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Augmentation of Extracellular Glutamate in the Ventromedial Prefrontal Cortex and its Role During the Incubation of Cocaine Craving in Rats

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

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by

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ABSTRACT

Augmentation of Extracellular Glutamate in the Ventromedial Prefrontal Cortex and its Role During the Incubation of Cocaine Craving in Rat

by

Christina Borom Shin

Incubation of craving is the phenomenon in which craving intensifies over the course of abstinence. Although understanding craving may be key in understanding why relapse occurs, not much is known about craving, especially the underlying mechanisms that occur in the brain. Clinical and preclinical models have recently implicated the ventromedial prefrontal cortex as one of the key regions in this phenomenon. In order to further characterize this region's role in incubation, a variety of behavioral, pharmacological, and molecular techniques are used to examine how withdrawal from long access cocaine self-administration directly affects the glutamatergic system. The series of experiments detailed in this dissertation aim to: (1) characterize extracellular glutamate fluctuations in the vmPFC during the incubation of cocaine-seeking, (2) characterize metabotropic glutamate receptor (mGlu) 2/3 changes during both short term and long term withdrawal, and (3) to determine the functional relevance of endogenous glutamate in the two subregions of the vmPFC. Taken together, these experiments suggest that although there seem to be no changes in mGlu2/3 at either point of withdrawal, glutamate in the prelimbic cortex of the vmPFC is functionally relevant in the incubation of cocaine seeking. Together, these results suggest

pharmacotherapeutic strategies geared toward damping excitatory glutamate drive within corticofugal projections from the prelimbic cortex may be effective to prevent incubated cue-induced craving during protracted abstinence.

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List of Abbreviations

3WD- 3 withdrawal days
30WD- 30 withdrawal days
ACC- Anterior cingulate cortex
AMPAR- AMPA receptors
ANOVA- Analysis of variance
AUC- Area under the curve
BA- Brodmann's area
BDNF- Brain-derived neurotrophic factor
BLA- Basolateral amygdala
B/M- Baclofen + muscimol
BNST- Bed nucleus of the stria terminalis
CEA- Central nucleus of the amygdala
CI- Calcium-impermeable
COC- Cocaine
CP- Calcium-permeable
DA- Dopamine
dlPFC- Dorsolateral prefrontal cortex
dmPFC- Dorsomedial prefrontal cortex
ERK- Extracellular signal-regulated kinases
Extinction Test- 2 hour cue-reinforced extinction-like drug-seeking test
fMRI- Functional magnetic resonance imaging
GLT- Glutamate glial transporter
GLU_{EC}- Extracellular glutamate
GLUT- Glutamate
IL- Infralimbic cortex
IM- Intramuscular
IP- Intraperitoneal
IV- Intravenous
LgA- Long access
LY38- LY379268
MDT- Mediodorsal nucleus of the thalamus
Meth- methamphetamine
mGlu- Metabotropic glutamate receptor
mPFC- Medial prefrontal cortex
MSN- Medium spiny neurons
NAC- Nucleus accumbens
NEUT- Neutral
OFC- Orbital frontal cortex
PCR- Polymerase chain reaction
PET- Positron emission tomography
PFC- Prefrontal cortex
PL- Prelimbic cortex
SA- Self-administration

SEM- Standard error of the mean
ShA- Short access
SUC- Sucrose
TBOA- Threo-β-hydroxyaspartate
TMS- Transcranial magnetic stimulation
TTX- Tetrodotoxin
WD- Days withdrawal
vmPFC- Ventromedial prefrontal cortex
VTA- Ventral tegmental area

Chapter 1:

General Introduction

1.1 Cocaine Addiction: A Public Health Concern

1.1.1 Prevalence of Cocaine Addiction

Cocaine addiction is a chronic brain disease characterized by compulsive drug use and drug-seeking, despite negative consequences. It involves a cycle of intoxication, bingeing, withdrawal, and craving (Goldstein & Volkow, 2011). In 2011, about 2.5 million emergency department visits involved drug misuse or abuse, a 52% increase since 2004 (Substance Abuse and Mental Health Services Administration, 2013a). Of the 2.5 million visits, over 40% were cocaine-related, the highest in the illicit drug category (Substance Abuse and Mental Health Services Administration, 2013a). In 2012, 8.0 million persons aged 12 or older needed treatment for an illicit drug use problem (Substance Abuse and Mental Health Services Administration, 2013a). Of these people, only 1.5% received the treatment they needed, leaving 6.5 million people (2.5% of the total population) untreated.

The latest results of the Substance Abuse and Mental Health Services Administration survey indicate that of the 21.7 million persons aged 12 or older with general substance abuse problems in 2015, just under 1 million people aged 12 or older were diagnosed with a cocaine use disorder, with about 0.1% of all adolescents aged 12 to 17 having a cocaine use disorder in the past year (Center for Behavioral Health Statistics and Quality, 2016). Of the 21.7 million persons with substance abuse issues, only 10.8% received the treatment they needed (Center for Behavioral Health Statistics and Quality, 2016). This is problematic as addicts relapse at least once on their road to recovery, with the probability of relapse remaining high even after months of abstinence. With this lack of treatment for addiction, especially any effective treatment for cocaine in particular, addicts have a difficulty journey

to full recovery. Cocaine addiction is a particularly interesting case as we know cocaine's mechanisms of action on the brain, yet we still do not have any effective treatments for Cocaine Use Disorder.

1.1.2 Cocaine Craving, Relapse, and its Triggers

One of the hallmark features of addiction is relapse - the resumption of drug-taking following a period of abstinence. In humans, cocaine relapse can be instigated by a number of stimuli, some of which include re-introduction to the drug itself (Jaffe et al., 1989), stressors (Sinha et al., 1999), cocaine-associated contexts, and cocaine-associated cues (Childress et al., 1993a; Childress et al., 1993b). Cocaine-associated cues and contexts are able to induce relapse as they elicit cocaine craving, or a powerful yearning for cocaine. One of the ways this intense craving manifests is an emotional state that is engulfed by a perseveration of the cocaine high. This fervent state of craving is also coupled with a loss of encumberment from the negative consequences of the drug (Childress et al., 1999). The two culminate to a point where the addict can no longer tolerate sobriety and eventually relapses. As cocaine craving brought on by cocaine-associated cues has the susceptibility of extending into prolonged abstinence (Childress et al., 1993; Grimm et al., 2001; Goldstein & Volkow, 2011), this dissertation will focus on this form of induced relapse.

1.1.3 Cocaine Use is Associated with Prefrontal Cortex Deficits in Humans

The prefrontal cortex (PFC) is responsible for a myriad of important behaviors, including (but not limited to): inhibition control, attention, working memory, emotion, and motivation. These important faculties, and the regions responsible for these faculties, are known to be disrupted in individuals addicted to cocaine and other drugs of abuse (e.g., Goldstein &

Volkow, 2011; Koob & Volkow, 2016), and this disruption may account for the compulsive, relapsing nature of addiction.

Cocaine addicts exhibit gross anatomical changes in their PFC. As measured by magnetic resonance imagining (MRI) scans, cocaine addicts have lower gray matter volumes in the orbital frontal cortex (OFC), as well as lower white matter volumes in the right anterior cingulate cortex and left inferior and medial frontal gyrus compared to healthy controls (Moreno-López et al., 2012). A positive correlation between impulsivity and gray matter volume in specifically the left inferior and middle frontal gyrus is observed in cocaine addicts but not in healthy controls (Moreno-López et al., 2012). Another study using MRI scans in healthy controls versus long-term cocaine users reported similar findings: cocaine use reduces gray matter volume in OFC and dorsolateral PFC (dlPFC) (Alia-Klein et al., 2011).

Those with cocaine use disorder also manifest different molecular changes in their PFC. For example, cocaine abusers are reported to have higher glucose and total creatine ratios in the pregenual anterior cingulate cortex (ACC) and the right dlPFC (Hulka et al., 2016). As higher cocaine hair concentrations were associated with lower glutamine/creatine ratios in the pregenual ACC, this suggested that cortical glutamate cycling is altered by cocaine use within the past 6 months (Hulka et al., 2016). A study using cDNA microarrays examined gene expression in the dlPFC from post-mortem male cocaine users and reported alterations in transcripts indicative of changes in mitochondria function, energy metabolism, and neuronal plasticity, among others, compared to healthy controls (Lehrmann et al., 2003). Chronic cocaine use was also associated with decreased expression of brain derived

neurotrophic factor (BDNF) 1 and 4 in the frontal cortex, as measured by BDNF mRNA levels through real time polymerase chain reaction (PCR) (Jiang et al., 2009).

Along with the observed PFC deficits, cocaine addicts experience increased impulsivity due, in part, to frontostriatal dysfunction (e.g., Jentsch & Taylor, 1999). Indeed, cocaine users have altered cerebral blood flow in the OFC as measured by positron emission tomography (PET) imaging during a decision-making task that measures the ability to weigh short-term rewards against long-term losses (Bolla et al., 2003). More specifically, cocaine users experience increased activation in the OFC and less activation in the right dlPFC and left medial PFC compared to healthy controls (Bolla et al., 2003). As these changes were recorded in addicts abstinent for 25 days, these alterations of the PFC seem to be enduring. Chronic cocaine abusers also have impaired performance on reward/punishment contingencies during a gambling task, yet these impairments seem specific to long-term versus short-term rewards as users do not suffer generalized deficits in regards to choice and planning as measured by a normal card sorting task (Grant et al., 2000). In the delay-discounting model of impulsivity, cocaine addicts have higher discount rates, or the reduction of the present value of a future reward, for real monetary rewards than do healthy controls, in conjunction with having higher discount rates for future rewards in general (Kirby et al., 2004). In addition, Bechara and colleagues report cocaine addicts closely resemble patients with bilateral lesions of the ventromedial PFC (vmPFC) on their ability to perform decision-making on electronic card gambling tasks (Bechara et al., 2001).

Cocaine use is linked to basal hypofunction of the PFC in addicts (e.g., Volkow et al., 2003), yet chronic cocaine users exhibit higher than usual PFC activation in response to cocaine-associated cues (e.g., Goldstein & Volkow, 2011). As regions of the PFC are known

to be involved in processing reward-associated cues (Kringelbach, 2005), higher activation of the PFC is thought to be associated with cocaine craving following cue exposure. Indeed, PFC activity has been positively correlated with self-reported craving (Wang et al., 1999; Garavan et al., 2000; Goldstein et al., 2007). A study using PET imaging reported PFC activation, along with increased heart rate and blood pressure, in 7-day abstinent cocaine addicts when discussing how they prepare their cocaine, versus a neutral theme interview (Wang et al., 1999). Another study using functional magnetic resonance imaging (fMRI) reported increased PFC activation when cocaine users watched a video of individuals smoking crack cocaine, compared to a sexually explicit film (Garavan et al., 2000). Using many different imaging techniques, numerous studies have since replicated the findings that the PFC exhibits increased activation in response to cocaine cues in addicts, relative to healthy controls (PET: Grant et al., 1996; Bonson et al., 2002; fMRI: Maas et al., 1998; functional connectivity MRI; Wilcox et al., 2011). In addition, transcranial magnetic stimulation (TMS) studies also indicate the PFC is a key region involved in craving, as repetitive TMS of the dlPFC significantly lowered instances of cocaine craving (Camprodon et al., 2007; Politi et al., 2008; Terraneo et al., 2016; Rapinesi et al., 2016), reduced overall cocaine intake 3-6 months after treatment (Bolloni et al., 2016), as well as decreased cocaine use up to 29 days compared to disulfiram-treated users (Terraneo et al., 2016).

Additionally, increased connectivity between regions responsible for processing reward and reward cues (i.e., ventral striatum and orbital frontal cortex) seen in the brains of cocaine addicts may explain why addicts place high salience on cocaine-associated cues (Wilcox et al., 2011). Together, the evidence points to neurological alterations occurring in the PFC of

cocaine addicts that may cause them to perseverate on drug cues over natural reward cues, which may ultimately lead to higher instances of relapse.

1.1.4 Incubation of Craving in a Clinical Setting

Although baseline craving dissipates in a time-dependent manner following cessation of drug-taking, drug-associated cues can elicit craving that intensifies during abstinence, a phenomenon dubbed “incubation of craving” (Gawin & Kleber, 1989; Tran-Nguyen et al., 1998; Grimm et al., 2001; Lu et al., 2004; Pickens et al., 2011). Gawin and Kleber first reported this phenomenon in 1986 in their monumental study delineating enduring psychiatric disorder symptoms (such as major depressive disorder and attention deficit hyperactivy disorder) from symptoms brought on by cocaine-use binges (Gawin & Kleber, 1986). This study described a three-phase sequence of post-cocaine abuse: 1) crash, immediate withdrawal symptoms such as dysphoria and anhedonia brought on by the end of a cocaine binge, 2) withdrawal, a short period of baseline functioning followed by a mild version of phase one in addition to perseveration on past cocaine binges and craving, and 3) extinction, a phase described by a baseline affective state before cocaine use dispersed with very few bouts of cocaine craving (Gawin & Kleber, 1986). After abstinence of drug-use and also the absence of craving for approximately 28 weeks, half the subjects reported feeling a fervent feeling of craving after exposure to cocaine-associated friends or environments (Gawin & Kleber, 1986). Since then, incubation of craving has been more comprehensively studied and it has been reported that in human cocaine addicts, craving in response to cocaine cues presents itself in an inverse U shape over the course of a year: peaking at 1 to 6 months before declining after one year of abstinence (Parvaz et al., 2016).

Along with cocaine, incubation of craving has been characterized in humans addicted to a variety of drugs. For example, along with eliciting craving, heroin-associated cues were also able to increase cardiovascular measures (e.g., heart rate, systolic pressure) and negatively impact decision-making by increasing impulsivity in a gambling task in former heroin addicts who were abstinent for 1-24 months (Wang, G.B. et al., 2011). Methamphetamine abusers also report decreased craving during abstinence, with cue-reinforced craving increasing until 3 months of abstinence and eventually decreasing and tapering off at 6 months of abstinence (Wang et al., 2013). Similar patterns have also been reported for nicotine (Bedi et al., 2011) and alcohol (Li et al., 2014).

Unfortunately, not much research has been done on specific brain regions involved in incubated drug craving. What we do know is that PFC activity in response to drug-associated cues is known to be enduring. During short-term abstinence in heroin users, fMRI scans show that the PFC was activated when users were presented heroin-associated cues (Li et al., 2012). These changes are also known to extend into long-term abstinence. Using fMRI imaging scans, Janes and colleagues (2009) reported the PFC is activated when smokers in extended abstinence are presented with cigarette-associated cues, but current smokers had little to no activation in these areas in response to smoking related cues (Janes et al., 2009). Another study focused on former heroin users on stable methadone treatment who were 5-24 months abstinent, a subject profile typically associated with low heroin craving. These subjects exhibited increased PFC activation in response to heroin-associated cues but not to neutral cues, indicating heroin-associated cues are able to elicit strong PFC activation despite no experience of craving (Wang, W. et al., 2011). Together, these data indicate that in drug abusers, neural adaptations are occurring in the PFC during extended abstinence that

may render addicts more sensitive to drug-associated cues. As drug-associated cues are known to be a powerful trigger of relapse, the above mentioned changes may indicate why addicts are so prone to relapse during protracted abstinence.

1.1.5 Drug Craving in Humans Versus Drug-Seeking in Rodents

Addiction studies using human subjects are not ideal due to a variety of reasons, some of which include complex ethical issues (e.g., Scott & White, 2005) to the massive number of confounding variables that are impossible to control (e.g., environmental differences, genetics, health/disease comorbidity etc.). Due to these reasons, results of human addiction studies can only be correlative and never causal. Thus, many addiction researchers turn to animal models, especially rodent models, in order to infer causation. However, craving is an internal state that cannot be examined directly in animals. Thus, craving or the wanting of drug must be inferred from the animal's behavior and the term "drug-seeking" is typically applied.

For the purposes of this dissertation, drug-seeking is operationally defined as emitting an operant response (lever pressing) for delivery of drug-associated stimuli (20 second light and tone), in the absence of any drug delivery (i.e., under extinction conditions; see section 1.3.1. for a more detailed description of the paradigm). Presumably, the number of times the animal presses the lever in the absence of cocaine is thought to represent the motivation of the animal to obtain cocaine or how much the animal is seeking the drug. Thus drug-seeking is used as a proxy to drug craving.

1.2 Ventromedial Prefrontal Cortex

1.2.1 PFC Homology: from Humans to Rodents

Humans and primates differ from all other mammals due to our evolution of the PFC, making the study of this region in non-human animal models arguably problematic. While homologous regions have been postulated in many species, the PFC still remains enigmatic. The little we know is due to our research on other species, the conclusions from which we carry over to infer into our own behavior. Conclusions drawn from other species are done with caution, as homology of the brain is not consistent across species. If homology is not taken into account, misconceptions may arise in regards to brain function between species.

One of the ways the PFC of primates has been defined over the years is by the presence of a granular layer (layer 4) in the cortex. Yet, this becomes problematic as the rodent brain completely lacks granular cortex and is only comprised of allocortex and agranular cortex (Wise, 2008). Clearly, no homolog exists between granular regions of primate PFC and rodent PFC, but homology between the species exists in the agranular regions of their PFCs. This has been argued for by cytoarchitectonics, topology, and corticostriatal organization between primate and rat PFCs (Wise, 2008). Thus, conclusions drawn from rat PFC studies, specifically regarding the delineation of the infralimbic, prelimbic, agranular insula, agranular orbital, and anterior cingulate cortices (ACC), can be cautiously generalized to humans, as they have presumed homologous areas within the primate PFC. For the purposes of this dissertation, this will suffice, as I will only be focusing on the ventromedial PFC (for reasoning see section 1.4.5).

This being said, in rodents, the PFC is located at the anterior tip of the frontal cortex and is comprised of the anterior cingulate, medial PFC, and orbital frontal cortex (OFC). Based on their thalamic inputs, the rodent prelimbic cortex (PL) is considered to be equivalent to Brodmann area (BA) 32, or the pregenual anterior cortex in humans, while the infralimbic cortex (IL) is equivalent to BA 25, or the subgenual anterior cortex in humans (Wise, 2008; Goldstein & Volkow, 2011; Gass & Chandler, 2013). The ACC corresponds to BA 32 and 24, while the medial OFC to BA 11, 13, and 14 (Wise, 2008).

1.2.2 Ventromedial Prefrontal Cortex Circuitry in the Rat

From dorsal to ventral, the medial PFC (mPFC) is subdivided into the ACC, the PL, and the IL. Unfortunately, as these regions do not have well demarcated boundaries, many researchers simply divide the PFC in half into the dorsomedial PFC (dmPFC; ACC plus the dorsal portion of the PL) and ventromedial PFC (vmPFC; ventral portion of the PL plus the IL), although ample research has shown that the ACC, PL and IL differ vastly in their function. For example, general anatomical studies report that the ACC is involved in eye movement control, the PL in cognitive processes, and the IL in visceromotor processes, to name a few (Vertes, 2004). Along with these properties, the PL and IL have been anatomically and functionally linked with the limbic system (Vertes, 2004), thus making these subregions prime candidates involved in the addiction process. For the purposes of this dissertation, the vmPFC will be divided into the PL and IL regions of the mPFC.

The PL and IL of the vmPFC are defined by their reciprocal connections with the mediodorsal nucleus of the thalamus (MDT): the central MDT projects to the PL while the ventromedial MDT projects to the IL (Vertes, 2004; Hoover & Vertes, 2007). Studies

injecting the retrograde tracer fluorogold (Hoover & Vertes, 2007), and the anterograde tracer *phaseolus vulgaris*-leucoagglutinin (Sesack et al., 1989; Vertes, 2004) have elucidated the intricate afferent and efferent connections of the PL and IL. As these two regions are interconnected to the whole brain, for simplicity's sake, I will only focus on their connections with brain regions implicated in addiction. With this said, these anatomical studies report cortico-cortical projections from the PL to the insular, OFC, cingulate, IL, and to itself, with the PL being reciprocally connected to all these regions but itself. The IL also sends efferent projections to the same regions, but only receives afferent cortical projections from the cingulate cortex and itself. Both PL and IL are reciprocally connected to basolateral amgydala (BLA), a region known to be involved in reinforcer valuation, and also the ventral tegmental area (VTA), a region that is involved in generation of motivation and goal directed behavior. The PL and IL also send efferent projections to the shell and core of the nucleus accumbens, and to the central nucleus of the amygdala (CEA) - two regions also heavily implicated in addiction - although these regions do not send reciprocal connections back to the vmPFC (see Figures 1 and 2 for more detailed overview). It must be said that although these areas have similar connections to regions within the brain, they differ in the strength and density of their projections to each region (Sesack et al., 1989; Vertes, 2004; Hoover & Vertes, 2007).

While extremely similar in their projections, there are distinct differences between these two regions. This becomes apparent in their projections to the amygdala, as the IL sends efferent projections to all the different nuclei of the amygdala, while the PL does not send efferents to specifically the anterior area, cortical, medial, and posterior amygdala Vertes, 2004). Interestingly, the IL receives reciprocal connections from all regions of the amygdala

excluding the CEA. Though the PL and IL both send projections to the two regions of the nucleus accumbens (NAC), they differ in their projection density. For example, the rostral-caudal and dorsal portions of the PL send a majority of their efferents to the NAC core, while the ventral PL and IL send their efferents to the NAC shell (Vertes, 2004). Thus, though there is light overlap, the PL seems to preferentially project to the NAC core while the IL to the NAC shell (Ma et al., 2014). In addition, the IL sends efferent projections to the bed nucleus of the stria terminalis (BNST) and ventral pallidum, while the PL does not. Due to its prime connections with the reward centers of the brain, along with clinical evidence of dysfunction within the PFC following chronic cocaine use, and recent findings tying the vmPFC to cocaine craving (see Section 1.4), the vmPFC of the rodent will be the target region of study for this dissertation.

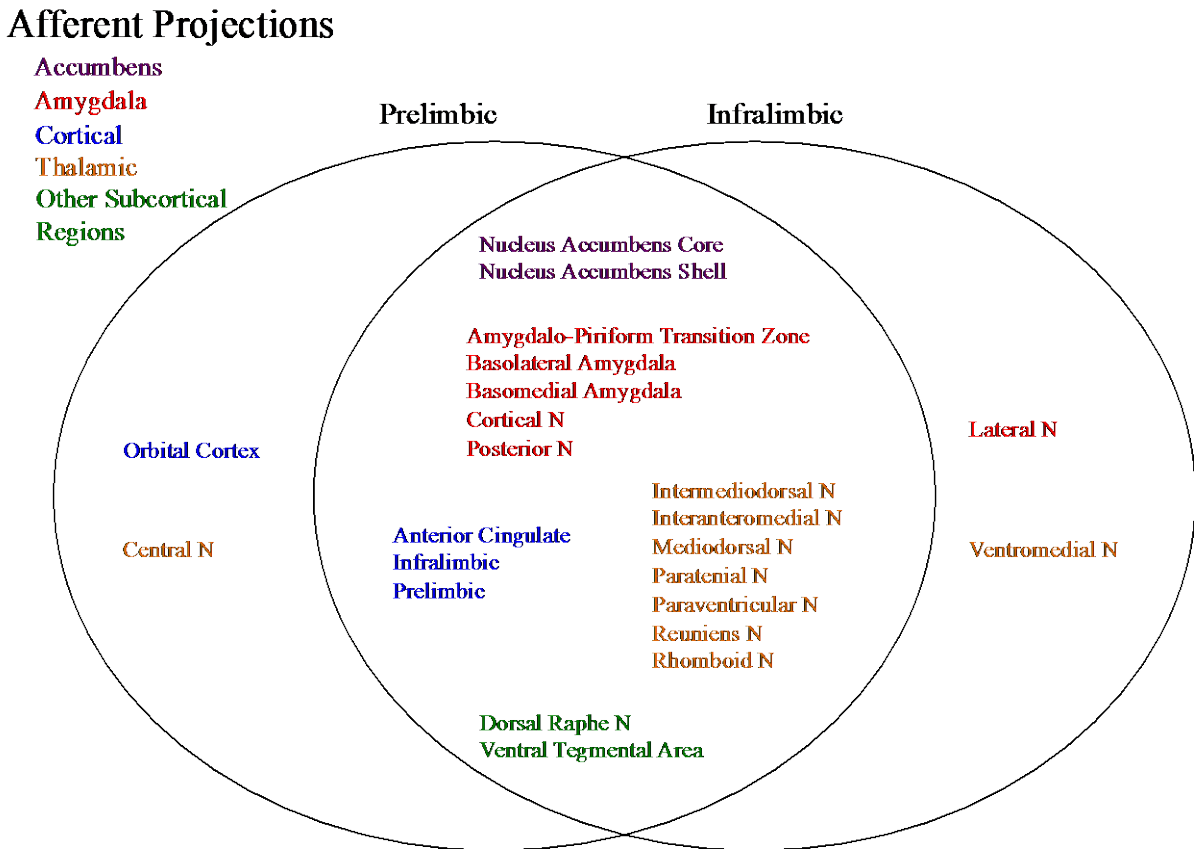

Figure 1. Afferent projections of the prelimbic and infralimbic cortex to different brain regions known to be involved in addiction. Adapted from results of Sesack et al., 1989 and Vertes, 2004. N: nucleus.

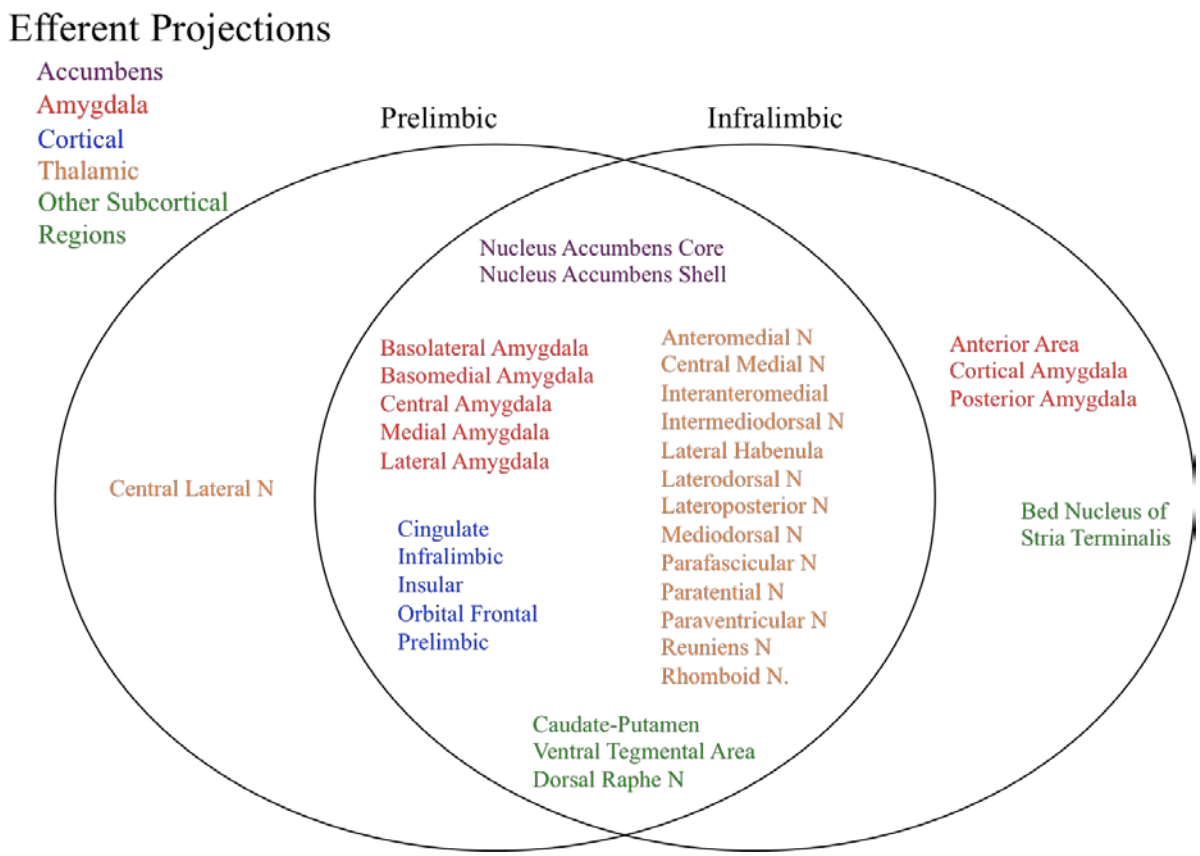

Figure 2. Efferent projections of the prelimbic and infralimbic cortex to different brain regions known to be involved in addiction. Adapted from results of Hoover & Vertes, 2007. N: nucleus.

1.3 Animal Models Implicating Glutamate in the Prefrontal Cortex in Addiction

As many barriers exist in studying addiction in the human brain, the bulk of addiction research is traditionally done in animal models, in particular to investigate the cellular and molecular mechanisms of cocaine addiction in the brain. These models are reliable and have become well established, with different paradigms existing to measure the many facets of drug addiction. Of the different models, intravenous self-administration has high predictive as well as high construct validity, and is a reliable predictor of abuse liability in humans (Horton, 2013). It also most closely resembles the human condition of drug-taking compared to other preclinical models, as it allows the animal to control their own drug intake. Thus, the main behavioral paradigm used in this dissertation will be the self-administration model, in conjunction with other procedures.

1.3.1 The Self-Administration Paradigm

In the rodent model of self-administration, an indwelling catheter is inserted into the jugular vein, which allows direct intravenous delivery of drug. The rat is subsequently introduced to an operant chamber where they are conditioned to perform a response (such as pressing a lever) that results in the delivery of drug. This model is particularly unique as it gives the researcher control over many different aspects of drug taking, such as different schedules of reinforcement, speed of drug delivery, and pairing of different discrete cues to drug delivery. This model is also multifaceted as it allows for different training schedules (e.g., progressive ratio, extinction, reinstatement, etc.). It is thus considered the “gold

standard" procedure for measuring reinforcing effects of a drug, as well as drug consumption.

Over the past few decades, the drug self-administration field has focused intense efforts on developing animal models of drug self-administration that better fit the clinical criterion for a diagnosis of substance use disorder/addiction. Such models should exhibit high levels of drug intake, an escalation of drug intake with subsequent drug experience, and continued drug intake in the face of negative consequences (e.g., punishment), as experienced by human addicts. One simple procedural modification that can accomplish these goals simply involves allowing animals longer access to the drug than has been historically employed in the literature (i.e., >2 hour/day). Indeed, ample evidence indicates that the pattern of drug intake observed under "long-access" (LgA; typically 4+ hours/day) paradigms is distinct from that observed under the more traditional "short-access" (ShA; ≤ 3 hours/day) paradigm. For instance, the ShA paradigm produces a stable, moderate intake of drug that is thought by some researchers in the field to best model recreational drug use, while the LgA paradigm more closely models addiction as rats exposed to this condition will show an escalated "binge-like" intake of drug, which is thought to model the more impulsive aspects of drug-use that is key to addiction (Ahmed & Koob, 1998). For these reasons, LgA self-administration (SA) will be utilized for the studies within this dissertation.

With the SA paradigm, one can model the different cycles of addiction, recovery, and relapse naturally seen in humans. One procedure that is used to model relapse in rodents is the extinction-reinstatement paradigm. In this paradigm, animals are trained to self-administer drug, quite often in the presence of drug-associated external neutral stimuli (e.g., tones and lights) that come to serve as conditioned reinforcers of the operant behavior. Then

the drug primary reinforcer (and sometimes the conditioned reinforcer) is removed and the operant response undergoes extinction. Extinction is a process of new inhibitory learning during which the animal learns that the operant response no longer results in the presentation of the primary and/or conditioned reinforcer. As such, the animal learns to inhibit its operant response and behavioral output processing decreases across trials. Once the operant response has been extinguished to come pre-determined criterion/criteria, the operant response is "reinstated" or re-instigated, typically by presenting animals with one of the major triggers of relapse in humans: including re-exposure to the operant context, the drug-associated cues, the drug itself, or by exposure to a pharmacological or physical stressor. This extinction-reinstatement paradigm has been the primary procedure for modeling drug relapse in animal models over the last two decades, and the good majority of our understanding of the psychobiological underpinnings of the chronic, relapsing nature of addiction, including deficits in PFC function, have been derived from studies using this particular animal model. (c.f., Bossert et al., 2013).

1.3.2 The Role for Glutamate in Craving

In animal models of addiction, drug context (e.g., Brown et al., 1992), drug cues (e.g. McFarland & Ettenberg, 1997), and re-exposure to cocaine (e.g., de Wit & Stewart, 1981) have all been reported to cause reinstatement of drug-seeking. Stressors, specifically the alpha 2 adrenoceptor antagonist, yohimbine (Shepard et al., 2004), or foot shock (Ahmed & Koob, 1997; Shaham et al., 2000), are also enough to induce reinstatement. As with humans, drug-associated cues are able to elicit intense drug-craving in rodent models of addiction, with the susceptibility to cue-elicited craving extending into prolonged abstinence (Childress et al., 1993; Grimm et al., 2001; Goldstein & Volkow, 2011).

Historically, the neurotransmitter dopamine has been the focus of the majority of addiction research as drugs of abuse increase its levels in the "reward centers" of the brain, such as the NAC, and thus is believed to underlie their rewarding effects (Kuhar, Ritz, & Boja, 1991; Di Chiara et al., 1993). Although dopamine may be critical in the addiction cycle, especially as it mediates incentive salience attribution to primary and conditioned reinforcers (Robinson & Berridge, 1993; Everitt & Robbins, 2006), dopamine appears to play a larger role in the early phases of the addiction process (Shultz, 1998; Jay, 2003; Kalivas & Volkow, 2005). The later stages of addiction are instead thought to be due to dysfunction of glutamate, as it is involved in long-term plasticity underpinning changes in cognitive dysfunction (e.g., inhibitory control deficits/impulsivity) that are known to occur during these stages (Kalivas & Volkow, 2005; Kalivas & O'Brien, 2008; Dalley, Everitt, & Robbins, 2011; Volkow et al., 2011). It is becoming increasingly clear that the processes underlying addiction is a combination of these two neurotransmitter systems. Indeed, the dopaminergic and glutamatergic systems are intimately interconnected as glutamate can modify the activity of the dopaminergic system, and vice versa (Calabresi et al., 1997; Tzschentke & Schmidt, 2003). In the case for addiction, these two systems converge onto medium spiny neurons (MSNs) located in the striatum (Cahill et al., 2014). In these striatal MSMs, co-localization of dopamine and glutamate receptors allow multiple possibilities for interactions (Cahill et al., 2014).

Of all the abundant glutamatergic interconnections within the brain, PFC efferents to the NAC are thought to be an integral pathway in the reward circuit, especially considering these two regions receive dopaminergic afferents from the ventral tegmental area (VTA) (Kalivas et al., 2005). The PFC is also the major source of glutamate for the VTA, BLA, and NAC,

and is a region that integrates addiction related information and routes it to the NAC for subsequent motor output (McFarland & Kalivas, 2001). Thus, glutamate originating in the PFC is a key candidate involved in addiction.

Some of the first evidence to support a role for glutamate in addiction-related neuroplasticity was derived from studies of cocaine behavioral sensitization (Pierce et al., 1998; Carlezon & Nestler, 2002), a phenomenon used to study neuroplasticity associated with drug exposure (Robinson & Berridge, 1993; Hyman et al., 2006). Neuropharmacology studies show that repeated intraperitoneal (IP) injections of cocaine increases glutamate transmission in the NAC in rats that are behaviorally sensitized to cocaine (Pierce et al., 1996; Cornish & Kalivas, 2001). Cocaine-induced stimulation of glutamate in the mPFC is also crucial to the development of behavioral sensitization, as sensitization is associated with increased extracellular glutamate in the mPFC as non-sensitized rats did not exhibit these changes (Williams & Steketee, 2004). Sensitization is also linked to the impairment of metabotropic glutamate receptor (mGlu) 2/3-mediated long-term depression (LTD), as *in vivo* repeated IP cocaine administration impaired mGlu2/3-mediated LTD in bath application of different mGlu2/3 agonists to layer 5 pyramidal neurons of the mPFC (Huang et al., 2007). Glutamate also plays a role in the rewarding effects of cocaine, as blocking it with the selective mGlu5 antagonist, MPEP, 10 minutes prior to cocaine exposure decreases conditioned place preference (McGeehan & Olive, 2003). More closely related to this dissertation, glutamate is thought to be involved in the reinstatement of cocaine-seeking as well, as antagonizing AMPA/kainate, NMDA, and mGlu5 all reduce reinstatement (Bäckström & Hyytiä, 2006; Bäckström & Hyytiä, 2007; Kumaresan et al., 2009; Novak et al., 2010; Wang et al., 2013).

1.3.3. Cellular and Molecular Changes During Reinstatement of Cocaine-Seeking

Efforts to identify the cellular and molecular changes within the mPFC that correlate with drug craving have revealed Fos expression, a marker of neuronal activation, is increased in the mPFC after re-exposure to cocaine cues (Ciccocioppo et al., 2001; Zavala et al., 2007; Kufahl et al., 2009), along with c-fos mRNA levels (Kufahl et al., 2009). Cue-induced reinstatement is also associated with increased mRNA levels of the plasticity-associated gene, Arc, in the PL (Zavala et al., 2008). The increased Fos expression in response to reinstatement of cocaine-seeking seems to be enduring, as Fos expression is still elevated in rats subjected to a test for cue-reinforced cocaine-seeking during protracted withdrawal of at least 22 days (Zavala et al., 2007). Along with PFC activation, this region experiences alterations of glutamate protein expression in response to drug-seeking.

Indeed, postsynaptic proteins responsible for trafficking glutamate receptors (i.e., Homer, PSD-95, and filamentous (F)-actin) are dysregulated after withdrawal from repeated cocaine exposure (Szumlinski et al., 2004; Kalivas & Volkow, 2005). More specifically, Homers and PSD-95 are downregulated, while actin levels are increased in either the NAC or in the PFC-NAC pathway (Szumlinski et al., 2004; Kalivas & Volkow, 2005). Along with altered glutamate receptor protein expression, extracellular glutamate in the PFC is dysregulated.

1.3.4. Microdialysis Studies of Reinstatement of Cocaine-Seeking

Cocaine induces glutamatergic changes in the NAC. For instance, both chronic non-contingent cocaine (Bell et al., 2000) and self-administered cocaine increases extracellular glutamate (GLU_{EC}) in the NAC (Miguéns et al., 2008; Suto et al., 2010). Increases in GLU_{EC} are known to last up to 5 days of extinction training (Miguéns et al., 2008; Suto et al., 2010),

as well as during cocaine-induced reinstatement (Miguéns et al., 2008; Berglind et al., 2009; Trantham-Davidson et al., 2012). Evidence show that the majority of cocaine-induced glutamate increases in the NAC originates from the PFC (Pierce et al., 1998; Park et al., 2002; McFarland et al., 2003; Kalivas et al., 2005; Ary et al., 2013). For example, ibotenic acid lesions into the dPFC prevents cocaine-induced GLU_{EC} increase in the NAC core (Pierce et al., 1998), while AAV-mediated increases of Homer2b, but knockdown of Homer1c, in the mPFC elicits increased GLU_{EC} in the NAC (Ary et al., 2013). Thus, the changes in GLU_{EC} in the NAC are likely due to changes in GLU_{EC} stemming from the PFC.

These glutamatergic projections are also thought to be important in the reinstatement of cocaine-seeking (McFarland et al., 2003; Kalivas et al., 2005; Kalivas & O'Brien, 2008). More specifically, cocaine-induced reinstatement elevates GLU_{EC} in the NAC (McFarland et al., 2003). This increase in GLU_{EC} is thought to be selective for cocaine-induced reinstatement, as a cocaine prime did not increase GLU_{EC} levels in a yoked control group and in a food-induced reinstatement study (in which rats were trained to SA food). This GLU_{EC} rise exhibited in the NAC is known to originate from the PFC, as inactivation of this region via GABAA+B agonists baclofen + muscimol (B/M) blocks the GLU_{EC} rise exhibited in the NAC (McFarland et al., 2003). Along with cocaine-induced reinstatement, GLU_{EC} is increased in the PFC to NAC pathway, particularly the PL to NAC core, during footshock-induced reinstatement of cocaine seeking (McFarland et al., 2004). These findings argue that an increased excitability of PFC glutamate projections to the NAC may be a critical neural substrate driving the propensity of relapse triggers to reinstate drug-seeking behavior in animal models.

1.3.5. Microinjection Studies of Reinstatement of Cocaine-Seeking

Further supporting a causal relation between the PFC and reinstatement, many studies demonstrate that inactivation of the PFC attenuates reinstatement of cocaine-seeking. For instance, cue- and cocaine-induced reinstatement is decreased after mPFC lesions (Fuchs et al., 2004; Fuchs et al., 2005). Further, B/M inhibition of the dorsomedial PFC (dmPFC) is implicated in footshock-induced reinstatement, while B/M had no effect when infused into the vmPFC (McFarland et al., 2004; McFarland & Kalivas, 2001). B/M into the dmPFC, but not vmPFC, dose-dependently blocked cocaine-induced reinstatement as well (McFarland & Kalivas, 2001).

Of the dmPFC subregions targeted in these prior extinction-reinstatement studies, it seems that the PL is more critically involved. For example, inactivation of the PL using tetrodotoxin (TTX), a sodium channel blocker, blocked footshock-, cue-, and cocaine-induced reinstatement, while infusion into the IL were ineffective for all three types of reinstatement (Capriles et al., 2003; McLaughlin & See, 2003). Inactivation of the PL using lidocane is also sufficient to block cue- and cocaine-induced reinstatement (Di Pietro et al., 2006). In contrast, TTX into the OFC reduced footshock-induced, but not cocaine-induced, reinstatement (Capriles et al., 2003).

Cocaine-associated cues are thought to initiate cocaine-seeking by increasing synaptic strength and inducing rapid, transient increases in dendritic spine size in the NAC, as these changes were not exhibited in rats similarly trained with sucrose (Gipson et al., 2013), a natural reinforcer that is also known to induce seeking (Grimm et al., 2005). Interestingly, these changes require neural activity from the PL subregion of the vmPFC, as inactivation of this region with B/M infusions both blocks spine head growth along with AMPA to NMDA

currents (Gipson 2013), implicating glutamate stemming from the PFC as a main component of relapse.

Indeed, blocking glutamate in the NAC also prevents reinstatement. For example, infusion of an AMPA receptor antagonist into the NAC reduces cocaine-induced reinstatement (McFarland & Kalivas, 2001). Inhibition of mGlu5 in the NAC core via infusion of MTEP prevents cue- and cocaine-induced reinstatement, while administering the mGlu5 agonist, CHPG, produces the opposite effect and induces reinstatement of cue- and cocaine-induced reinstatement (Wang et al., 2012). This is likely due to blocking the glutamate projections stemming from the PFC in the PFC-NAC pathway, based on the afore cited work of McFarland and colleagues (2003). B/M inactivation of either the PFC or NAC is also reported to block the increase in GLU_{EC} in the NAC and PFC, respectively, along with blocking footshock-induced reinstatement (McFarland et al., 2004). BDNF infusion into the dmPFC prevents GLU_{EC} increases in the NAC that is normally caused by cocaine-induced reinstatement, along with drug-seeking behavior (Berglind et al., 2009).

Along with direct manipulation of glutamate, manipulation of glial glutamate transporter (GLT) 1, a sodium-dependent transporter found on astrocytes that is responsible for reuptake of most of the glutamate accumulation in the extracellular fluid (Anderson & Swanson, 2000), impacts cue-induced cocaine reinstatement. Sari and colleagues report that multiple IP injections of ceftriaxone, a beta-lactam antibiotic known to increase GLT1 expression, attenuates cue-induced cocaine reinstatement, but not cue-induced food relapse (Sari et al., 2009). Moreover, the attenuation of cue-induced reinstatement is correlated with increases in GLT1 expression in the PFC and NAC (Sari et al., 2009). mGlu5 is also known to be involved in both cue- and cocaine-induced reinstatement (Kumaresan et al., 2009; Novak et

al., 2010; Wang et al., 2013), further solidifying glutamate's role in relapse. In conclusion, cellular, neurochemical, molecular, and pharmacological evidence all point to glutamate emanating from the PFC as critical for the reinstatement of cocaine-seeking. But despite this, and the fact that the PFC subregions receive large glutamate inputs from various sources (see section 1.2.2), the vast majority of the research to date pertaining to corticoaccumbens glutamate and addiction has focused on the NAC, with little work focusing on the PFC proper.

1.4 Preclinical Studies of Incubation of Cocaine-Seeking and the Role of Glutamate

1.4.1 Incubation vs. Reinstatement: The Difference Between Paradigms

Drug craving is a difficult phenomenon to model in rats and can ultimately only be inferred through their behavior. As discussed above, the most popular preclinical model of craving is the extinction/reinstatement model. However, some have argued that this model has low face validity, as rarely do recovering addict undergo explicit extinction training, especially in their drug-taking context. Instead, addicts tend to abstain from drug-taking and avoid the drug and drug-associated stimuli/environment completely.

The incubation model mirrors many aspects of the extinction/reinstatement model as it involves operant training for drug infusions, but instead of extinction training, the rat is given a period of abstinence at the end of which the rat is given a drug-seeking test. This abstinence, or withdrawal period, more closely models the human condition. The drug-seeking test, during which the previously learned operant response still delivers drug-associated cues but not the drug itself, provides a measure of cue-reinforced drug-seeking,

which is thought to more closely mirror craving. Thus, the preclinical model of incubation is thought to be a closer model of relapse and drug-craving than the reinstatement model. In support of the differences between the models, studies describe molecular differences between rats that were left in their home cage, versus rats that received extinction training.

Zavala and colleagues report increased levels of Fos labeled cells in both the PL and IL of rats left in their homecage for 22 days of withdrawal, compared to rats that underwent extinction (Zavala et al., 2007). Ghasemzadeh and colleagues report glutamate receptor protein changes between three types of post SA withdrawal regimens; extinction training, exposure to the operant box, and home cage abstinence regimens all differed in redistribution of glutamate receptors in the PFC (Ghasemzadeh et al., 2011). Specifically, extinction training was associated with the most plasticity in glutamate receptor expression as this group experienced increases in AMPA receptor subunit GluR1, PSD-95, and actin, while mGlu5 levels were decreased. Rats exposed to the operant box with retracted levers and lights off only experienced decreases in mGlu5 and had increases in actin. The group re-exposed to the operant chamber did not go through true extinction training per se, and can be thought as a partial extinction training as they were re-exposed to the drug-associated environment and thus unpaired the context with drug. In contrast, rats left in their homecage did not experience any significant changes in glutamate receptors in the PFC. These results indicate glutamatergic alterations occur due to extinction training: the more explicit the extinction training becomes (i.e., extinction group vs. re-exposure group), the more changes in glutamate protein expression (Ghasemzadeh et al., 2011). Similar results were reported to occur in the NAC core, as rats that undergo extinction training had increased levels of PSD-95, Homer 1b/c, and mGlu5, compared to rats kept in their homecage (Knackstedt et al.,

2010). Together, these studies support the claim that extinction training causes different neuronal changes than would plain abstinence before exposure to a drug-seeking test (i.e., incubation model), and thus the extinction model would not be the most ideal to compare to human drug relapse. Although research on relapse through extinction/reinstatement models have vastly enlightened the field, these differences in protein expression prove not all information can be carried over and generalized to relapse in humans. Thus, follow up studies need to be done on the incubation model.

1.4.2 Incubation of Cue-Reinforced Drug-Seeking in Rodent Models

An animal model of incubated craving has been developed (Grimm et al., 2001) that serves as a relatively facile model with which to study the time-dependent, as well as enduring, changes in the brain that underpin high levels of cue-reinforced drug-seeking behavior. In animals, incubation of cue-reinforced craving is modeled by a withdrawal-dependent increase in the conditioned reinforcing properties of drug-paired discrete stimuli assessed in a drug-free state (Pickens et al., 2011), and is reliably replicated across procedural variations. At the beginning of my dissertation work, little about the underpinning mechanisms of incubated craving was known as it has relatively recent focus of intense research.

Pre-clinical studies on male rats have established incubated drug-seeking, similar to humans, involves low responding up to the first 30 days of abstinence, with cocaine-seeking peaking from 1-3 months, and tapering off after 6 months (Lu et al., 2004b). This is specific to incubated cue-reinforced cocaine-seeking, as rates of cocaine-induced drug-seeking remain unchanged over 6 months (Lu et al., 2004b). As baboons trained to self-administer

alcohol also exhibit incubated alcohol seeking (Weerts et al., 2006), incubation of drug-seeking expressed in other species can be assumed to be a generalizable human condition.

Many factors have the ability to impact incubation of drug-seeking. For example, age is an important component as although adult and adolescent rats had similar cocaine SA intake, adolescent rats had lower rates of incubated cocaine-seeking, along with cue-induced reinstatement, compared to adult rats (Li & Frantz, 2009). Along with age, sex also has an impact on incubation. Kerstetter et al. (2008) tested both male and females for incubated cocaine-seeking on either 1, 14, 60, or 180 days of withdrawal in a hybrid drug-seeking test of 8, 1-hour blocks: the first five 1-hour blocks involved extinction (no cues presented); the sixth block involved cue-induced reinstatement (light and tone cues presented); the seventh block a saline injection (control for cocaine prime; no cues presented); and lastly, the eighth block tested for drug-induced reinstatement using a cocaine prime (no cues presented). During extinction, both sexes respond similarly on the active lever at 1, 14, and 60 days withdrawal, but as male responding tapered down at 180 days, females still responded highly. Interestingly, females in estrus respond considerably more than both males and non-estrus females during 1, 60, and 180 days of withdrawal. When presented with cocaine-associated cues, both sexes only reinstated cocaine-seeking behaviors at 60 days of withdrawal. For the cocaine prime challenge, estrus females responded almost two-fold higher while non-estrus females and males exhibiting similar responding. These results suggest that incubation of cocaine-seeking dissipates at 180 days of withdrawal in males but not in females, indicating that these changes are more enduring in females, especially depending on the reproductive cycle (Kerstetter et al., 2008).

Different types of self-administration regimens (LgA [4+ hours] vs. ShA [≤ 3 hours]) are known to differentially impact incubation as well. For example, rats trained in 2 hour sessions conducted over 11 days did not exhibit increases in CP-AMPA in the NAC core, a molecular marker of incubation of craving (Conrad et al., 2008; Ma et al., 2014; Loweth et al., 2014), after 40 days of abstinence, but these increase in CP-AMPA were exhibited in rats trained in 6 hour sessions given over 10 days (Purgianto et al., 2013). This study also tested rats trained in 2 hour sessions over 24 days and rats trained in 6 hour sessions over 24 days (first 10 days were 2 hour session after which they were bumped to 6 hours). Only the two LgA regimens induced increases of CP-AMPA in MSN of the NAC core. As 6hr/10days and 2hr/11days rats received about the same number of sessions, and 2hr/24days and 6hr/10days had similar overall cocaine infusions, it was concluded that session duration was key in inducing increases of CP-AMPA, not the number of sessions or infusions received (Purgianto et al., 2013). Yet as this study did not test for incubated cocaine-seeking behavior and only incubation of CP-AMPAR, it cannot be said that these shorter regimens do not induce incubated cocaine-seeking behaviors.

Indeed, other studies have found that 2 hour sessions were able to induce incubated cocaine-seeking behaviors (Sorge & Stewart 2005; Hollander & Carelli, 2007). Another subset of studies provide evidence that 2 hour sessions is enough to induce both incubated behavior and CP-AMPAR, as long as rats were given one overnight session at the beginning of training (Suska et al., 2013; Lee et al., 2013; Ma et al., 2014). Interestingly, incubation of cocaine-seeking along with increased mGlu1 mRNA expression in the PL and IL can be induced in mice after only one 6 hour session (43 days withdrawal; Halbout et al., 2014). In concert with incubated behavior, time-dependent alterations in NMDA and AMPA

regulation were shown to occur: AMPA binding was decreased in the PL and IL at 9WD, while NMDA binding was decreased only in the IL at both 9 and 43WD, as measured by receptor autoradiography (Halbout et al., 2014).

A voluntary abstinence paradigm defined as discrete choice between palatable food or drug has been established in methamphetamine (meth) incubation studies (Caprioli et al., 2015; Caprioli et al., 2017; Venniro et al., 2017). Rats in voluntary abstinence are given access to 200-minute discrete choice trails between palatable food and meth, whereas forced abstinence rats were kept in their homecages (Caprioli et al., 2015). Interestingly, meth-seeking is reported to be higher after 21 days withdrawal in either voluntary or forced abstinence in male and female rats (Venniro et al., 2017). This was not the case with heroin, as seeking only incubated at 21 days of withdrawal after forced abstinence in both sexes (Venniro et al., 2017). Yet as this paradigm is still new, it has not yet been tested on incubation of cocaine-seeking.

Environmental enrichment, such as group housing, access to running wheels, and novel toys, may also influence incubation of drug-seeking, although studies report mixed results. One report demonstrates environmental enrichment has no effect on incubated drug-seeking, though there was a trend showing it did lower drug-seeking compared to an isolated condition (Thiel et al., 2011). On the other hand, Chauvet and colleagues found the opposite effect and that environmental enrichment does prevent the development of incubated cocaine-seeking, and can even reverse already developed incubation (Chauvet et al., 2012). Unfortunately, the effects of environmental enrichment are transient as it has been observed that when stopped, its positive effects on drug craving dissipate (Chauvet et al., 2012).

1.4.3 Incubation of Drug-Seeking: Other Reinforcers

Although much less studied in comparison to cocaine, incubation of drug-seeking has been found to envelop other types of drugs of abuse (see Table 1). For instance, meth has been reported to induce incubated seeking after both ShA and LgA paradigms (Theberge et al., 2013; Li et al., 2015; Caprioli et al., 2015; Caprioli et al., 2017; Venniro et al., 2017). A subcutaneous (SC) injection of the mGlu2 positive allosteric modulator, ZD8529, decreases meth-seeking at 21 but not 1 day of withdraw after either forced or voluntary abstinence (Caprioli et al., 2015). Incubation of meth-seeking is associated with increased fos expression in the dorsomedial striatum (Caprioli et al., 2017). The CEA is also thought to play a role, as B/M inactivation abolishes incubated meth-seeking, but does not have the same effect when infused into the BLA, vmPFC, dmPFC, or OFC (Li et al., 2015).

Heroin is another drug that also induces time-dependent changes of drug-seeking. Studies have reported that in both ShA and LgA SA paradigms, heroin is able to elicit time-dependent changes of drug-seeking (Theberge et al., 2012; Fanous et al., 2012). In both male and female rats, incubated heroin-seeking is induced at 21 days withdrawal by forced, but not voluntary, abstinence (Venniro et al., 2017). Chronic delivery of naltrexone through an osmotic minipump, but not an acute SC injection, decreases incubated seeking (Theberge et al., 2013). As inactivation of the OFC with B/M infusion abolishes drug-seeking only at 14 but not 1 day of withdrawal, and the OFC exhibits increased levels of fos expression only at 14 day withdrawal, the OFC is thought to play a salient role in this phenomenon (Fanous et al., 2012).

Although less studied, the rewarding effects of morphine as measured by conditioned place preference also seem to incubate from 1 to 14 days withdrawal, with phospho-extracellular signal-regulated kinases (p-ERK) and phosopho-cAMP response element-binding protein (p-CREB) in the CEA thought to be involved as infusion of an extracellular signal-regulated kinases (ERK) inhibitor (U0126) decreases their levels, along with abolishing morphine-seeking behavior at 14 days withdrawal (Li et al., 2008). As NMDA stimulation of ERK and cAMP response element-binding protein (CREB) in the CEA at 1 day withdrawal was able to increase morphine-seeking behaviors, which was subsequently reversed with U0126 infusion, it was concluded that the ERK pathway in the CEA is functionally relevant in incubated morphine-seeking (Li et al., 2008).

More recently, incubation of nicotine-seeking has been reported after nicotine training after a ShA SA paradigm (Funk et al., 2016; Markou et al., 2016). Different from other drugs of abuse, incubated craving for nicotine peaks earlier at 7 to 14 days of withdrawal, tapering off by 21 and 42 days of withdrawal (Markou et al., 2016). Both adult and adolescent age range rats develop incubation, with fos expression increased in the dmPFC, OFC, NAC core, and CEA, but adult rats seek nicotine at a higher magnitude than adolescents (Funk et al., 2016).

Surprisingly, incubation of alcohol-seeking has not been very well studied in rats. What is known is that cue-induced alcohol-seeking has been reported to time-dependently increase from 1 to 28 days, with seeking peaking at 56 days withdrawal (Bienkowski et al., 2004). Using a hybrid of extinction (no cues; 20 min) and cue-induced reinstatement (cues; last 10 min) into one 30-min testing session, Bienkowski and colleagues report at 56 days withdrawal but not 28 days, alcohol cues are able to rescue previously extinguished seeking-

behavior learned during extinction (Bienkowski et al., 2004). These results indicate that alcohol cues, and perhaps drug cues in general, are extremely powerful during protracted abstinence.

Interestingly, drugs of abuse are not the only reinforcers to cause incubation of craving: sucrose also has the power to elicit incubated craving. Rats trained to self-administer sucrose exhibit incubated sucrose-seeking, although this behavior is less robust and has a shorter duration (only up to 30 day withdrawal) compared to incubated cocaine-seeking (Grimm et al., 2003; Grimm et al., 2005; Grimm et al., 2006; Uejima et al., 2007). These two phenomena, although behaviorally similar, involves different neuronal mechanisms as there were no changes in BDNF levels in the NAC, VTA, or amygdala in incubated sucrose-seeking rats (Grimm et al., 2003). Though as systemic or direct infusion of LY379268 into the CEA attenuates incubated sucrose-seeking, along with incubated cocaine-seeking, it seems likely that similar glutamatergic mechanisms are at play in general reward craving (Uejima et al., 2007; Lu et al., 2007). Recently, Aoyama and colleagues have reported saccharin, a non-caloric sweetener, also has the ability to induce incubated craving (Aoyama et al., 2014). Although similar patterns of incubation exists for different types of reinforcers, one needs to be careful to make comparisons across reinforces as they all have different brain regions involved (see Table 1; Ettenberg et al., 1982; Ettenberg et al., 2009; Badiani et al., 2011).

Study	Species	Reinforcer	Dose	Paradigm	WD	Changes Compared to Controls & Brain Region (when applicable)
Grimm et al., 2003	Long-Evans rats	Sucrose; Cocaine	1 mg/kg/ infusion; 10% sucrose solution	LgA self-administration (6x1hr blocks separated by 5 min)	1, 30, 90	- U-shaped curve established for sucrose-seeking, peaking at 30WD - BDNF in VTA, NAC, and amygdala progressively increased after cocaine withdrawal, but not sucrose
Grimm et al., 2005	Long-Evans rats	Sucrose	10% sucrose solution	ShA self-administration & LgA self-administration (6x1hr blocks separated by 5 min)	1, 7, 30	- ShA and LgA exhibit similar incubated seeking responding - Sucrose-seeking abolished when free access to sucrose given for 17hr before extinction responding (no cues) on 1 or 30WD; no effect on 7WD - Sucrose access did not influence cue-induced incubated seeking
Grimm et al., 2006	Long-Evans rats	Sucrose	10% sucrose solution	LgA self-administration	1, 30	- Incubated seeking accompanied by increase in locomotor activity - IP cocaine (5 mg/kg) increased sucrose-seeking at 1WD while at 30WD, only 10 & 20 mg/kg cocaine increased sucrose-seeking
Grimm et al., 2007	Long-Evans rats	Sucrose	10% sucrose solution	ShA self-administration	1, 30	- IP naloxone (0.0001, 0.01, 1, 10 mg/kg) reduced incubated seeking
Uejima et al., 2007	Long-Evans rats	Sucrose	10% sucrose solution	LgA self-administration (6x1hr blocks separated by 5 min)	1, 21	- Systemic mGlu2/3 agonist (LY379268) prevents incubated seeking - CEA: infusion of LY379268 prevents incubated seeking
Aoyama et al., 2014	Long-Evans rats	Saccharin	0.3% saccharin solution	ShA self-administration	1, 30	- 30WD rats had higher responded throughout the whole session compared to 1WD
Markou et al., 2016	Wistar rats	Nicotine	0.03 mg/kg	ShA self-administration	1, 7, 14, 21, 42	- U-shaped curve, peaking at 7 and 14WD, pattern established for incubated nicotine-seeking - In rats with extinction training, a U-shaped curve peaking at 7WD was also exhibited, but lower magnitude of responding compared to no extinction training
Funk et al., 2016	Fos-lacZ transgenic rats	Nicotine	0.03 mg/kg/ infusion	ShA self-administration	1, 7, 14, 28	- Incubated seeking only at 14WD in both adults and adolescents, though adults responded more in general - Increased fos expression in dmPFC, OFC, NAC core, and CEA for both adults and adolescents at 14WD - Increased fos in NAC shell for adolescents at 14WD - Increased fos in BLA for adults at 14WD - Inactivation of nicotine cue activated fos neurons in CEA, but not OFC, decreased incubated seeking at 14WD in adults
Li et al., 2008	Sprague Dawley rats	Morphine	1; 3; 10 mg/kg	Conditioned place preference (8 days)	1, 7, 14	- Established 10 mg/kg morphine is too high and does not result in incubated seeking - CEA: p-ERK & p-CREB elevated at 14WD in Test rats; U0126 (ERK inhibitor) infusion decreases seeking and decrease p-ERK & p-CREB levels at 14WD; NMDA infusion at 1WD increased seeking behavior and p-ERK & p-CREB - BLA: p-ERK & p-CREB elevated at 1WD in both Test and No Test; U0126 (ERK inhibitor) infusion only decreases p-ERK & p-CREB levels at 14WD

Table 1. Summary of studies on incubation of cue-induced seeking for different types of reinforcers. All male rats were used, unless noted by (M/F). All paradigms, unless noted, were generic and lasted for 5-15 days, with 1-3 hours for short access (ShA) and 4-6 hours for long access (LgA). WD, days of withdrawal; Test, extinction test; Meth: methamphetamine; B/M: baclofen+muscimol; CEA, central nucleus of the amygdala; dmPFC, dorsomedial prefrontal cortex; vmPFC, ventromedial prefrontal cortex; NAC, nucleus accumbens; OFC, orbitofrontal cortex; DMS, dorsomedial striatum; BLA, basolateral amygdala.

Study	Species	Reinforcer	Dose	Paradigm	WD	Changes Compared to Controls & Brain Region (when applicable)
Bienkowski et al., 2004	Wistar rats	Alcohol	8% alcohol solution	ShA self-administration (30min/30 days)	1, 28, 56	- The first 20 min of Test involved extinction (no cues), while the last 10 min involved cue-induced reinstatement (cues). - Extinction was highest at 28 days, then 56 days. - Cue-induced reinstatement time-dependently increased from 1 to 56 days. At 56WD, cue-induced reinstatement intensified as it was able to bring responding back to pre-extinguished levels in rats that underwent 20 min extinction
Theberge et al., 2012	Sprague-Dawley rats	Heroin	0.075 mg/kg/ infusion	ShA self-administration	1, 11/15, 30	- Systemic naloxone (1 mg/kg) decreased incubated seeking at 15WD (30WD not tested) - NAC: MOR mRNA is decreased at 1WD - Incubated seeking is not associated with changes in BDNF, TrkB, MeCP2 mRNA in NAC, DS, or mPFC
Fanous et al., 2012	Sprague-Dawley rats	Heroin	0.075 mg/kg/ infusion	LgA self-administration (6x1hr blocks separated by 5 min)	1, 14,	- OFC: Increased Fos expression at 14WD; inactivation (B/M) decrease seeking at 14WD; Daun02 inactivation decreases lever pressing in response to heroin cues but not novel cues
Theberge et al., 2013	Sprague-Dawley rats	Meth; Heroin	0.1 mg/kg/ infusion (both drugs)	LgA self-administration (9 hrs)	1, 13	- Chronic (13 days), but not acute (day of) SC delivery of naltrexone decreased incubated heroin-seeking (7.5, 15, 30 mg/kg/day in mini osmotic pump) - Chronic or acute delivery of naltrexone did not alter incubated meth-seeking
Venniro et al., 2017	Sprague-Dawley rats (M/F)	Meth; Heroin	0.1 mg/kg	LgA self-administration (6x1hr blocks separated by 10 min)	1, 21	- Compared forced abstinence (home cage) vs. new "voluntary abstinence" paradigm, defined as discrete choice between palatable food or drug - No sex differences in meth SA or palatable food choice over meth - No sex differences in heroin SA or palatable food choice over heroin - Meth seeking higher after 21WD in both voluntary or forced abstinence compared to 1WD, no sex differences - Heroin seeking higher after 21WD in forced, but not voluntary, abstinence compared to 1WD, no sex differences
Li et al., 2015	Sprague-Dawley rats	Meth	0.1 mg/kg/ infusion	LgA self-administration (3x3hr blocks separated by 1 hr)	2, 30	- CEA: B/M inactivation abolished incubated meth-seeking - B/M inactivation did not effect incubated meth-seeking in BLA, vmPFC, dmPFC, OFC
Caprioli et al., 2015	Sprague-Dawley rats	Meth	0.1 mg/kg	ShA self-administration (3x1hr blocks separated by 10 min) & LgA self-administration (3x1hr blocks separated by 10 min)	1, 21	- Compared forced abstinence (home cage) vs. new "voluntary abstinence" paradigm, defined as discrete choice between palatable food or drug - Forced abstinence increases magnitude of meth-seeking compared to voluntary abstinence on LgA, but seeking is comparable at ShA - Subcutaneous injection of a mGlu2 positive allosteric modulator (AZD8529; 20 and 40 mg/kg) decreased meth-seeking at 21, but not 1WD, on both forced and voluntary abstinence
Caprioli et al., 2017	Sprague-Dawley rats & Fos-lacZ transgenic rats (M/F)	Meth	0.1 mg/kg	LgA self-administration (6x1hr blocks separated by 10 min)	1, 21	- Utilized the "voluntary abstinence" paradigm, defined as discrete choice between palatable food or drug - DMS: Increased Fos expression colabeled with DA1 and DA2. DA antagonists decreased meth-seeking at 21WD - Fos-lacZ rats: selective inactivation at 18WD of DMS fos neurons known to be activated during incubated seeking decreased seeking at 21WD

Table 1 (continued). Summary of studies on incubation of cue-induced seeking for different types of reinforcers. All male rats were used, unless noted by (M/F). All paradigms, unless noted, were generic and lasted for 5-15 days, with 1-3 hours for short access (ShA) and 4-6 hours for long access (LgA). WD, days of withdrawal; Test, extinction test; Meth: methamphetamine; B/M: baclofen+muscimol; CEA, central nucleus of the amygdala; dmPFC, dorsomedial prefrontal cortex; vmPFC, ventromedial prefrontal cortex; NAC, nucleus accumbens; OFC, orbitofrontal cortex; DMS, dorsomedial striatum; BLA, basolateral amygdala.

1.4.4 Neural Mechanisms of Incubated Cocaine-Seeking

In regards to preclinical literature on the neural mechanisms of incubation of cocaine craving, most studies focus on the NAC, VTA, or the amygdala (Conrad et al., 2008; Chen et al., 2008; Lu et al., 2005; Lu et al., 2007; Pelloux et al., 2013; Wolf, 2016), with very little investigating the role of the PFC. In the CEA, increased ERK levels are associated with incubated drug-seeking (Lu et al., 2005). Inhibition of ERK phosphorylation at protracted withdrawal inhibits drug-seeking, while stimulation of ERK phosphorylation during short-term withdrawal, a time where there is normally low drug-seeking behavior, increases drug-seeking, further pointing to a causal role of ERK in the CEA during incubation (Lu et al., 2005). As glutamate is known to activate the ERK pathway, it was hypothesized that this neurotransmitter is involved. Consequently, Lu and colleagues tested this theory by infusing the mGlu2/3 agonist LY379268 either systemically or into the CEA, reporting that both were enough to attenuate incubated cocaine-seeking behavior at protracted withdrawal, but had no effect during short-term withdrawal (Lu et al., 2007). Interestingly, there were no changes of ERK levels in the BLA, nor did LY379268 infusions into the BLA change incubated behavior (Lu et al., 2005; Lu et al., 2007). Yet, disconnection of the BLA to dorsal hippocampus inhibits incubated drug-seeking (Wells et al., 2011), suggesting the BLA may not play a direct role in incubation.

In the NAC, important changes in different types of AMPA receptors (AMPAR) are known to occur. This receptor class is classified into two different categories: calcium-permeable (CP) vs. calcium-impermeable (CI). The large majority of AMPARs are CI due to the presence of the GluA2 subunit, which possesses a positively charged arginine within the channel pore that repels divalent cations (Dingledine et al., 1999). AMPAR lacking the

GluA2 subunit are CP and therefore have higher conductance than their CI counterpart, enabling them to increase synaptic strength. CP-AMPAR in the NAC core are highly implicated in cue-reinforced drug-seeking (Conrad et al., 2008). For instance, CP-AMPAR accumulate in the NAC core during protracted withdrawal after exposure to extended-access cocaine (Conrad et al., 2008), but not after short-access cocaine (Purgianto et al., 2013). CP-AMPAR accumulate beginning around 30 days of withdrawal and stay elevated until 70 days withdrawal, the latest time point measured to date (Issac et al., 1995). Blockade of CP-AMPAR with Naspm (Conrad et al., 2008) or indirectly through the upregulation of mGlu1, a glutamate receptor known to express long-term depression by removal of CP-AMPARs, attenuates incubation of cocaine craving (Loweth et al., 2014). Moreover, inhibiting CP-AMPAR accumulation in protracted withdrawal prevented incubated drug-seeking, while potentiating CP-AMPAR accumulation in short-term withdrawal increased incubated behaviors (Loweth et al., 2014), further indicating functional relevance of glutamate transmission through CP-AMPAR in incubation of cocaine craving.

1.4.5 Ventromedial Prefrontal Cortex Glutamatergic Projections and Their Role in Incubation

Exposure to LgA of cocaine has been found to reduce basal levels of glutamate within the mPFC, as well as diminishing glutamatergic response to self-administered cocaine (Ben-Shahar et al., 2012). Along with dysregulation of glutamate during cocaine-taking, glutamate transmission is altered in response to cocaine-associated cues. The presentation of drug-associated cues elicits a surge of extracellular glutamate (GLU_{EC}) within both the cell body and terminal regions of the mesocorticolimbic dopamine system, independent of cocaine (Suto et al., 2010; Suto et al., 2013; You et al., 2007). Moreover, GLU_{EC} in the NAC is

elevated during an extinction session (Suto et al., 2010), along with in the VTA during the first extinction session but not the 13th session (You et al., 2007). This fluctuation of GLU_{EC} seems to depend on cues that signal the availability of cocaine, as GLU_{EC} decreases in the NAC when cues associated with cocaine unavailability are presented, but increases when cues associated with cocaine availability are presented (Suto et al., 2013). As yoked cocaine controls did not exhibit GLU_{EC} fluctuations, these changes seem to be selective for cocaine cues after chronic cocaine exposure (Suto et al., 2013). In parallel, clinical imaging studies have shown that drug-associated cues mimic the effects of drug administration on PFC activity in drug-experienced individuals (Goldstein & Volkow, 2011), and that metabolic activity in the PFC is increased during cue primed craving (Grant et al., 1996).

One of the first studies on incubation to focus on the PFC proper was done by Koya and colleagues. In this study, Koya's group report a doubling increase in fluorescence staining for p-ERK cells, a neural marker for activity, via immunohistochemistry in the vmPFC of rats re-exposed to drug-associated context and cues (Koya et al., 2009). As this was not found in the dmPFC or in rats not tested for incubated drug-seeking, it suggests that the vmPFC is activated during incubation and may be a critical component in this phenomenon. In support of a cause and effect relation between craving and the PFC, the same study reports inactivation of the vmPFC via microinjection of B/M was able to block incubated drug-seeking without having an effect during short-term withdrawal. Activation of this region with a $GABA_a$+$GABA_b$ antagonist cocktail, bicuculline+saclofen, increased drug-seeking behavior at short-term withdrawal. These results seem to be cocaine selective, as no effects of any kind were exhibited by rats trained to self-administer for sucrose, and also region selective, as injection into the dmPFC had no effect on drug-seeking.

Another study on linking incubation to glutamate within the vmPFC found that exposure to drug-associated cues after protracted withdrawal from LgA cocaine SA causes a reduction of mGlu1/5 in the vmPFC (Ben-Shahar et al., 2013). Importantly, a change in mGluR expression is not observed in animals with equivalent cocaine self-administration history, but without the opportunity to engage in cocaine-seeking. Thus, reduced vmPFC Group 1 mGluR expression is not a mere pharmacodynamic response to cocaine withdrawal, but reflects some interaction between withdrawal from cocaine-taking history and re-exposure to the drug-paired context/cues (Ben-Shahar et al., 2013). Of relevance to behavior, the reduction in vmPFC Group1 mGluR expression is correlated with incubated craving. Moreover, mimicking (via intra-vmPFC antagonist infusion) and reversing (via intra-vmPFC agonist infusion) a deficiency in Group1 mGluR function, promotes and attenuates, respectively, cue-maintained drug-seeking in rats (Ben-Shahar et al., 2013). As this receptor adaptation does not occur in cocaine-experienced rats that have not been tested for cue-induced craving, or in rats tested during short-term withdrawal, this indicates that some neurochemical event is occurring during the test for cocaine-seeking that is driving mGlu1/5 receptor expression down. Together, these two studies led me to the conclusion that glutamate in the vmPFC may be directly involved in incubated drug-seeking.

1.5 Specific Aims

The studies presented in this dissertation characterize the role of glutamate in the vmPFC during the incubation of cocaine-craving in rats using the LgA self-administration procedure. The aims of this dissertation were to: 1) clarify the neurochemical changes that occur in the vmPFC during a test for drug-seeking during short-term and protracted

withdrawal, 2) determine the protein changes that occur in the vmPFC during a test for drug-seeking during short-term and protracted withdrawal, and 3) directly manipulate endogenous glutamate in the two regions of the vmPFC in order to determine its functional relevance for incubated drug-seeking.

Chapter 2:

Neurochemical Adaptations in the Ventromedial Prefrontal Cortex During Incubation of Cocaine, but Not Sucrose, Craving

2.1 Introduction

Cocaine addiction is a chronic relapsing disorder, characterized by a high propensity for relapse even during protracted abstinence. Re-exposure to drug-associated cues and contexts are known to trigger drug craving and can even promote relapse (Childress et al., 1999; Volkow et al., 1999). The capacity of cues to elicit craving in humans and drug-seeking in laboratory animals increases or "incubates" with the passage of time in drug withdrawal (Gawin & Kleber, 1986; Grimm et al., 2001). This phenomenon, termed the incubation of craving, renders addicts highly susceptible to relapse even following prolonged periods of abstinence and has thus become a model of interest. The incubation model serves as a relatively facile model with which to study the time-dependent, as well as enduring, changes in the brain that underpin high levels of drug-seeking behavior.

In rodent models, ventromedial aspects of the prefrontal cortex (vmPFC) are known to critically regulate the manifestation of incubated drug-craving (Koya et al., 2009; Ma et al., 2014), yet no studies have investigated the neurochemical anomalies that occur in this area during the incubated cocaine craving. Extracellular dopamine and glutamate levels are dysregulated in the medial PFC of animals exposed to chronic cocaine use (Ben-Shahar et al., 2012), which may be critical in cue-induced drug-seeking. Dopamine itself has not yet been implicated in the incubation of craving, but pharmacologically inhibiting dopamine in the medial PFC via D1 receptors antagonists attenuates cue-induced reinstatement during protracted withdrawal (Ciccocioppo et al., 2001). On the other hand, incubated cocaine-craving has been correlated with changes in the expression of glutamate receptor-related proteins, as well as increased activation of downstream effectors within this subregion (Ben-

Shahar et al., 2013; Gould et al., 2014; Koya et al., 2009). Indeed, the presentation of drug-associated cues after chronic cocaine exposure elicits a surge of extracellular glutamate within both the cell body and terminal regions of the mesocorticolimbic dopamine system (Suto et al., 2010; Suto et al., 2013; You et al., 2007), with decreases in glutamate associated with cue-unavailability and increases with cue-availability (Suto et al., 2013). Such findings have led us to hypothesize that the incubation of cue-reinforced drug-seeking might reflect heightened dopamine and glutamate release within the vmPFC. Using *in vivo* microdialysis procedures, this hypothesis was tested by examining the patterns of extracellular glutamate and dopamine within the vmPFC during cue-reinforced responding at early versus later withdrawal. To determine the reinforcer-specificity of our observed effects, parallel studies were conducted in animals with a history of sucrose-pellet self-administration or in animals allowed to respond for the presentation of neutral cues in the absence of any primary reinforcer.

2.2 Materials & Methods

2.2.1 Subjects, Lever-Response Training, and Surgery

All procedures were approved by the Institutional Animal Care and Use Committee of the University of California Santa Barbara and were consistent with the guidelines of the NIH Guide for Care and Use of Laboratory Animals. Male Sprague-Dawley rats (275-325g; Charles River Laboratories, Hollister, CA) were pair-housed under standard reverse light-cycle conditions (lights off: 0700 h), with ad libitum food/water except during lever-response training, during which food was restricted (16 g/day), 24 h prior to 16-h overnight operant sessions (FR1 schedule of reinforcement; 45 mg food pellet; Bio Serv, Frenchtown,

NJ; acquisition criterion=100 responses on the active lever/session). Self-administration training was conducted in standard 2-lever operant chambers (Med Associates Inc., St. Albans, VT). Under ketamine/xylazine anesthesia (respectively 56.25 and 7.5mg/kg, IM; 2mg/kg banamine analgesic, SC, for post-operative pain), animals were implanted with a unilateral microdialysis guide cannula (20-gauge; 8mm long; Synaptech, Marquette, MI) aimed 2 mm above the vmPFC (AP: +3.0; ML ± 0.75; DV: –3.0, in mm from Bregma), with the placement counterbalanced across hemisphere within each group. Animals slated to self-administer cocaine were also implanted with a chronic indwelling jugular catheter as described previously by our group (see Ben-Shahar et al., 2013; Kersetter et al., 2008). A minimum of 4 days was allowed for recovery, with jugular catheter patency maintained by daily flushing of sterile heparin/timentin/saline (60 IU/ml and 100 mg/ml, respectively; vol=0.1 ml) and confirmed weekly by intravenous infusion of 5 mg/kg brevital (JHP Pharmaceuticals, Parsippany, NJ).

2.2.2 Self-Administration and In Vivo *Microdialysis during Cue-Testing Procedures*

Animals were trained to lever-press under an FI20 schedule of reinforcement for intravenous cocaine (0.25 mg in 0.1 ml saline infusion; NIDA, Bethesda, MD) or a 45 mg sucrose pellet (Bio Serv), with delivery of either reinforcer signaled by a 20-second light-tone compound stimulus. For control rats, active lever-presses resulted in the light-tone stimulus only. Depression of the “inactive lever” had no programmed consequences for any group. During training of the initial cohorts of rats, cocaine animals received an average of 102 reinforcer-stimulus pairings/6-hour session. Thus, the total maximum number of reinforcer-stimulus pairings earned by sucrose-trained animals was capped at 102 to equate associative learning across groups. On average, sucrose-trained animals earned 102

reinforcers within 3 hours. Thus, control rats were permitted to respond for the neutral cues for 3 hours/day. Animals were trained under the above conditions once daily across 10 days, and were then left undisturbed in their home cages for either 3 or 30 days, at which time *in vivo* microdialysis procedures were conducted (e.g., Ben-Shahar et al., 2012) during a 2-hour cue-reinforced extinction-like drug-seeking test (Extinction Test). For these Extinction Tests, active lever-presses resulted in presentation of the light-tone stimulus only. A minimum of 4 hours prior to the Extinction Test, a microdialysis probe (8 mm long with 2 mm membrane; Synaptech) was inserted into the guide cannula, the animals were placed into their operant chamber with levers retracted and house lights off, and probes were perfused with artificial cerebral spinal fluid (2.0 µl/min; see Ben-Shahar et al., 2012). Dialysate collection occurred, in 20-min intervals, for 1 hour prior to the Extinction Test and then throughout the duration of the 2-hour Extinction Test session. 10µl of preservative (4.76 mM citric acid, 150 mM NaH2PO4, 50 µM EDTA, 3 mM sodium dodecyl sulfate, 10% methanol (v/v), 15% acetonitrile (v/v), pH 5.6) was added into each dialysate sample to prevent oxidation of dopamine. Upon completion of the Extinction Test, probes were removed, animals were anesthetized with 4% isoflurane, brains extracted and then stored in 4% paraformaldehyde for later determination of probe placement within the PFC by standard histological methods. Only data from rats exhibiting probe placement within the boundaries of the vmPFC (prelimbic and/or infralimbic subregions) were employed in the statistical analyses of the data. Dialysate content of dopamine (27µl) and glutamate (20µl) was determined for each sample using high pressure liquid chromatography with electrochemical detection as described previously (Ben-Shahar et al., 2012). As the studies of cocaine-trained, sucrose-trained and control rats were conducted in series, the data were analyzed by

ANOVAs separately for each self-administration group, followed by post-hoc tests when appropriate.

2.3. Results

2.3.1 Self-Administration Training

Relative to both sucrose-trained and control rats, cocaine-trained animals exhibited the highest active-lever responding. However, due to our capping procedure, the number of reinforcers/cue presentations earned by cocaine- and sucrose-trained animals was comparable (see Table 1). Importantly, the lever-responding, as well as the number of reinforcers/cue presentations earned, over the last 3 days of self-administration training was equivalent between rats who were slated to be tested at 3 versus 30 day withdrawal, within each self-administration group (t-tests, p's>0.05).

	Active Lever Presses		Reinforcers/Cue Presentations	
Group	**3 days WD**	**30 days WD**	**3 days WD**	**30 days WD**
Neutral	25.3 ± 4.6	27.3 ± 4.7	19.1 ± 3.8	18.5 ± 2.6
Sucrose	135.4 ± 15.2	142.0 ± 9.2	92.8 ± 6.5	96.4 ± 3.8
Cocaine	182.0 ± 41.3	146.5 ± 19.6	100.4 ± 6.1	95.6 ± 5.2

Table 2. Summary of the average number of active lever-presses emitted and number of reinforcer/cue presentations earned (± SEMs) over the last 3 days of self-administration training by rats reinforced by neutral cues (Control), by sucrose pellets paired with neutral cues (Sucrose) or by cocaine infusions paired with neutral cues (Cocaine), slated to be tested for cue-reinforced lever-pressing behavior at either 3 or 30 days withdrawal (WD).

2.3.2 Neurochemical Correlates of Incubated Cocaine-Seeking

When tested for cue-reinforced cocaine-seeking at 3 or 30 days withdrawal, cocaine-trained rats exhibited a withdrawal-dependent increase in active lever-pressing that manifested throughout the 2-hour Extinction Test session (Fig.3A) [Withdrawal X Time: $F(5,80)=3.51$, $p=0.006$; post-hoc t-tests: p's<0.04]. Cocaine rats responded primarily on the active lever and the total number of active lever-presses increased as a function of drug withdrawal (Fig.3B) [Lever X Withdrawal: $F(1,19)=8.01$, $p=0.01$], indicative of incubated cue-reinforced cocaine-seeking.

Under our conventional microdialysis procedures, no time-dependent differences were apparent for baseline extracellular levels of glutamate (t-test, $p>0.10$), although baseline dopamine levels were lower in dialysate collected from Cocaine rats tested at 3 vs. 30 days withdrawal [$t(16)=4.00$, $p=0.001$] (data not shown). The difference in baseline dopamine levels likely reflects two issues: 1) withdrawal from chronic cocaine exposure is correlated with decreased basal dopamine in the NAC (Weiss et al., 1992) and PFC (Ben-Shahar et al., 2012) and 2) individual probe recovery rather than probe localization within PFC as histology revealed comparable placements within the prelimbic or infralimbic cortices between rats tested at 3 vs. 30 days withdrawal (Fig.3C). Responding for cocaine-associated cues elicited dopamine release, but the magnitude of this effect was greater in early vs. later withdrawal, most notably during the 2nd hour of testing (Fig.3D) [Withdrawal X Time: $F(8,136)=4.33$, $p<0.0001$; post-hoc t-tests, p's<0.05]. The time-dependent reduction in the cocaine cue-reactivity of vmPFC dopamine was made even more apparent by an analysis of the area under the curve (AUC) for dopamine release during the Extinction Test session (Fig.3E) [$t(16)= 2.09$, $p=0.05$], however, there was no significant correlation between the

magnitude of cue-reinforced dopamine release within vmPFC and cocaine-seeking behavior (Fig.3F). However, opposite dopamine (Fig.3D), responding for cocaine-associated cues induced a rise in vmPFC glutamate only in later withdrawal, as revealed by analyses of either the time-course (Fig.3G) [Withdrawal X Time: $F(8,128)=2.11$, $p=0.04$; post-hoc t-tests: p's<0.05] (with elevated vmPFC glutamate primarily in the 2nd hour of testing) or the magnitude of the response (Fig.3H) [$t(16)= 2.90$, $p=0.01$], the latter of which did predict cocaine-seeking behavior (Fig.3I).

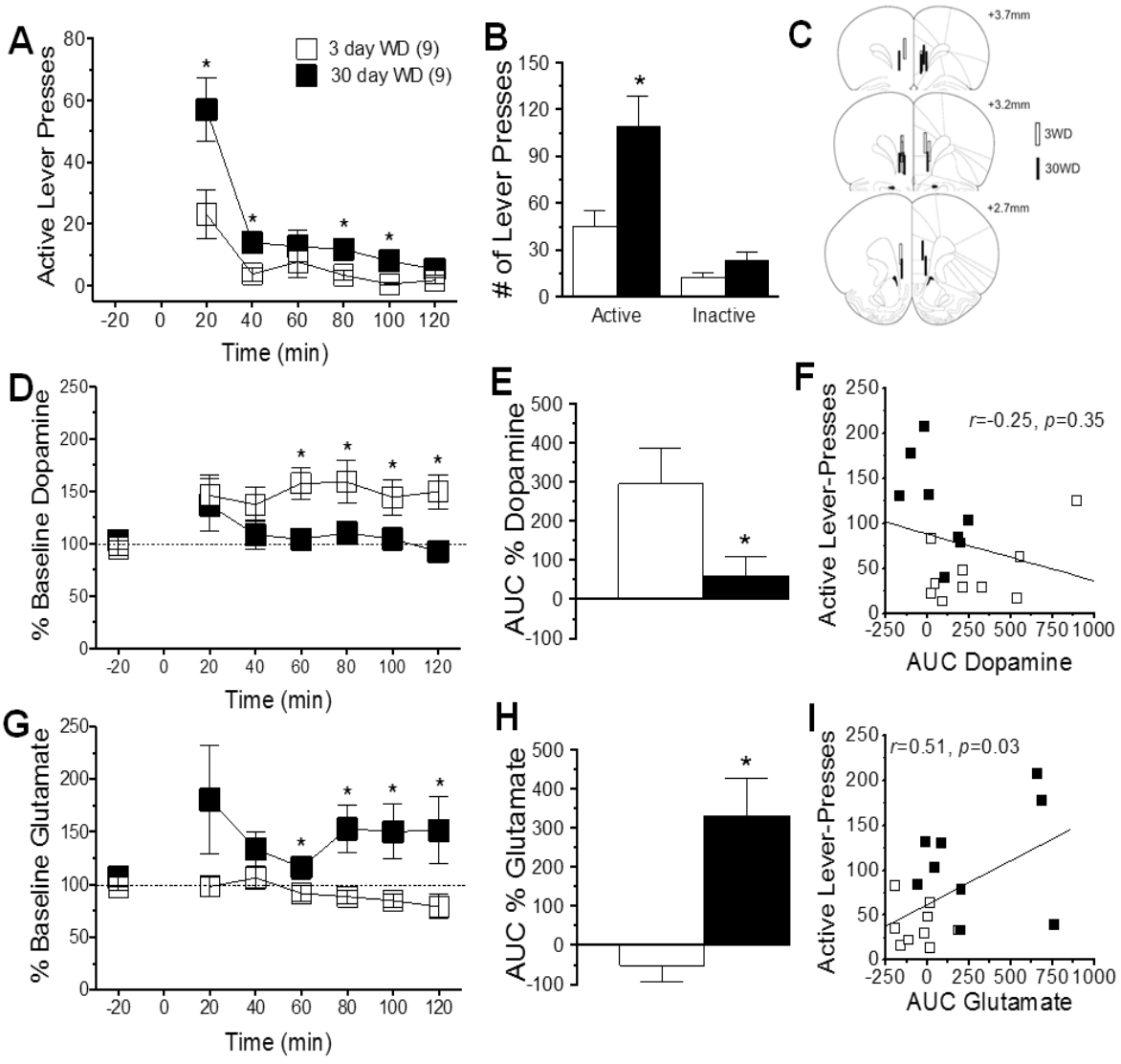

Figure 3. Summary of the effects of short- (3 day) versus long-term (30 day) withdrawal (WD) from self-administered cocaine upon cue-reinforced behavior and neurochemistry within the vmPFC when animals were tested for 2 h in a cocaine-free state. *(A)* Time-course of active lever presses (in 20-min bins) emitted by rats during the 2-h session, illustrating greater responding throughout testing at 30 vs. 3 days WD, indicative of incubation. *(B)* Comparison of the total number of active and inactive lever-presses at 3 vs. 30 days WD, indicating that incubated behavior was goal-directed. *(C)* Summary of unilateral placements of microdialysis probes within the vmPFC. *(D-F)* Summary of the time-course and area under the curve (AUC) for vmPFC extracellular dopamine, illustrating a waning of cue-reinforced dopamine release at 30 days WD and an inverse relation between cue-reinforced dopamine release and cocaine-seeking. *(G-I)* Comparable results for vmPFC extracellular levels of glutamate, illustrating an incubation of cue-reinforced glutamate release and a predictive relation between cue-reinforced glutamate release and cocaine-seeking. Data represent the means ± SEMs of the number of rats indicated in parentheses. *$p<0.05$ vs. 3 days WD (t-tests).

2.3.3 Neurochemical Correlates of Sucrose-Seeking

When tested for cue-reinforced sucrose-seeking at 3 or 30 days withdrawal, sucrose-trained rats exhibited a withdrawal-dependent increase in active lever-pressing, which manifested only during the 1st 20 min of the 2-hour Extinction Test session (Fig.4A) [Withdrawal X Time: $F(5,80)=3.24$, $p=0.01$; post-hoc t-tests]. Sucrose-trained rats responded selectively on the active lever, but the time-dependent increase in total active lever-responding did not reach statistical significance (Fig.4B) [Lever effect: $F(1,15)=32.17$,$p>0.001$; Lever X Withdrawal, $p=0.16$], suggesting that reinforcer capping, employment of pellets, or allowing *ad libitum* homecage feeding may blunt incubation (for comparison, see e.g. Grimm et al., 2002; 2011).

Microdialysis probes were localized to both the prelimbic and infralimbic cortices in sucrose-trained animals (Fig.4C). However, responding for sucrose-paired cues failed to elicit a significant rise in vmPFC dopamine (Fig.4D-E; p's>0.20) and there was no relation between sucrose-seeking and the magnitude of vmPFC dopamine release (Fig.4F). Engaging in sucrose-seeking elevated vmPFC extracellular glutamate [Time effect: $F(8,104)= 2.28$, $p=0.03$]; however, this effect did not vary significantly with sucrose withdrawal (Fig.4G, Withdrawal X Time: $p=0.10$; Fig.4H, t-test: $p=0.15$) and there was no predictive relation between sucrose-seeking and the magnitude of cue-reinforced glutamate release (Fig.4I).

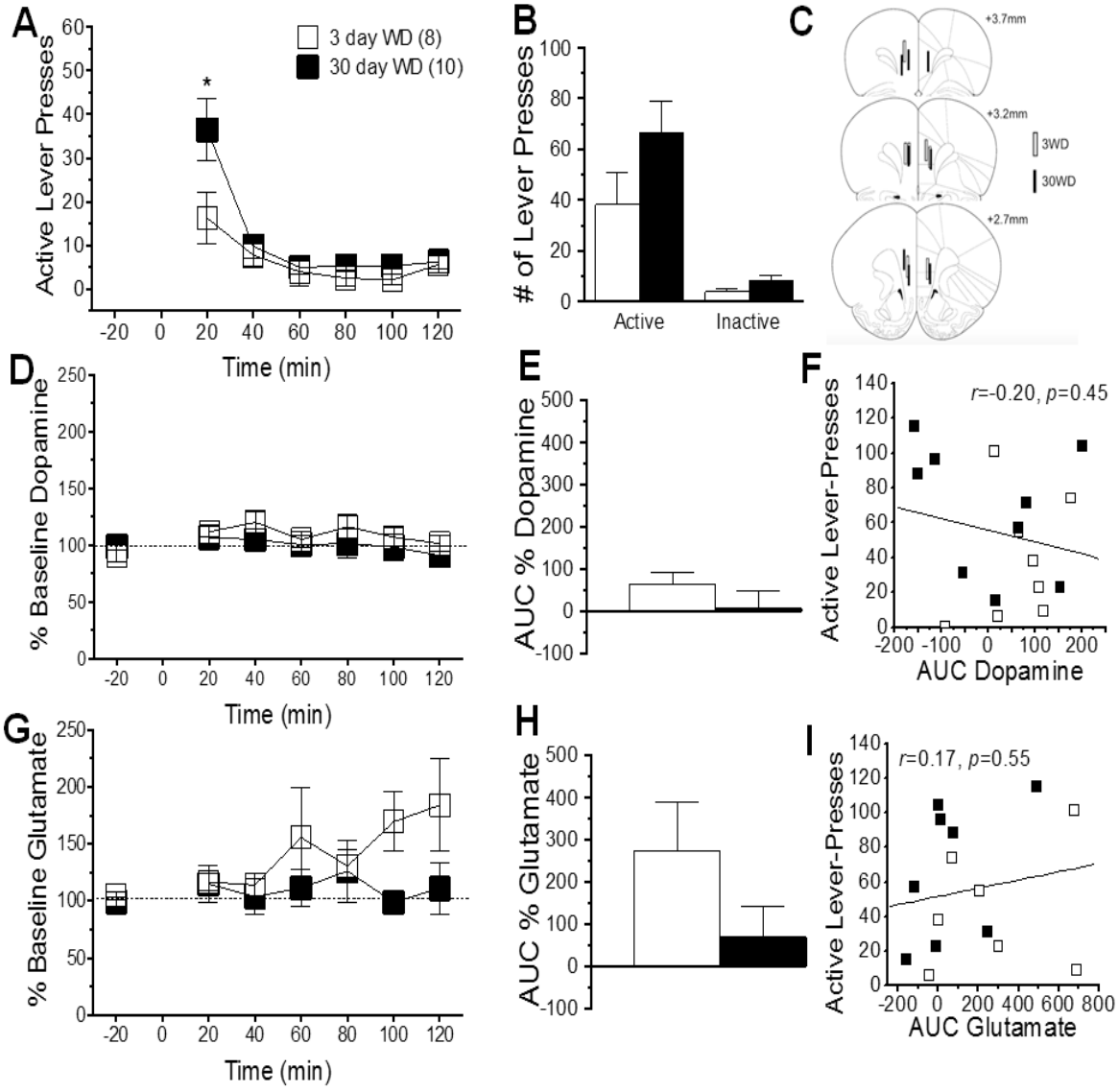

Figure 4. Summary of the effects of short- (3 day) versus long-term (30 day) withdrawal (WD) from self-administered sucrose upon cue-reinforced behavior and neurochemistry within the vmPFC when animals were tested for 2 h in a sucrose-free state. (A) Time-course of active lever presses (in 20-min bins) emitted by rats during the 2-h session, illustrating greater responding during the first 20min bin at 3 vs. 30 days WD, indicative of a weak incubation. (B) Comparison of the total number of active and inactive lever-presses at 3 vs. 30 days WD, which failed to support an incubation of responding. (C) Summary of unilateral placements of microdialysis probes within the vmPFC. (D-F) Summary of the time-course and area under the curve (AUC) for vmPFC extracellular dopamine, illustrating no cue-reinforced dopamine release and no relation to sucrose-seeking. (G-I) Comparable results for vmPFC extracellular levels of glutamate, illustrating no cue-reinforced glutamate release and no relation to sucrose-seeking. Data represent the means ± SEMs of the number of rats indicated in parentheses. *p<0.05 vs. 3 days WD (t-tests).

2.3.4 Neurochemical Correlates of Neutral Cue-Seeking

Control rats exhibited very low and stable rates of lever-pressing for the neutral cues across the 2-h Extinction Test session (Fig.5A) [Withdrawal X Time ANOVA, all *p*'s>0.06]. Control rats did selectively allocate their responding towards the "active" lever, most likely due to residual learning from lever-response training, however cue-reinforced behavior did not vary significantly between the Extinction Tests (Fig.5B) [Lever effect: $F(1,15)=30.45$, $p<0.0001$; interaction, $p>0.07$]. The localization of the microdialysis probes within the vmPFC of the control rats was comparable to that for the other 2 self-administration groups (Fig.5C). Inspection of Fig.5D and 5E suggested that the opportunity to lever-press for neutral cues elevated vmPFC dopamine levels in control rats, particularly at the 3-day time-point. However, the results of the statistical analyses of these data failed to confirm group differences (Fig.5D, Withdrawal X Time ANOVA: *p*'s>0.08; Fig.5E, t-test: $p=0.61$) and there was no significant relation between cue-reinforced responding for neutral cues and the magnitude of dopamine release in control animals (Fig.5F). Lever-pressing for neutral cues did elevate vmPFC glutamate levels, but this effect did not vary across Extinction Tests [Fig.5G, Time effect: $F(8,88)= 3.68$, $p=0.001$; other *p*'s>0.50; Fig.5H, t-test: $p=0.91$] and did not predict cue-reinforced responding (Fig.5I). These data argue that while the mere presentation of neutral cues is reinforcing and can elicit glutamate release within the vmPFC, neither behavior nor glutamate release incubates with the passage of time since last cue exposure.

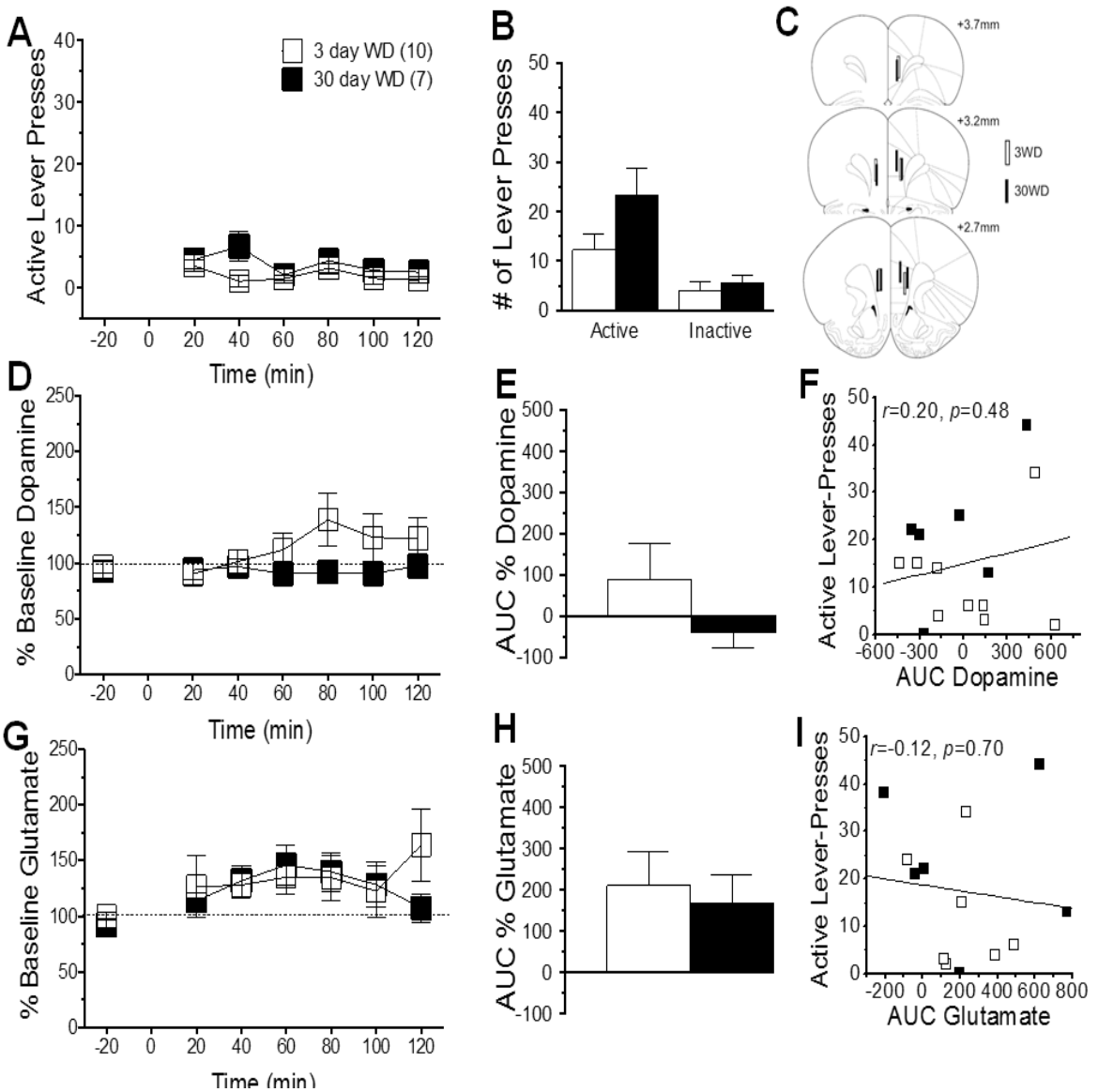

Figure 5. Summary of the effects of short- (3 day) versus long-term (30 day) "withdrawal" (WD) from operant sessions in which rats responded for neutral cues in the absence of any primary reinforcer upon cue-reinforced behavior and neurochemistry within the vmPFC when animals were tested for 2 h. (A) Time-course of active lever presses (in 20-min bins) emitted by rats during the 2-h session, illustrating stable, low levels of responding at both WD time-points. (B) Comparison of the total number of active and inactive lever-presses at 3 vs. 30 days WD, which failed to support an incubation of responding. (C) Summary of unilateral placements of microdialysis probes within the vmPFC. (D-F) Summary of the time-course and area under the curve (AUC) for vmPFC extracellular dopamine, illustrating no cue-reinforced dopamine release and no relation to cue-seeking. (G-I) Comparable results for vmPFC extracellular levels of glutamate, illustrating no cue-reinforced glutamate release and no relation to cue-seeking. Data represent the means ± SEMs of the number of rats indicated in parentheses. *$p<0.05$ vs. 3 days WD (t-tests).

2.4 Discussion

Neuronal activity within the vmPFC is critical for incubated cocaine-craving as derived from studies of animal models (Koya et al., 2009; Ma et al., 2014). Using *in vivo* microdialysis procedures, the present results demonstrate that incubated cocaine-seeking is associated with a withdrawal-dependent increase in cue-reinforced glutamate release within the vmPFC, concomitant with a waning of dopamine release. To the best of our knowledge, this study is the first to examine by *in vivo* microdialysis the changes in extracellular neurotransmitter content within vmPFC as cocaine-free animals engage in cue-reinforced responding at different time-points during cocaine withdrawal. The inverse relation between vmPFC extracellular dopamine and glutamate levels is contrary to our original hypothesis, but is in line with the results from an earlier *in vivo* microdialysis study of the neurochemical effects of cocaine-taking conducted by our group (Ben-Shahar et al., 2012). The diametrically opposed cue-reactivity of vmPFC dopamine and glutamate during both early and later cocaine withdrawal suggest an antagonistic relation between these two neurotransmitter systems in the regulation of cue-reinforced cocaine-seeking, with dopamine suppressing and glutamate facilitating behavioral hyper-reactivity to cocaine-paired cues. Moreover, these results point to time-dependent dysregulation of the balance between these two neurotransmitter systems within the vmPFC as a neurochemical correlate of incubated cocaine-craving during protracted withdrawal.

The precise nature of the dopamine-glutamate interaction at play within the vmPFC to regulate cue-reinforced drug-seeking is a topic of current investigation in our laboratory. One theory under investigation poses that a time-dependent dysregulation of autoinhibitory

mechanisms occur within vmPFC glutamate terminals. The resultant increase in vmPFC glutamate hyper-activates corticofugal afferents to the nucleus accumbens (NAC) and/or amygdala to increase the incentive salience of drug-associated cues and invigorate drug-seeking behavior. Supporting excitatory corticofugal drive in craving, cue/imagery-elicited craving in human psychomotor stimulant addicts is associated with a coordinate increase in metabolic hyperactivity within frontal cortex, striatum and amygdala, which is theorized to reflect cue/imagery-elicited hyper-activation of corticofugal afferents (c.f., Kalivas et al., 2005). More relevant to the incubation of craving phenomenon, (1) optogenetic inhibition of vmPFC glutamate afferents to the NAC prevents incubated cocaine-seeking in rats (Ma et al., 2014), (2) incubated cocaine-seeking is associated with an incubated or sensitized rise in extracellular glutamate within both the vmPFC (Fig.3G,H) and interconnected NAC (Suto et al., 2010) and (3) incubated cocaine-seeking can be inhibited by the local infusion of mGlu2/3 autoreceptor agonists into the central nucleus of the amygdala (Lu et al., 2007). Indeed, mGlu2/3 autoreceptor function is down-regulated within mPFC during protracted withdrawal in rats with a history of repeated cocaine injections (Xie & Steketee, 2009). Although the present observation of heightened cue-reinforced glutamate release in cocaine-incubated rats is consistent with a deficit in autoregulatory mechanisms within vmPFC, we do not yet know how mGlu2/3 function and expression is impacted within PFC subregions by a history of cocaine-taking. While it is tempting to generalize across models, our prior immunoblotting research indicates clearly that withdrawal from IV self-administered cocaine produces changes in the expression of certain glutamate receptor proteins that are distinct from those produced by classical, cocaine sensitization, injection protocols (Ary & Szumlinski, 2007; Ben-Shahar et al., 2009, 2013). Moreover, and important for our understanding of the neural substrates of incubated drug-craving, the expression pattern of

glutamate receptor-related proteins varies with the opportunity to engage in cue-reinforced cocaine-seeking behavior during protracted withdrawal (e.g., Ben-Shahar et al., 2013). For example, repeated cocaine-injected rodents exhibit increased PFC expression of mGlu1/5 receptors during protracted withdrawal (Ary & Szumlinski, 2007), while mGlu1/5 receptor expression is down-regulated within vmPFC of rats exhibiting incubated cocaine-seeking, but not in similarly cocaine-experienced and –withdrawn rats not afforded the opportunity to drug-seek (Ben-Shahar et al., 2013). mGlu1/5 receptors desensitize rapidly upon stimulation and exhibit slow recovery (e.g., Gereau & Heinemann, 1998), raising the possibility that the reduction in vmPFC mGlu1/5 expression observed in incubated cocaine-seeking rats (Ben-Shahar et al., 2013) results from the incubation of cue-reinforced glutamate release within this region. As reduced vmPFC mGlu1/5 function produces cognitive impairments that promote cue-reinforced drug-seeking (Ben-Shahar et al., 2013), current research in the laboratory seeks to replicate the results of Xie and Steketee (2009) within the context of incubated cocaine-seeking to test the hypothesis that incubated cue-reinforced glutamate release within vmPFC might reflect a down-regulation of autoreceptor function.

An alternative, but not necessarily mutually exclusive, theory under investigation relates to the observation that dopamine activation of D1 receptors, localized to GABAergic interneurons within PFC, inhibits local glutamate release in drug-naïve subjects via GABA-mediated heteroinhibition of glutamate terminals (e.g., Abekawa et al., 2000). Completely hypothetical at this point, we propose that the withdrawal-dependent waning of the cocaine cue-reactivity of presumed mesocortical dopamine projections (Fig.3D,E) relieves inhibitory GABA tone upon glutamate terminals within vmPFC, thereby disinhibiting local glutamate release. The withdrawal-dependent waning of the cue-reactivity of vmPFC dopamine

observed herein is a finding in line with clinical evidence for dysregulated frontal cortex dopamine in human cocaine addicts (e.g., Kalivas & Volkow, 2011), and is consistent with earlier work indicating reduced cocaine-cue reactivity of PFC dopamine in rats with a prolonged history of cocaine self-administration (40 days) (Ikegami et al., 2007). The molecular underpinnings of the withdrawal-dependent waning of dopamine cue-reactivity are unclear at the present time, but could theoretically relate also to anomalies in autoinhibitory mechanisms. At present, we surmise that blunted cue-reinforced dopamine release reflects a progressive hyper-sensitivity of D3 dopamine autoreceptors on vmPFC dopamine terminals. Although D3 receptor expression is relatively low within PFC, the local infusion of D3 receptor antagonists is sufficient to influence different aspects of social behavior in rodents, supporting their relevance in motivated behavior (e.g., Watson et al., 2012). While no study to date has examined directly the role for vmPFC D3 receptors in regulating drug-seeking, systemic pretreatment with D3 receptor antagonists or partial agonists attenuate drug-seeking behavior under various procedures, including the cue-induced reinstatement model of relapse (c.f., Keck et al., 2015). Such findings further the notion that postsynaptic (presumably D1) receptor stimulation within vmPFC normally serves to inhibit drug-seeking behavior, rendering D3 autoreceptors as an intriguing candidate for further exploration as a neural substrate of incubated drug-seeking.

Interestingly, neither dopamine nor glutamate within the vmPFC responded in any significant manner in animals trained to respond for sucrose-paired (Fig. 4) or neutral cues (Fig. 5). However, compared to prior studies of sucrose reinforcement (e.g., Grimm et al., 2002), we observed a relatively modest, albeit significant, incubation of sucrose-seeking; whether the magnitude of these effects were due to capping of the number of reinforcers,

differences in sucrose delivery (pellet vs solution), or ad libitum vs restricted homecage feeding is unclear. Nevertheless, it is clear from the present data that when rats are subjected to comparable self-administration training, sucrose-paired cues are less potent than cocaine-paired cues at eliciting both an incubation of reinforcer-seeking (see also Grimm et al., 2002) and dopamine/glutamate release within the vmPFC (Fig. 4). Interestingly, the differential impact of time on the ability of cocaine- vs sucrose-paired cues to elicit behavior and neurochemical changes are consistent with recent data indicating that cocaine generates strong secondary, but not primary, reinforcement relative to sucrose (Turnstall & Kearns, 2014). Such observations are consistent with previous reports indicating that drugs and natural rewards produce different biochemical effects within PFC (e.g., Grimm et al., 2002; Koya et al., 2009) and argue that certain biochemical underpinnings of incubated craving may be reinforcer-specific.

2.4.1 Conclusions

The results of the present study indicate that incubated drug-seeking is associated with a time-dependent increase in cue-reinforced glutamate elevations within the vmPFC but a blunted dopamine rise within the same region under the same conditions. This neurochemical adaptation is not observed in sucrose-seeking animals or cocaine-naïve controls responding for cues, arguing that it is a pharmacodynamic response produced by withdrawal from cocaine use. These data implicate an incubation of cue-reinforced glutamate release and dopamine dysfunction within vmPFC as neurochemical cordons to relapse prevention and addiction recovery. If relevant to humans, these results pose pharmacotherapeutic strategies that curb corticofugal glutamate responsiveness to cocaine-paired cues as a viable strategy for facilitating addiction recovery.

Chapter 3:

mGlu2/3 Adaptations in the Ventromedial Prefrontal Cortex During Incubation of Cocaine-Seeking

3.1 Introduction

As discussed in the previous two chapters, prefrontal glutamate plays a key role in the reinstatement of cocaine-seeking (McFarland et al., 2003; McFarland et al., 2004). More recently, prefrontal glutamate has also been examined within the context of the incubation of cocaine-seeking (Shin et al., 2016). The previous chapter established that GLU_{EC} in the vmPFC is elevated in response to cocaine-associated cues during protracted withdrawal, but not during short-term withdrawal. Furthermore, this increase was selective for cocaine-associated cues as it was not observed in rats responding for sucrose-associated or neutral cues. These results suggest that elevated GLU_{EC} is functionally relevant for incubated cocaine-seeking. Thus, the next question addressed in this dissertation is related to understanding the mechanism(s) behind this cue-induced augmentation of prefrontal glutamate in cocaine-experienced animals.

Glutamate transmission is partially regulated through mGlu receptors (Conn & Pin, 1997; Niswender & Pinn, 2010). These receptor types and their role in drug addiction have been gaining attention, with particular emphasis on the mGlu1/5 and mGlu2/3 subtypes (Kenny & Markou 2004; Moussawi & Kalivas, 2010). Our group has previously shown that mGlu1/5 expression in the vmPFC is downregulated after an Extinction Test given during long-term withdrawal (Ben-Shahar et al., 2013). However, neuropharmacological studies indicated that these receptor subtypes are not involved in cue-induced drug-seeking behavior directly, as the local infusion of neither mGlu1/5 agonists nor antagonists was effective at reducing lever-pressing during either early or later withdrawal (Ben-Shahar et al., 2013). As mGlu1/5 are not directly involved in incubated responding, the next potential glutamate

receptor class is the group 2 mGlu receptors. This class of receptors consists of mGlu subtypes 2 and 3, whose activation causes a decrease in glutamate signaling via their coupling to Gi/o proteins (Conn & Pin, 1997). As they are mainly found presynaptically on both neurons as well as on glia, mGlu2/3 function mainly as glutamate autoreceptors (Conn & Pin, 1997; Niswender & Pinn, 2010). As incubated drug-seeking is associated with increased glutamate, and since there is an abundance of mGlu2/3 in the mPFC (Gu et al., 2008), we hypothesized that these receptors may be involved in the neural underpinnings of incubated craving.

Indeed, of all the glutamate-targeted addiction treatments used in preclinical studies, mGlu2/3 agonists have been the most successful. For example, systemically administered mGlu2/3 agonists reduce the reinforcing properties of cocaine, as evidenced by lower cocaine self-administration in both rats (Baptista et al., 2004) and squirrel monkeys (Adewale et al., 2006). Further, mGlu2/3 agonists reduced cocaine-primed reinstatement of drug-seeking in both species (Baptista et al., 2004; Peters and Kalivas, 2006; Adewale et al., 2006). Systemic mGlu2/3 agonists are also able to block cue-induced reinstatement (Baptista et al., 2004; Canella et al., 2013), as well as foot shock-induced reinstatement (Martin Fardon & Weiss, 2012) of cocaine-seeking. Of relevance to this chapter, systemic mGlu2/3 agonist administration impairs incubated cocaine-seeking in rats (Lu et al., 2006). The sites of the “anti-addictive” action of mGlu2/3 agonists are thought to lie within the mesocorticolimbic system as microinjecting mGlu2/3 agonists into the NAC core (Peters & Kalivas, 2006) or CEA (Lu et al., 2006) reduces drug-primed cocaine reinstatement and incubation, respectively. These data argue that changes in mGlu2/3 expression or function

within the mPFC may contribute not only to drug-seeking behavior, but its incubation during protracted withdrawal.

To date, no study has yet examined the relation between mGlu2/3 expression within the mPFC and the manifestation of incubated craving. However, a few studies have examined the effects of cocaine withdrawal on mGlu2/3 expression and/or function. Huang et al. (2007) reported that, after 5 consecutive days of IP cocaine administration, mGlu2/3-induced long-term depression (LTD) is impaired as early as 3WD. In rats treated repeatedly with non-contingent cocaine, mGlu2/3 function and signaling is reduced within the mPFC during protracted withdrawal (Xi et al., 2002; Bowers et al., 2004; Xie & Steketee, 2009). Fewer still have examined the effects of chronic self-administered cocaine on mGlu2/3 function within the mPFC, with studies reporting mixed results, likely due to procedural differences. For example, mGlu2/3 levels in the mPFC of rats chronically self-administering cocaine under either short- or long-access procedures were reported to be no different compared to saline-treated controls, when assessed at less than 24 hours withdrawal (Hao et al., 2010). On the other hand, Kasanetz et al. state that mGlu2/3 are downregulated in the dorsomedial aspect of the PFC after chronic self-administration training in their rat model of cocaine addiction (Kasanetz et al., 2013). Cocaine-induced changes in mGlu2/3 expression may be subregionally selective within the PFC as a different study established that mGlu2/3 receptors are up-regulated both in terms of expression and functional coupling in the prelimbic cortex (PL) of the vmPFC in rats administered an intravenous bolus of cocaine (Allian et al., 2017). However, another study reports mRNA expression levels do not differ in the vmPFC between naïve rats or rats exposed to chronic self-administered cocaine,

though this is hypothesized by the group to be mainly due to translational and not transcriptional regulation (Canella et al., 2013).

While no cocaine self-administration study has examined for enduring changes in mGlu2/3 expresssion and/or function within the PFC, Schwendt et al. (2012) reported a reduction in PFC surface expression of mGlu2/3 in rats at 14 WD from self-administered methamphetamine (Schwendt et al., 2012). Rats exposed to subcutaneous injections of nicotine have shown differential effects on prefrontal expression of mGlu2, depending on developmental factors: 35WD from nicotine during adulthood does not alter mGlu2 levels in the mPFC, although mGlu2 levels are downregulated during this withdrawal time-point in rats exposed to nicotine during adolescence (Counotte et al., 2011). Collectively, these studies raise the possibility that a history of cocaine self-administration may also induce enduring changes in mGlu2/3 function/expression that relate to incubated craving.

The evidence presented above supports the notion that mGlu2/3 plays an important role in cocaine addiction, yet these receptors have not yet been studied within the context of incubated cocaine-seeking. The present chapter aims to characterize the relation between incubated cocaine-seeking and mGlu2/3 protein levels in the vmPFC via immunoblotting procedures. As an increase in GLU_{EC} is observed during protracted withdrawal, it is hypothesized that mGlu2/3 autoreceptor levels would be decreased after both 3WD and 30WD from chronic cocaine exposure, but to a greater extent during 30WD, corresponding with the manifestation of intensified drug-seeking.

3.2 Materials & Methods

3.2.1 Subjects and Cocaine Self-Administration Procedures

As described in Chapter 2, rats were trained to self-administer cocaine (0.25mg/kg/infusion) on an LgA paradigm (6hr/day) for 10 consecutive days. At either 3 or 30WD, rats underwent a 2 hour Extinction Test in concert with microdialysis procedures on one hemisphere of the brain (see Section 2.2 for detailed procedures).

3.2.2 Western Immunoblotting

Two experiments were conducted in the same cohort of rats in order to minimize the number of animal subjects needed to run experiments in Chapter 2 and 3. The hemisphere free of microdialysis cannula implantation of rats used in Chapter 2 (see Section 2.2) was immediately dissected after the completion of the Extinction Test in order to perform Western blotting procedures. Rats were lightly anesthetized with 4% isoflurane (~5 minute exposure) and the vmPFC was dissected out over ice. Tissue was immediately stored in dry ice in the -80°C freezer until processing.

As previously described by our group (Obara et al., 2009; Ben-Shahar et al., 2013), brain tissue was homogenized in a medium consisting of 0.32 M sucrose, 2 mM EDTA, 1% w/v sodium dodecyl sulfate, 50 µM phenyl methyl sulfonyl fluoride, 1 µg/ml leupeptin (pH=7.2), 1 mM sodium fluoride, 50 mM sodium pyrophosphate, 20 mM 2-glycerol phosphate, 1 nM p-nitrophenyl phosphate, 1 mM orthovanadate, and 2 µM microcystin LR, in order to inhibit phosphatase activity. Samples were subjected to centrifugation at 10,000 X g for 20 minutes. Protein content was determined using Bio-Rad DC protein assay (Bio-Rad, Hercules, CA).

Protein samples (15 µg/lane) were subjected to SDS-polyacrylamide gel electrophoresis, reduced, on Tris-acetate gradient gels (3-8%; Invitrogen, Carlsbad, CA). 3 µL of Odyssey protein molecular weight marker (LiCor, Lincoln, NE) consisting of 10-250 kDa markers was also loaded in order to visualize weight of proteins detected. Proteins were wet transferred onto hydrophobic polyvinylidene difluoride membranes (Immobilon-FL, Millipore, Billerica, MA) then pre-blocked with phosphate-buffered saline containing 0.1% (v/v) Tween-20 and nonfat dried milk powder for no less than 2 hours at room temperature, followed by overnight incubation with primary antibody. Anti-mGlu2/3 rabbit polyclongal (1:1000; Upstate Cell Signaling Solutions; Lake Placid, NY) was used as the primary antibody for mGlu2/3 receptor detection. For all gels, anti-calnexin rabbit polyclonal primary antibody (1:1000 dilution; Enzo Life Sciences; Farmingdale, NY) was used to confirm even protein loading and transfer. As calnexin is also a common house-keeping gene, it was also used as a reference protein for comparison, as discussed later.

After primary incubation, membranes were washed 3x for 5 minutes then incubated with fluorescent secondary IRDye 800CW goat anti-rabbit (1:10,000 dilution; LiCor) for 90 minutes at room temperature. As the secondary antibody is light sensitive, each step from here on forth was light protected. Membranes were again washed 3x for 5 minutes, then placed into an Odessey Fc (LiCor) in order to visual florescent imaging of immunoreactive bands, and also to digitally analyze said bands. Immunoreactivity of each protein band of interest was normalized to their respective calnexin band, with the relative change in protein immunoreactivity expressed as a percent of the average reactivity of neutral cue group on the corresponding gel (n=3-4/gel).

3.2.3 Statistical Analyses

As the main goal of this experiment was to compare protein levels of rats trained to lever press for cocaine (COC), sucrose (SUC), or neutral light and tone cues (NEUT) at 3 and 30WD, each gel was run separately by Withdrawal group. Although conventional methods for testing for incubation would be to compare the immunoreactive signal at 3 and 30WD, the present study had 3 treatment groups for each withdrawal period. Using gels with the maximum number of lanes possible (15), it was not feasible to run 6 different treatment groups on one gel as an n=3-4 for each treatment group is needed to reliably run statistical analyses. Thus, we opted to compare across samples from each of the three treatment groups (COC, SUC, or NEUT), separately for each withdrawal period. The data for 3 or 30WD were analyzed separately using an univariate ANOVA across the three different treatment group. $\alpha = 0.05$ for all analyses.

3.3 Results

3.3.1 Cue-Reinforced Cocaine-Seeking Does Not Affect mGlu2/3 Expression in the vmPFC at Either Short or Long-Term Withdrawal

There were no differences between the SUC and NEUT groups at either short [$t(24) = 1.07$, $p=0.30$] or long-term [$t(21)=0.79$, $p=0.44$] withdrawal. Thus, the data from these two control groups were combined together into a control group to increase the statistical power of the analysis. When compared to the control group, exposure to the cocaine-associated cues did not affect mGlu2/3 levels within the vmPFC at either short [$F(1,35) = 0.87$, $p=0.36$; Figure 6] or long term [$F(1,31) = 0.7$, $p=0.41$; Figure 7] withdrawal. COC rats tested during

3WD exhibited an approximate 40% reduction in mGlu2/3 [*M*= 100, *SEM*= 23.37], compared to 3WD control animals [*M*= 65.21, *SEM*= 14.27], however, this change was not statistically significant.

3.3.2 Correlational Analysis of the Relation Between vmPFC mGlu2/3 Levels and Active Lever-Pressing

When all animals were considered, there was no relation between mGlu2/3 levels within vmPFC and the number of active lever-presses emitted by the rats during cue-testing in Chapter 2 (*r*= -0.20, *p*= 0.19, *N*= 44). When only cocaine-experienced animals were considered, the correlation between mGlu2/3 levels and lever-pressing was stronger, but not statistically significant (*r*= -0.39, *p*= 0.13, *N*= 16; Figure 8). In contrast, there was absolutely no correlation between mGlu2/3 levels and responding exhibited by control animals (*r*= 0.01, *p*= 0.95, *N*= 28; Figure 8). When examining the data from the animals tested at 3WD, there was also no significant correlation between mGlu2/3 levels and lever-presssing (*r*= -0.06, *p*= 0.79, *N*= 22) nor was there a significant correlation between these variables for animals tested at 30WD (*r*= -0.20, *p*= 0.36, *N*= 22).

3.3.3 Correlational Analysis of the Relation Between vmPFC mGlu2/3 Levels and AUC for GLU_{EC}

Given that mGlu2/3 receptors regulate GLU_{EC}, the relationship between vmPFC mGlu2/3 expression and the AUC for GLU_{EC} observed in Chapter 2 was also determined. When all animals were considered, there was no correlation between mGlu2/3 levels within vmPFC and the AUC for GLU_{EC}, when measured from the opposite hemisphere (*r*= 0.08, *p*= 0.61, *N*= 44; data not shown). The correlation did not improve when only COC animals were

considered (r= 0.07, p= 0.80, N= 16) or when only the collapsed controls (SUC + NEUT) were considered (r= 0.09, p= 0.64, N= 28). When examining the rats tested at 3WD, there was no relation between vmPFC mGlu2/3 levels and GLU_{EC} (r= -0.09, p= 0.68, N= 22; data not shown). However, for the rats tested at 30 WD, the correlation between mGlu2/3 levels and GLU_{EC} was shy of significance (r= 0.37, p= 0.09, N= 22; data not shown), but, unexpectedly, the relationship was positive and not negative, as would be expected given the autoreceptor function of mGlu2/3.

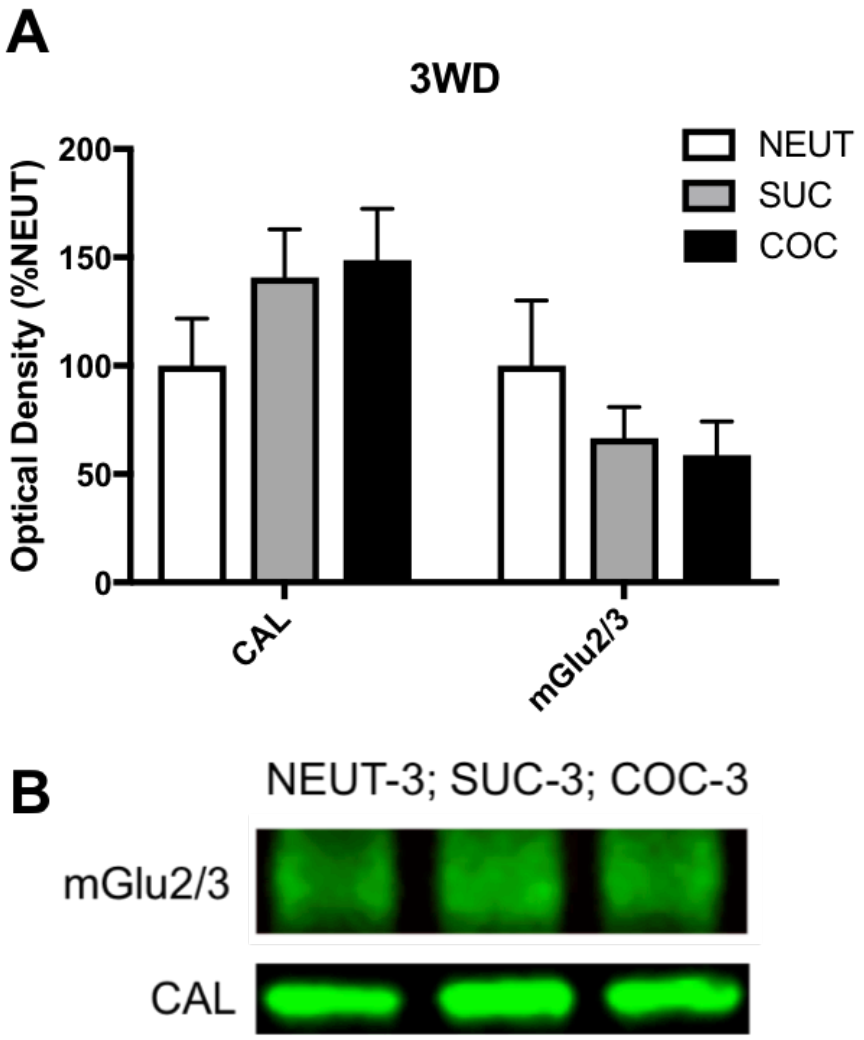

Figure 6. vmPFC immunoblotting results. Rats were trained to self-administer either cocaine (COC) or sucrose (SUC) for for 10 days, with each reinforcer delivery associated with a light/tone cue. Cue-neutral (NEUT) rats were placed into the self-administration chambers daily but their lever-pressing resulted in no reinforcer delivery. Animals then experienced a 2-hr extinction-like test of cue-induced reinforcer-seeking, at 3 days withdrawal (WD). (A) No group differences were observed for calnexin (left) or mGlu2/3 levels (right) within the mPFC. (B) Representative immunoblots for calnexin and mGlu2/3from NEUT, SUC, and COC treatment conditions at 3 WD.

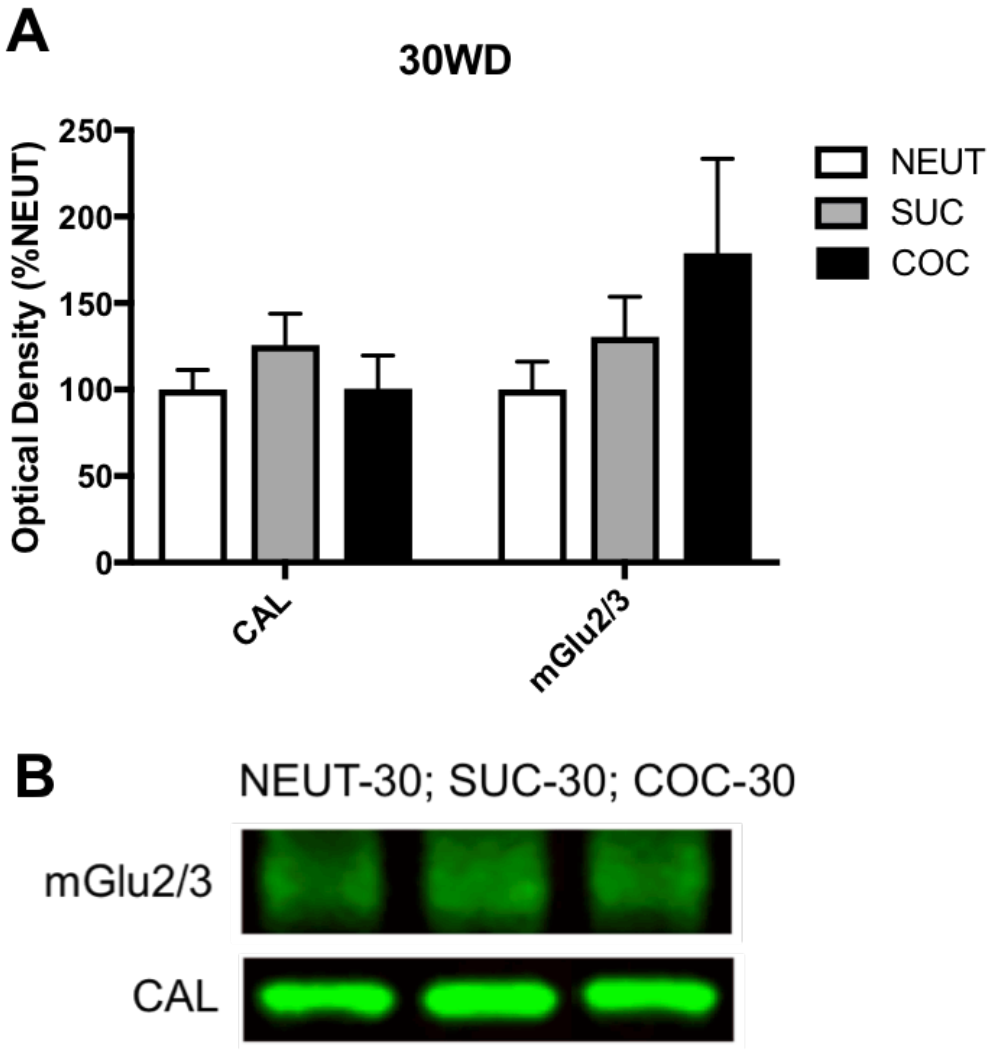

Figure 7. vmPFC immunoblotting results. Rats self-administered for either cocaine (COC), sucrose (SUC), or neutral (NEUT) rewards for 6hr/10days then experienced a 2hr cue-induced extinction-like seeking test after 30 days withdrawal (WD) exhibited no change in mGlu2/3 levels. (A) The optical density for calnexin (CAL), a reference protein to control for differences in gel loading, or mGlu2/3. (B) Representative immunoblots from NEUT, SUC, and COC treatment conditions.

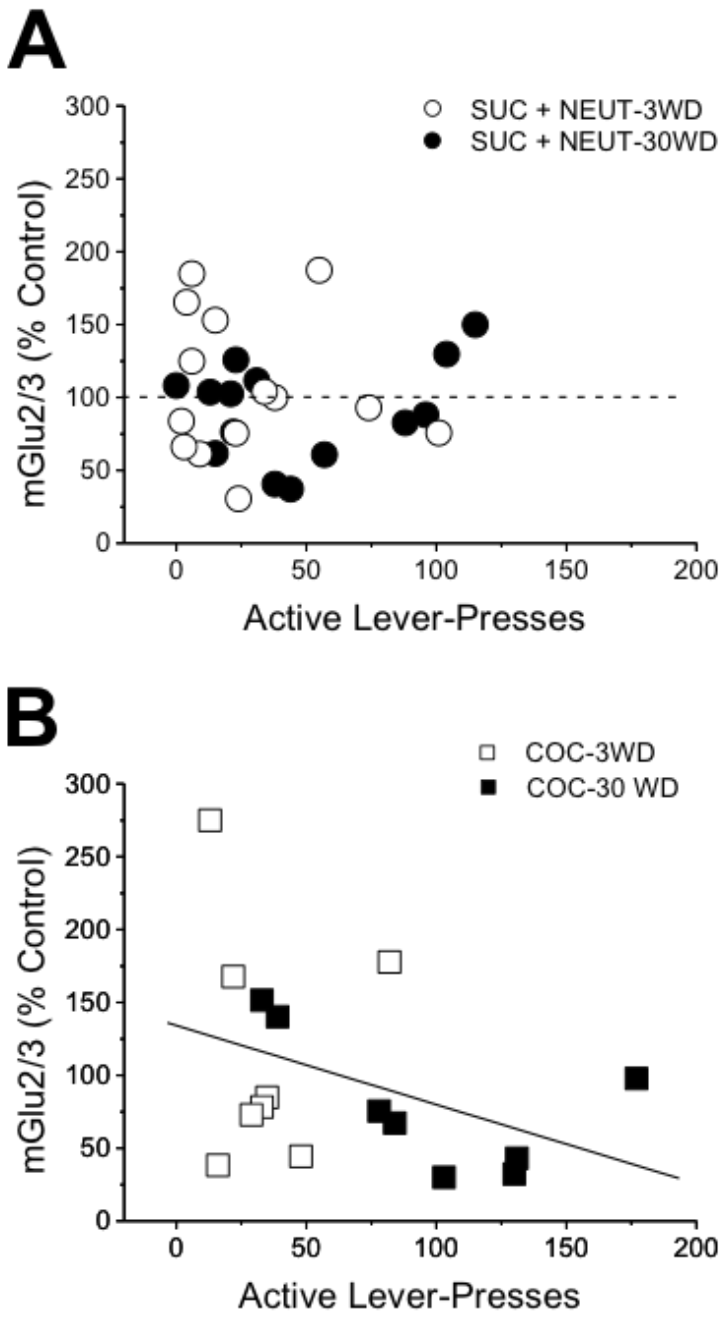

Figure 8. Pearson's Correlations conducted on the data for mGlu2/3 levels and active lever-presses during a 2hr cue-induced extinction-like seeking test at either 3 or 30 days withdrawal (WD). There was no correlation between treatment group [neutral (NEUT), sucrose (SUC), or cocaine (COC)] and active lever-presses at either WD time point. All *p*'s<0.13.

3.4 Discussion

As 30WD is a time period where GLU_{EC} is elevated in response to cue-induced drug-seeking (Shin et al., 2016), it was originally hypothesized that mGlu2/3 expression would be downregulated at this time, and to a lesser extent, at 3WD. However, cue-induced cocaine-seeking did not affect mGlu2/3 levels in the vmPFC at either short- or long-term withdrawal, as compared to sucrose-seeking rats or rats lever pressing for neutral cues. In addition, there was no correlation between vmPFC mGlu2/3 levels and active lever-pressing at either 3 or 30WD. There was also no correlation between mGlu2/3 expression and AUC GLU_{EC} at either withdrawal time points. The present findings are consistent with some of the existing literature pertaining to the effects of cocaine self-administration upon mGlu2/3 expression, which also failed to observe any effects upon mGlu2/3 total expression within mPFC (Hao et al., 2010). Taken together, these data argue little relationship between the manifestation of incubated cocaine-seeking, GLU_{EC}, and mGlu2/3 expression within mPFC.

The present results do not negate the possibility that mGlu2/3 within other regions may contribute to this phenomenon. To reiterate, mGlu2/3 has reduced receptor function and is downregulated in many brain regions such as the PFC and NAC after exposure to self-administered cocaine (Kasanetz et al., 2013; Moussawi & Kalivas, 2010). As systemic mGlu2/3 agonists impair incubated cocaine-seeking (Lu et al., 2007), it is plausible that along with a loss of function after exposure to cocaine, these receptors also downregulate following protracted withdrawal from chronic cocaine and their function is rescued by mGlu2/3 agonists. One region in which mGlu2/3 appears to contribute to incubated drug-seeking is the CEA, as microinjecting the mGlu2/3 agonist LY379268 directly into the CEA decreases incubated drug-seeking (Lu et al., 2007). In addition, mGlu2/3 in the CEA lose

their ability to modulate synaptic transmission after chronic IP cocaine treatment, with these decreases persisting for least 7 days withdrawal (Neugebauer et al., 2008). Interestingly, synaptic transmission is enhanced in the CEA (Neugebauer et al., 2000) after cocaine treatment, suggesting that the downregulation of mGlu2/3, and their loss of ability to control synaptic transmission, may be a driving factor behind the propensity for relapse. As PFC synaptic transmission is also enhanced after cocaine treatment (Luís et al., 2016), and much evidence exists in support of a cocaine-induced mGlu2/3 reduction in the PFC (Baptista et al., 2004; Peters & Kalivas 2006; Lu et al., 2007), it was surprising that the present study did not find any evidence to support the claim that mGlu2/3 in the PFC also contributes to incubated drug-seeking.

The lack of any time-dependent change in mGlu2/3 expression, coupled with the failure to observe a correlation between mGlu2/3 levels and GLU_{EC}, may indicate that incubation and elevated GLU_{EC} exhibited during protracted withdrawal are not due to a downregulation of mGlu2/3 in the vmPFC. Although the present findings argue that mGlu2/3 in the vmPFC is not directly involved in incubation, other studies have found the opposite. For example, a recently published study found that mGlu2 knock out (KO) mutant rats show reduced instances of cue-induced drug-seeking at 2, 7, and 14WD (Yang et al., 2017), suggesting that mGlu2 is involved in craving during withdrawal. More specifically, this study showed that mGlu2 KO rats have reduced reward efficacy of cocaine, show faster extinction, and have lower rates of cocaine-induced reinstatement. Hence, it is plausible that these receptors are indeed involved in incubated drug-seeking, as well as cue-induced reinstatement as many studies indicate (Baptista et al., 2004; Peters & Kalivas 2006; Lu et al., 2007), and the present study did not find mGlu2/3 involvement due to study limitations.

First and foremost, all rats examined in this experiment underwent a 5-hr microdialysis session on the contralateral hemisphere (see Section 2.2 for more details). Thus, there is the possibility that mGlu2/3 level detection was influenced by this microdialysis procedure. Another potential contributing factor to our negative immunoblotting results might relate to the experimenter-related differences in the free-hand dissection of the mPFC as two experimenter were involved in this process and I was naïve to brain dissection procedures at the time of tissue collection.

Another limitation of the present study relates to the specific immunoblotting technique employed. For one, this study examined only total protein expression and not cell surface expression. Therefore, it is possible that although we did not detect overall changes in mGlu2/3 protein levels during drug-seeking, the cell surface expression of mGlu2/3 function may still have been altered, but any such effect could be masked by inclusion of cytosolic/intracellularly retained proteins. Indeed, studies have found that mRNA expression for mGlu2's are not altered, but the level of functional mGlu2 are dysregulated in both cocaine- (Canella et al., 2013) and nicotine-treated animals (Counotte et al., 2011), arguing that withdrawal somehow changes mGlu2/3 function but not expression, per se. Lastly, the present study unfortunately did not examine the active, dimer form of mGlu2/3 (Pin et al., 2003) and instead examined the monomer form of mGlu2/3 at 100-110kDa due to the gel employed. In addition, mGlu2/3 are notorious for being hard to detect due to high levels of background staining, which prevented me from being able to examine the dimer form at 200-10kDa. Thus, future work should focus on refining our immunoblotting approaches to facilitate detection of both the monomer and dimer forms of mGlu2/3.

Lastly, null results may have been due to the fact that the vmPFC can be subdivided into two cortical subareas involved in different aspects in drug-seeking: the prelimbic and infralimbic cortices (respectively, PL and IL). Although juxtaposed, the PL and IL oppositely control aspects of drug-seeking, particularly during protracted withdrawal. Specifically, neuropharmacological (Koya et al., 2009; Pelloux et al., 2013) and optogenetic (Stefanik et al., 2013; Ma et al., 2014; Stefanik et al., 2015) evidence points to PL involvement in driving drug-seeking, as its inactivation prevents incubated drug-seeking. In contrast, IL activity inhibits aspects of drug-seeking in the extinction-reinstatement model of relapse (Peters et al., 2008; LaLumiere et al., 2012). Along with opposing roles in regulating drug-seeking, the glutamatergic projections from the PL and IL undergo differential remodeling based on circuitry as well (Ma et al., 2014).

As briefly discussed in Section 1.4.2., optogenetically reversing the maturation of silent synapses – a phenomenon linked to incubated drug-seeking – in the IL-NAC projections attenuates incubated cocaine-seeking, while reversing maturation in the PL-NAC projections potentiates incubated cocaine-seeking (Ma et al., 2014). In this influential study, Ma and colleagues report that silent synapses are generated in the projections from both the PL and IL to the NAC core and shell, respectively, following cocaine self-administration, with these synapses maturing during protracted withdrawal in both cortical projections (Ma et al., 2014). However, the time-dependent unsilencing of synapses in the two opposing pathways are done distinctively. PL-NAC projections involve the recruitment of CI-AMPAR within the NAC core as it is blocked by a non-selective AMPAR antagonist, but not a GluA2-selective antagonist. In contrast, the time-dependent unsilencing of synapses within the IL-NAC shell projections are due to CP-AMPAR insertion within the NAC shell, as the

unsilencing can be reversed by both selective and non-selective GluA2-containing AMPAR antagonists.

Just as the PL and IL undergo differential remodeling, mGlu2/3 may be regulated differently subregionally. Indeed, Meinhardt et al. has reported subregion-selective regulation of mGlu2/3 during protracted alcohol withdrawal in alcohol-dependent rats, which exhibit a persistent (at least 21 days) reduction in the expression of the GRM2 transcript encoding mGlu2 that was selective for the IL, but not the PL of the vmPFC (Meinhardt et al., 2013). As the goal of the present study was to obtain a protein correlate to relate directly to results of Experiment 1 (Chapter 2), the entire vmPFC was examined to stay consistent with Experiment 1 as it was not able to distinguish dialysate origin between subregions. It is therefore my hypothesis that subregion-specific changes in mGlu2/3 may have gone unnoticed as the entire vmPFC was taken. As it is now clear that the two subregions of the vmPFC differ in remodeling, mGlu2/3 regulation, and their role in drug-seeking, studies moving forward will delineate between the two subregions.

Future studies will need to assess the impact of incubated drug-seeking on the expression of mGlu2/3 without the confounds of microdialysis procedures. The expression of mGlu2/3 would ideally be measured following subcellular fractionation or cell-surface biotinylation assays to index for changes in the amount of cell-surface receptors. Further, examination of the relative expression of the dimer versus monomer forms of mGlu2/3 would also provide important information regarding the effects of cocaine-taking and withdrawal upon the amount of active receptor. Finally, GTPγS binding assays and/or neuropharmacological approaches could be employed to index more directly changes in mGlu2/3 function and the functional relevant of this receptor in incubated cocaine-seeking. For all of these assays,

future studies should assess the differences of mGlu2/3 levels in the PL vs. the IL, given that they play opposing roles in regulating incubated drug-seeking and relapse in animal models and given the evidence that different glutamatergic adaptations can occur within the projections from these vmPFC subregions. To this end, the next chapter aims to examine the functional relevance of endogenous glutamate within the PL versus IL, in cue-induced cocaine-seeking and its incubation during protracted withdrawal.

Chapter 4:

Endogenous Glutamate Within the Prelimbic and Infralimbic Cortices Oppositely Regulate the Incubation of Cocaine-Seeking in Rats

4.1 Introduction

Drug-associated cues elicit intense drug-craving (Childress et al., 1993; Goldstein et al., 2011), and this ability strengthens during prolonged abstinence (Tran-Nguyen et al., 1998; Grimm et al., 2001; Lu et al., 2004; Pickens et al., 2011). In human cocaine addicts, craving in response to drug-associated cues presents itself in an inverse-U shape curve over time (Gawin & Kleber, 1986; Parvaz et al., 2016). This phenomenon, termed the incubation of craving (Grimm et al., 2001; Lu et al., 2004; Pickens et al., 2011), also manifests with other types of drugs of abuse (Nicotine: Bedi et al., 2011; Meth: Wang et al., 2013; Alcohol: Li et al., 2014) and may help elucidate a period of time where addicts are most vulnerable to cue-induced relapse. The functional neuroanatomy involved in the incubation of cocaine craving remains to be fully elucidated. However, it likely relates to neuroadaptations within the prefrontal cortex (PFC), based on evidence that cocaine abusers consistently exhibit increased metabolic indices of hyperactivity within this region during cue-induced craving (Grant et al., 1996; Garavan et al., 2000; Bonson et al., 2002).

In laboratory rodents, the incubation of cue-induced craving is modeled by a withdrawal-dependent increase in the conditioned reinforcing properties of drug-paired discrete stimuli, typically assessed when the animal is in a drug-free state (Grimm et al., 2001; Pickens et al., 2011). Indeed, evidence from such preclinical studies also implicates the PFC, notably its more ventromedial aspects (vmPFC), as underpinning incubated craving. For one, neuropharmacological inactivation of the vmPFC, via infusion of a GABA agonist cocktail, decreases incubated drug-seeking (Koya et al., 2009). Cues signaling cocaine availability promote cocaine-seeking by elevating glutamatergic transmission in the nucleus accumbens

(Suto et al., 2013), a major downstream projection of the vmPFC. Further, the expression of incubated cocaine-seeking is positively correlated with higher expression of neural activity markers within the vmPFC, such as p-ERK (Koya et al., 2009) and p-PKCε (Miller et al., 2016), as well as increases in extracellular glutamate during incubated drug-seeking (Shin et al., 2016). This cue-reinforced rise in extracellular glutamate in the vmPFC appears to be cocaine-selective as it is not apparent in sucrose- or neutral cue-reinforced rats (Shin et al., 2016). Put together, these data have led to the hypothesis that endogenous glutamate from the vmPFC may be a driving factor in incubated drug-seeking.

The vmPFC is divided into two subregions: the prelimbic and infralimbic cortices (respectively, PL and IL). Although some neuroanatomical overlap exists with respect to their innervation of nucleus accumbens (NAc) subregions, the PL mainly projects to the NAc core, while the IL mainly projects to the NAc shell (Vertes et al., 2004). Although adjacent to each other, these two regions oppositely control aspects of drug-seeking, particularly during protracted withdrawal. Specifically, neuropharmacological (Pelloux et al., 2013) and optogenetic (Stefanik et al., 2013; Ma et al., 2014; Stefanik et al., 2015) evidence argues that PL activity drives drug-seeking behavior. In contrast, IL activity inhibits aspects of cocaine-seeking in the extinction-reinstatement model of relapse (Peters et al., 2008; LaLumiere et al., 2012) and also during incubated cocaine-seeking (Ma et al., 2014). Likewise, 3,4-methylenedioxymethamphetamine (MDMA) reinstatement studies also point to dichotomous roles for the IL and PL in regulating drug-seeking behavior, as inactivation of the PL, but not the IL, completely blocked cue-induced reinstatement of MDMA-seeking behavior (Ball et al., 2012). Further, optogenetic and electrophysiological studies indicate that AMPA receptors in the NAc undergo differential remodeling during protracted

withdrawal from cocaine self-administration to influence the incubation process (Conrad et al., 2008; Ma et al., 2014). More specifically, optogenetically reversing the maturation of silent glutamatergic synapses – a phenomenon linked to incubated drug-seeking – in IL-NAC projections attenuates incubated cocaine-seeking, while reversing maturation in the PL-NAC projections potentiates incubated cocaine-seeking (Ma et al., 2014). As optogenetic approaches cannot inform as to the biochemical bases of incubation, we sought to delineate the relative role for glutamate within the PL versus IL in the incubation of cocaine-seeking using bidirectional neuropharmacological approaches. More specifically, endogenous glutamate within each vmPFC subregion was raised using the non-selective excitatory amino acid transporter (EAAT) reuptake inhibitor DL-threo-β-benzyloxyaspartate (TBOA) and lowered using the mGlu2/3 autoreceptor agonist LY379268. As the PL putatively drives drug-seeking behavior, we hypothesized that mimicking the cue-reinforced increase in glutamate (Shin et al., 2016) within this subregion upon TBOA infusion would augment incubated drug-seeking behavior, while inhibiting cue-reinforced glutamate release with LY379268 would produce the opposite effect. Conversely, as IL activity tends to inhibit drug-seeking, we hypothesized that increasing and lowering glutamate within this more ventral subregion would attenuate and promote incubated drug-seeking, respectively.

4.2. Material and Methods

4.2.1 Subjects

Male Sprague-Dawley (*N*= 160) rats weighing 275-325g were obtained from Charles River Laboratories (Hollister, CA, USA) and allowed 2 days to acclimate to the colony. Rats were pair-housed in a colony room that was temperature (25°C) and humidity (71%)

controlled under a 12-hr reverse light cycle (lights off: 0700 h). Food and water was available ad libitum except during food training. All experimental protocols were consistent with the National Institute of Health Guide for Care and Use of Laboratory Animals and approved by the University of California, Santa Barbara, Institutional Animal Care and Use Committee.

4.2.2 Lever-Response Training

To be consistent with the procedures employed in our prior microdialysis study (Shin et al., 2016), rats were food-deprived to 16 grams/day in order to promote lever-response training. Rats were placed into sound-attenuated operant-conditioning chambers (30x20x24 cm; Med Associates Inc., St. Albans, VT) for 16-h overnight. Each chamber contained two retractable levers, a stimulus light above each lever, a food trough between the levers, a house light on the wall opposite the levers, and a speaker connected to a tone generator (ANL-926, Med Associates). A lever press on the active lever resulted in the delivery of a 45 mg food pellet (Bio Serv, Frenchtown, NJ), along with a 1-sec presentation of light above the active lever. Once lever-response training was completed (acquisition criterion= 100 responses per active lever/session), rats were taken off food restriction, then slated to undergo surgery.

4.2.3 Jugular Implantation and vmPFC Cannulae Surgeries

Rats were anesthetized with a ketamine/xylazine cocktail (56.25 and 7.5mg/kg, respectively, intramuscular) and then implanted with a chronic indwelling catheter into the right jugular vein, as previously described by our group (Ben-Shahar et al., 2013; Shin et al., 2016; Miller et al., 2016). Each catheter was comprised of Silastic tubing (13 cm long; 0.3

mm inner diameter, 0.64 mm outer diameter; Dow Corning, Midland, MI), attached to a threaded 22 gauge metal guide cannula (Plastics One, Roanoke, VA) that was cemented to a small square of polypropylene mesh (Bard Mesh, C.R. Bard, Cranston, RI), which ensured adherence to tissue around the animal's back.

Immediately following intravenous catheter implantation, rats were transferred to a stereotaxic apparatus and implanted with a bilateral guide cannula (22 gauge, 1 mm c/c, 13mm long; Plastics One) aimed 2 mm above either the PL (AP: +3 mm; ML: ±1.0; DV: -1.5mm) or the IL (AP: +3 mm; ML: ±1.0; DV: -3 mm; Paxinos & Watson, 2007). Four small stainless steel screws and cranioplastic cement secured the guide cannulae to the skull. Stylets (Plastics One) were placed into each cannula in order to prevent occlusion.

Rats were given banamine (2 mg/kg; non-opiate analgesic; subcutaneous) to reduce postsurgical pain before surgeries and for 2 days post-surgery. On the subsequent days until the end of the experiment, rats were flushed intravenously with 0.1 ml of sterile gentamycin (2 mg/kg) and heparin+cefazolin (60 IU/ml and 1 mg/ml, respectively) in order to maintain catheter patency. All catheters were tested every week for patency using sodium brevitol (5 mg/kg, intravenous; JHP Pharmaceuticals, Parsippany, NJ, USA).

4.2.4 Self-Administration

Allowing a minimum of 5 days for recovery after surgeries, rats were allowed to self-administer cocaine (0.25 mg/0.1 ml/infusion; Sigma-Aldrich, St. Louis, MO) on a fixed ratio 1 schedule of reinforcement, 6 h/day, for 10 consecutive days. This was done in the same operant chambers as lever-response training described above. Depression of the active lever activated a 20-sec light and tone (78 dB, 2kHz) compound stimulus, which also served as a

time-out period in which lever presses were recorded but had no consequences. The first two days of self-administration were capped at 100 and 102 infusions, respectively, to prevent overdose. The next 8 days of self-administration was unhindered, after which rats were left undisturbed in their home-cage for either 3 or 30 days, depending on withdrawal group. Only rats that exhibited stable levels of drug taking over the last 3 days of the self-administration phase of the study were tested for the effects of our neuropharmacological manipulations upon drug-seeking. Rats were randomly assigned to a microinjection treatment group (TBOA vs. vehicle; LY379268 vs. vehicle), with all groups exhibiting equivalent drug intake prior to testing.

4.2.5 Microinjection and Test for Cue-Reinforced Cocaine-Seeking

DL-threo-β-Benzyloxyaspartic acid (TBOA; Tocris, Minneapolis, MN) was dissolved into 0.1 N NaOH and neutralized with 0.1 N HCl, then diluted with sterile water to a working concentration of 300 µM. This TBOA concentration was selected for study as it elevates extracellular glutamate within the PFC of rats (Melendez et al., 2005) and augments a cocaine-conditioned place-preference when infused intra-mPFC in mice (Lominac et al., 2016). LY379268 (Tocris) was mixed with sterile water to a concentration of 20 mM, a dose comparable to that demonstrated previously to lower cocaine-induced behavioral sensitization during protracted withdrawal when infused intra-mPFC (Lupinsky et al., 2010). Sterile water served for vehicle injections.

At either 3 or 30 days withdrawal, rats were microinjected bilaterally with 0.5 µl of their assigned drug (0.5 µl/min) for one minute. Injectors were then left in place for an additional minute to allow drug diffusion. The microinjectors were removed and rats were tethered as

per usual, then given a 30-minute test for cue-reinforced responding (Extinction Test), during which each lever press resulted in the presentation of the cues associated with self-administration (i.e., light and tone), but no drug. TBOA infused rats were placed into the chambers immediately upon microinjection in a manner consistent with prior neuropharmacological studies using this drug (e.g., Kapasova & Szumlinski, 2008; Lominac et al., 2016). Rats slated for the LY379268 and LY379268's corresponding vehicle group were left inside the operant chamber (levers retracted, doors open) for ~10 minutes in order for the drug to take effect, as conducted in prior neuropharmacological studies (Bossert et al., 2004; Counette et al., 2011; Myal et al., 2015), before starting the 30-minute Extinction Test.

At the end of the Extinction Test, rats were anesthetized with 4% isoflurane and decapitated in order to extract the brain. Tissue was sliced on a vibratome (Leica, Nussloch, Germany) then stained with 0.1% cresyl violet acetate to visualize microinjector placement. Only data from rats exhibiting injector placement within the boundaries of the vmPFC (prelimbic and/or infralimbic areas) were employed in the statistical analyses.

4.2.6 Statistical Analyses

As the four studies of the effects of intra-PL and intra-IL infusion of TBOA and LY379268 on incubated drug-seeking were each conducted independently, in series, the data from each experiment were analyzed separately. To confirm equivalent cocaine intake across the four experimental conditions within each study, the data for the average number of cocaine infusions earned during the last 3 days of self-administration training were analyzed using a Withdrawal (3 vs. 30 days withdrawal) X Treatment (TBOA/LY379268 vs. vehicle)

ANOVA. The behavioral data for the Extinction Test was analyzed by a Withdrawal (3 vs. 30 days withdrawal) X Treatment (TBOA/LY379268 vs. vehicle) ANOVA. When appropriate, interactions were deconstructed using simple effects analyses of group differences between 3 and 30 days withdrawal rats. $\alpha = 0.05$ for all analyses.

4.3. Results

4.3.1 Self-Administration Training

All rats were trained to self-administer cocaine, then randomly assigned into their respective withdrawal time-point and intracranial drug treatment groups. An analysis of the number of cocaine infusions earned over the last 3 days of self-administration training failed to indicate any group differences in cocaine intake in any of the studies (Withdrawal X Treatment ANOVAs, for PL-TBOA: all *p*'s > 0.08; for PL-LY379268: all *p*'s > 0.10; for IL-TBOA: all *p*'s > 0.30; for IL-LY379268: all *p*'s > 0.09; see Table 3).

Group	Cocaine Infusions	
	3WD	30WD
PL-TBOA	90.19±5.59	97.30±8.12
IL-TBOA	93.14±6.67	92.43±5.86
PL-LY38	96.24±29.81	103.63±29.65
IL-LY38	91.73±4.10	107.75±7.90

Table 3. Mean and SEMs of the number of cocaine infusions earned over the last 3 days of cocaine self-administration across treatment groups.

4.3.2 TBOA Infusion into the Infralimbic, but Not the Prelimbic Cortex, Attenuates the Incubation of Cocaine-Seeking

Localization of the microinjection sites for both vehicle- and TBOA-infused rats are presented in Figure 9. In the PL experiment, infusion of TBOA did not affect cue-reinforced cocaine-seeking at either 3 or 30 days withdrawal, compared to vehicle [Withdrawal effect: p= 0.07; Withdrawal X Treatment: $F(1,45)$= 0.00, p= 0.99; see Figure 10]. On the other hand, TBOA infusion into the IL significantly altered the time-dependent change in cue-reinforced cocaine-seeking behavior [Withdrawal X Treatment: $F(1,38)$= 4.18, p= 0.05]. Deconstruction of the interaction along the Withdrawal factor indicated that TBOA significantly decreased cocaine-seeking at 30 days withdrawal [$F(1,19)$= 6.23, p= 0.02] but not 3 days withdrawal [$F(1,19)$= 0.55, p= 0.47; Figure 10]. No effect of TBOA infusion was observed for inactive lever presses, irrespective of subregion (data not shown) [Treatment X Withdrawal ANOVAs, for PL: all p's> 0.30; for IL: all p's> 0.40]. These latter results argue that TBOA infusion did not produce any adverse motor side-effects that might interfere with responding. Further, these data indicate that the intra-IL TBOA effect was selective for the formerly cocaine-reinforced lever.

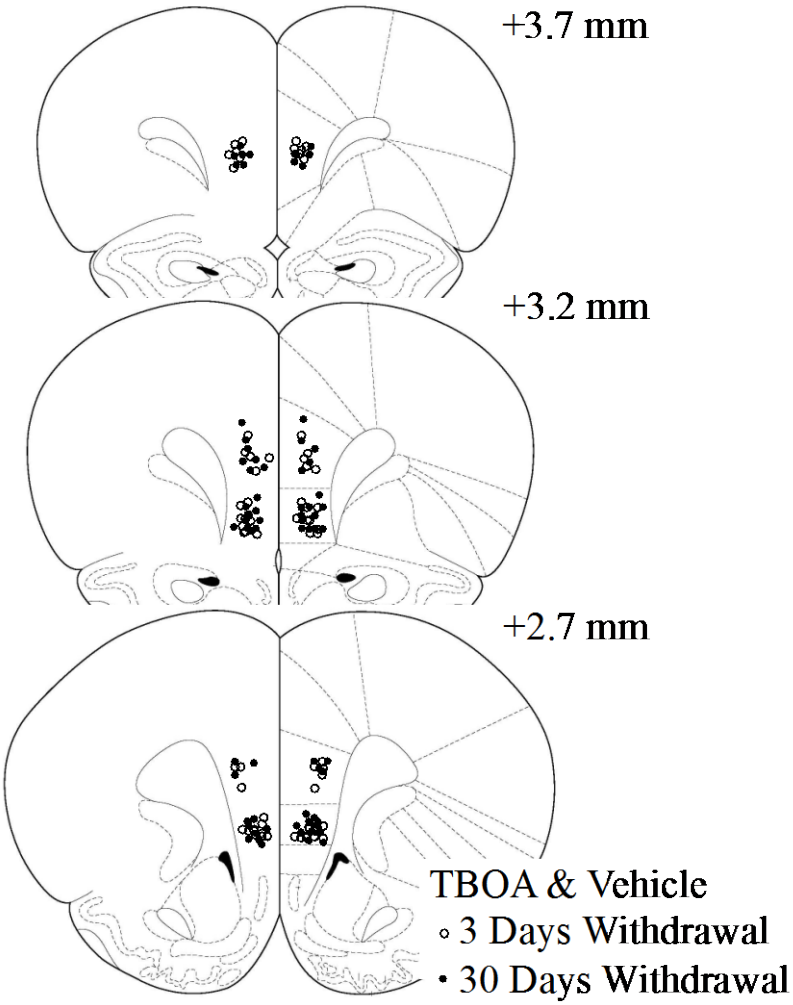

Figure 9. Summary of bilateral placements of TBOA & vehicle microinjection cannulae within the PL and IL.

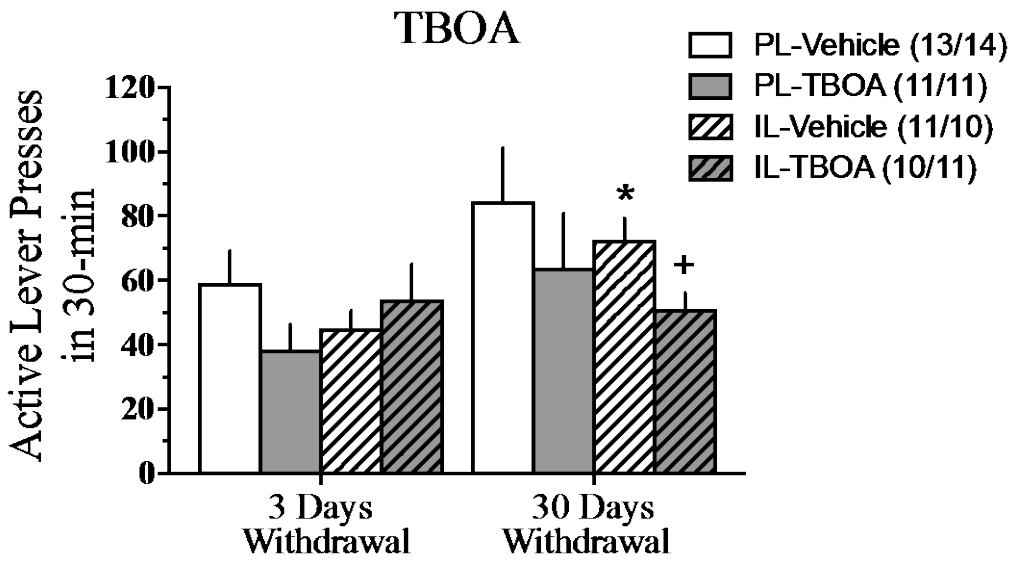

Figure 10. Comparison of the total number of active lever presses at 3 vs. 30 days withdrawal during the Extinction Test for TBOA- or vehicle-infusion into either the PL or IL. Data represents the means ± SEMs of the number of rats indicated in parentheses. *$p<0.05$ vs. IL-3 days withdrawal vehicle, illustrating an incubation of drug-seeking. +$p<0.05$ vs. IL-30 days withdrawal vehicle.

4.3.3 LY379268 Infusion into the Prelimbic, but Not the Infralimbic Cortex, Attenuates the Incubation of Cocaine-Seeking

Localization of the microinjection sites for both vehicle- and LY379268-infused rats are presented in Figure 11. In the PL experiment, LY379268 infusion significantly influenced the time-dependent change in cue-reinforced cocaine-seeking [Withdrawal X Treatment: $F(1,30)= 6.66$, $p= 0.02$; main effect of withdrawal: $F(1,30)= 16.10$, $p< 0.001$; Figure 12]. Deconstruction of the interaction along the Withdrawal factor demonstrated that LY379268 significantly decreased drug-seeking behavior at 30 days withdrawal [$F(1,17)= 9.80$, $p= 0.006$], but did not affect behavior at 3 days withdrawal [$F(1,13)= 0.65$, $p= 0.44$]. In contrast, intra-IL infusion of LY379268 had no statistically significant effect on drug-seeking at either time point [Treatment X Withdrawal ANOVA, all p's > 0.08; Figure 12]. No effect of LY379268 was apparent for inactive lever presses (data not shown) [Treatment X Withdrawal ANOVAs, for PL: all p's> 0.06; for IL: all p's> 0.20]. Thus, LY379268 infusion did not produce any overt motor side-effects that might interfere with responding. Further, these data indicate that the intra-PL LY379268 effect was selective for the formerly cocaine-reinforced lever.

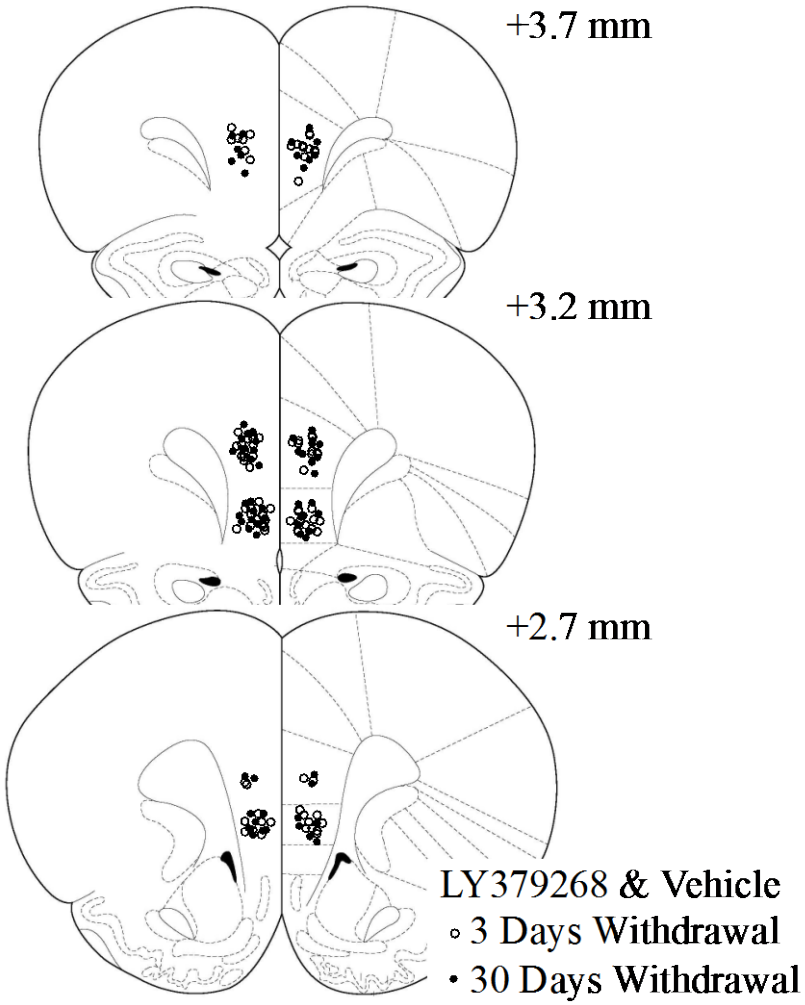

Figure 11. Summary of bilateral placements of LY379268 & vehicle microinjection cannulae within the PL and IL.

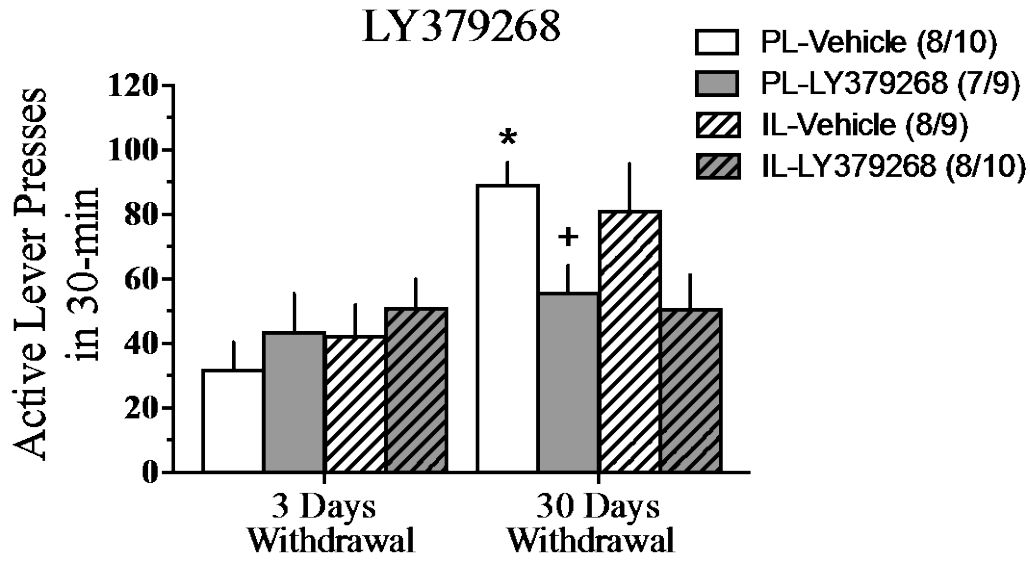

Figure 12. Comparison of the total number of active lever presses at 3 vs. 30 days withdrawal during the Extinction Test for LY379268- or vehicle-infusion into either the PL or IL. Data represents the means ± SEMs of the number of rats indicated in parentheses. *p<0.05 vs. PL-3 days withdrawal vehicle, illustrating an incubation of drug-seeking. +p<0.05 vs. PL-30 days withdrawal vehicle.

4.4. Discussion

Extracellular glutamate levels are elevated in the vmPFC during incubated cocaine-seeking (Shin et al., 2016). However, at the outset of this study, the functional relevance that elevated glutamate plays in incubated drug-seeking was unknown. The vmPFC is subdivided into the PL and IL subregions, which are theorized to play opposing roles in regulating drug-seeking behavior, including the incubation of drug-seeking during protracted withdrawal (Ma et al., 2014; Ball et al., 2012). Through neuropharmacological manipulation of endogenous glutamate within the PL and IL, the present study demonstrated that endogenous glutamate in the PL is necessary, but not sufficient, for incubated drug-seeking as the local application of a mGlu2/3 autoreceptor agonist attenuated, while infusion of the non-selective neuronal EAAT inhibitor TBOA did not affect, incubated drug-seeking. Conversely, in the IL, local application of TBOA abolished incubated drug-seeking, while mGlu2/3 agonism produced no significant effect. Lastly, these neuropharmacological manipulations of endogenous GLU did not affect drug-seeking during short-term withdrawal, indicating a more critical role for excitatory glutamate neurotransmission within the vmPFC in regulating the increased cue-reactivity observed during protracted withdrawal.

4.4.1 Glutamate Manipulations Influence Drug-Seeking Behavior During Protracted, But Not Short-Term, Withdrawal

All drug effects observed in the present study were selective for protracted withdrawal. As none of our manipulations altered behavior during early withdrawal, it is unlikely that the reduction in incubated drug-seeking produced by our manipulations reflects gross impairments in general motivation, motor activity or cognitive function (i.e., the ability to

recall the drug-cue/context association or the operant response). The temporal specificity of our drug effects for later withdrawal supports previous incubation studies in which neuropharmacological or peptide manipulations of either the cell body (Ben-Shahar et al., 2013; Miller et al., 2016; present study) or terminal (Conrad et al., 2008; Fischer et al., 2013; Li et al., 2013; Loweth et al., 2014) regions of corticoaccumbens projections, along with the general CEA (Lu et al., 2007), exerted effects on drug-seeking only in protracted withdrawal. These behavioral results, coupled with prior *in vivo* microdialysis (Shin et al., 2016), immunoblotting (Boudreau & Wolf, 2005; Lu et al., 2005; Ghasemzadeh et al., 2011; Ben-Shahar et al., 2013; Miller al., 2016) and electrophysiological (Lee et al., 2013; Ma et al., 2014; Scheyer et al., 2016) evidence, further support the argument that the neural substrates of incubated cue-reinforced drug-seeking are distinct from those underpinning drug-seeking per se and that the passage of time in withdrawal is critical for the neuroadaptations within vmPFC that bring this structure "on-line" in protracted withdrawal to augment behavioral reactivity to drug-associated cues (Wolf, 2016; Shin et al., 2016). Indeed, others have also hypothesized glutamate-mediated mechanisms are involved in incubated versus non-incubated cocaine-seeking (Lu et al., 2005; Lu et al., 2007).

The neurophysiological properties of terminals within specific corticoaccumbens and amygdaloaccumbens projections are markedly different in early versus later withdrawal (Lee et al., 2013; Ma et al., 2014). Specifically, glutamate projections from the vmPFC to the NAC (Ma et al., 2014), as well as projections from the basolateral amygdala to NAC (Lee et al., 2013), undergo silencing or dematuration during early drug withdrawal. However, with the passage of time in withdrawal, these synapses re-mature and become "unsilenced", owing to the insertion of specific AMPA receptor subunits, and possibly other yet unknown

molecular adaptations that augment synapse excitability (Lee et al., 2013; Ma et al., 2014). Currently, it is not known whether or not synapses within the vmPFC proper also undergo similar forms of time-dependent metaplasticity. However, if synapse silencing does occur during early withdrawal in glutamate synapses within vmPFC, such a phenomenon might account for the relative ineffectiveness of our glutamate manipulations, as well as other neuropharmacological manipulations of the vmPFC (e.g., Ben-Shahar et al., 2013; Miller et al., 2016; present study), at earlier withdrawal time-points.

4.4.2 Endogenous Glutamate in the Prelimbic Cortex is Necessary, but Not Sufficient, for Incubated Drug-Seeking

Stimulation of mGlu2/3 autoreceptors via LY379268 infusion into the PL significantly attenuated incubated drug-seeking, implicating endogenous glutamate within this subregion as a critical mediator of incubated responding. Our neuropharmacological result is consistent with evidence that the PL is implicated in cue-induced reinstatement (Capriles et al., 2003; Zavala et al., 2008; McGlinchey et al., 2016), with PL projections to the NAc core being recruited during, and in proportion to, cue-induced reinstatement of cocaine-seeking (McGlinchey et al., 2016). Further, inactivation of the PL is known to attenuate stress-induced, cue-induced, and cocaine-induced reinstatement of drug-seeking (Capriles et al., 2003; McLaughlin & See, 2003; Fuchs et al., 2004; Di Pietro et al., 2006), supporting a critical role for this vmPFC subregion in these animal models of relapse. More directly relevant to incubated cocaine-seeking, the PL exhibits enhanced coding of cocaine-associated stimuli (West et al., 2014), and the PL projection to the NAC core is strengthened (Suska et al., 2013; Ma et al., 2014; Luís et al., 2016) during protracted withdrawal from chronic cocaine self-administration. Further, the strengthening of this pathway coincides

with increased electrophysiological indices of presynaptic glutamate release from PL-NAC projections (Suska et al., 2013; Luís et al., 2016). Taken together, we propose that a time-dependent increase in glutamate release within the PL is at least one neurochemical substrate driving the enhanced encoding of cocaine-associated cues by this subregion, as well as the resultant metaplasticity within the NAC (and other terminal regions) culminating in greater cue-reactivity and incubated responding. Important questions for future work relate to (1) the source of the glutamate within the PL that incubates during protracted withdrawal and is critical for the manifestation of incubated responding, (2) the neuronal or glial adaptations that occur within the PL resulting in increased cue-reactivity of glutmate and (3) the specific glutamate receptors activated by incubated drug-seeking glutamate release within the PL that promote the synaptic strengthening of corticoaccumbens projections to drive incubated responding.

At the outset of study, we hypothesized that if the cue-induced increase in glutamate within the PL drives incubated responding, then augmenting endogenous glutamate levels within this subregion via an infusion of the EAAT inhibitor TBOA might increase the magnitude of the incubated response. In contrast to our hypothesis, intra-PL TBOA infusion did not significantly affect drug-seeking in later withdrawal. This negative result does not likely reflect the dose of TBOA employed as 300 μM TBOA is within the range reported to produce a 2- to 3-fold increase in glutamate within PFC (Melendez et al., 2005) and, as discussed below, this dose was effective at altering incubated responding when infused into the IL. Given that responding for cocaine-associated cues during protracted withdrawal results in a near-doubling of glutamate within the vmPFC that is maintained throughout a 2-h cue-reinforced extinction-like test (Shin et al., 2016), it is likely that the failure to observe

a potentiation of incubated drug-seeking by intra-PL TBOA infusion reflects a ceiling effect upon behavior. This being said, intra-PL TBOA infusion did not augment cocaine-seeking expressed by rats tested during early withdrawal – a time when cocaine-associated cues do not influence glutamate within vmPFC (Shin et al., 2016) – and thus a time point where an effect of TBOA should be apparent. Alternatively, given our findings for LY379268, the possibility exists that elevating endogenous glutamate prior to the test for incubated drug-seeking engaged autoregulatory and/or presynaptic axoaxonal inhibitory mechanisms that occluded our ability to detect behavioral potentiation. Indeed, incubated cocaine-seeking tended to be suppressed by intra-PL TBOA infusion in the present study, suggesting the possibility that inhibitory mechanisms may have been engaged by TBOA pretreatment.

The PFC expresses the glial transporters EAAT1 and EAAT2, as well as the neuronal transporter EAAT3 (Danbolt, 2001). The reported IC50 of TBOA for each of these transporters is 70 μM, 6 μM and 6 μM, respectively (Jabaudon et al., 1999) and thus, the 300 μM TBOA dose employed in the present study is well above that predicted to inhibit all three transporters. Indeed, intra-PFC infusion of TBOA doses between 250 and 500 μM is sufficient to produce a robust local increase in extracellular glutamate (while manipulations of sodium-independent transporters are without effect) (Melendez et al., 2005), the relative contribution of glutamate release from glia versus neuronal sources and the role for sodium-dependent glial transporters in the maintenance and regulation of vmPFC glutamate remains to be determined, to the best of our knowledge. Thus, while it is presumed that the incubated glutamate release observed when rats respond for cocaine-associated cues (Shin et al., 2016) is derived from neuronal sources (a likely candidate being thalamocortical projections), there is no evidence negating the contribution of glia to this phenomenon. Thus, the intriguing,

although speculative, possibility exists that the failure of TBOA to influence incubated drug-seeking reflects a greater involvement of glia-derived glutamate, which will be tested in future studies by manipulating EAAT1 expression.

4.4.3 Increased Endogenous Glutamate in the Infralimbic Cortex is Sufficient to Block Incubated Drug-Seeking

In contrast to the PL, inhibition of glutamate re-uptake via TBOA infusion within the IL blocked incubated drug-seeking, arguing an inhibitory role for endogenous glutamate within this subregion. These neuropharmacological results complement the extant literature pertaining to the reinstatement of drug-seeking demonstrating that IL activation with AMPA agonists reduces cocaine-primed reinstatement of drug-seeking (Peters et al., 2008; LaLumiere et al., 2010). Further, our results are in line with optogenetic evidence demonstrating that activation of IL projections to the NAC shell inhibits incubated cocaine-seeking (Ma et al., 2014) and extend these findings by implicating glutamate as an upstream mediator of this effect. It is noteworthy that in an earlier study by Koya et al. (2009), the local infusion of an inhibitory GABA agonist cocktail into the more ventral aspect of the vmPFC decreased incubated cocaine-seeking. While this result may seem contrary to the present findings, the microinjection sites in this prior study were not localized exclusively to the IL, with a fraction of the microinjections localized more dorsally within the PL (Koya et al., 2009). Given the present observations for LY379268's effects within the PL and earlier optogenetics work (Ma et al., 2014), the inhibitory effect of the GABA agonist cocktail observed in Koya et al. (2009) could reflect the effects of inhibiting PL projections.

The above being said, an inspection of Figure 12 indicates that the magnitude of the intra-IL LY379268 effect upon incubated behavior was comparable to that produced by an intra-PL infusion of this mGlu2/3 agonist. Further inspection of Figure 12 suggests that the failure to detect a statistically significant effect of intra-IL LY379268 infusion likely reflected the variability in cue-reinforced responding observed in the vehicle-infused controls in this particular experiment. The reduction in incubated drug-seeking upon intra-IL LY379268 infusion is peculiar in light of the discussion presented above, as well as prior work indicating little to no effect of IL inactivation upon stress- or cocaine-primed reinstatement of drug-seeking (Caprilles et al., 2003), or upon cue-primed reinstatement of MDMA-seeking (Ball et al., 2012). Further, in contrast to the PL, IL neurons do not exhibit a withdrawal-dependent enhancement of the encoding of cocaine-associated stimuli (West et al., 2014). Although a body of literature argues an important role for IL activity in suppressing drug-seeking behavior, other data argue that IL activation may, in fact, drive such behavior (see Moorman et al., 2015 for detailed review). As one example, lesions of either the PL or the IL are reported to reduce cocaine-seeking after 7 days of withdrawal (Pelloux et al., 2013). Notably, however, the effect of the IL lesion was more modest than that observed for the PL, which is a finding consistent with the data in Figure 12. Furthermore, immunohistochemical data indicate high levels of cellular activation within the IL when animals are exhibiting drug-seeking or drug-conditioned behaviors (e.g., Franklin and Druhan, 2000; Hamlin et al., 2008; Koya et al., 2009; Moorman and Aston-Jones, 2015; Nic Dhonnchadha et al., 2012; Zavala et al., 2007, 2008). Given the complexity of the vmPFC and the large overlap in projections from the PL and IL (Heidbreder and Groenewegen, 2003; Moorman et al., 2015a; Vertes, 2004, 2006), it is entirely possible that the increased cue-reinforced glutamate release observed in our prior study (Shin et al., 2016)

emanated from either the PL or the IL (or both) to drive incubated drug-seeking. This hypothesis remains to be addressed empirically.

4.4.4 Conclusions

In conclusion, the present study demonstrates that endogenous glutamate in the vmPFC is necessary for the manifestation of incubated drug-seeking during protracted withdrawal, while elevating endogenous glutamate within the IL is sufficient to attenuate this behavioral phenomenon. In contrast, manipulations of endogenous glutamate within neither region altered, in any obvious manner, drug-seeking expressed during early withdrawal. These neuropharmacological data extend prior correlative evidence of a relationship between vmPFC glutamate and the incubation of cocaine-seeking and argue a critical role for the incubation of glutamate release within "pro-relapse circuits" involving the PL (and perhaps also the IL) in the incubation of cue-reactivity during extended drug abstinence. If relevant to the human condition, these data highlight the potential pharmacotherapeutic utility of glutamate autoreceptor agonists as a viable strategy for curbing excessive cue-reinforced craving during protracted withdrawal and facilitating addiction recovery.

Chapter 5:

General Discussion

5.1 Summary of Studies & Findings

The data presented in this dissertation are the first to elucidate the role of endogenous glutamate in the PL and IL subregions of the vmPFC during incubated cocaine-seeking. In Experiment 1, adult male rats were trained to self-administer IV cocaine in a LgA paradigm for ten consecutive days, after which they were kept in their home cage for either 3 or 30 days of withdrawal (WD). At either 3 or 30WD, rats underwent microdialysis procedures on one hemisphere of the brain during a cue-reinforced cocaine-seeking test (Extinction Test). This experiment determined that there was a time-dependent increase in extracellular glutamate (GLU_{EC}) in the vmPFC selective for cocaine-trained rats, as these changes were not apparent in sucrose- or neutral cue-trained rats. These time-dependent increases in GLU_{EC} were positively correlated to incubated cocaine-seeking.

As mGlu2/3 receptors are essential in the regulation of GLU_{EC}, it was hypothesized that mGlu2/3 receptors internalize during protracted withdrawal, thus producing the increase in GLU_{EC} observed during protracted withdrawal in Experiment 1. Thus, Experiment 2 was designed to begin to test this hypothesis. Western immunoblotting was performed on the other, manipulation-free, hemisphere from the rats in Experiment 1, in order to ascertain mGlu2/3 levels in the vmPFC during short (3) or protracted (30) withdrawal. However, no changes in total mGlu2/3 expression were found at either time point. Hitherto, I ascertained that elevated GLU_{EC} in the vmPFC is correlated with incubated cocaine-seeking, but that this effect is likely unrelated to a change in the total protein expression of mGlu2/3 receptors.

As the results from Experiments 1 and 2 were correlational in nature, the question of vmPFC glutamate's functional relevance for cocaine-seeking, and its incubation, remained. Thus, Experiment 3 was designed to explore this question, using neuropharmacological approaches. After stable LgA cocaine SA was achieved, rats were again withdrawn from cocaine for either 3 or 30 days, at which time point they were exposed to an Extinction Test. As the PL is implicated in driving drug-seeking, while the IL is implicated in its supression, each subregion was targeted separately. Before the Extinction Test, endogenous glutamate was manipulated in either the PL or IL through microinjection of the EAAT inhibitor, TBOA (to raise glutamate levels), or the mGlu2/3 agonist, LY379268 (to lower glutamate levels/prevent release). In the PL, preventing the normal rise in glutamate found during 30WD via LY379268 infusion blocked incubated cocaine-seeking behavior, while increasing glutamate via TBOA infusion had no significant effects on behavior. In the IL, the results were less clear-cut; decreasing endogenous glutamate at 30WD reduced drug-seeking to a similar extent as that observed for the PL, but the effect was not statistically significant. In contrast, increasing glutamate in the IL via TBOA infusion significantly blunted incubated behavior. These results are consistent with the theory that the PL plays a necessary, but not a sufficient, role in driving cue-reinforced drug-seeking behavior, while the IL plays a more complicated role in regulating incubated drug-seeking behavior. Together, the three experiments in this dissertation point to glutamate within both subregions of the vmPFC as critical driving factors for incubation of cocaine craving.

5.2 Implications of Findings

As discussed in the General Introduction, the state of our knowledge regarding the PFC's role in the incubation of cocaine craving at the outset of this dissertation was limited. At that time, it was known that the vmPFC was involved in incubated cocaine-seeking, as inactivating this region abolished drug-seeking at protracted, but not short-term withdrawal (Koya et al., 2009). Over the course of the past five years (2012-2017), research on the incubation of cocaine seeking has exponentially increased, but most work has predominately focused on the NAC, as indicated in various reviews of incubation of cocaine seeking (e.g., Wolf, 2016; Li, Caprioli, & Marchant, 2015; Pickens et al., 2011). Despite some progress, the PFC is still clearly understudied within the context of incubated drug-seeking and more research needs to be done in order to have a more solid understanding of how PFC subregions contribute to this clinically relevant phenomenon.

5.2.1. Challenging the Anatomical Dichotomy of the Prelimbic and Infralimbic Cortex in Incubated Drug-Seeking

Along with the pro- and anti-relapse circuit of incubated craving not being clear cut, the PL and IL are also not so clearly defined, at least if based on anatomical connectivity alone. As mentioned in section 1.2.2, the PL and IL have extensive overlap in their projections, but they also have clear differences. Using the anterograde anatomical tracer, *phaseolus vulgaris*-leucoagglutinin (PHA-L), Vertes was able to record the projections of the PL and IL (Vertes, 2004). As much of the literature on incubated cocaine-seeking points to the heavy involvement of the mPFC to NAC circuit, I will focus on these projections.

It is known that the PL and IL both project to the NAC core and shell, respectively, but what is not usually described are the differing pattern and extent of their projections. In reality, the mPFC projections to the NAC mainly originate from the PL as the NAC is one of the main projection sites of the PL. The PL has fibers distributing throughout both the core and shell, but these fibers tend to terminate more heavily in the core. The rostral PL is known to distribute more heavily to the NAC core, as well as a slight tendency for the dorsal PL to project more dominantly to the core and the ventral PL to the shell. In contrast, the IL has significantly less labeling of fibers to the NAC, as the NAC is not one of the main projections of this region. The few projections the IL extends to the NAC terminate in both the NAC core and shell, with a slight preference to the shell.

The anatomical complexity increases as both the PL and IL project to each other, as well as projecting to itself. For example, the PL to NAC projection is the first step of the PL cortical loop to itself, called the PL-ventral striatopallidal-thalamocortical circuit. More specifically, the loop consists of the PL projecting to the NAC, which projects to the ventral pallidum and substantia nigra, to the mediodorsal nucleus, and back to itself. Thus, even though functional dichotomy exists between the two regions, it is clear that it will be hard to parse apart and producing mixed results are inevitable due the complexity of connections (e.g., Pelloux et al., 2013 results of IL compared to anterior IL; see Section 5.2.2.).

5.2.2. Imperfect Functional Mapping of the Infralimbic Cortex in Drug-Seeking

The pro-/anti-relapse dichotomy of the PL and IL cortices is likely too much of an overly simplistic view of the functional neurocircuitry underpinning any behavior or psychological process. Indeed, evidence calls into question if the anti-relapse IL region is truly involved in

the suppression of cocaine-seeking (see Moorman et al., 2015 for a comprehensive review). For example, inactivation of the IL (as well as the PL) decreased cocaine-induced reinstatement (Vassoler et al., 2013). Pelloux and colleagues report that after 7 days forced abstinence, lesions of the PL decreased context-induced cocaine-seeking after a battery of tests, some of which included progressive ratio schedule and intermittent punishment, with lesions of the IL exhibiting a trend toward decreased seeking as well (Pelloux et al., 2013). Interestingly, *anterior* IL lesions were able to significantly increase seeking responses compared to controls (Pelloux et al., 2013), which indicate that within the same subregion, functionality may differ.

Indeed, this may partially explain the unconventional results reported in Chapter 4. In the IL, either increasing (TBOA infusions) or decreasing endogenous glutamate (LY379268 infusions) seemed to produce similar behavioral results: cue-induced cocaine-seeking was blunted during protracted withdrawal (see Figures 10 and 12). Although not statistically significant compared to controls, decreasing glutamate in the IL during protracted withdrawal seemed to blunt incubated drug-seeking. Unfortunately, the thickness of my brain sections were too thick (75µM) to accurately deduce where injectors were placed. Figures 9 and 11 suggest they ever so slightly tend to land more posterior, but as I still have a significant number of injections in both anterior and posterior IL, the data is difficult to interpret in regards to the functional split between regions within the IL. Though both studies study cocaine relapse in the context of drug-seeking, these studies still differed methodologically to the incubation model used in my experiments so these results must be taken into consideration with caution. Of course, it may as well be possible that the evidence

for extinction/reinstatement and incubation may not differ so much in regards to the IL driving drug-seeking, but as of yet, no empirical evidence has tested this theory.

On the other hand, evidence arguing against the role of the PL in some general role in driving drug-seeking is less compelling. For example, inactivation of the PL enhanced cue-induced reinstatement of heroin-seeking (Schmidt et al., 2005), while inactivation of the PL also did not disrupt seeking induced by a cocaine-associated conditioned reinforcer (Di Ciano et al., 2007). It should be noted, however, the studies providing conflicting roles were mostly carried out in extinction/reinstatement models and not in the context of incubated drug-seeking. As these two paradigms are known to differentially impact both cellular and molecular changes in the brain (see section 1.4.1), it is possible that the results pertaining to the do not extend to the phenomena of incubation. In addition to utilization of different paradigms, most studies supporting this role of the PL focus on other reinforcers (e.g., heroin, sucrose, etc.) and not cocaine. Indeed, within the same incubation of craving paradigm, different reinforcers are known to recruit different brain regions (see section 1.4.3 and Table 1). Even evidence from my own work shows neurochemical differences between incubated sucrose versus cocaine-seeking (Chapter 3). Thus in the context of incubation of cocaine-seeking, the theory of the pro-relapse PL region may still hold and must be tested empirically.

5.3 Limitations & Future Directions

The data presented in this dissertation elucidate the functional role of glutamate in the PL and IL subregions of the vmPFC during incubated cocaine-seeking, yet many questions still remain. To address these questions, the following experiments are proposed:

(1) Characterize the molecular regulation of incubated glutamate release in the PL and IL. Along with bifurcating the role of GLU_{EC} in the PL and IL, the same needs to be done on the observed immunoblotting results of mGlu2/3 in the vmPFC (Chapter 3) before claiming mGlu2/3 are not involved in incubated cocaine-seeking. While research has shown that the PL and IL differentially regulate mGlu2/3 receptors during protracted withdrawal in alcohol-dependent rats (Meinhardt et al., 2013), further testing needs to be done on mGlu2/3 levels in the specific subregions of the vmPFC to ensure the same is not occurring during protracted withdrawal from chronic cocaine. As I dissected the whole vmPFC without separating PL and IL for the immunoblots, it is possible that changes in mGlu2/3 levels were not detected as they negated each other due to opposite regulation of the protein in the PL and IL. In addition, other assays can be used in order to further probe mGlu2/3 such as dimers, cell surface expression, post-translational modifications, etc.

(2) Characterize the role of calcium permeable (CP) and calcium impermeable (CI) AMPAR in the PL and IL. AMPAR within both the BLA and NAC play an important role in the incubation of cocaine craving (Conrad et al, 2008; Loweth et al., 2014; McCutcheon et al., 2011), raising the possibility that stimulation of these receptors within vmPFC, specifically the PL, by cue-reinforced glutamate release (Shin et al., 2016) might drive incubated drug-seeking behavior. Indeed, recent optogenetic work shows that silent glutamatergic synapses are generated in the vmPFC projections to the NAC during withdrawal from chronic cocaine, with these synapses eventually maturing/unsilencing via insertion of CI-AMPAR in the PL and CP-AMPAR in the IL during protracted withdrawal (Ma et al., 2014). The optogenetic reversal of this synapse maturation within PL-NAC projections decreased incubated drug-seeking, while that within the IL-NAC projections

potentiated incubated drug-seeking behavior. Similar work has been done in the BLA to NAC shell pathway, as reversing the maturation of silent synapses through downregulation of CP-AMPAR decreases incubated drug-seeking behavior (Lee et al., 2013). CP-AMPA are also implicated in the VTA as they accumulate in this region as well (Mameli et al., 2007; Chen et al., 2008). Despite this recent work, no neuropharmacological evidence exists directly linking AMPAR subtypes in the vmPFC to incubation of craving. Thus, future work should address this question by pharmacologically blocking CP- and CI-AMPAR using 1-naphthylacetylspermine (Naspm) and 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), respectively, in order to test the hypothesis that CI-AMPAR, but not CP-AMPAR, in the PL are functionally relevant for the incubation of cocaine-craving.

(3) Characterize the time-course of GLU_{EC} in the PL and IL, individually, during the incubation of cocaine-seeking. While Chapter 2 examined GLU_{EC} levels in the overall vmPFC during incubated cocaine-seeking, Chapter 4 makes clear that the PL and IL of the vmPFC are differentially involved in incubation. While we were limited by the length of our dialysis probes (2mm long), which precluded the possibility to be able to parse out dialysate stemming from the PL or IL (regions that are approximately 1mm), future studies utilizing a more refined microdialysis technique using 1 mm probes need to be run in order to determine the individual contribution of the PL and IL to elevated GLU_{EC} in the vmPFC. Results from Chapter 4 suggest that the elevated GLU_{EC} reported from Chapter 1 mainly stems from the PL during incubated cocaine-seeking, but this has not yet been confirmed empirically.

(4) Characterize the origin of glutamate that drives the time-dependent changes in vmPFC glutamate. Chapter 4 illustrated glutamate in the PL is functionally relevant in

incubated drug-seeking, which most likely drives the similar increase of GLU_{EC} exhibited in the NAC (McFarland et al., 2003; Suto et al., 2010) as the PL is one of the main glutamate afferents of the NAC core. Yet the question of where the increase of GLU_{EC} in the vmPFC originates arises. It can be that it all originates from the vmPFC itself, but another equally likely hypothesis is that it comes the thalamus. Indeed, the PL and IL both have strong afferent projections stemming from the mediodorsal nucleus (MDT), paratenial nucleus, paraventricular thalamic nucleus (PVT), nucleus reuniens, and rhomboid nucleus of the thalamus (Hoover & Vertes, 2007), making these nuclei a likely candidate. Although limited pre-clinical research has been done on the thalamus in the scope of drug addition, compared to the PFC, emerging evidence makes it clear this region is involved, particularly the PVT. For example, the thalamus of cocaine-dependent human subjects are known to exhibit lower gray matter volume (Sim et al., 2007; Garza-Villarreal et al., 2017). Pre-clinical reports also mirror this conclusion as lesions to the PVT block cocaine sensitization (Young and Deutch, 1998), which is another time-dependent phenomenon in which behavior intensifies in drug-experienced animals. Further, re-exposure to a cocaine-paired environment activates PVT neurons (James et al., 2011; Pelloux et al., 2017), and baclofen+muscimol (B/M) inactivation of the PVT blocks expression of a cocaine-conditioned place preference (Browning et al., 2014). Of more direct relevance to the work presented in this thesis, B/M inactivation of the PVT blocks cue-induced reinstatement (Matzeu et al., 2015), along with cocaine-primed reinstatement of drug-seeking (James et al., 2010), while having no effects on sweetened condensed milk seeking behaviors (Matzeu et al., 2015). Other regions of the thalamus may also play a role in cocaine addiction as lesions to the MDT decreases the rate of IV cocaine self-administration (Weissenborn et al., 1998). However, other thalamic nuclei

have not yet been extensively studied in the scope of cocaine addiction. However, the evidence implicates the thalamus and its nuclei in addiction, warranting further study.

(5) Characterize the sex differences of incubation of cocaine-seeking. As this dissertation utilized adult male rats, all studies should be extended to include females as well, as females are known to be more sensitive to relapse in both human and animal models. Clinical studies report female addicts are more reactive to drug-associated stimuli and have higher rates of relapse compared to males (e.g., Becker, 2016; Bobzean et al., 2014). These findings extend to rodent models, as female rats are more sensitive to cocaine-primed reinstatement (Lynch et al., 2000), exhibit more activity on the active lever during reinstatement (Lynch et al., 2000b; Kippin et al., 2005; Kersetter et al., 2008), and exhibit more enduring incubated cocaine-craving (Kersetter et al., 2008) compared to their male counterparts. Parsing the source of sex-specific differences in cocaine incubation will be the task of future researchers.

5.4 Conclusion

In summary, this dissertation provides novel insight into the role of glutamate in the vmPFC during incubated drug-seeking following a history of long-access cocaine self-administration. The results are the first of their kind to indicate that glutamate in the PL (and perhaps also the IL) is functionally relevant for cue-induced cocaine-seeking during protracted withdrawal. The data complements existing literature that PL-NAC core glutamate projections may be driving the enhanced encoding of cocaine-associated cues by this subregion (Suska et al., 2013; Luís et al., 2017), as well as the resultant metaplasticity within the NAC (and other terminal regions) culminating in greater cue-reactivity and

incubated responding. Together, the data within this dissertation add to the limited literature highlighting the importance of the PFC during incubated cocaine craving, and to our general understanding of the neurobiology of cocaine addiction.

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