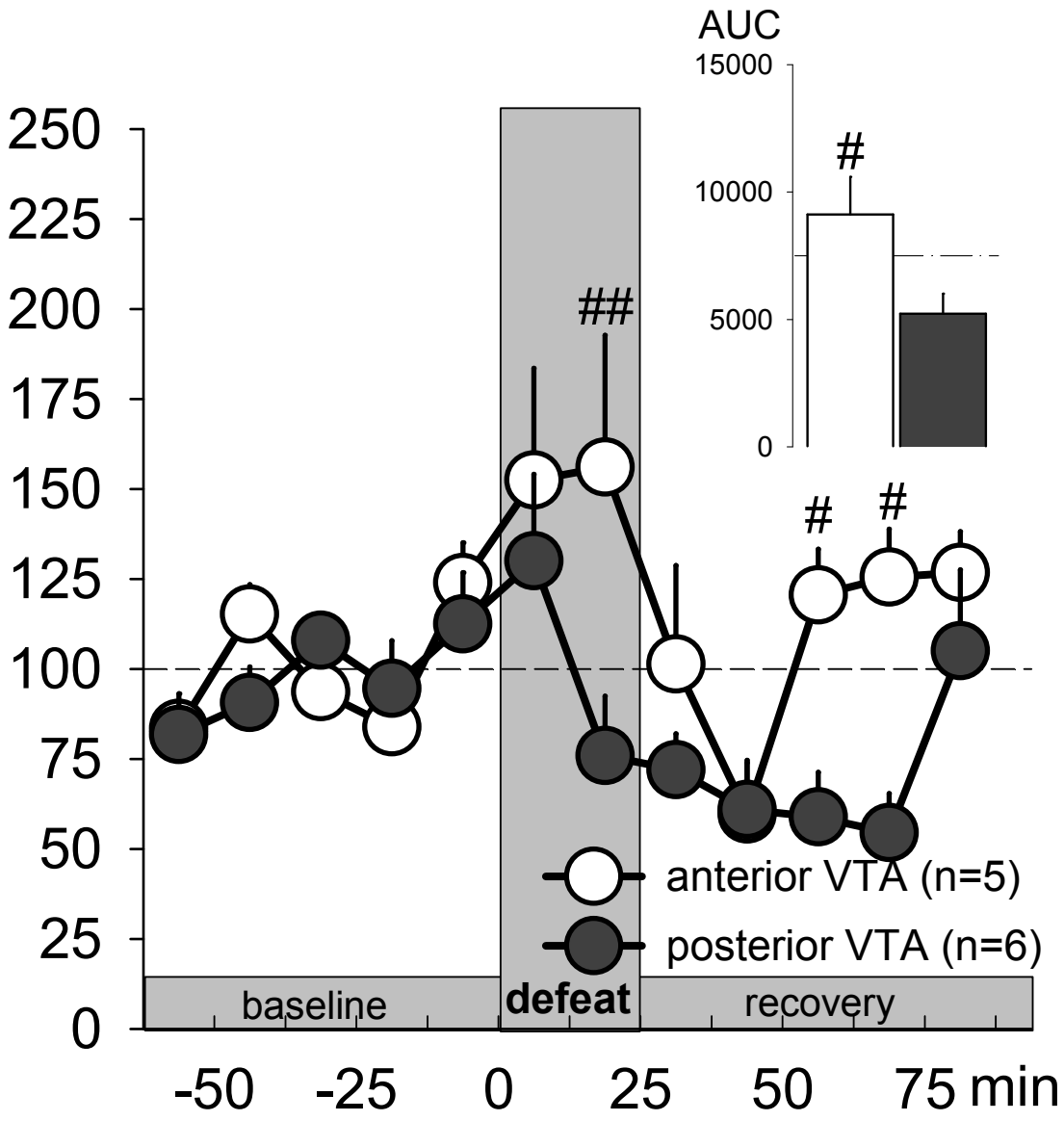
Repeated stress phasically increases extracellular CRF in the anterior, but not posterior, VTA

After exposure to repeated stress, extracellular CRF is altered within both the anterior and posterior VTA during the last defeat. During the final defeat, CRF is slightly elevated above baseline in both the anterior and posterior during the first half of social defeat stress, after which they diverge (Figure 21). Within the anterior VTA, CRF remains elevated, after which it returns to baseline. However, within the posterior VTA, CRF becomes suppressed below baseline. Two way repeated measures ANOVA revealed main effects of VTA subregion ($F_{1,7}$=5.752, p=0.048) as well as sample ($F_{7,49}$=3.764, p=0.002), but no interaction.

The pattern of extracellular increase in CRF during defeat during repeated stress reflects a shift from the pattern during acute stress. There was a significant main effect of sample ($F_{7,49}$=2.419, p=0.033), as well as an interaction between defeat day and sample ($F_{7,49}$=3.116, p=0.008). Overall the initial response to stress did not vary from day 1 to day 10, but there was a significant difference between day 1 and 10 during the second and third samples of the recovery period (sample 2: Holm-Sidak t=3.609, p=0.002; sample 3: Holm-Sidak t=2.151, p=0.044). Within the anterior VTA in particular, there was an interaction between sample and day ($F_{7,21}$=2.781, p=0.033). Within the posterior VTA, however, there was a main effect of day ($F_{1,3}$=16.385, p=0.027) and sample ($F_{7,21}$=7.033, p<0.001), with significant differences between day 1 and day 10 observed during the second half of social defeat stress (Holm-Sidak t=2.898,

Figure 21. Extracellular CRF in the VTA during repeated social defeat.

On the final day of social defeat, corticotropin releasing factor (CRF) is increased in the anterior (white) ventral tegmental area (VTA), while it is initially increased, then suppressed below baseline, within the posterior (dark gray) VTA. Data are represented as group means±SEM of percent change from individual baseline CRF concentration across 12.5 min samples. Inset depicts area under the curve (AUC) after the initiation of social defeat in the anterior (white) and posterior (dark gray) VTA. #=$p<0.05$, ##=$p<0.01$ vs. posterior VTA.


AUC
15000
10000
5000
0
#
250
225
200
175
150
125
100
75
50
25
0
##
# #
anterior VTA (n=5)
posterior VTA (n=6)
baseline
defeat
recovery
-50 -25 0 25 50 75 min

p=0.009), and the first four samples within the recovery period (sample 1: Holm-Sidak t=2.374, p=0.028; sample 2: Holm-Sidak t=3.957, p<0.001; sample 3: Holm-Sidak t=2.987, p=0.007; sample 4: Holm-Sidak t=2.640, p=0.016).

Aim 3: Investigating long-term effects of CRF-dopamine interactions in the VTA during repeated stress on cocaine taking and seeking

Intra-VTA antagonism of CRF-R1 or CRF-R2 during repeated stress prevents the induction of dopaminergic cross-sensitization to cocaine.

Baseline levels of dopamine did not differ across stress or treatment groups (mean±SEM pmol control-aCSF=3.416±1.436 pmol; control-500 ng/side CP376395 (CP)=4.384±1.756 pmol; control-1000ng/side Astressin2B (A2B)=1.548±0.242; stressed-aCSF=1.773±0.385; stressed-50 ng/side CP=1.054±0.148; stressed-500 ng/side CP=3.263±0.845; stressed-100 ng/side A2B=2.198±0.913; stressed-1000 ng/side A2B=2.198±0.913).

Three-way repeated measures ANOVAs revealed no significant difference between neither the five baseline samples and the post-saline injection sample, nor between baseline and all samples collected after 65 min post-cocaine injection. Therefore, only samples from the saline injection to 65 min after the cocaine injection were used in further analysis.

Intermittent social defeat stress induced dopaminergic cross-sensitization to cocaine in aCSF pretreated animals and was prevented by

both CRF-R1 (Figure 22) and CRF-R2 (Figure 23) antagonism in the VTA prior to each defeat. A three-way repeated measures ANOVA revealed significant main effects of drug pretreatment ($F_{4,33}$=6.27, p<0.001) and sample ($F_{7,231}$=23.74, p<0.0001). Additionally, significant interactions were observed between drug pretreatment and stress condition ($F_{2,33}$=3.78, p=0.0333) and between drug pretreatment and sample ($F_{28,231}$=4.51, p<0.0001), although no three way interaction between drug pretreatment, stress condition, and sample was observed. Additionally, all animals with accurate microdialysis probe placements in the NAcSh also had correct intra-VTA microinjection cannulae placements, so no analysis of anatomical controls was possible.

To examine the effects of cocaine within treatment groups, *a priori* driven post hoc two-way repeated measures ANOVAs with Holm-Sidak corrections for multiple comparisons were performed. Non-stressed rats previously pretreated with aCSF and A2B showed significantly elevated extracellular dopamine in the NAcSh 25-55 min after cocaine injection (p<0.05), while those pretreated with CP showed no significant change from baseline. Within stressed animals, dopamine was significantly increased from baseline within both the aCSF and CP pretreated groups, although the time course varied. Stressed rats pretreated with aCSF exhibited a prolonged elevation in NAcSh dopamine (25-55 min after injection, p<0.001), while those pretreated with CP showed more transient elevations (50 ng/side CP: 25-35 min, p<0.05; 500 ng/side CP 35 min,

Figure 22. Effect of intra-VTA CRF-R1 antagonism on the induction of neural sensitization

Dopamine (DA) in the nucleus accumbens shell (NAcSh) in response to saline and cocaine (10 mg/kg, ip) in rats pretreated with the CRF-R1 antagonist CP-376395 (CP, 50 or 500 ng) before social defeat stress or handling. Data are represented as group mean±SEM of the percent change from individual baseline. *=$p<0.05$ vs. non-stressed aCSF group.

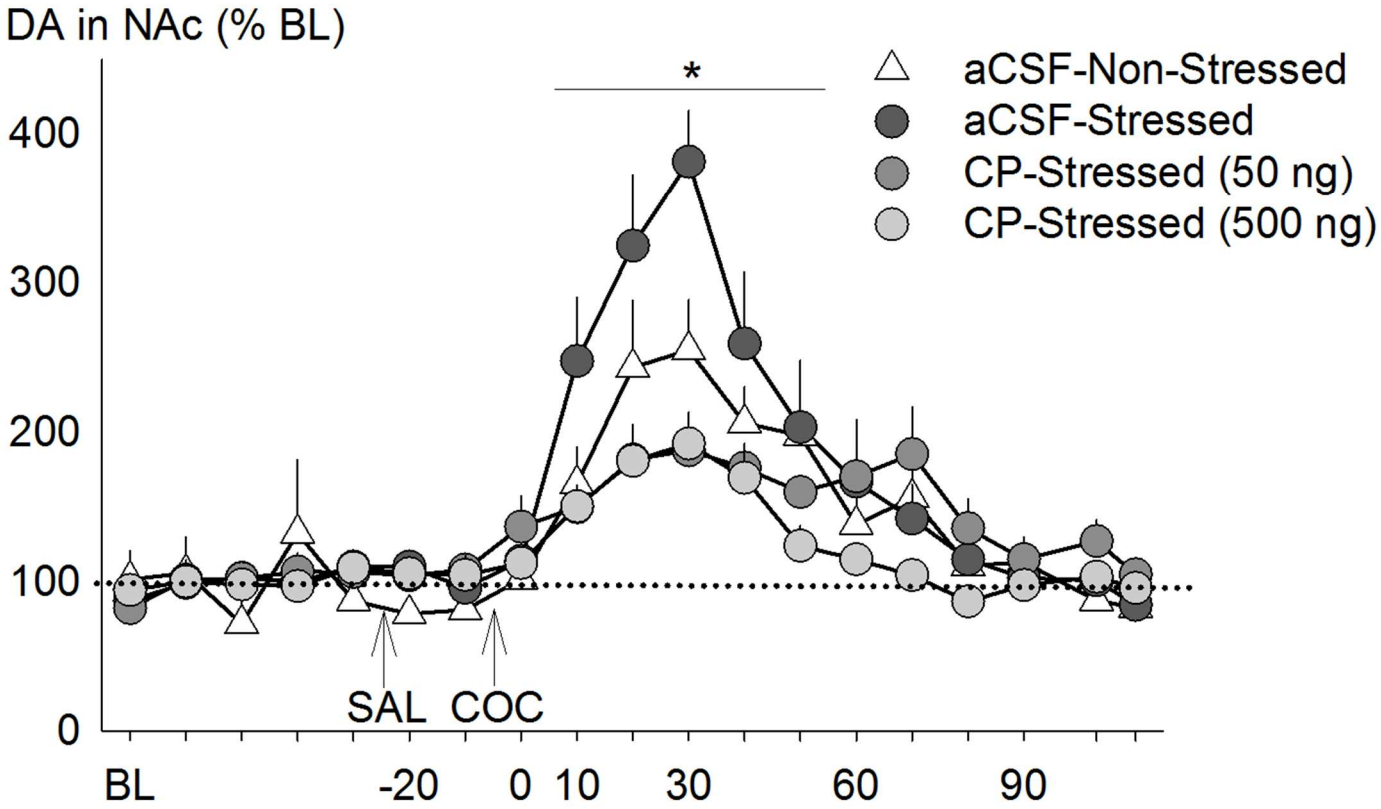
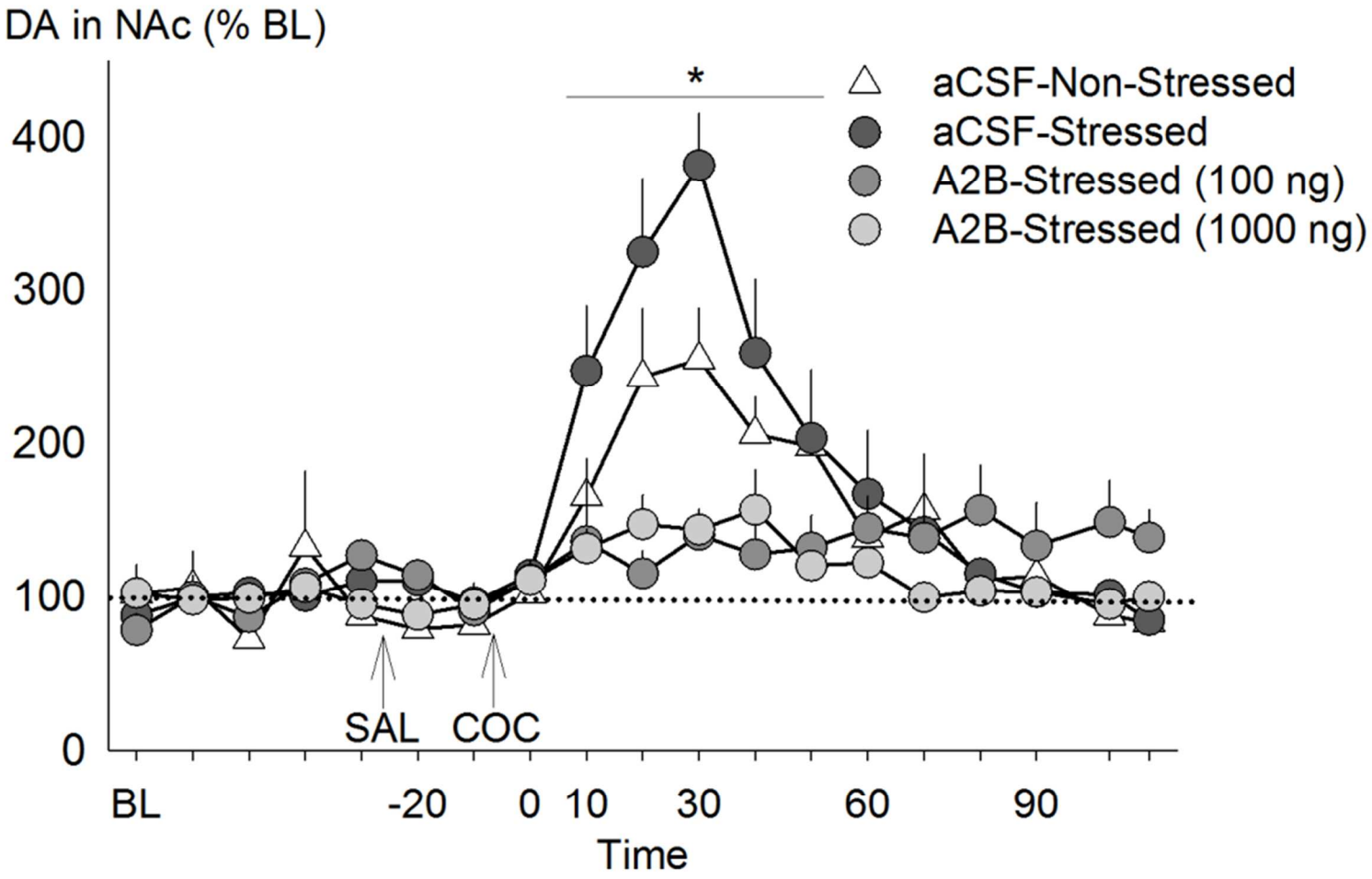

DA in NAc (% BL)
400
300
200
100
0
*
aCSF-Non-Stressed
aCSF-Stressed
CP-Stressed (50 ng)
CP-Stressed (500 ng)
SAL
COC
BL
-20
0
10
30
60
90

Figure 23. Effect of intra-VTA CRF-R2 antagonism on the induction of neural sensitization

Dopamine (DA) in the nucleus accumbens shell (NAcSh) in response to saline and cocaine (10 mg/kg, ip) in rats pretreated with the CRF-R2 antagonist Astressin2B (A2B, 100 or 1000 ng) before social defeat stress or handling. Data are represented as group mean±SEM of the percent change from individual baseline. *=$p<0.05$ vs. non-stressed aCSF group.


DA in NAc (% BL)
400
300
200
100
0
*
aCSF-Non-Stressed
aCSF-Stressed
A2B-Stressed (100 ng)
A2B-Stressed (1000 ng)
SAL
COC
BL
-20
0
10
30
60
90
Time

$p<0.05$). Conversely, animals pretreated with both doses of A2B prior to each defeat did exhibit significant differences in extracellular NAcSh dopamine in response to cocaine.

Within vehicle pretreated animals, intermittently socially defeated rats had a significantly greater extracellular NAcSh dopamine response to cocaine compared with non-stressed controls 15-35 min after cocaine ($p<0.041$). This was prevented with pretreatment of both CP and A2B prior to each social defeat. Stressed rats showed significant main effects of drug pretreatment ($F_{4,154}=6.518$, $p=0.001$) and sample ($F_{7,154}=16.439$, $p<0.001$), with an interaction between drug pretreatment and sample ($F_{28,154}=4.135$, $p<0.001$). Stressed rats pretreated with aCSF had significantly greater extracellular dopamine in the NAcSh after cocaine compared with those pretreated with CP (50 ng/side Holm-Sidak $t=3.166$, $p=0.031$; 500 ng/side Holm-Sidak $t=3.875$, $p=.007$) and A2B (100 ng/side Holm-Sidak $t=4.046$, $p=0.005$; 1000 ng/side Holm-Sidak $t=4.450$, $p=0.002$), whereas there was no difference between CP and A2B groups. The cocaine-induced dopamine increase in extracellular dopamine was significantly attenuated by pretreatment of both doses of both drugs 15-45 min after cocaine injection ($p<0.05$). Within the non-stressed controls, CP pretreatment also significantly blunted the dopaminergic response to cocaine. Two-way repeated measures ANOVA revealed a significant main effect of sample ($F_{7,77}=14.503$, $p<0.001$) and interaction of drug pretreatment and sample ($F_{14,77}=1.954$, $p=0.033$), but there was no overall

main effect of drug pretreatment. Compared to aCSF and A2B pretreated controls, non-stressed rats pretreated with CP showed significantly attenuated cocaine-induced extracellular dopamine efflux in the NAcSh from 35-55 min after cocaine injection ($p<0.045$).

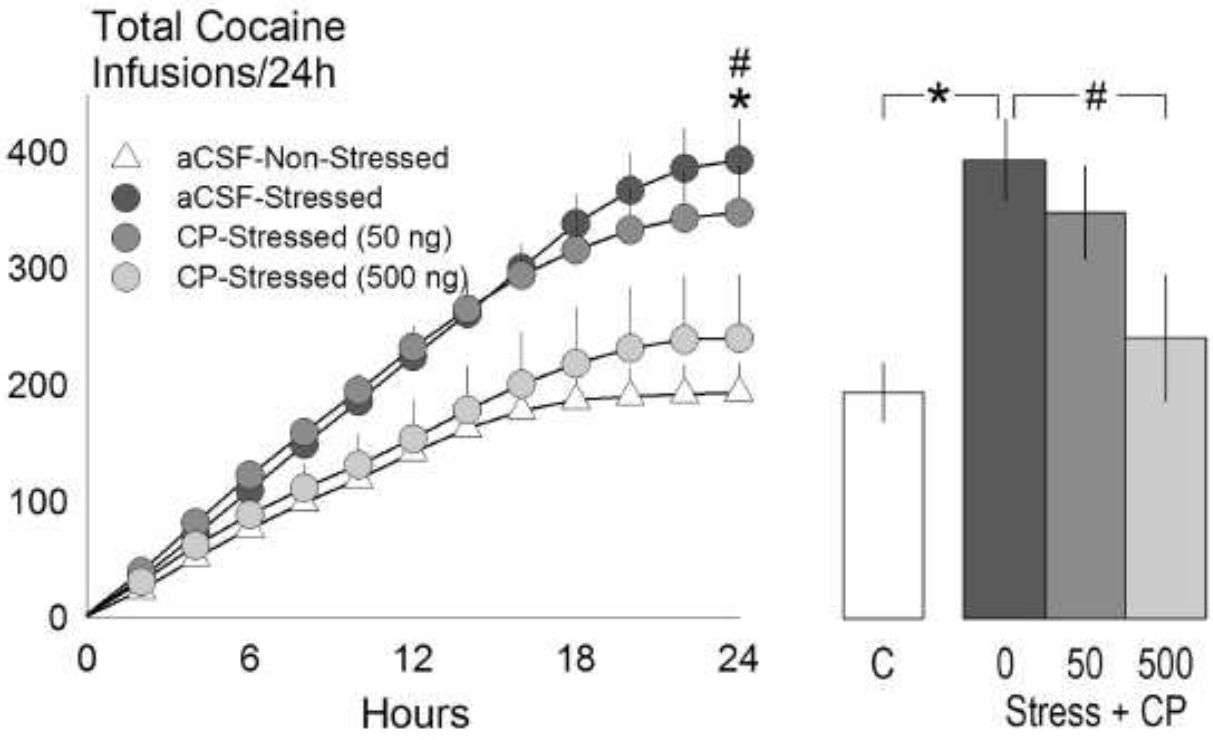
Intra-VTA antagonism of CRF-R1 or CRF-R2 during repeated stress also prevents later escalated cocaine self-administration

All rats acquired cocaine self-administration within 7 d, and reliably self-administered cocaine under a FR5 schedule of reinforcement within 15 d of acquisition. Pretreatment of non-stressed controls with both doses of CP significantly increased the FR5 performance rate, defined as the mean infusions per minute across the last three days of stable FR5 performance (Table 15; $F_{2,46}=4.926$, $p=0.012$). However, there were no statistically significant effects of drug pretreatment or stress condition on responding during a progressive ratio schedule of reinforcement (Table 15).

Intra-VTA antagonism of both CRF-R1 prior to each social defeat encounter significantly and dose-dependently prevented the long-term effects of stress on cocaine self-administration during a 24 h “binge” (Figure 24). There was a significant main effect of drug pretreatment ($F_{2,46}=4.282$, $p=0.020$) as well as a drug pretreatment x stress condition interaction ($F_{2,46}=4.576$, $p=0.015$). Within aCSF pretreated rats, previously stressed animals self-administered significantly more cocaine during the “binge” compared to non-stressed controls (Holm-Sidak $t=3.819$, $p<0.001$).

Figure 24. Effects of intra-VTA CRF-R1 antagonism during social defeat on later cocaine self-administration during a 24 h "binge".

Intra-ventral tegmental area (VTA) antagonism of CRF-R1 with CP376395 (CP) before each defeat dose dependently prevents escalated cocaine self-administration during a 24 h "binge". Left: Average cumulative infusions in 2 h bins across the 24 h session. Right: Total infusions self-administered during the 24 h session. Data represent mean ± SEM. *=$p<0.05$ vs. non-stressed aCSF group; #=$p<0.05$ vs. stressed-aCSF group. (Boyson et al., 2014)


Total Cocaine Infusions/24h
aCSF-Non-Stressed
aCSF-Stressed
CP-Stressed (50 ng)
CP-Stressed (500 ng)
400
300
200
100
0
0
6
12
18
24
Hours
#
*
*
#
C
0
50
500
Stress + CP

The higher (500 ng/side) dose of CP was able to prevent this escalated cocaine self-administration (Holm-Sidak t=2.741, p=0.026), while the lower (50 ng/side) dose did not.

Antagonism of VTA CRF-R2 prior to each social defeat was also able to significantly and dose-dependently prevent the escalated cocaine self-administration during the “binge” (Figure 25). Overall, there was a significant interaction between drug pretreatment group and stress condition ($F_{2,51}$=3.907, p=0.026). As with CP, the higher dose of A2B (1000 ng/side) was able to prevent the increased “binge” cocaine self-administration (Holm-Sidak t=3.016, p=0.012), while the lower dose (100 ng/side) approached but did not reach statistical significance (Holm-Sidak t=1.833, p=0.140).

Rats with a history of repeated stress exhibit greater context-induced reinstatement after forced abstinence compared with non-stressed controls

All rats acquired and reliably self-administered cocaine at an FR3 schedule of reinforcement, with a two-way repeated measures ANOVA revealing a main effect of self-administration day ($F_{9,459}$=177.259, p<0.001) but no effect of prior stress history or stress x day interaction. Furthermore, there was no significant difference between previously stressed and non-stressed rats on cumulative cocaine earned prior to forced abstinence (Figure 26, left panel).

Previous intermittent social defeat stress resulted in significantly more lever pressing during context-induced reinstatement compared to

Figure 25. Effects of intra-VTA CRF-R2 antagonism during social defeat on later cocaine self-administration during a 24 h "binge".

Intra-ventral tegmental area (VTA) antagonism of CRF-R2 with Astressin2B (A2B) before each defeat dose dependently prevents escalated cocaine self-administration during a 24 h "binge". Left: Average cumulative infusions in 2 h bins across the 24 h session. Right: Total infusions self-administered during the 24 h session. Data represent mean ± SEM. *=$p<0.05$ vs. non-stressed aCSF group; #=$p<0.05$ vs. stressed-aCSF group. (Boyson et al., 2014)

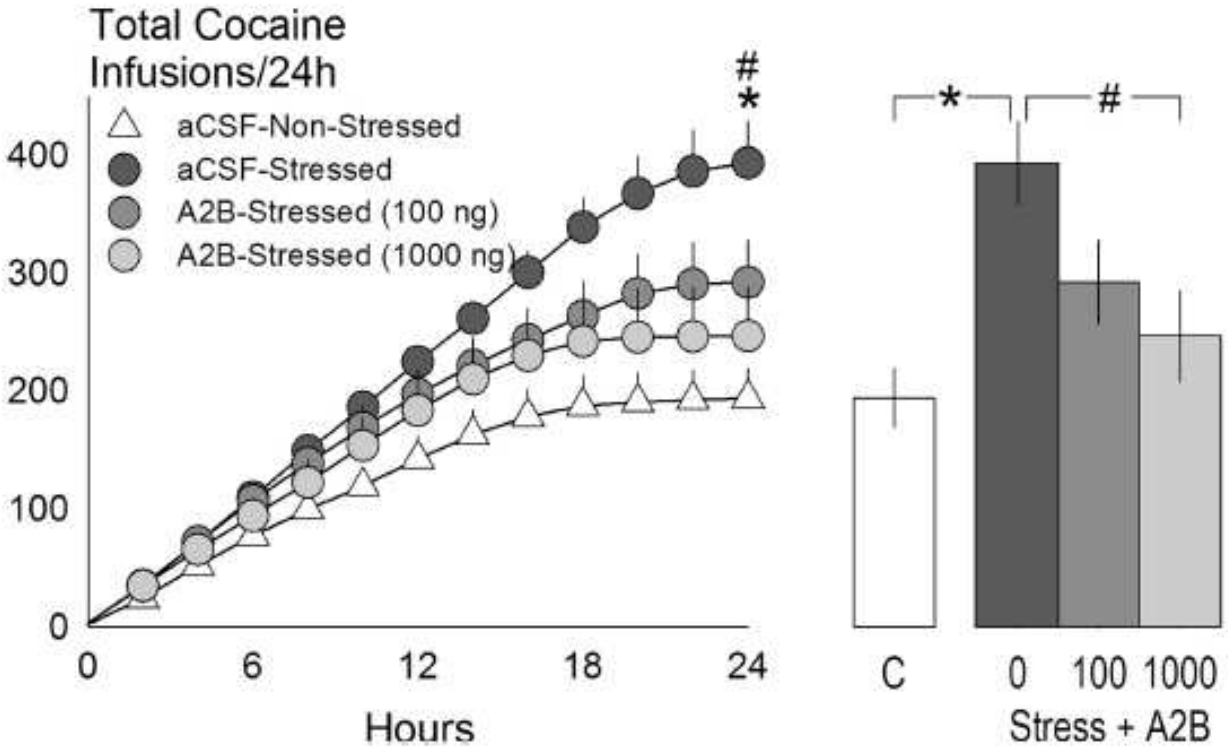
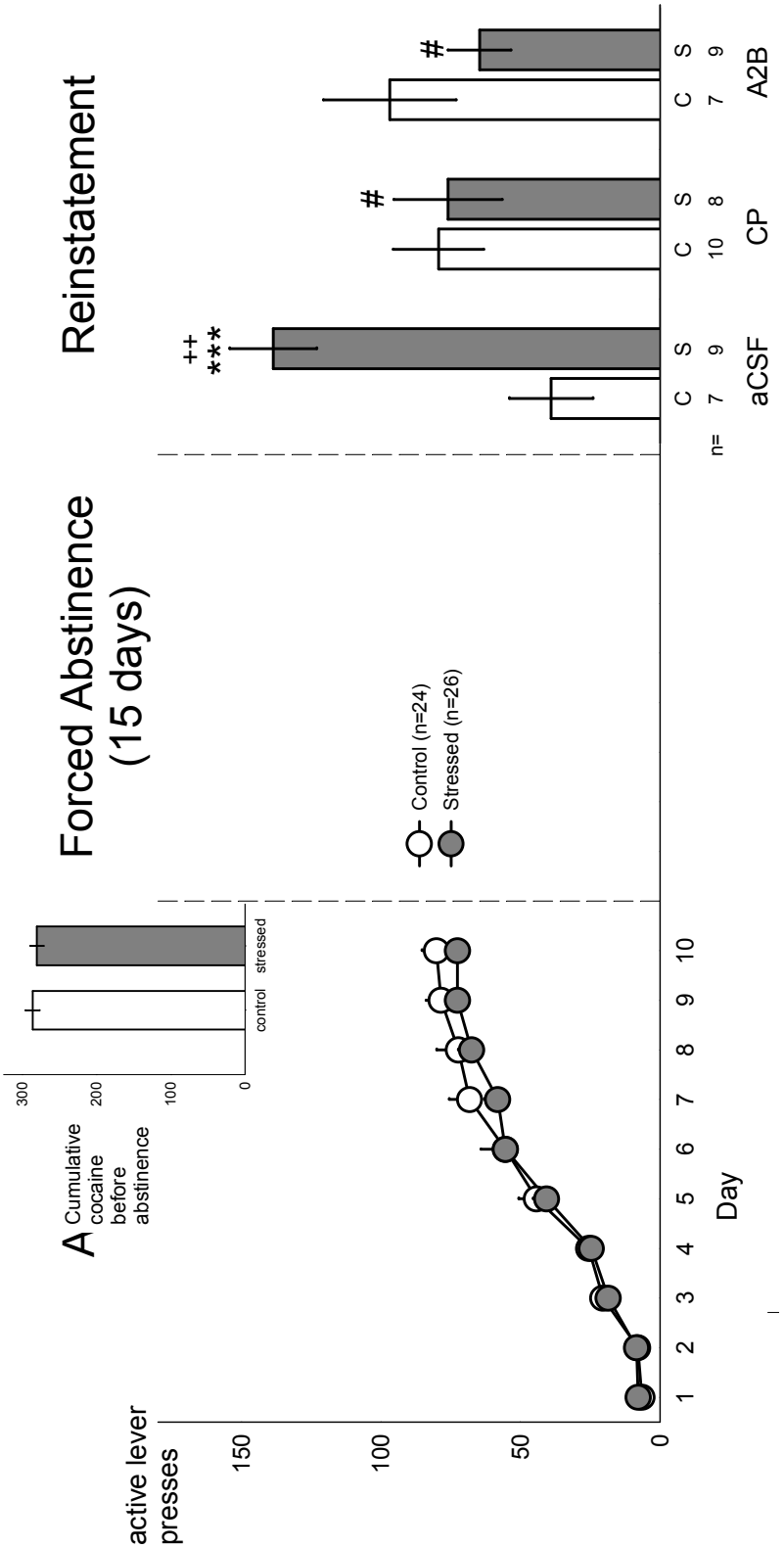

Total Cocaine
Infusions/24h
aCSF-Non-Stressed
aCSF-Stressed
A2B-Stressed (100 ng)
A2B-Stressed (1000 ng)
400
300
200
100
0
0
6
12
18
24
Hours
#
*
*
#
C
0
100
1000
Stress + A2B

Figure 26. Intra-VTA CRF-R1 or CRF-R2 antagonism and context-induced reinstatement to cocaine seeking.

No differences between control (white) and previously stressed (gray) rats were observed in active lever presses across the first 10 d of cocaine self-administration (left panel) or cumulative cocaine self-administered prior to abstinence (inset). After 15 d forced abstinence, previously stressed animals microinjected with vehicle (aCSF) into the VTA exhibited significantly more lever presses than non-stressed controls as well as their last day of cocaine self-administration prior to abstinence. This effect was blocked by intra-VTA antagonism of CRF-R1 (CP376395, CP, 500 ng/side) and CRF-R2 (Astressin2B, A2B, 1000 ng/side), while having no effect on non-stressed controls. ++$p<0.01$ vs last day cocaine self-administration; ***$p<0.001$ vs control-aCSF; #$p<0.05$ vs stressed-aCSF


Forced Abstinence
(15 days)
Reinstatement
A
Cumulative
cocaine
before
abstinence
control
stressed
Control (n=24)
Stressed (n=26)
active lever
presses
Day
n=
aCSF
CP
A2B
C S
++
***
#

non-stressed controls (Figure 26, right panel). Previously stressed rats microinjected with aCSF 20 min prior to reinstatement pressed the lever significantly more than their last day of cocaine self-administration (one-way repeated measures ANOVA $F_{1,17}$=10.549, p=0.012), an effect not seen in aCSF treated rats with no stress history. The stressed-aCSF group also exhibited significantly more cocaine seeking during reinstatement compared to the non-stressed-aCSF group (Holm-Sidak t=3.879, p<0.001).

Heightened context-induced reinstatement in previously stressed animals is associated with increased tonic CRF in the VTA acting on CRF-R1 and CRF-R2.

The augmented context-induced reinstatement observed in previously stressed rats could be reversed by intra-VTA antagonism of CRF-R1 and CRF-R2 20 min prior to the reinstatement test (Figure 26, right panel). While there were no main effects of stress history or drug treatment on lever responding during reinstatement, there was a significant stress history x drug treatment interaction ($F_{2,43}$=7.565, p=0.002), such that the stress effect was dependent on drug treatment. Aside from the stressed-aCSF group, no other group lever pressed significantly more on the reinstatement day compared to the last day of cocaine self-administration, and within the stressed group, lever pressing during reinstatement was significantly reduced with CP (Holm-Sidak t=3.220, p=0.005) and A2B (Holm-Sidak t=2.645, p=0.011) compared to aCSF.

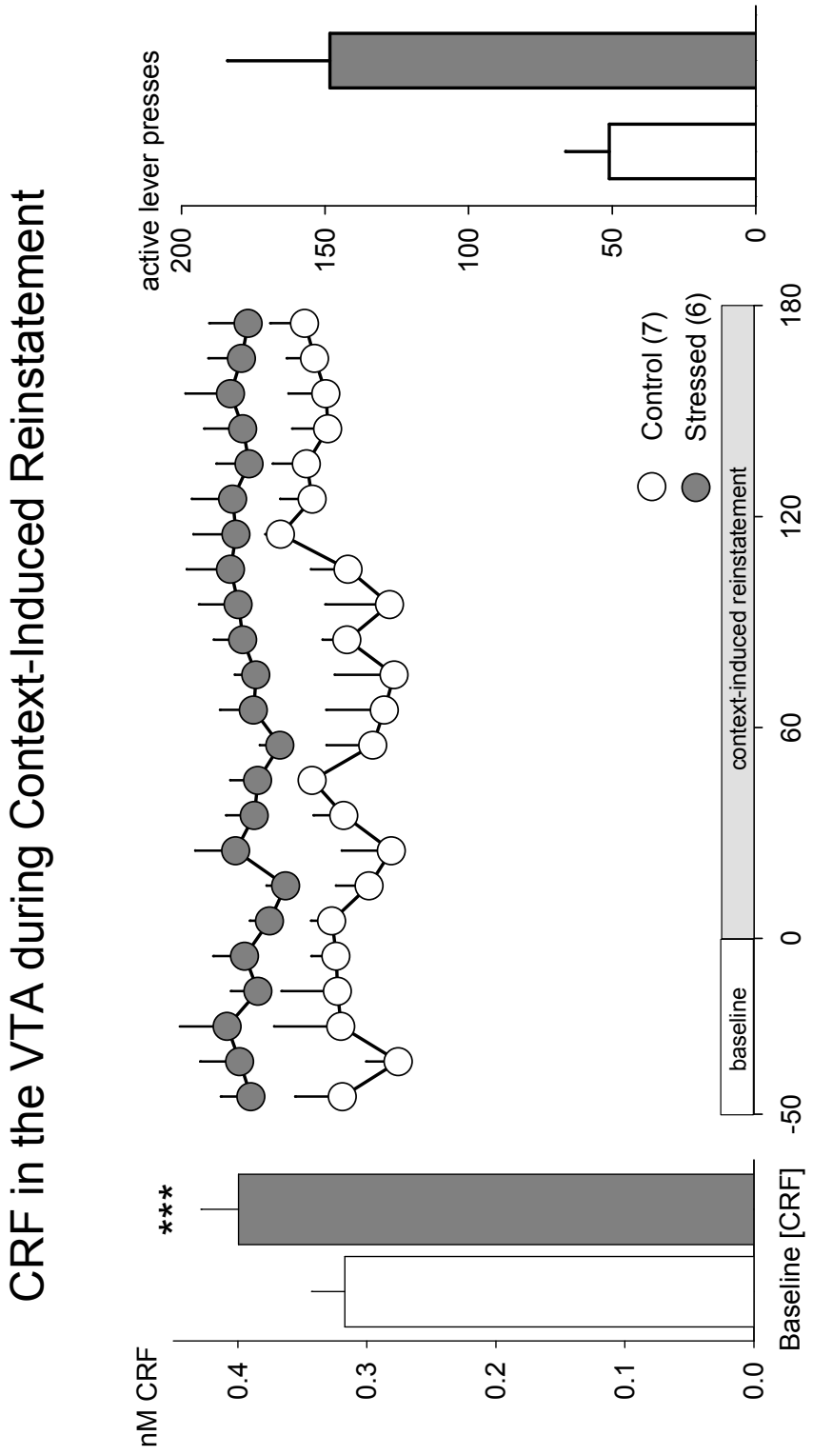
Unexpectedly, there was no phasic increase from baseline in extracellular CRF in the VTA during context-induced reinstatement in either previously stressed or non-stressed group (Figure 27). However, nM concentrations of CRF were significantly greater in previously stressed compared to non-stressed rats (one-way ANOVA $F_{1,11}$=6.529, p=0.027).

IV. DISCUSSION

Intermittent social defeat increases dopaminergic activity during stress and in response to cocaine, as well as promotes later intense cocaine taking and seeking behavior in rats. The current studies demonstrate that CRF and its receptors within the VTA play a fundamental role in these stress-induced maladaptive effects. Both acute and repeated social defeat stress cause similar phasic increases in mPFC and NAcSh dopamine, which requires CRF-R2 activation within the VTA (Figures 12-18). However, this dopaminergic increase is at least partially dissociated from subsequent drug taking and seeking behavior, as blockade of either CRF-R1 or CRF-R2 during stress exposure prevents the induction of neural sensitization and subsequent escalated cocaine self-administration during a 24 h “binge” (Figures 22-25). Importantly, tonic levels of CRF are persistently increased with repeated stress exposure, such that stressed rats show significantly elevated tone for over 1 month after cessation of social stress (Figures 19, 20, 27). This elevated tone may also play a role in heightened cocaine seeking in previously stressed animals during

Figure 27. CRF in the VTA during context-induced reinstatement.

Tonic baseline concentrations of CRF in the VTA as assessed by *in vivo* microdialysis were significantly higher in previously stressed (gray) compared to non-stressed (white) rats (far left). CRF did not significantly fluctuate from baseline in either group during context-induced reinstatement (center), but previously stressed animals exhibited more active lever presses than non-stressed controls (far right). ***=$p<0.001$ vs. non-stressed controls.


CRF in the VTA during Context-Induced Reinstatement
nM CRF
0.0
0.1
0.2
0.3
0.4
***
Baseline [CRF]
-50
0
60
120
180
baseline
context-induced reinstatement
Control (7)
Stressed (6)
active lever presses
0
50
100
150
200

context-induced reinstatement. While microdialysis does not detect a phasic change in VTA CRF during reinstatement testing, antagonism of both CRF-R1 and CRF-R2 within the VTA is able to prevent augmented lever pressing (Figures 26-27).

CRF-dopamine interactions during acute stress

Phasic extracellular dopamine increases

Consistent with previous findings using other stressors (Figure 3, Tables 1-9), social defeat stress rapidly and potently increases extracellular dopamine within both the mPFC and NAcSh. 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 (Column 5 of Tables 1-9), 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 throws his body against the protective barrier, while the intruder often vocalizes audibly (personal observation; not measured or recorded). While it could be argued that the concurrent dopaminergic increase is due to novelty, Feenstra and colleagues (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, Roques, & Dauge, 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, Verheij, Mele, & Cools, 2006). As such, the most likely interpretation of the observed extracellular dopamine increase in both the mPFC and NAcSh during the instigation and subsequent phases of social defeat is a stressful, aversive reaction to the aggressive display of the resident rat.

Phasic extracellular CRF increases

In addition to phasic dopamine increases during acute defeat, phasic changes in extracellular CRF within the VTA were also observed. To date, there has only been one other report of phasic extracellular CRF changes in the VTA during stress. B. Wang et al. (2005) demonstrated that mild footshock (0.3-0.6 mA, 500 ms, 30±40s intervals, across 20 min) similarly increases CRF within the VTA. While histological verification of probe placement was not presented, stereotaxic coordinates were aimed

at the posterior VTA in this previous study. Thus, the findings of Wang and colleagues appear to be consistent with the present findings of sustained, increased extracellular CRF in the posterior VTA during acute stress.

The distribution of CRF peptide/mRNA expression and afferent connectivity has not been carefully described in terms of VTA heterogeneity (Section 5 of Introduction), so the findings of a clear difference in CRF efflux between the anterior and posterior VTA during acute stress in the present study are novel. Future work should elucidate where CRF input to both the anterior and posterior VTA originates and determine which neural circuit is responsible for the observed CRF efflux during acute stress. Preliminary evidence shows that in Cre-CRH mice, the posterior VTA may receive CRF innervation from the anterior VTA and dorsal raphe nucleus (Gobrogge, Figure 4). However, other preliminary, unreported findings with Cre-CRH rats find additional connectivity between the BNST and PVN (George, personal communication).

Role of CRF receptors

Within the posterior VTA in particular, the observed phasic CRF release during acute stress appears to be acting upon CRF-R2 to directly or indirectly influence the dopaminergic increase in the NAcSh, but not in the mPFC. While the heterogeneity in the projections of VTA dopamine neurons to the mPFC has not been adequately described, efferent connections between VTA dopamine neurons and the striatum have been well characterized (Ikemoto, 2007). Anterior VTA dopamine neurons

predominately project to the dorsal striatum, NAc core and lateral NAc shell, whereas VTA dopamine neurons in the posterior VTA predominately project to the medial OT and NAcSh (Ikemoto, 2007), where microdialysis probes were placed. Therefore, CRF release within the posterior VTA is particularly poised to affect medial NAcSh dopamine efflux.

Although CRF may be released in a region of the VTA associated with stress-induced dopaminergic neuron firing, the anatomical distribution of CRF receptor expression within the VTA has also not yet been established. Current antibodies for CRF-R1 and CRF-R2 peptide are not sufficiently selective or reliable. Although antibodies for the mRNA of both CRF receptors are substantially better, they clearly do not capture the expression of the receptors on the cell membrane. CRF receptors are trafficked into and out of the cell membrane of both locus coeruleus and dorsal raphe nucleus neurons after exposure to acute stress (Bangasser & Valentino, 2012; Wood et al., 2013), so merely examining mRNA expression is not sufficient to explain how these receptors affects dopaminergic activity in the VTA and subsequent behavior.

During the first episode of stress, CRF is phasically released within the VTA, where it may act upon both CRF-R1 and CRF-R2. However, the function and synaptic location of both of these receptors has been the subject of considerable debate (see Section 5 of Introduction). The present work demonstrates that during the initial exposure to social defeat stress, CRF-R2 activation is required for stress-induced dopaminergic

efflux within the NAcSh, but not mPFC. This effect on dopaminergic activity could be induced by synergistic action of CRF and 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 & 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 shows that CRF-R1 activation in particular enhances VTA dopaminergic neuron firing rate (Wanat et al., 2008). However, the use of 1 μM CRF in this study may be a significant confound. The greatest evoked CRF concentration observed in the current experiments was 1.047 nM, which with an average 15% probe recovery rate translates to approximately 6.98 nM evoked concentration *in vivo*. While concentrations of CRF used in electrophysiology cannot be directly compared to those obtained from *in vivo* microdialysis, the CRF dose used in this study is still quite substantial. Admittedly, CRF is lost in the electrophysiology apparatus and does not completely penetrate into tissue. However, dose response work from both the central amygdala (Roberto et al., 2010) and VTA (Williams et al., 2014) find that such high doses of CRF are not 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 does not directly and immediately increase dopamine neuron firing rate.

The present results find a more significant role of CRF-R2. Although the exact synaptic location of CRF-R2 is also unknown, the current work indicates that in naïve 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. Although current antibodies may prove unsuitable for elucidating the location of CRF-R2, future electrophysiological work may be able to provide some insight. 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).

Unfortunately, none of the prior studies properly confirmed the neurochemical identity of the neurons studied, and as such the data are

conflated by the previously described (Section 2.3) and well-validated issues with dopamine neuron identification in electrophysiology studies (Ungless & Grace, 2012). Using classical criteria for identifying dopamine neurons yields approximately 70% accuracy, and given that dopamine neurons only comprise approximately 60% of the VTA (Nair-Roberts et al., 2008; Sesack & Grace, 2010), this prior work may merely be describing the response of the VTA as a whole to CRF bath application. Future studies should investigate CRF activity on both dopamine and GABA neurons in the VTA that have been identified through single cell RT-PCR, staining methods, or optogenetic activation. With these criteria met, experiments may be designed to investigate whether CRF-R1 or CRF-R2 activation directly activates VTA dopamine neurons, GABA neurons, or other tertiary neurons.

Together, the current experiments do indicate a clear phasic increase of extracellular CRF in the posterior VTA during acute stress, and that CRF-R2 antagonism can prevent acute stress-induced dopamine efflux in the NAcSh. Dopamine is increased in both the mPFC and NAcSh, to such significant levels to preclude a function of novelty or social interaction (Figures 12 and 13). Notably, CRF is increased exclusively in the posterior, but not anterior, VTA (Figure 14), highlighting the importance of VTA heterogeneity in future studies. Future work should clarify the role of the urocortins (Ucns) and CRF-BP in this stress-induced

dopamine efflux, as well as how CRF-R2, but not CRF-R1, enhances dopaminergic activity during acute stress.

CRF-dopamine interactions during repeated stress

The extracellular mPFC and NAcSh dopamine response to social defeat stress was not altered with repeated experience (Figures 15-16). However, there was a shift in the phasic CRF response within the VTA, such that there was a robust increase in extracellular CRF in the anterior VTA during the last defeat, and the increase in the posterior VTA was smaller than during acute defeat, and was suppressed below baseline levels following the fight (Figure 21). 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 (Figures 17 and 18). These shifts in CRF release and the role of CRF-R2 during defeat reflect neuroadaptive changes occurring with repeated intermittent social stress.

Phasic extracellular dopamine

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, van Erp, Tornatzky, & Miczek, 2003).

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 and colleagues (1992) demonstrated that repeated daily 120 min restraint stress resulted in a habituated extracellular dopamine response in the NAc when repeated for six 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 hours 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 five 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 severity of the stressor.

Tonic extracellular CRF

Unlike the dopaminergic response, the CRF response in the VTA is altered across the course of intermittent social defeat stress. During acute defeat, CRF was unchanged in the anterior VTA, while there was a significant, sustained increase in extracellular CRF within the posterior VTA (Figure 14). Over the ten days of intermittent defeat, tonic baseline levels of CRF were significantly elevated, compared with a significant decrease within the non-stressed controls (Figure 21). The reduced basal concentration of CRF within the non-stressed controls likely represents damage from repeated insertion of the probe. A separate group of non-stressed controls underwent microdialysis on days 10 and 20 to exclude the possibility of age-related decline in tonic CRF concentration within the VTA, and their day 10 baseline CRF concentrations were no different than those of stressed and non-stressed rats on day 1 (data collapsed within non-stressed control group).

Due to damage-induced decline in basal CRF concentration within non-stressed controls, the ~200% increase observed in the stressed animals is particularly impressive. This increase in CRF tone could be due to the recruitment of previously silent CRF neurons, increased vesicular storage of CRF by CRF neurons, or increased firing rate of CRF neurons projecting to or passing through the VTA. As of yet, the activity of CRF-containing neurons terminating in the VTA has not been studied at

baseline or after stress exposure, which should be the subject of future work.

Phasic extracellular CRF

Altered phasic CRF responses occur on the background of the elevated tone in the VTA during repeated stress exposure. During the first defeat, there was no change from baseline CRF levels within the anterior VTA, however, by the last day of defeat, CRF was significantly elevated during stress. This may reflect the activation of previously silent neurons, or neuroadaptations resulting in a phasic response of already active CRF neurons that do not respond to acute stress, but again, changes in CRF neuronal activity due to stress have not been investigated. Additionally, there was a shift in the phasic response of CRF within the posterior VTA, such that after an initial elevation, CRF is significantly decreased from baseline after the social defeat. Neuropeptides are much slower to regenerate within vesicular stores than traditional neurotransmitters (van den Pol, 2012), so this depression from baseline may represent complete depletion of vesicular stores at the beginning of the social defeat procedure.

While the modulation of VTA dopamine neuron activity by CRF has seen a burst in attention over recent years, the source of CRF into the VTA and nature of normal activity remain unreported. Emerging evidence is elucidating the source of CRF into the VTA. Prior work found direct connections between CRF containing neurons in the PVN, CeA, and

BNST to the VTA (Rodaros et al., 2007), but recent AAV tracing work in Cre-CRH mice and rats is finding little evidence for these monosynaptic connections (K. Gobrogge, unpublished, and O. George, unpublished, personal communication). Within naïve mice, the ventral posteromedial VTA receives CRF innervation from both the anterior VTA and dorsal raphe nucleus, which may be the source of phasic CRF increases observed in the posterior VTA during acute stress (K. Gobrogge, unpublished, Figures 4 and 14). However, the source of CRF increases into the anterior VTA during a repeated stress exposure remains unresolved. ChR/CRH mice and rats may provide some illumination as to how repeated stress alters CRF signaling within different subregions of the VTA. Additionally, these mice and rats can be used in electrophysiology preparations to identify CRF afferents to the VTA and investigate both normal and stress-altered physiology.

Role of CRF receptors

In addition to a shift in the basal and evoked activity of CRF neurons projecting into the VTA, there was also 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 was able to prevent 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 & 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.

Secondly, 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.

Together, these data show that despite no change in dopamine efflux across repeated intermittent stress experience, there are distinct alterations in CRF efflux as well as its putative interactions with CRF-R2. Tonic levels of CRF are increased with repeated stress (Figures 19 and 20), and there is a shift from phasic increase in the posterior VTA during acute defeat to a phasic increase in the anterior VTA by the last defeat (Figure 21). Moreover, CRF-R1 continues to play a limited role in stress-induced mPFC and NAcSh dopamine increases, but CRF-R2 is recruited or functionally altered to mediate the increase in both the mPFC and NAcSh with repeated stress experience (Figures 17 and 18).

Role of CRF receptors *during stress* on subsequent cross-sensitization to cocaine and escalate cocaine self-administration

Although CRF-R1 and CRF-R2 had differential effects on dopamine efflux during acute and repeated social defeat, both CRF-R1 and CRF-R2 in the VTA contribute to long-lasting neuroadaptations following intermittent social defeat. Intra-VTA antagonism of either CRF receptor prior to each social defeat successfully and dose-dependently prevented the development of dopaminergic cross-sensitization to cocaine (Figures 22 and 23) as well as escalated cocaine self-administration during a 24 h “binge” (Figures 24 and 25). These experiments extend the findings from the prior two experiments, demonstrating that CRF receptor activation during repeated social stress is necessary and required for long lasting maladaptive changes related cocaine addiction.

Intermittent stress induces neural cross-sensitization and escalated cocaine self-administration

The present results confirm prior findings that intermittent social defeat stress enhances subsequent cocaine self-administration. Rats pretreated with aCSF prior to each social defeat self-administered significantly more cocaine over a 24 hour unlimited access period than similarly pretreated non-stressed controls, consistent with prior work from this laboratory (Boyson et al., 2011; Covington et al., 2005; Covington & Miczek, 2001; Covington, Tropea, Rajadhyaksha, Kosofsky, & Miczek, 2008; Cruz, Quadros, Hogenelst, Planeta, & Miczek, 2011; Holly et al., 2012; Miczek et al., 2011; Quadros & Miczek, 2009). This escalated cocaine self-administration represents a maladaptive dysregulation of cocaine intake in a manner analogous to dysregulated binge drug taking observed in human addicts (Gawin, 1991).

Sensitization to cocaine may be one factor contributing to escalated cocaine self-administration. Sensitization can be induced by repeated psychostimulant treatment, and observed as an increased response to treatment with the same psychostimulant compared to previously non-treated controls (Robinson & Berridge, 1993). With cross-sensitization, another drug or stimulus, in the current case stress, serves as the sensitizing agent (Kalivas, Richardson-Carlson, & Van Orden, 1986). Sensitization can be observed either behaviorally, manifested as increased locomotor activity, or neurally, manifested as increased dopaminergic response in the NAc. There have been multiple compelling

reports of dissociation between locomotor sensitization and psychostimulant self-administration (Ahmed & Cador, 2006; Cador, Bjijou, & Stinus, 1995), indicating that behavioral sensitization may play a limited role in later drug taking and seeking behavior. However, locomotor sensitization can also be dissociated from dopaminergic sensitization, and dopaminergic sensitization may be more closely linked to increased drug self-administration (Balfour, Benwell, Birrell, Kelly, & Al-Aloul, 1998).

The present results confirm previous findings that exposure to some types of repeated stress can induce dopaminergic cross-sensitization to cocaine, such that acute, experimenter administered cocaine yields augmented dopaminergic increases in the NAcSh in previously stressed compared with non-stressed rats (Garcia-Keller et al., 2013; Han et al., 2015; Holly et al., 2012; Miczek et al., 2011; Pacchioni, Gioino, Assis, & Cancela, 2002; Sorg & Kalivas, 1991).

It is hypothesized that dopaminergic cross-sensitization to subsequent psychostimulants may be due to neuroadaptations resulting from repeated VTA dopamine neuronal activation during stress. Indeed, as described in Sections 4 and 5 of the Introduction, repeated stress persistently increases burst firing of VTA dopamine neurons (Anstrom et al., 2009; Anstrom & Woodward, 2005; Brischoux et al., 2009), increases the AMPA/NMDA ratio on VTA dopamine neurons (Dong et al., 2004; Saal et al., 2003), and causes substantial phasic dopamine increases in the mPFC and NAcSh (Experiment 1). This stress-induced facilitation of

enhanced excitatory neurotransmission in these VTA dopaminergic cells may drive subsequent neural cross-sensitization to psychostimulants such as cocaine.

Role of CRF receptors

Notably, CRF has been implicated in many of these stress-induced effects (Borgland et al., 2010; Korotkova et al., 2006; Saal et al., 2003; Ungless et al., 2010; Wanat et al., 2008; B. Wang et al., 2005; B. Wang et al., 2007; Williams et al., 2014; Wise & Morales, 2010). However, although CRF-R2, but not CRF-R1, antagonism in the VTA prevented stress-induced increases in extracellular mPFC and NAcSh dopamine (Experiment 1), antagonism of both CRF receptor subtypes prior to each defeat prevented the induction of neural cross-sensitization to cocaine ten days later.

These findings demonstrate that CRF-R1 activation is inducing long-lasting modulatory changes on VTA dopamine neuronal activity that are likely indirect and dissociated from dopamine neuronal activation during stress itself. One possibility may be that CRF-R1 activation directly or indirectly (through GABAergic interneurons) modulates the activity of VTA dopamine cells that are responsive to reward as opposed to aversion. Neuropeptides do not behave like classical neurotransmitters; they are often not released directly into synapses, can be released from dendrites, somas, or all along the axon, and are not quickly metabolized so can diffuse much greater distances (van den Pol, 2012). Thus, the

phasic CRF release observed in experiments 1 and 2 may be affecting more than just aversion-responsive VTA dopamine neurons, and through a CRF-R1-PKC pathway may produce long-lasting changes on neighboring reward-responsive VTA dopamine neurons that are not active during social defeat stress.

Similarly, intra-VTA antagonism of both CRF receptor subtypes prior to each defeat prevented the development of later escalated cocaine self-administration. One surprising finding was that intra-VTA CRF-R1 antagonism enhanced stable FR5 performance rate within non-stressed controls. While these results may be conflated by a low response rate within the aCSF pretreated non-stressed controls, chronic CRF-R1 antagonism blunts the NAc dopamine response to systemic cocaine (Lodge & Grace, 2005), so rats may be compensating for reduced accumbal dopaminergic transmission by increasing response rate.

Repeated stress was unable to increase the breakpoint in the PR schedule of reinforcement within aCSF-pretreated rats, and repeated intra-VTA CRF receptor antagonism similarly had no effect on the PR breakpoint. Results from this laboratory on breakpoint in a PR schedule of reinforcement have been inconsistent, with some studies showing a significant increase in breakpoints within previously stressed animals (Covington & Miczek, 2005; Covington et al., 2008; Quadros & Miczek, 2009), while others have found no effect (Boyson et al., 2011; Covington & Miczek, 2001; Cruz et al., 2011). This discrepancy could be a result of a

non-optimal cocaine dose (0.3 mg/kg/infusion), and as such future studies could incorporate a dose-response curve in PR performance between stressed and non-stressed animals.

The most intriguing finding of these experiments, however, was that intra-VTA antagonism of either CRF-R1 or CRF-R2 prior to each defeat completely prevented stress-induced escalation in cocaine consumption during a 24 hr “binge” more than one month after the last defeat. This is in agreement with a previous study showing both systemic and intra-VTA CRF-R1 antagonism prior to stress can protect against augmented “binge” cocaine self-administration (Boyson et al., 2011). The current finding adds to the growing literature demonstrating both CRF-R1 and CRF-R2 subtypes are involved in escalated cocaine self-administration (Chen et al., 2014; Specio et al., 2008) and stress-induced reinstatement (Blacktop et al., 2011; Sarnyai et al., 2001; Shaham, Erb, Leung, Buczek, & Stewart, 1998; B. Wang et al., 2005; B. Wang et al., 2007). However, in contrast to these previous experiments, the present study administered intra-VTA CRF antagonists prior to each stress to evaluate prevention of long term as opposed to transient effects.

These findings demonstrate that an intact CRF receptor system is necessary for long-lasting effects of stress on cocaine-related neurochemistry and self-administration. This is dissociated from the role of CRF receptors in mediating extracellular dopaminergic increases in the mPFC and NAcSh during stress (Experiment 1), as VTA CRF-R1 is

necessary for the induction of neural sensitization and escalated “binge” cocaine consumption, but not increased stress-induced dopamine efflux.

Role of VTA CRF receptors *after stress* on cocaine seeking

A translational model of cocaine self-administration and reinstatement

While the previous experiments demonstrate a clear role of VTA CRF and its receptors during stress on extracellular dopamine and the development of later maladaptive neural cross-sensitization and escalated cocaine self-administration, the final experiment demonstrates that CRF and its receptors still play an integral role long after the termination of stress. CRF tone remains elevated in previously stressed animals, such that baseline CRF concentrations in the VTA are significantly elevated compared to non-stressed controls over one month after the last social defeat. Furthermore, although there is no phasic change in VTA CRF during context-induced reinstatement, blockade of both CRF-R1 and CRF-R2 prior to reinstatement can prevent augmented cocaine seeking in previously stressed rats.

Clinical data demonstrate that individuals with a history of high levels of stress initiate drug use earlier (Karlsgodt, Lukas, & Elman, 2003), show escalated levels of cocaine taking (Barrett & Turner, 2006; Newcomb & Bentler, 1988; Newcomb & Harlow, 1986; Wills & Cleary, 1996; Wills, Vaccaro, & McNamara, 1992), and are more prone to relapse (Fox, Hong, Siedlarz, & Sinha, 2008; Sinha, 2001, 2007; Sinha, Catapano,

& O'Malley, 1999; Sinha, Fuse, Aubin, & O'Malley, 2000) than those with lower levels of prior stress.

Previous work from this laboratory has translated much of this work to rodents, clearly demonstrating that repeated social defeat stress accelerates the acquisition of cocaine self-administration (Tidey & Miczek, 1997), increases breakpoints in a progressive ratio schedule of reinforcement (Covington & Miczek, 2005; Covington et al., 2008; Quadros & Miczek, 2009), and escalates cocaine self-administration during a 24 hr “binge” (Boyson et al., 2014; Boyson et al., 2011; Covington et al., 2005; Covington & Miczek, 2001, 2005; Covington et al., 2008; Cruz et al., 2011; Holly et al., 2012; Miczek et al., 2011; Quadros & Miczek, 2009). However, to date, we have not reported any effects of social defeat history on reinstatement, an animal correlate of relapse.

Most studies investigating reinstatement in rodents first train animals to reliably self-administer cocaine and then begin an extinction protocol, where the cocaine is replaced with saline (Reichel & Bevins, 2009). In this method, animals must learn to inhibit previous responding. In addition to learning a new behavior, this model is not translationally relevant (Reichel & Bevins, 2009); although a similar model of cue exposure is being attempted as an addiction therapy in humans, it is not consistently effective (Santa Ana et al., 2015). Instead, most human addicts undergo abstinence—either voluntary or forced. Thus, the current study attempted to provide a more translational model, through repeated

stress prior to cocaine self-administration, followed by forced abstinence for two weeks, and finally reintroducing animals to the prior cocaine self-administration context to assess responding on the lever previously associated with cocaine, interpreted as cocaine seeking or craving.

Here, we report the first evidence that a history of social stress causes significant context-induced reinstatement more than one month after the last defeat. As previously stressed animals microinjected with aCSF into the VTA immediately prior to reinstatement pressed the formerly active lever significantly more than the last cocaine self-administration session, this may reflect an incubation effect (Grimm, Hope, Wise, & Shaham, 2001).

However, no incubation effect is present in previously non-stressed controls. This is likely a result of not using discrete cocaine-associated cues during reinstatement. Nearly all reports of an incubation of cocaine seeking following abstinence use cue-induced reinstatement (Calu et al., 2007; Chauvet, Goldberg, Jaber, & Solinas, 2012; Conrad et al., 2008; Dikshtein et al., 2013; Freeman et al., 2008; Karlsson, Kircher, Shaham, & O'Donnell, 2013; Koya et al., 2009; Lee et al., 2013; Li & Frantz, 2009; Lu, Grimm, Dempsey, & Shaham, 2004; Lu, Uejima, Gray, Bossert, & Shaham, 2007; Lu et al., 2009; Sorge & Stewart, 2005; Thiel et al., 2012; Xi et al., 2013). While certainly context could be contributing to the cue-induced reinstatement observed in these studies, drug-induced reinstatement does not show incubation following abstinence (Deroche-

Gamonet, Martinez, Le Moal, & Piazza, 2003; Lu et al., 2004; Marinelli, Cooper, Baker, & White, 2003). As context would be present in drug-induced reinstatement procedures as well, it is likely that incubation effect is predominately due to lever press-contingent cue presentation during reinstatement.

While Kelamangalath and Wagner (2009) reported a time-dependent incubation effect due to context only in abstinent rats, there were significant flaws with the interpretation of their data. On the last day of cocaine self-administration, rats pressed the active lever approximately 75 times across 90 min. However, to assess context-induced reinstatement, only responding during the first 10 min of the session was used as rats quickly stopped pressing the lever with no CS or cocaine delivery. While 30 day abstinent rats may have pressed the lever significantly more than 1 day abstinent rats, they did not show similar responding to their last day of cocaine self-administration, a critical component of reinstatement and the incubation effect (Grimm et al., 2001; Pickens et al., 2011). Furthermore, statistics in this study were flawed, as proper analysis should have been a one-way ANOVA comparing all abstinent groups as opposed to multiple t-tests.

The sizeable context-induced reinstatement observed in previously stressed rats in this translational model of cocaine self-administration parallel clinical observations (Sinha, 2008). The methods are different than other context-induced reinstatement paradigms in rodents, in which

animals are trained in one context, undergo extinction in a second, markedly different context, and are reinstated in their previous cocaine associated context (Crombag, Bossert, Koya, & Shaham, 2008). In those models, all animals reinstate lever pressing in their cocaine context, likely because they have only learned to inhibit responding in their extinction context (but see Crombag et al. (2008) for excellent review of various psychological, behavioral, and neurobiological interpretations and explanations of context-induced reinstatement). Using the abstinence procedure, we could elicit a significant, clinically relevant stress effect.

Role of the VTA in context-induced reinstatement

The role of the VTA has received only limited attention in context-induced reinstatement to cocaine seeking. Systemic D1-like (SCH-23390) or D2-like (raclopride) antagonism decreases context-induced reinstatement of cocaine seeking after extinction in a separate context. In a similar protocol for context-induced reinstatement to heroin seeking, intra-NAcSh SCH-23390 can also prevent increased lever pressing when reintroduced to the drug paired context compared to vehicle controls (Bossert, Poles, Wihbey, Koya, & Shaham, 2007; Bossert et al., 2012). Recent evidence indicates a functional role of theta rhythms in hippocampal CA3 glutamatergic neurons projecting to GABAergic caudodorsal lateral septum, which in turn project to GABAergic interneurons within the VTA inhibiting dopamine neurons (Luo, Tahsili-Fahadan, Wise, Lupica, & Aston-Jones, 2011). Disrupting this circuit

prevents context-induced reinstatement of cocaine seeking (Luo et al., 2011). Further evidence points to a pivotal role of VTA dopamine neurons, showing a similar attenuation of context-induced reinstatement by disrupting a circuit of VTA dopamine neurons projecting to the orbitofrontal cortex, which sends bihemispheric connections to the basolateral amygdala (Lasseter et al., 2014). Thus, CRF-related neuroadaptations in the VTA resulting from intermittent social defeat may drive the observed context-induced reinstatement after abstinence in previously stressed rats.

Role of VTA CRF and its receptors during context-induced reinstatement

Indeed, intra-VTA antagonism of either CRF-R1 or CRF-R2 prior to reinstatement, after any stress-induced neuroadaptation had already occurred, was able to significantly prevent augmented cocaine seeking in previously stressed rats. VTA CRF-R1 and CRF-R2 have both been implicated in stress-induced reinstatement to cocaine seeking after extinction (Blacktop et al., 2011; B. Wang et al., 2007), so it was initially hypothesized that context-induced reinstatement in previously stressed animals was serving as an additional stressor, and CRF receptor antagonism was preventing the effect of a phasic increase in extracellular CRF within the VTA. However, the present results indicate that while previously stressed animals still had significantly greater CRF tone in the VTA compared to previously non-stressed rats, there were no phasic CRF changes in the VTA within either group. Thus, it appears that intra-VTA

CRF receptor antagonism, rather than preventing any phasic CRF effect, may prevent the increased extracellular tonic CRF from exerting effects, driving context-induced reinstatement in previously stressed animals.

Conclusions

Dynamic interactions of CRF and its receptors within the VTA to promote increased extracellular dopamine in VTA projection targets during acute social defeat stress (Figure 28A) shift over the course of repeated intermittent social defeat stress (Figure 28B), and are integral in the development of neural cross-sensitization to cocaine and escalated cocaine self-administration during a 24 hr “binge” (Figure 28C). Furthermore, tonic levels of CRF increase with repeated stress exposure, and this increase is persistent for at least one month, driving later context-induced cocaine seeking (Figure 28C).

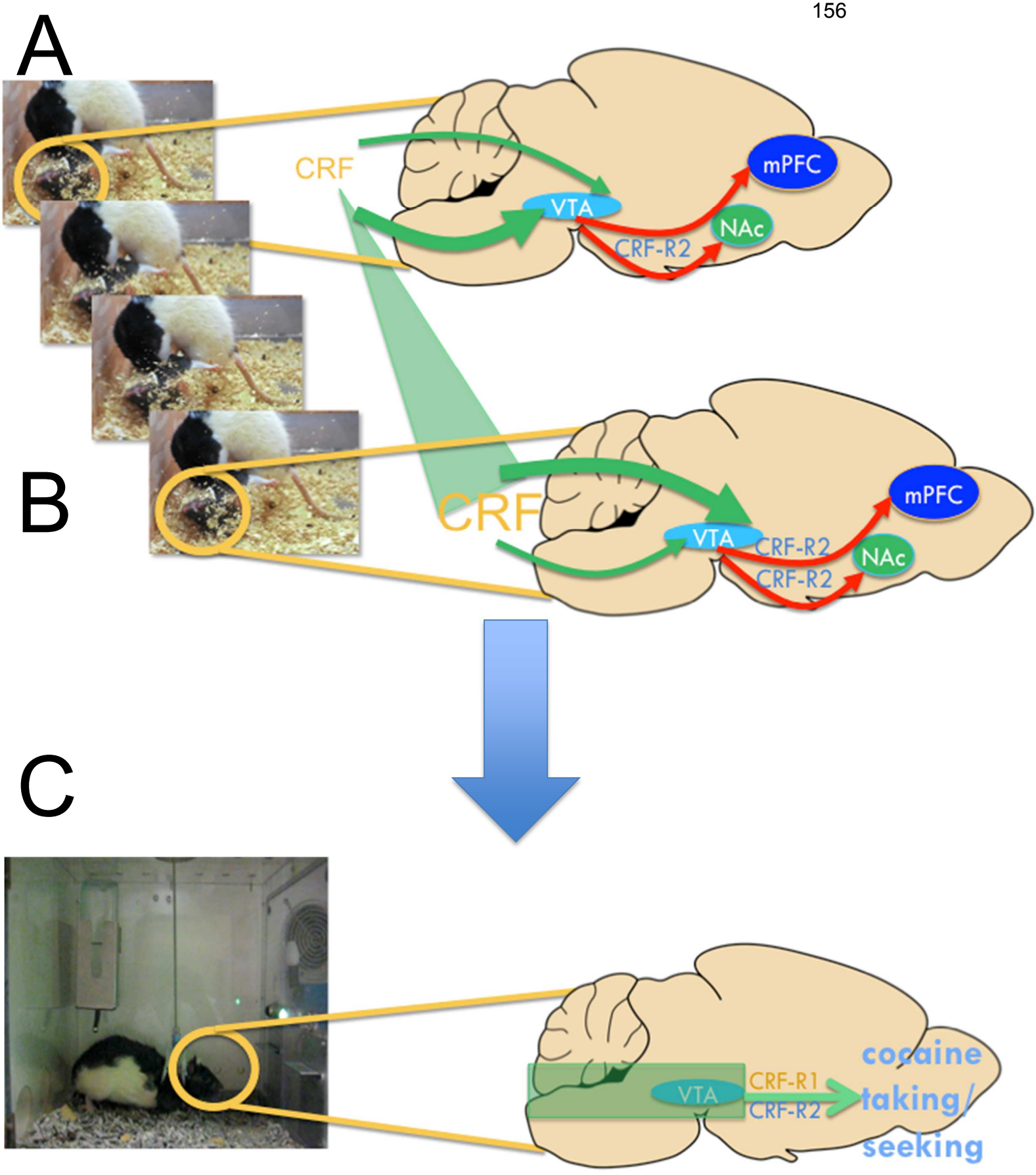
It is imperative to consider that these experiments do not demonstrate a causal role of VTA CRF in mesocorticolimbic dopamine increases. First, the direct effect of intra-VTA CRF on mPFC and NAcSh dopamine has never been examined. While intracerebroventricular microinjection of CRF dose-dependently elevates extracellular dopamine in the mPFC (Lavicky & Dunn, 1993), the role of other dopaminergic sources aside from the VTA cannot be ruled out. CRF is not the only endogenous ligand for CRF receptors within the brain; urocortins (Ucns) also exhibit affinity for CRF receptors. Specifically, Ucn1 shows high affinity for both CRF-R1 and CRF-R2, while Ucns 2 and 3 show selective

Figure 28. Summary of findings.

A. During acute social defeat stress, CRF is released into the posterior VTA, where its putative actions on CRF-R2 are required for the stress-induced dopamine efflux in the NAcSh, but not mPFC.

B. With repeated stress, CRF tone in the VTA increases, and CRF becomes phasically increased in the anterior as opposed to posterior VTA, where its putative actions on CRF-R2 are now required for the stress-induced dopamine efflux in both the mPFC and NAcSh.

C. CRF-R1 and CRF-R2 activation are required both during and after strss for stress-induced escalation in cocaine taking and seeking.


A
CRF
mPFC
VTA
NAc
CRF-R2
B
CRF
mPFC
VTA
CRF-R2
CRF-R2
NAc
C
VTA
CRF-R1
CRF-R2
cocaine
taking/
seeking

affinity for CRF-R2 (Ryabinin et al., 2012). As such, it is possible that while CRF is phasically increased into the VTA during acute stress, Ucns are as well, and it is their action on CRF-R2 mediating the increase in extracellular dopamine in the NAcSh. Future work should verify that Ucns are not also phasically released into the VTA during stress, and that microinjection of CRF at physiologically relevant concentrations directly into the VTA can produce similar effects on dopamine efflux in the mPFC and NAcSh as observed during social defeat stress.

Additionally, actions of CRF in VTA terminal regions, rather than the VTA itself, may be driving the phasic dopamine increases during acute and/or repeated stress. Recent work demonstrates that co-activation of both CRF-R1 and CRF-R2 in the NAcSh by CRF bath application can increase evoked dopamine release in the NAcSh (Lemos et al., 2012). Similarly, as CRF and its receptors have been isolated in layer V of the prefrontal cortex (Yan, Baram, Gerth, Schultz, & Ribak, 1998), they may influence dopaminergic release in the mPFC as well.

Acute stress causes a significant phasic rise in CRF within the posterior, but not anterior, VTA. CRF putatively interacts with CRF-R2 to stimulate the extracellular increase in NAcSh, but not mPFC, dopamine. CRF may simultaneously act upon CRF-R1 to promote other changes unrelated to phasic dopamine increases. Repeated stress shifts the phasic CRF response to the anterior VTA, likely recruiting previously silent CRF neurons, and engages a CRF-R2 dependent mechanism to drive phasic

dopamine responses in VTA projection targets. Due to issues with supraphysiological doses of CRF used in some electrophysiological preparations (Ungless et al., 2003; Wanat et al., 2008), it is not clear what physiologically relevant CRF receptor activation does, but in general, stress exposure enhances NMDAR and mGluR function, and increases the AMPA/NMDA ratio, engendering a hyperexcitable dopaminergic system (Korotkova et al., 2006; Saal et al., 2003; Ungless et al., 2003; Wanat et al., 2008). With a hyperexcitable VTA, future stimulation produces more robust, sensitized effects to different stressors or rewards. This effect can be prevented by blocking either CRF-R1 or CRF-R2 in the VTA prior to each defeat, indicating a likely synergistic function of the two receptors.

Finally, repeated stress substantially increases extracellular CRF tone in the VTA, which may drive subsequent reward-related behaviors long after the termination of stress. Future work can elucidate a causal as opposed to correlational role of augmented CRF tone by selective under- or overexpression of CRF within the VTA.

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TABLES

Table 1. Effects of restraint stress on extracellular dopamine concentrations in the NAc

Reference	Species	Stressor parameters	Stressor pattern	Sample length (min)	Max DA (%BL)	Timecourse of DA efflux	Increase at termination?	Additional findings
Imperato et al. (1989)	SD rat	90 min	acute	10	145%	immediate increase, returned to baseline by 70 min	not measured	corticosterone also increased DA
Imperato et al. (1990)	SD rat	120 min	acute	10	150%	immediate increase, peaked at 30-40 min, returned to baseline by 80 min	not measured	prevented by 5HT3 antagonist but not diazepam
Imperato et al. (1991)	SD rat	120 min	acute	10	150%	immediate increase, peaked at 30 min, gradual decrease to baseline by 80 min, increase at release	yes	exogenous corticosterone did not increase DA
Puglisi-Allegra et al. (1991)	SD rat	240 min	acute	10	140%	immediate increase, peaked at 30 min, gradual return to baseline by 80 min, increase at release	yes	
Imperato et al. (1992)	SD rat	60 min for 6 consecutive days, repeated after 3 days	repeated	10	150%	Day 1: immediate rise, peak at 20 min, gradual decrease towards baseline, but increase at release. Day 2, 3, 4: blunted initial response, no change at termination response. Day 7: Same as Day 1	yes	decrease in dopaminergic tone as well
Imperato et al. (1993)	SD rat	120 min, with 5 prior days of 60 min	acute and repeated	10	CTRL 150%, prev. stress 70%	Previously non-stressed: immediate increase, peaks at 20 and 30 min, gradual return to baseline by 50 min. Previously stressed: initially stay at baseline, drop below baseline 80-120 min into restraint	not measured	
Lillrank et al. (1999)	SD rat	30 min	acute	15	130%	no changes during restraint, peak only observed 60 min after termination	yes	NAc core, not shell, and probe too long (included more than core)
Jackson and Moghaddam (2004)	SD rat	10 min	twice, 3h apart	10	125%	both exposures showed similar increase, peaking at 20 and 30 min, return to baseline by 60 min, increase at termination	yes	

*dopamine did not return to baseline prior to stressor termination, so cannot assess; NAc=nucleus accumbens, DA=dopamine, BL=baseline, SD=Sprague Dawley

Table 2. Effects of restraint and immobilization stress on extracellular dopamine in the mPFC

Reference	Species	Stressor parameters	Stressor pattern	Sample length (min)	Max DA (%BL)	Timecourse of DA efflux	Increase at termination?	Additional findings
Matuszewich, Filon, Finn, and Yamamoto (2002)	SD rat	immobilization 60 min	acute	20	175%	immediate maximal increase during first 20 min, then back to baseline for duration	no	MDMA pretreatment blocked effect
Pozzi et al. (2002)	SD rat	immobilization 120 min	acute	20	250%	immediate maximal increase, returned to baseline within 100 min; increase again 20-60 min after release, although not as high as before	yes	
C. J. Swanson et al. (2004)	SD rat	immobilization 30 min	acute	30	189%	increased to 150% during stress, but peaked at 189% after termination, with gradual return to baseline by 90 min after initiation of stressor.	yes	mglu2/3 agonist blocks increases in both dopamine and noradrenaline
Renoldi and Invernizzi (2006)	CD-COBS rats, Mongolian Gerbils	immobilization 40 min	acute	20	188%, 316%	Rats showed immediate increase during immobilization, which remained elevated 40 min after stressor termination. Gerbils showed immediate increase, peaking in second half of stressor presentation, and returning to baseline 40 min after stressor termination	*	
Arriaga-Avila et al. (2014)	Wistar rat, female	immobilization 30 min	acute	15	200%, n/a	increased to 200% in second half of stress in virgin females, returning to baseline by 45 min after termination. No effect observed in non-virgins (lactating dams)	*	
Imperato et	SD rat	restraint 120	acute	10	180%	immediate increase, peaking 30	yes	looked at corticosterone—

al. (1991)		min				min into restraint, and returning to baseline after 90 min. increase again at termination		adrenalectomy had no effect, and exogenous corticosterone did not affect dopamine release.
Cuadra et al. (1999)	Wistar rat	restraint 60 min	acute, with one week of chronic variable stress	30	146%, 177%	no CVS group increased dopamine beginning at 60 min, with maximal increase at 120 min, never returning to baseline. CVS group showed maximal (177%) increase at 120 min, returning to baseline at 300 min.	*	reversed by naloxone
Cuadra et al. (2001)	Wistar rat	restraint 60 min	acute, one week chronic variable stress	30	139%, 189%	without CVS, dopamine increased gradually during restraint, peaking (139%) and sustained for duration of sampling. CVS group also increased gradually and peaked (189%) 30 min after termination without returning to baseline	*	
Mokler et al. (2007)	SD rat	restraint 20 min	acute, some with prior prenatal malnourishment	20	150%, n/a	controls 150% during stress, immediately back to baseline on termination. Malnourished did not increase dopamine during stress, but were significantly attenuated 100-160 min after release	no	
Garrido et al. (2013)	Wistar rat	restraint 20 min	acute	20	165%	immediate increase in response to stress, remained elevated, back to baseline by 40 min after termination	*	
Jackson and Moghaddam (2004)	SD rat	restraint 10 min	repeated after 3 hours	10	140%	immediate increase during first exposure, sustained for one sample after termination, then back to baseline. Second exposure showed habituated DA response	*	

Ventura, de Mello, and de Melo Reis (2013)	NMRI outbred female mice	restraint 180 min	acute	20	165%	remained elevated for 120 min of restraint	did not measure

*dopamine did not return to baseline prior to stressor termination, so cannot assess; mPFC=medial prefrontal cortex, DA=dopamine, BL=baseline, SD=Sprague Dawley

Table 3. Effects of footshock stress on extracellular dopamine in the NAc

Reference	Species	Stressor parameters	Stressor pattern	Sample length (min)	Max DA (%BL)	Timecourse of DA efflux	Increase at termination?	Additional findings
Sorg and Kalivas (1991)	SD rat	0.55 mA/200ms/s for 20 min	acute	20	150%	increase with stressor, sustained for 60 min after termination	*	stress cross-sensitized to increased response to cocaine, but not vice versa
Young et al. (1993)	SD rat	0.33mA, 1s train of 6ms pulses, 25 Hz, 5 min intervals	acute and conditioned	10	193%	in non-conditioned rats, immediate increase during footshock, returning to baseline immediately after. Efflux augmented (to above 300%) with CS pairing	no	
Kalivas and Duffy (1995)	SD rat	0.35mA/200ms/s for 20 min	acute	20	230%	increased only at termination, sustained for an additional 20 min	yes	no increases observed in NAc core
Saulskaya and Marsden (1995)	Lister rat	0.5mA for 1sx10, 1 min apart, paired with tone	acute	20	135%	increased during footshock, remained elevated for 20 min after termination	*	intra-NAc dizocilpine did not affect initia response, but prevented sustained increase. AMPA antagonist no effect.
Motzo et al. (1996)	SD rat	0.2mA for 500 ms every s, 8 min	acute	10	160%	increased to 120% during stressor, maximal increase at termination, remained elevated for 40 min after termination of stressor	*	ICV allopregnanalone dose-dependently reduced basal dopamine and prevented mPFC and NAc footshock-induced increases. ICV midazolam similar effect but with greater potency
Fulford and Marsden (1998)	Lister rat	0.5mA for 1sx10, 1 min apart, paired with tone	acute, one group reared in isolation	20	173%, 200%	group reared rats showed delayed dopamine response to footshock, reaching max (173%) 60 min after stress initiation. Isolation reared showed immediate increase in NAc dopamine (200%), which remained elevated for 120 min. Both showed conditioned response, but more so in isolation reared	*	
Takahashi, Takada, Nagai, Urano, and Takada (1998)	Wistar rat	0.1mA for 10s, once per minute for 30 min	acute	30	167%	did not increase during stress, but sustained increase at termination	yes	prevented by chronic nicotine; stress resulted in nicotine cross-sensitization
Yamanashi,	SD rat	0.4mA, 200ms,	acute	20	140%	immediate increase during stress,	*	pretreatment with mecamylamine and

Miyamae, Misu, and Goshima (2001)		1Hz, 20 min				gradually increased, return to baseline by 120 min after stress initiation		diazepam each attenuated dopamine release
Young (2004)	SD rat	0.3mA, 1s train of 6ms pulses, 25 Hz; 4 presentations at 5 min intervals, repeated day 2. Separate group footshock paired with tone, and tone alone on day 2	repeated	1	175%, 225%	day 1: immediate increase during footshock (max 175%), which returned to baseline immediately upon termination, no habituation. Effect identical on day 2. Augmented when paired with tone (225%), and tone itself elicited 40% increase.	no	

*dopamine did not return to baseline prior to stressor termination, so cannot assess; NAc=nucleus accumbens, DA=dopamine, BL=baseline, SD=Sprague Dawley

Table 4. Effects of footshock stress on extracellular dopamine in the mPFC

Reference	Species	Stressor parameters	Stressor pattern	Sample length (min)	Max DA (%BL)	Timecourse of DA efflux	Increase at termination?	Additional findings
Feenstra et al. (2001)	Wistar rat	aversive conditioning: 10s white noise (25dB) immediately followed by 0.3mA footshock repeatedly presented 9x (conditioned group), or non-paired presentations (pseudo group) or no conditioning (control group). Later tested just CS	acute	16	250%, 200%, n/a	Significantly increased immediately in aversive conditioning (250%) and pseudo conditioning (200%) groups, gradually returning to baseline, with no changes in control group. Presentation of CS alone resulted in 150% increase in aversive group only	*	
Hamamura and Fibiger (1993)	Wistar rat	0.4mA, 10s duration, 50s interval, 20 min	acute, with possible prior injection stress (14d)	20	225%	immediate increase during footshock, slowly returning to baseline by 40 min after termination	*	

Sorg and Kalivas (1993)	SD rat	0.55mA/200ms/ s, 20 min	acute	20	200%	initial increase to 150% baseline, 200% in sample after termination, returning to baseline 40 min after termination	*	cocaine pretreatment abolished stress-induced DA response, and footshock reduced response to subsequent acute cocaine
Dazzi et al. (1995)	SD rat	0.2mA for 500ms every s for 8 min	acute	10	190%	initial increase to 140% baseline, peaking at termination, returning to baseline 20 min after termination. Repetition one hour later resulted in smaller increase (125%)	*	
Motzo et al. (1996)	SD rat	0.2mA for 500 ms, every s, for 8 min	acute	10	165%	immediate rise to 125% during footshock, peaking at termination, returning to baseline 30 min after termination	*	ICV allopregnanalone and midazolam dose dependently reduced basal DA and prevented stress-induced DA increase, midazolam with a greater potency
Dazzi, Serra, et al. (2001)	SD rat	0.2mA for 500ms, every 2, for 8 min	acute	20	190%	immediate increase during stress, no longer statistically significant 10 min later	sample included both stress and termination	2 week (but not single dose) imipramin or mirtazapine reduced and completely antagonized (respectively) increase in DA during footshock
Wedzony, Mackowiak, Fijal, and Golembiowska (1996)	Wistar rat	0.5mA/200ms for 5s twice during one 25 min session, then removal, brought back to context 25 min later with no shocks	acute	25	150%, 140%	increase to 150% during footshock, immediately returning to baseline, and increase to 140% basal levels in response to context	no	diazepam decreased outflow and blunted conditioned stress response. Ipsapirone and buspirone abolished stress-evoked elevation in dopamine
Dazzi, Spiga, et al. (2001)	SD rat	0.2mA for 500ms every s for 8 min	acute	20	190%	increased during stressor, immediately returned to baseline	sample included both stress and termination	2 week imipramine or mirtazapine inhibited or prevented (respectively) stress-induced DA increase.
Dazzi, Seu, Cherchi, and Biggio (2004)	SD rat	0.2mA for 500ms every s for 8 min	acute	20	190%	increased during stress, immediately returning to baseline in next sample	sample included both stress and termination	2 week olanzapine or clozapine prevented or significantly inhibited, respectively, stress-induced DA increase; haloperidol had no effect

*dopamine did not return to baseline prior to stressor termination, so cannot assess; mPFC=medial prefrontal cortex, DA=dopamine, BL=baseline, SD=Sprague Dawley

Table 5. Effect of tail pinch stress on extracellular dopamine in the NAc

Reference	Species	Stressor parameters	Stressor pattern	Sample length (min)	Max DA (%BL)	Timecourse of DA efflux	Increase at termination?	Additional findings
Klitenick, Taber, and Fibiger (1996)	SD rat	10 min	acute	10	121%	increase during and sample after release, gradual return to baseline	*	corticosterone increased DA response by 50%
King, Zigmond, and Finlay (1997)	SD rat	30 min	acute	15	120%	peaked during tail pressure, slow return to baseline after removal	*	no change in NAc core; DA efflux potentiated with mPFC lesions
Rouge-Pont, Deroche, Le Moal, and Piazza (1998)	SD rat	10 min	acute	20	130%	immediate rise during stress, gradual decrease back to baseline	*	blocking corticosterone decreased stress-induced DA release
Di Chiara et al. (1999)	SD rat	10 min, repeated after 120 min	acute, one group with 4wks CMS	10	75%, 130%	non-stressed showed 25% decrease immediately after tail first tail pinch, no change after second. prior CMS peak DA during first tail pinch, returned to baseline 80 min after release, similar effect during second tail pinch	*	
Naef et al. (2013)	SD rat	30 min	repeated 5 days	15	175%, 240%	day 1: immediate increase, slightly decreased after release, back to baseline following sample. day 5: sensitized response, peak during stressor, return to baseline 45 min after termination, but spiked again 90 min later	*	

*dopamine did not return to baseline prior to stressor termination, so cannot assess; NAc=nucleus accumbens, DA=dopamine, BL=baseline, SD=Sprague Dawley

Table 6. Effect of tailpinch stress on extracellular dopamine in the mPFC

Reference	Species	Stressor parameters	Stressor pattern	Sample length (min)	Max DA (%BL)	Timecourse of DA efflux	Increase at termination?	Additional findings
Jedema and Grace (2003)	SD rat	20 min	acute	20	180%	increased during stress, peaked immediately after termination, returned to baseline by 60 min after termination	*	AP5 did not blunt response, but CNQX did
Finlay et al. (1995)	SD rat	30 min	acute, with prior chronic cold exposure	30	154%	increased during stressor, remained elevated for 30 min after termination, no difference between controls and CCE	*	diazepam decreased basal DA and attenuated stress evoked increase in control rats only (no effect of diazepam in CCE group)
Venator, Lewis, and Finlay (1999)	SD rat	30 min	acute	15	200%	immediate increase, remained elevated after cessation, returning to baseline 60 min later	*	
Mendlin, Martin, and Jacobs (1999)	SD rat	20 min	acute, repeated once	20	144%	immediate increase, returned to baseline 40 min after sample termination	*	raclopride augmented effect
Di Chiara et al. (1999)	SD rat	10 min	acute, repeated once, one group with prior CMS	10	175%, 225%	Controls showed significant increase (175%) during tail pinch, slowly decreasing back to baseline by 30 min after release, same time course and magnitude with second m pinch. CMS animals showed significantly greater magnitude (225%) with similar time course	*	
Page and Lucki (2002)	SD rat	20 min	acute	20	n/a	no change	n/a	
Butts, Weinberg, Young, and Phillips (2011)	SD rat	15 min	acute	15	300%	immediate increase, gradual return to baseline by 90 min after termination of stressor	*	GR antagonism in the LV prevented increase
Butts and Phillips (2013)	SD rat	15 min	acute	15	225%	increase during stress, reduced upon termination and back to baseline by 30 min later	*	GR antagonists prevented increase

*dopamine did not return to baseline prior to stressor termination, so cannot assess; mPFC=medial prefrontal cortex, DA=dopamine, BL=baseline, SD=Sprague Dawley

Table 7. Effect of tail shock stress on extracellular dopamine in the mPFC

Reference	Species	Stressor parameters	Stressor pattern	Sample length (min)	Max DA (%BL)	Timecourse of DA efflux	Increase at termination?	Additional findings
Abercrombie, Keefe, DiFrischia, and Zigmond (1989)	SD rat	1.0mA pulses for 1s every 10s for duration of 1 min, repeated every 5 min for 30 min	acute	20	195%	immediate increase, peaking in 2nd half of stressor immediately returned to baseline after termination	no	
Gresch et al. (1994)	SD rat	1.0mA pulses for 1s every 10s for duration of 45s, repeated every 5 min for 30 min	acute, with 17-28d prior chronic cold exposure	30	150%, 271%	immediate increases in naïve (150% max) and CCE (271%), sustained for 60 min after termination	*	
Bland et al. (2003)	SD rat	1.0mA, 100 trials, ITI avg 60 s, terminated by escapable shock (ES) rat turning wheel	acute, escapable (ES) or inescapable (IS)	20	150%, 275%	ES showed initial immediate increase to 150%, returning to baseline after the first sample. IS increased to 150% initially, peaking at 275% subsequently and gradually returned to baseline by 200 min after initiation of stress	no	
Murphy et al. (2003)	SD rat	1.0mA constant pulse for 1s every 10s for duration of 45s, repeated every 5 min for 30 min	acute, with prior 14-20d chronic cold exposure	15	183%, 258%	naïve rats immediately increased mPFC DA (183%), returning to baseline immediately upon shock termination. Prior CCE rats: immediate increase to 258%, while also immediately returning to baseline on termination	no	ICV CRH antagonist did not alter evoked dopamine increase, but attenuated CRF-induced dopamine increase

*dopamine did not return to baseline prior to stressor termination, so cannot assess; mPFC=medial prefrontal cortex, DA=dopamine, BL=baseline, SD=Sprague Dawley

Table 8. Effect of other stressors on extracellular dopamine in the NAc

Reference	Species	Stressor parameters	Stressor pattern	Sample length (min)	Max DA (%BL)	Timecourse of DA efflux	Increase at termination?	Additional findings
Azzi et al. (1998)	Wistar rat	10 min forced swim	acute	20	n/a	no effect in NAcSh, although the sample was twice as long as the stressor	no	
Y. L. Wu et al. (1999)	Wistar rat	"psychological"-rat in center compartment of a 9 compartment chamber divided by plexiglass, all other compartments received shocks at 3.0mA for 5s in 30s intervals for 20 min	acute	20	155%	significantly increased during stress, remained elevated after termination, returned to baseline 40 min after termination.	*	no effect in NAc core
Merali et al. (1997)	SD rat	airpuff and/or cytokine (IL-8) injection	acute	30	n/a	no effects	n/a	
Feenstra, Botterblom, and van Uum (1998)	Wistar rat	16 min handling	acute	15	130%	peaks during handling, sustained 15 min after termination	*	local inhibition (reverse dialysis) of ionotropic glutamate receptors did not affect handling induced corticosterone, dopamine, or noradrenaline release, nor did an mGluR antagonist or GABAE agonist.
Inglis and Moghaddam (1999)	SD rat	20 min handling	acute	20	150%	not significantly elevated during handling (125%), but significant increase at release (150%), which was sustained for 40 min	yes	
Cenci, Kalen, Mandel, and Bjorklund (1992)	SD rat, female	15 min handling	acute	15	n/a	no effect	n/a	
Tidey and	Long	social threat; 40	acute, with	20	160%	initial response to cage without	yes, not seen in	

Miczek (1996)	Evans rat	min in aggressors homecage without aggressor, 60 min with aggressor behind screen, 40 min again with no aggressor	prior history of 4 social defeats			aggressor (137%), with peak in response to introduction of aggressor (160%), returned to 130% when aggressor was removed, and increased again (148%) when returned to homecage	controls	
Tidey and Miczek (1997)	Long Evans rat	social threat; 40 min in aggressors homecage without aggressor, 60 min with aggressor behind screen, 40 min again with no aggressor	acute, with prior history of 4 social defeats	20	143%	initial response to aggressor's homecage (133.5%), with peak in response to resident aggressor (143%). Gradual return to baseline, but did not measure return to homecage	did not measure	faster acquisition of cocaine self-administration compared to rats with no history of defeat
Jezierski, Zehle, Bock, Braun, and Gruss (2007)	juvenile degu	60 min isolation, with or without 3 weeks daily maternal separation	acute	20	169%, 150%	larger increase in control compared to early separation group, both groups returned to baseline immediately upon reunion	no	chronic methylphenidate cross-sensitizes

*dopamine did not return to baseline prior to stressor termination, so cannot assess; NAc=nucleus accumbens, DA=dopamine, BL=baseline, SD=Sprague Dawley

Table 9. Effect of other stressors on extracellular dopamine in the mPFC

Reference	Species	Stressor parameters	Stressor pattern	Sample length (min)	Max DA (%BL)	Timecourse of DA efflux	Increase at termination?	Additional findings
Merali et al. (1997)	SD rat	air puff and/or cytokine (IL-8) injection	acute	30	n/a	no effect	n/a	
Azzi et al. (1998)	Wistar rat	10 min forced swim	acute	20	n/a	marginal increase, sustained at least 200 min, but does not report baseline	*	repeated administration of neurotensin antagonist has no effect
Jordan et al. (1994)	SD rat	8 min forced swim, repeated 24 h later	repeated once	30	n/a, 441%	no effect on day 1, but second day significant increase, persisting for 60 min after termination	*	
Petty et al. (1997)	SD rat	8 min forced swim, repeated 24 h later	repeated once	30	n/a, 200%	Day 1: no effect on dopamine. Day 2: increased to 200% during stress, peaked after termination at approximately 300%, sustained for 90 min.	*	flumazenil increased stress response on day 1; diazepam attenuated stress response on day 2.
Cenci et al. (1992)	SD rat, female	15 min handling	acute	15	n/a	no effect	n/a	
Enrico, Bouma, de Vries, and Westerink (1998)	Wistar rat	15 min handling	acute	15	225%	150% during stress, increased to maximal 225% after release, gradually decreased back to baseline by 90 min after termination	*	intra-VTA baclofen, CPP, AP5, CNQX suppressed handling induced increases, while intra-VTA muscimol, atropine, mecamylamine, and +-HA-966 did not
Feenstra et al. (1998)	Wistar rat	16 min handling	acute	15	300%	peaked during handling, gradual return to baseline by 60 min after release	*	local inhibition (reverse dialysis) of ionotropic glutamate receptors did not affect handling induced corticosterone, dopamine, or noradrenaline release, nor did an mGluR antagonist or GABAE agonist.
Takahata and Moghaddam (1998)	SD rat	20 min handling	acute	20	150%	increased during handling, immediate return to baseline after termination	no	blockade of AMPA and NMDA receptors in the VTA during handling reduced dopaminergic response
Inglis and Moghaddam (1999)	SD rat	20 min handling	acute	20	150%	immediate increase, sustained 20 min after release	*	
Del Arco and Mora (2001)	Wistar rat	40 min handling	acute	20	200%	increase sustained during handling, decreased slightly at termination and return to baseline by 20 min after release	*	no effects on GABA or glutamate in mPFC

Del Arco, Segovia, and Mora (2001)	Wistar rat	40 min handling	acute	20	189%	increased during handling, immediate return to baseline after termination	no	
Marsteller et al. (2002)	SD rat	15 min handling	acute	15	155%	increase during handling, peak after cessation, rapid return to baseline	*	
Del Arco, Segovia, Garrido, de Blas, and Mora (2007)	Wistar rat	40 min handling	acute	20	150%	increase during handling, remained elevated at release, return to baseline by 40 min after termination	*	no effects of prior environmental enrichment
Kawahara, Kawahara, and Westerink (1999)	Wistar rat	10 min handling	acute	15	175%	increased during handling, slow return to baseline	*	intravenous infusion of sodium nitroprusside (induces hypotension) also potently increases mPFC DA
Pehek, Nocjar, Roth, Byrd, and Mabrouk (2006)	SD rat	20 min handling	acute	20	182%	increased during handling, immediate return to baseline	no	
Tidey and Miczek (1996)	Long Evans rat	social threat; 40 min in aggressors homecage without aggressor, 60 min with aggressor behind screen, 40 min again with no aggressor	acute, with prior history of 4 social defeats	20	160%	initial response to cage without aggressor (136%), with peak in response to introduction of aggressor (162%), returned to 130% when aggressor was removed, and increased again (148%) when returned to homecage	yes, not seen in controls	
Watt et al. (2014)	SD rat	adolescent social defeat; 20 min exposure to resident	acute, with three prior social defeats	20	150%	increased during encounter, slowly returned to baseline by 60 min after termination	*	
Jezierski et al. (2007)	juvenile degu	60 min isolation, with or without 3 weeks daily maternal separation	acute	20	171%, 146%	larger increase in control compared to early separation group, both groups returned to baseline immediately upon reunion	no	chronic methylphenidate cross-sensitizes

W. R. Wu et al. (2003)	SD rat	predator odor (fox) for 20 min	acute	20	205%	gradual increase in dopamine that was maximal 120min after beginning of odor presentation	*

*dopamine did not return to baseline prior to stressor termination, so cannot assess; mPFC=medial prefrontal cortex, DA=dopamine, BL=baseline, SD=Sprague Dawley

Table 10. Group sizes for Experiment 1, microdialysis during defeat for dopamine in the mPFC and NAcSh after CRF-R1 or CRF-R2 antagonism

	Day 1		Day 10	
Pretreatment	**mPFC**	**NAcSh**	**mPFC**	**NAcSh**
aCSF	7	9	5	5
CP	4	5	6	4
A2B	6	6	5	5

Table 11. Group sizes for Experiment 2, microdialysis during defeat for CRF in the VTA

	Day 1		Day 10	
Group	**aVTA**	**pVTA**	**aVTA**	**pVTA**
Stressed	5	5	5	6
Control	3	3	3	3

Table 12. Group sizes for Experiment 3A, role of VTA CRF receptors during stress on the induction of dopaminergic cross-sensitization to cocaine

Pretreatment	Control	Stressed
aCSF	5	5
CP-500 ng/side	5	5
CP-50 ng/side	--	7
A2B-1000 ng/side	5	6
A2B-100 ng/side	--	4

Table 13. Group sizes for Experiment 3B, role of VTA CRF receptors during stress on escalated cocaine self-administration

Pretreatment	Control	Stressed
aCSF	13	11
CP-500 ng/side	7	10
CP-50 ng/side	3	8
A2B-1000 ng/side	9	13
A2B-100 ng/side	8	8

Table 14: Group sizes for Experiment 4, role of VTA CRF and its receptors after stress on context-induced reinstatement after forced abstinence

	Microinjection			
	aCSF	**CP**	**A2B**	**Microdialysis**
Control	7	10	7	7
Stressed	9	8	9	6

Table 15. Effects of social defeat stress on stable cocaine self-administration fixed ratio 5 (FR 5) rate (response/minute) and progressive ratio (PR) infusions

Group	Drug	n	FR 5[a]	PR[b]
Control	aCSF	13	0.64 ± 0.05	8.96 ± 0.48
Control	CP 50	7	0.90 ± 0.14 [1]	10.0 ± 0.83
Control	CP 500	3	1.13 ± 0.18 [1]	10.9 ± 0.29
Control	A2B 100	9	0.92 ± 0.11	11.0 ± 0.80
Control	A2B 1000	3	0.71 ± 0.11	12.7 ± 1.17
Stress	aCSF	11	0.80 ± 0.08	10.7 ± 0.69
Stress	CP 50	8	0.87 ± 0.07	11.9 ± 0.52
Stress	CP 500	10	0.99 ± 0.11	9.75 ± 0.67
Stress	A2B 100	8	0.93 ± 0.11	11.2 ± 0.55
Stress	A2B 1000	13	0.77 ± 0.06	10.8 ± 0.77

[a]Data represent the mean ± SEM of the last 3 d of stable FR 5 rate (responses/min) on the active lever for cocaine delivery.
[b]Data represent the mean ± SEM number of infusions self-administered during PR sessions.
*p<0.05 vs aCSF