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Prefrontal Synaptic Glutamate Transmission Dynamics across Psychostimulants and Behavioral Paradigms of Drug Addiction

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Prefrontal Synaptic Glutamate Transmission Dynamics Across Psychostimulants and Behavioral Paradigms of Drug Addiction

by

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Abbreviation Key

5-HT3A	-	serotonin receptor subclass 3 subunit A
Abs.	-	abstinence
ACSF	-	artificial cerebrospinal fluid
AMPA	-	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMPAR	-	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor
B/S	-	baclofen/muscimol
BLA	-	basolateral amygdala
CA1	-	hippocampal *Cornu Ammonis* area 1
CNQX	-	6-cyano-7-nitroquinoxaline-2,3-dione
CP-AMPARs	-	calcium-permeable AMPA receptor
CPP	-	conditioned place preference
CS	-	conditioned stimulus
CS-	-	unpaired compartment
CS+	-	paired compartment
Ctrl	-	saline control
D1	-	dopamine receptor 1
DA	-	dopamine
DALYs	-	disability-adjusted life years
DAT	-	dopamine transporter
dlCPu	-	dorsolateral caudate-putamen
dmPFC	-	dorsomedial PFC
DREADDs	-	designer receptors exclusively activated by designer drugs
EAAT	-	excitatory amino acid transporter

eEPSC	-	evoked EPSC
EPSC	-	excitatory postsynaptic current
Ext	-	extinction
GABA	-	γ-aminobutyric acid
GluA	-	AMPA receptor subunit
GluK	-	kainate receptor subunit
GluN	-	NMDA receptor subunit
I/O	-	input - output curve
iGluR	-	ionotropic glutamate receptor
IL	-	infralimbic mPFC
LTD	-	long-term depression
LTP	-	long-term potentiation
MDMA	-	3,4-methylenedioxymethamphetamine
mEPSC	-	miniature EPSC
meth	-	methamphetamine
mGluR2/3	-	metabotropic glutamate receptor 2/3
mGluR5	-	metabotropic glutamate receptor 5
mPFC	-	medial prefrontal cortex
MSN	-	medium spiny neuron
NAc	-	nucleus accumbens
NMDA	-	N-methyl-D-aspartate
NMDAR	-	N-methyl-D-aspartate receptor
PA	-	prolonged abstinence
PEPA	-	4-[2-(phenylsulfonylamino)ethylthio]-2,6-difluorophenoxyacetamide
PL	-	prelimbic mPFC

$PN^{5/6}$	-	deep layer 5/6 pyramidal neuron
PPR	-	paired-pulse ratio
PVN	-	paraventricular nucleus
RI	-	rectification index
SA	-	self-administration
SA	-	short abstinence
sEPSC	-	spontaneous EPSC
TTX	-	tetrodotoxin
VGLUT	-	vesicular glutamate transporter
vmPFC	-	ventromedial PFC

Abstract

JOSE I. PEÑA BRAVO. Prefrontal Synaptic Glutamate Transmission Dynamics Across Psychostimulants and Behavioral Paradigms of Drug Addiction. (Under the direction of ANTONIETA LAVIN, Ph.D.)

The medial prefrontal cortex (mPFC) is an important node in the brain's reward-seeking circuit and neuronal activity within this region is modulated by exposure to discrete cues and contexts previously associated with a drug experience. The mPFC is anatomically divided into the dorsal mPFC (dmPFC: containing the cingulate and dorsal prelimbic areas) and ventral mPFC (vmPFC: ventral prelimbic and infralimbic areas). Several studies have explored the functional distinctions between the dorsal and ventral mPFC with pharmacologic or genetic manipulations of its afferent glutamatergic projections to the ventral striatum. This line of research has uncovered opposing roles between these two mPFC subregions. Specifically, increases in activity within the prelimbic area (PL-mPFC) have been shown to drive drug-seeking behavior while excitatory drive of the infralimbic area (IL-mPFC) inhibits this behavior following extinction training. However, the basal features of glutamatergic synaptic transmission that underlie this functional distinction and the synaptic plasticity generated by drug experience or exposure to drug-associated stimuli in PL- and IL-mPFC pyramidal projection neurons are not known. This dissertation addresses the hypothesis that glutamate synaptic transmission in deep layer 5/6 pyramidal neurons of the mPFC exhibits basal differences between mPFC subregions that are altered in response to drug-related cues and context, and that drug-seeking behavior, specifically psychostimulants such as cocaine and methamphetamine, is in part regulated by these plastic changes in ionotropic excitatory synaptic transmission. To test this, we made brain slice recordings in two widely used models of drug addiction in rats: the conditioned place preference paradigm (CPP) and the

reinstatement model of drug self-administration. Following self-administration or experimenter administration (in the CPP paradigm) of psychostimulants (i.e. cocaine or methamphetamine), we tested whether exposure to discrete cues (reinstatement model) or the context (CPP) previously associated with the drug, produced alterations in synaptic excitatory ionotropic glutamate receptor transmission that could account for the following behavioral responses: retention of CPP after different abstinence intervals or cue-induced reinstatement of drug seeking. Our results suggest that cocaine produces different effects on PL and IL neurons that are dependent on the behavioral paradigm that is utilized. Specifically, cocaine self-administration followed by extinction alone, or cue-induced reinstatement did not produce any measurable differences in glutamate transmission compared to saline yoked rats. Cocaine-CPP on the other hand, produced several changes in glutamate transmission in both PL and IL neurons. These neuroadaptations were dependent on the length of abstinence and were reversed by context re-exposure. Lastly, contrary to the effects of cocaine self-administration, methamphetamine self-administration followed by 8 days of abstinence produced pre- and postsynaptic changes in glutamate transmission in mPFC neurons. In summary, these results provide evidence that general changes in mPFC synaptic glutamate transmission account for aspects of drug-seeking behavior that are not responsive to exposure to drug-associated cues or context, while other alterations in synaptic transmission that meet the functional distinction between mPFC subregions are sensitive to drug cue- or context-associations.

Chapter 1:

Introduction

The medial prefrontal cortex (mPFC), an important node in the brain reward circuit, is modulated by exposure to discrete cues and contexts previously associated with a drug experience. The mPFC is anatomically divided into the dorsal (dmPFC: containing the cingulate and dorsal prelimbic areas) and ventral mPFC (vmPFC: ventral prelimbic and infralimbic areas)(Sesack et al., 1989; Heidbreder and Groenewegen, 2003; Hoover and Vertes, 2007). Several studies in the field of drug addiction have explored the functional distinctions between the dorsal and ventral mPFC with pharmacologic or genetic manipulations of its afferent glutamatergic projections to the ventral striatum(Berendse et al., 1992; Peters et al., 2009; Moorman et al., 2015; Gourley and Taylor, 2016; Garcia et al., 2017). This line of research has uncovered opposing roles in the control of drug-conditioned behavioral responses between these two mPFC subregions. More specifically, increases in activity within the prelimbic area (PL-mPFC) have been shown to drive drug-seeking behavior while the excitatory drive of the infralimbic area (IL-mPFC) inhibits this behavior following extinction training (McLaughlin and See, 2003; Peters et al., 2008; Rocha and Kalivas, 2010; LaLumiere et al., 2012; Moorman et al., 2015; Augur et al., 2016; McGlinchey et al., 2016).

However, several questions about the psychostimulant-induced synaptic alterations in the mPFC proper remain unanswered. Mainly, questions about the changes in deep layer 5/6 pyramidal neuron ($PN^{5/6}$) synaptic ionotropic glutamate receptor (iGluR) transmission); and among these: 1) what are the basal features of glutamatergic synaptic transmission that underlie the functional distinction in drug reward and relapse between the PL and IL-mPFC regions? 2) Are there changes in iGluR-

mediated spontaneous or electrically evoked postsynaptic currents that could account for the expression of addiction-related behaviors? 3) Which synaptic iGluR alterations emerge from the experience of drug-associated stimuli?

The dissertation research described herein addresses two hypotheses. First, **glutamate synaptic transmission in $PN^{5/6}$ of the mPFC exhibits basal differences between mPFC subregions.** These basal differences are necessary for efficient mPFC functioning. Repeated exposure to psychostimulants facilitates associative learning processes and the generation of long-term rewarding memories (Torregrossa et al., 2011; Volkow and Morales, 2015). Therefore, disrupting these aberrant associative learning processes can be beneficial for addicted individuals with a high relapse vulnerability. This approach requires the characterization of the glutamatergic synaptic elements that promote this differential mPFC subregion functioning. That leads to the second hypothesis of this dissertation: **psychostimulant seeking behavior (e.g., cocaine and methamphetamine) induced by drug-associated cues and contexts, is regulated by plastic changes in ionotropic glutamate receptor (iGluR) synaptic transmission in the mPFC**. To test these hypotheses, we made brain slice recordings in two widely used models of drug addiction in rats: the conditioned place preference paradigm (CPP) and the reinstatement model of drug self-administration. Following self-administration (SA) or experimenter administration (CPP) of psychostimulants (cocaine or methamphetamine), we tested whether exposure to discrete cues (reinstatement model) or the context (CPP) previously associated with the drug, produced alterations in $PN^{5/6}$ iGluR transmission that could account for the expression of drug-seeking behavior. Specifically, these synaptic alterations represent the underlying changes in $PN^{5/6}$ iGluR synaptic transmission that mediate the retention of CPP memories after different abstinence intervals; or, drug-seeking behavior after a cue-induced reinstatement test.

The expression of psychostimulant-conditioned behavioral responses in our studies serves as a pre-requisite for the electrophysiological characterization of iGluR synaptic transmission in mPFC brains slices. With a greater understanding of the overlapping neuronal adaptations produced by psychostimulants and psychostimulant-associated stimuli, we can complement the available cognitive behavioral treatments for recovering addicts and attempt to curve the trends of drug use disorders amongst the population.

Addiction as a public health problem

In addition to mechanistic preclinical studies, an understanding of the global and national burden of drug abuse disorders allows researchers to substantiate the need for more access to research funding. These preclinical studies can also inform public health and government policy making with the aim of tackling this global epidemic. According to the United Nations Office of Drugs and Crimes (UNODC): World Drug Report 2017, an estimated 5% of the global adult population used drugs at least once in 2015. Out of this sample, about 0.6% of individuals suffer from drug use disorders(UNODC, 2017). Psychostimulants, a class of drugs that includes cocaine and amphetamines, produce increases in arousal, attention, wakefulness among other physiological responses. Interestingly, disorders related to the use of amphetamines are second after opioids at their shared global burden of drug use [calculated as disability-adjusted life years (DALYs) or numbers of years of life lost and early deaths due to drug use-related disability] (UNODC, 2017). Data from the Substance Abuse and Mental Health Services Administration (SAMHSA) revealed that in 2015, there were 27.7 million people (aged 12 and up) who used an illicit drug in the prior month (current users), which corresponds to about 1 in 10 Americans. Out of these, 1.9 million and 0.9 million are active cocaine and methamphetamine users, respectively. Moreover, approximately 21.7 million Americans (aged 12 and up) received substance abuse treatment(Bose et al., 2016). For the US,

the substance abuse epidemic costs roughly $600 billion (this estimate includes alcohol and nicotine abuse disorders), an amount of money that points out the need to invest in research for effective treatments thereby reducing the economic burden of this disease(NIDA, 2012).

Cocaine use and abuse statistics

Epidemiological reports on cocaine use show that after years of declining use, the numbers of users in North America and Europe appear to be increasing. This trend is represented in the cases of drug overdose involving cocaine, which has shown a marked increase in the US between 2012-2015(UNODC, 2017). Worldwide cocaine use is estimated at 17 million users, which accounts for about 0.4 percent of the global population within the ages of 15-64(UNODC, 2017). North America has the highest prevalence of cocaine use at 5.3 million people (or 1.7% of the population 14 years and older).

A prospective study surveying the prevalence of use of various drugs for 8th, 10th and 12th graders in the US (Monitoring the Future: revised in 2017) showed that lifetime cocaine use for the year 2016 accounted for 1.4%, 2.1%, and 3.7%, respectively, of the student population(Johnston et al., 2017). The numbers for this population have decreased from the previous three years but suggest that a significant portion of the student population are at risk of cocaine abuse disorder as early as 13-14 years of age(Johnston et al., 2017).

The National Survey on Drug Use and Health (US) for the year 2015 estimates the lifetime prevalence of cocaine use at 14.5% for ages 12 and older and 16.6% for ages 26 and older. Despite a decrease in cocaine use in the previous years, these statistics represent the illicit drugs with the highest use levels, compared to

hallucinogens at 16.2%, LSD at 10.7% and methamphetamine at 6.4% for ages 26 and older. Importantly, cocaine use is associated with the most hospital emergency room visits for causes related to illicit drugs in the US. There were 505,000 visits in 2011 due to cocaine-related emergencies. Among the patients visiting the emergency room for withdrawal symptoms, one-fourth of these cases were due to cocaine(SAMHSA, 2011).

Methamphetamine use and abuse statistics

Methamphetamine use appears to be increasing as is the number of users seeking treatment. Amphetamine-type stimulants are among the fastest growing abused drugs worldwide. An estimated 4.7 million Americans have tried methamphetamine at some time in their lives, with similar rates of use among males at 0.32% and 0.23% for females(Durell et al., 2008). Methamphetamine use in the US reached epidemic levels in the early 2000s in the East and Midwest. Even though recent reports estimate a stable rate of methamphetamine use, law enforcement agencies and substance abuse treatment programs indicate that the drug problem continues to be a significant public health problem.

Recent epidemiological data on relapse rates suggest that about two-thirds of addicts relapse within weeks to months of treatment initiation(Sinha, 2011). Moreover, there is no effective addiction treatment currently available. Therefore, basic science research should play a protagonist role in informing public health policy on drug use disorders. Among the approaches that can be taken to treat this complex disorder are: abuse-deterrent or tamper-resistant formulations, substitution therapy (replacing an illegal drug with a legal alternative that can promote abstinence), antagonist therapies (such as vaccines, that eliminate the euphoric and reinforcing effects of the drugs), aversion therapies (drugs that make the subsequent illicit drug use aversive). Lastly, the

approach that we are interested in taking is the diverse novel approach, where researchers aim to disrupt drug craving and relapse behavior by altering the drug-associated memories and behavioral responses(Tzschentke, 2014).

The relevance of mPFC activity (glutamate output) in mediating relapse and cravings in response to stimuli previously associated with a drug experience has been supported by preclinical and clinical studies(Bauer et al., 2013; Chen et al., 2013; Parsegian and See, 2014; Hanlon et al., 2015). Therefore, targeting this enhancement in mPFC glutamatergic output during a relapse event, either by directly inhibiting neuronal output or by targeting discrete synaptic receptors that influence the intrinsic excitability of these neurons is an important strategy in the development of relapse prevention therapeutics.

Glutamate synaptic transmission: A focus on ionotropic glutamate receptors and their role in drug-induced plasticity

Glutamate constitutes approximately 80-90% of the excitatory synaptic transmission in the brain. The following diagram depicts several steps in glutamate signaling, with each step briefly explained below (**Fig. 1**). This overview of the life cycle of glutamate signaling starts with the synthesis of glutamate in the presynaptic nerve terminal where glucose, produced via the Krebs cycle, is converted into glutamate. Additionally, glial cells synthesize glutamine, release it to the extracellular space, where it is actively taken up at the terminals and enzymatically converted into glutamate by glutaminase. At the nerve terminals, glutamate is stored in synaptic vesicles via proton antiporters named vesicular glutamate transporters (VGLUTs). Currently, three identified VGLUTs perform this function in the CNS. VGLUT 1 and 2 show similar patterns of expression, predominantly in excitatory neurons. On the other hand, VGLUT3 is mainly expressed in non-glutamatergic neurons. Upon nerve terminal depolarization (i.e., in a

Ca^{2+}- and activity-dependent manner) glutamate is exocytosed to the synaptic cleft, where it is available to bind to pre-, post- and extrasynaptic receptors. Two major families of glutamate-binding receptors exist: ionotropic and metabotropic receptors.

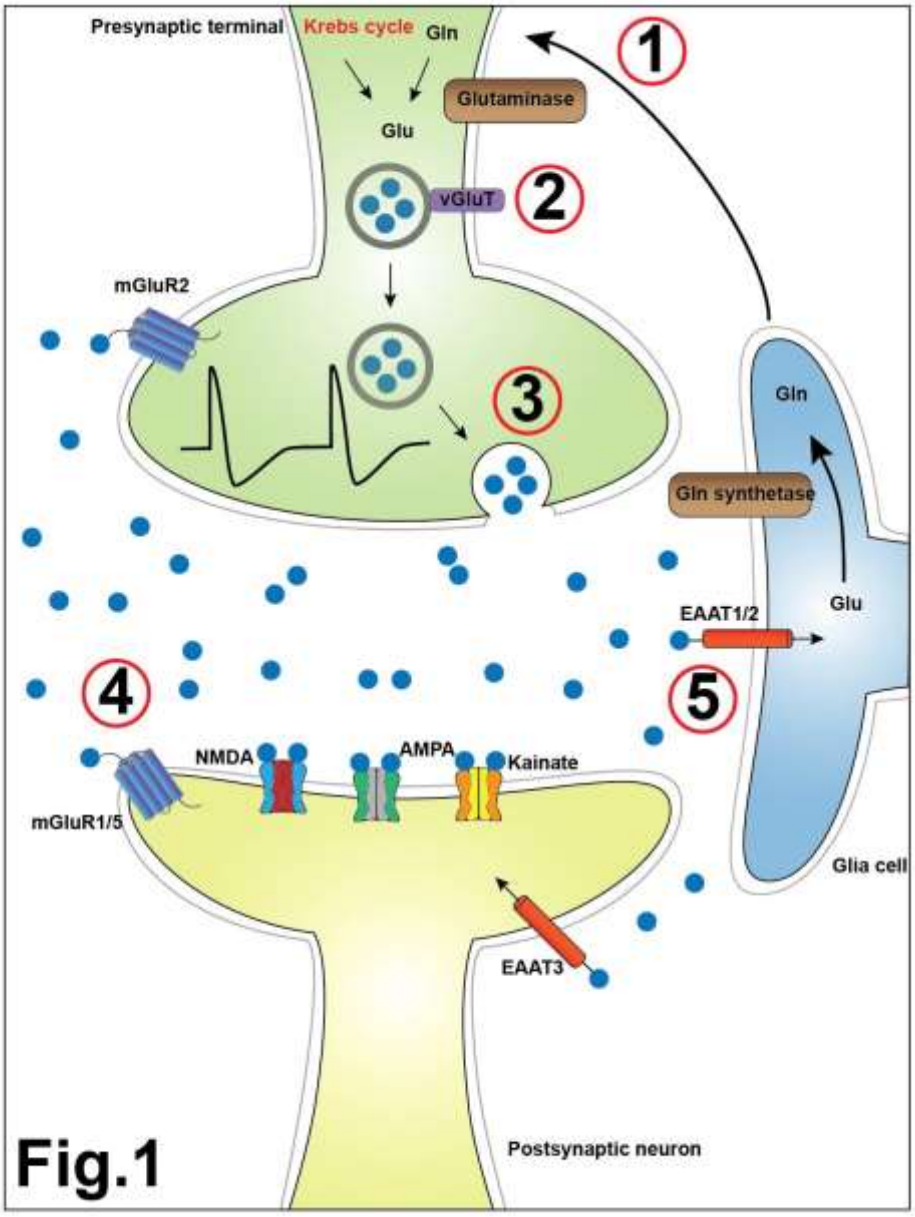

Figure 1. Components of glutamatergic synaptic transmission. Diagram depicts the steps of glutamate synaptic transmission: 1) Glutamate synthesis and glutamine (Gln) conversion to glutamate (Glu). 2) Transport into synaptic vesicles 3) Exocytosis 4) Receptor binding 5) Synaptic transporters at different synaptic locations and in glial cells.

These receptors mediate fast excitatory transmission and G protein-mediated intracellular signaling, respectively (more on this topic in the next paragraph). Finally, the glutamate signal is terminated by re-uptake of glutamate by excitatory amino acid transporters (EAATs). EAATs 2,5 are located pre- and EAATs 3,4, postsynaptic. Additionally, glutamate is taken up by glial EAATs 1 and 2. More information on the main families of glutamate binding ion channels is provided in the following section as an introduction to the focus of this study: the psychostimulant-mediated effects on different forms of plasticity mediated by fast excitatory postsynaptic glutamate transmission.

The glutamate-binding ionotropic receptor (iGluR) family consists of three subfamilies of tetrameric receptors: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and kainate receptors. Each receptor family exists in the brain as different subtypes composed of a combination of subunits that dictate the pharmacological and kinetic profiles of the receptors. NMDA receptors (NMDARs) are made up of three subunits: GluN1-3 (GluN1, four subtypes: GluN2A-D and two subtypes: GluN3A and B) and the pore formed by different combinations of these subunits are permeable to Ca^{2+}, Na^{+}, and K^{+}. Another feature of this receptor is that, in addition to glutamate binding, glycine or D-serine co-agonist activation is a prerequisite for NMDAR activation. Lastly, the NMDAR ion channel pore is blocked by Mg^{2+} at rest, giving the receptor the function of coincidence detector in the synapse. Removal of this block requires pre- and postsynaptic membrane depolarization to achieve maximum channel conductance. AMPA receptors (AMPARs) are made up of a combination of four subunits: GluA1-4. Alternative splicing of the AMPAR subunits' RNA generates different variants. AMPARs containing edited GluA2 subunit RNA (codon substitution from glutamine (Q) to arginine (R)) yields receptors that are Ca^{2+}-impermeable. Most receptors contain the edited form of the GluA2; therefore, receptors

lacking the GluA2 subunit are Ca^{2+}-permeable. This characteristic will be further explored as it relates to cocaine-evoked alterations in AMPAR subunit composition and synaptic transmission. The last group of iGluRs is kainate receptors. Kainate receptors and their respective subunits (GluK1-3) form functional homomers except for subunits (GluK4,5) that only form functional receptors as heteromers in combination with the other subunits. Similar to the GluA2 subunit, GluK 1 and 2 show differences in Ca^{2+} permeability depending on the alternative splicing. The lack of compounds that selectively target these receptors has made it difficult to distinguish them from AMPARs in mediating drug-induced plasticity and other physiological roles in synaptic transmission. Glutamate signaling beyond ionotropic and metabotropic receptor activation (i.e., intracellular signaling, posttranslational modifications and gene transcription) is intrinsically tied to the long-term effects of synaptic plasticity but will not be expanded on. This dissertation will focus on iGluR synaptic transmission dynamics and understanding first how this form of neuronal communication occurs under healthy circumstances, and how drugs of abuse, particularly psychostimulants, usurp synaptic plasticity mechanisms and transiently or constitutively alter synaptic transmission. It is worth mentioning that drugs of abuse can alter glutamate synaptic transmission and signaling at many levels that go beyond the scope of this dissertation, for a review of the literature see: (Bowers et al., 2010; Bellone and Luscher, 2012; van Huijstee and Mansvelder, 2014; Mulholland et al., 2016).

Functional Dichotomy between dmPFC and vmPFC

The dorsomedial (dmPFC) and ventromedial prefrontal cortex (vmPFC) are functionally heterogeneous brain regions and their projections follow this functional dichotomy(Van den Oever et al., 2010; Moorman et al., 2015; Gourley and Taylor,

2016). This section, summarized in **(Table. 1)** provides a brief overview of the dorsoventral axis distinction of mPFC neurons in drug-associated behavioral responses.

Anatomical organization and neuronal physiology of prefrontal cortical pyramidal neurons

Information processing in the cortex relies on the integration of signals between distinct neuronal cell types and the activation dynamics between cortical layers and areas. Cortical layers are organized in an inside-out pattern with the innermost layer 6 forming first and outermost layer 1 forming last. Pyramidal neurons localize to all layer of the cortex except for layer 1. The PFC is composed of excitatory neurons (pyramidal and spiny stellate neurons) that project to cortical and subcortical regions. The other population of cortical neurons are inhibitory GABAergic interneurons which make up about 10-15% of all cortical neurons (Meyer et al., 2011). These inhibitory neurons can be subdivided into multiple subgroups based on morphological, physiological and connectivity parameters. The major groups include parvalbumin positive (40%), somatostatin-positive (30%) and 5-HT3A receptor positive inhibitory neurons(Tremblay et al., 2016). Although the focus of this dissertation is on pyramidal neurons in the mPFC, this small population of inhibitory neurons plays a crucial role in the modulation of cortical outputs and information processing(Kubota et al., 2016; Tremblay et al., 2016).

Anatomical distinction along the dorsoventral axis of the mPFC

The mPFC can be divided along its dorsoventral axis into dorsal mPFC that includes the anterior cingulate cortex and the prelimbic (PL) cortices, and the

ventral mPFC including the ventral part of the PL cortex and the IL cortex (Heidbreder and Groenewegen, 2003; Vertes, 2004; Hoover and Vertes, 2007; Moorman et al., 2015).

The PFC receives connections from the amygdala and the hypothalamus to the ventral and medial regions(Fuster, 2001). The PFC is connected largely to high order association areas but not directly to primary sensory or motor regions of the cortex. Moreover, the different subregions of the PFC heavily connect within and between each other. Connections between association cortex regions initiate in layers 2/3 and their projections terminate back in the same layers.

Prelimbic Cortex afferent and efferent connections

Among the regions that project to the PL cortex are: the orbital cortex (medial and ventral), the IL cortex, agranular insular, perirhinal and enthtorhinal cortices. These corticocortical projections originate from layer 5. The CA1 and subiculum subregions of the hippocampus send dense projections to the PL. However, Hoover and Vertes found, that the mPFC does not project back to the hippocampus directly, it does so through connections with the nucleus runiens of the midline thalamus (Hoover and Vertes, 2007). Thalamic projections of the PL originate from the medial and mediodorsal thalamus. Among the major innervations of the PFC is the DA/glutamatergic projection originated from the VTA(Seamans and Yang, 2004). The PL region projects to the nucleus accumbens (NAc) core region of the ventral striatum. These projections originate from layer 5 of the cortex and innervate contra and ipsilaterally.

Similarly, downstream targets of the PL cortex to the amygdala target specifically the basolateral and lateral nuclei(Gourley and Taylor, 2016).

Infralimbic Cortex afferent and efferent connections

PL cortex projections are among the major source of prefrontal cortex input to the IL subregion. Similarly, the IL projects back to the PL as well as the hippocampal formation. Interestingly, the input from sensorimotor and association cortex regions to the IL is almost negligible. Among the principal efferent connections of the IL are: the agranular insular cortex, olfactory tubercle, perirhinal cortex, the whole amygdaloid complex, caudate putamen, nucleus accumbens, bed nucleus of the stria terminalis, midline thalamic nuclei, the lateral preoptic nucleus, paraventricular nucleus, supramammillary nucleus, medial mammillary nucleus, dorsal and posterior areas of the hypothalamus, ventral tegmental area, central gray, interpeduncular nucleus, dorsal raphe, lateral parabrachial nucleus, locus coeruleus, the anterior olfactory nucleus, piriform cortex, anterior hypothalamic area and lateroanterior hypothalamic nucleus were observed(Takagishi and Chiba, 1991). The IL also sends specific projections to the amygdala that innervate the basal, central and medial subregions particularly the intercalated cells that created clusters of GABAergic divisions between these regions(Gourley and Taylor, 2016). Another feature of IL connectivity is its connection to visceral and autonomic brainstem nuclei that control cardiovascular, respiratory and gastrointestinal functions.

Manipulation of the medial prefrontal cortex activity to investigate addiction-related behaviors

Studies of context-induced conditioned hyperactivity revealed that intra-mPFC muscimol (GABA-A agonist) infusion blocked conditioned hyperactivity in response to cocaine. Indeed, immediately or 4 months after extinction training, cocaine discriminative stimulus elicited a strong recovery of responding and increased Fos immunoreactivity in the mPFC. These effects were selectively reversed by D1 antagonists (Ciccocioppo et al., 2001) suggesting that conditioned hyperactivity (locomotor activity test under drug-free conditions) was associated with increased Fos expression in the mPFC(Franklin and Druhan, 2000b). In a different study, Fos expression in PL and NAc core neurons was positively correlated with cue-induced cocaine-seeking behavior(McGlinchey et al., 2016). These results show that pharmacological manipulations that alter the activity of the PFC (without specific targeting of subdivisions in this region) can alter behavioral responses associated with drug seeking and the rewarding effects of drugs.

Drug-related responses following manipulations of dorsomedial prefrontal cortex (anterior cingulate cortex and prelimbic cortex) activity

The role of PL-mPFC in mediating reinstatement to psychostimulants and other drugs of abuse has been extensively investigated. Ball and colleagues have shown that lidocaine inactivation of PL-mPFC prevents conditioned stimulus (CS)-induced reinstatement of MDMA seeking(Ball and Slane, 2012). Similarly, Di Pietron and colleagues showed that inactivation of the PL-mPFC by local application of lidocaine reduced drug seeking during the maintenance phase of the behavior and disrupted responding for cocaine-associated cues but not odorant cues (Di Pietro et al., 2006). In another set of studies, TTX inactivation of the dmPFC blocked context-induced

reinstatement(Fuchs et al., 2005). Interestingly, another study showed that rostral basolateral amygdala (BLA) and PL-mPFC asymmetric lidocaine inactivation attenuated reinstatement elicited by discrete and contextual cocaine-paired cues. Unilateral inactivation was not sufficient for disruption of reinstatement to cocaine seeking, suggesting an interplay between these two regions (Mashhoon et al., 2010). Asymmetric pharmacological manipulation (blockade of DA signaling in PL-mPFC and glutamate signaling in the NAc core) attenuated cue-induced reinstatement of cocaine seeking but had no effect on reinstatement of sucrose seeking (McGlinchey et al., 2016). Similarly, intra-PL TTX reversible inactivation impaired cue-induced reinstatement of cocaine seeking (McLaughlin and See, 2003). Also, inactivation of the PL-mPFC or the nucleus accumbens core (NAc core) with inhibitory cocktails such as baclofen/muscimol (B/M) eliminated cue or meth primed reinstatement(Rocha and Kalivas, 2010). A recent student used a viral strategy to express an inhibitory opsin in PL-mPFC neurons and revealed that bilateral optical inhibition of the PL-mPFC inhibited the reinstatement of cocaine seeking(Stefanik et al., 2013).

Drug-related responses following manipulations of ventromedial prefrontal cortex (ventral prelimbic cortex and infralimbic cortex) activity

The role of the IL-mPFC in addiction is less clear. Studies using retrograde labeling from the NAC shell to vmPFC neurons expressing excitatory designer receptors exclusively activated by designer drugs (DREADDs) show that activation of these neurons reduced cue-induced reinstatement of cocaine seeking following extinction training but did not alter cocaine seeking in rats that underwent abstinence(Augur et al., 2016). Different from these effects, studies using lidocaine inactivation of IL-mPFC show similar levels of response to a conditioned stimulus (CS) -induced reinstatement of MDMA seeking, suggesting a lack of influence of the IL-mPFC on seeking behavior(Ball

and Slane, 2012). Other studies have shown that asymmetrical disconnection by B/S infusion into vmPFC and SCH 23390 (D1-like antagonist) infusion into the NAc shell decrease context-induced reinstatement (Bossert et al., 2012). In a recent study using PEPA, an AMPA receptor positive allosteric modulator was microinjected into the IL-PFC and it suppressed cue-induced reinstatement of cocaine seeking. This effect was reversed by local infusion of dopamine (DA) into the PL-mPFC or BLA. The effect of intra-IL injections of PEPA was also reversed by DA or CNQX (AMPA/kainate receptor antagonist) injections into the NAc-shell (LaLumiere et al., 2012); however, reversible inactivation of the IL with TTX had no effect on cue-induced reinstatement of cocaine seeking (McLaughlin and See, 2003). On the other hand, inactivation of IL or NAc shell inhibited meth primed and cue-induced reinstatement of methamphetamine seeking(Rocha and Kalivas, 2010).

While there is still much to investigate regarding the role of IL-mPFC in addiction, a picture that suggests that IL-mPFC may be involved in decreasing drug seeking or reinstatement is beginning to emerge.

Manipulating <u>mPFC terminals in the NAc</u> as evidence of a role of PFC glutamate input in drug-associated behavioral responses

Adding to the evidence that shows a specific role of the prefrontal cortex, in addition to the literature on the NAc role as a limbic-motor interface, it is important to list the studies that have selectively targeted PFC terminal innervating NAc neurons. Studies have shown that infusion of baclofen (GABA-B agonist) in the NAc blocked conditioned hyperactivity in response to a cocaine-associated context(Franklin and Druhan, 2000a). Interestingly, optogenetic reversal of cocaine-evoked plasticity and seeking responses was achieved by applying a 13Hz light stimulation protocol (LTD) at

mPFC to NAc synapses 4 hours prior to a cue-induced reinstatement test(Pascoli et al., 2014) and bilateral optical inhibition of NAc core or the PL-mPFC fibers in the NAc core inhibited the reinstatement of cocaine seeking(Stefanik et al., 2013). Intriguingly, optical inhibition of the PL-mPFC fibers in the NA core was associated with a decrease in MSNs spine head diameter and AMPA to NMDA ratio(Stefanik et al., 2016).

Conditioned hyperactivity (locomotor activity test under drug-free conditions) in response to a cocaine-associated stimulus is associated with increased Fos expression in the NAc(Franklin and Druhan, 2000b) and microdialysis studies show increases in glutamate release in the NAc in response to exposure to a cocaine-associated cue. Indeed, following long access cocaine self-administration and withdrawal for either 3 or 30 days show that the long-lasting withdrawal (30 days) resulted in an increase and decrease in cue-elicited vmPFC glutamate and dopamine release, respectively. These results suggest that the effect occurs only after long periods of abstinence following cocaine experience (Shin et al., 2016).

Methamphetamine self-administration reduces basal glutamate levels in the dmPFC and NAc. However, reinstatement of methamphetamine self-administration increased dmPFC and NAc glutamate efflux. Additionally, there were increases in dopamine efflux in the dmPFC but not NAc under different reinstatement conditions. Interestingly, combined methamphetamine prime and cue-induced reinstatement produced the highest increases in glutamate efflux in mPFC and NAc glutamate (Parsegian et al., 2011b).

target region	manipulation	blocked behavioral response	references
mPFC	TTX	context-induced cocaine seeking	Fuchs, RA 2005
	bac/mus	conditioned context-induced hyperactivity	Franklin, TR 2000
dmPFC (**PL**)	lidocaine	cue-induced MDMA seeking; context and discrete cue-induced cocaine seeking	Ball, KT 2012; Di Pietro, NC 2006; Mashoon, Y 2010
	bac/mus	cue-induced methamphetamine seeking	Rocha, A 2010
	genetic	cocaine seeking (opto)	Stefanik, MT 2013
	DA antagonist	cue-induced cocaine seeking	McGlinchey, EM 2016
vmPFC (vPL + **IL**)	bac/mus	context-induced cocaine seeking; cue-induced methamphetamine seeking	Bossert, JM 2012, Rocha, A 2010
	genetic	cue-induced cocaine seeking (DREADDs)	Augur, IF 2016
mPFC nerve terminal (NAc)	bac/mus	conditioned context-induced hyperactivity	Franklin, TR 2000
	genetic	cue-induced cocaine seeking (opto); cocaine seeking (opto)	Pascoli, V 2014; Stefanik, MT 2013, 2016

Table.1 Behavioral pharmacology, viral-mediated optogenetic (opto) and chemogenetic manipulations of mPFC target regions in drug-conditioned behavioral responses.

Hypothesis

Characterizing synaptic glutamate transmission in the same population of neurons, using different rodent models of addiction as well as different types of psychostimulants, allows for the identification of the overlapping drug-evoked adaptations and differentiation of adaptations that are unique to a particular drug or model. This type of study will inform the development of therapies that target common mechanisms between multiple drugs of abuse as well as instances where individualized approaches are advantageous.

The dichotomy over control of drug-related behavioral responses between the dorsal and ventral aspects of the mPFC encompasses experience-dependent alterations in synaptic glutamate inputs to deep layer (5/6) pyramidal neurons in response to different cue modalities associated with a previous drug experience. Drug-induced alterations in synaptic glutamate transmission are dependent on multiple factors that can be controlled experimentally (such as the type of drug, length of drug exposure, dosage, route of administration, and others). Nonetheless, there are global or overlapping synaptic changes that occur independently of the factors above and identifying these global alterations in synaptic glutamate transmission will serve as the basis for research on drug abuse and relapse. For our studies, we used the functional dichotomy between the dorsal and ventral mPFC in an effort to show that regardless of the type of drug that was administered, and the behavioral paradigm employed to create drug-stimuli associations, the dorsal aspect of the mPFC will increase its measures of synaptic glutamate transmission and promote drug seeking or the rewarding effects of drugs, while the ventral aspect will show no changes.

We set out to explore this ambitious hypothesis by the integration of two widely used behavioral paradigms in the addiction field: the conditioned place preference paradigm and the reinstatement model of drug self-administration. Also, we compared the actions of two different types of stimulants, cocaine and methamphetamine, to expand our knowledge of the psychostimulant-evoked adaptations in mPFC deep layer (5/6) pyramidal neurons. The following sets of aims were developed to engage different phases of the addiction process:

Aim1: Characterize the changes in glutamatergic synapses onto deep layer 5/6 neurons from prelimbic and infralimbic cortex produced by abstinence from cocaine-induced conditioned place preference.

Aim 2: Identify markers of plasticity in excitatory synapses innervating prelimbic and infralimbic deep layer 5/6 pyramidal neurons surrounding cue-induced reinstatement of cocaine seeking.

Aim 3: Characterize the changes produced in glutamate neurophysiology by methamphetamine self-administration.

Chapter 2:

Experimental Procedures

Laboratory Animals

Subjects used for cocaine-induced cocaine place preference studies (Chapter 3) were adult male Sprague–Dawley rats (Harlan), weighing 250–275 g upon arrival. Rats were pair-housed in a temperature-controlled colony room on a 12-hour light/dark cycle with food and water available *ad libitum*. Subjects used for cocaine (Chapter 4) and methamphetamine (Chapter 5) studies were adult male Sprague-Dawley rats (Charles Rivers) weighing 225-250 g upon arrival. Rats were individually housed with free access to food and water on 12 hours reversed light/dark cycle. All procedures were conducted in accordance with the 'Guide for the Care and Use of Laboratory Rats' (Institute of Laboratory Animal Resources on Life Sciences, National Research Council) and approved by the IACUC of the Medical University of South Carolina.

CPP Apparatus (Chapter 3)

A 3-compartment chamber (68 × 21 × 21 cm; ENV-013; MED Associates) was used to assess CPP. The chamber had manual sliding guillotine doors to separate the three compartments. The neutral compartment in the middle (12 × 21 × 21 cm) had gray walls and floor. The end compartments had the same dimensions (28 × 21 × 21 cm), with the right having black walls with a stainless steel grid rod floor and the right compartment having white walls with a stainless steel mesh floor. A computer controlled the CPP test using Med-IV software. A series of infrared photobeams (6 beams in the black and white compartments and 3 beams in the gray compartment) were used to record the amount of time spent in each compartment.

Cocaine Place Conditioning (Chapter 3)

On habituation day (day 0), all rats were allowed to roam the three compartments of the CPP apparatus for 10 minutes. The pre-conditioning test (PC) was used to verify the unbiased construction of the apparatus. Conditioning compartments were assigned in an unbiased manner such that each rat had equal opportunity to receive cocaine in their naturally least or most preferred side (Mueller and Stewart, 2000; Tzschentke, 2007; Reichel et al., 2010a). Placements were counterbalanced according to chamber color (black/white) and whether the rats received the cocaine injection on the first or second day of conditioning. Multiple cohorts of rats were used in this study and a group of saline-treated (Ctrl: cocaine naïve) rats was included in each cohort. Rats were conditioned for 8 days with 24 hr intervals between sessions. During odd days (days 1,3,5,7) of conditioning, rats were either restricted to the compartment with (black) rod floors or to the compartment with mesh floors (white) for 25 min. Rats placed in their paired compartment (CS+) were injected with cocaine (20 mg/kg) and rats placed in their unpaired compartment (CS -) received a saline injection immediately before placement. During even days (days 2,4,6,8) of conditioning, rats were placed in the opposite compartment for 25 minutes. On the test day (day 9), rats were injected with saline and were allowed to explore all chambers for 10 minutes. Time spent in each compartment was recorded and evaluated. Following the preference test, rats entered a short (SA: 8 days) or prolonged (PA: 30 days) abstinence period. Rats that failed to show a preference ratio greater than 50% (see data collection and analysis section below for calculation) were excluded from the study. Upon successful expression of a place preference, rats were randomly assigned to an abstinence group (SA or PA) and were tested for CPP (+) or remained in their home cages (-) until 24 hours after when the brains were dissected and mPFC slices were prepared for patch-clamp electrophysiology experiments (Fig.1 A). Saline rats (Ctrl) remained in their home cages for 8 days (SA) following the initial preference test and were allowed to explore the CPP

apparatus 15 minutes before tissue processing for electrophysiology experiments (Fig.1 A).

Catheter implantation surgery (Chapter 4 and 5)

The Neurobiology of Addiction Research Center (NARC) core technicians performed all surgeries for cocaine (Chapter 4) and methamphetamine (Chapter 5) self-administration experiments. Rats were anesthetized with intraperitoneal injections of ketamine (66 mg/kg; Vedco Inc, St Joseph, MO, USA), xylazine (1.3 mg/kg; Lloyd Laboratories, Shenandoah, IA, USA), and equithesin (0.5 ml/kg; sodium pentobarbital 4 mg/kg, chloral hydrate 17 mg/kg, and 21.3 mg/kg magnesium sulfate heptahydrate dissolved in 44% propylene glycol, 10 % ethanol solution). Ketorolac (2.0 mg/kg, intraperitoneal; Sigma, St. Louis, MO, USA) was given just prior to surgery as an analgesic. One end of a silastic catheter was inserted 33 mm into the external right jugular and secured with 4.0 silk sutures. The other end ran subcutaneously and exited from a small incision just below the scapula. This end was attached to an infusion harness (Instech Solomon, Plymouth Meeting, PA, USA) that provides access to an external port for IV drug delivery. Following this surgical procedure rats were given a subcutaneous injection of an antibiotic solution Cefazolin (10 mg/0.1 ml; Schein Pharmaceuticals, Florham Park, NJ, USA) and allowed to recover for 5 days.

Cocaine Self-Administration (Chapter 4)

Animals received surgery for intra-jugular catheter implantation (described in detail in (Reichel et al., 2011a) and were trained to self-administer cocaine or saline for 10 days until criterion was met. In brief, rats were trained for 10 days (daily/2-hour sessions) on a fixed ratio schedule (FR-1 schedule) of i.v. cocaine or saline self-administration (active lever press resulted in a 2mg/50µL infusion and a 5 second light/tone presentation), followed by 2 weeks of extinction training ending with a reinstatement session. Electrophysiological experiments were conducted either 15 minutes or 24 hours after a 2

hrs cue-induced reinstatement test. For extinction only rats, experiments were carried out 24 hours after the last session.

Methamphetamine Self-administration (Chapter 5)

Self-administration was conducted as previously described (Scofield et al., 2015b). In brief, methamphetamine self-administration began no less than five days following surgery. Methamphetamine was delivered intravenously on an FR1 schedule at a volume of 20 µg/50 µl infusion. Rats self-administered methamphetamine over 21 days with one-hour sessions for seven days, followed by six-hour sessions for 14 days. Another group of rats received yoked saline infusions as a control group. That is, every time a methamphetamine rat received an infusion of drug, a control rat received saline. Following the self-administration sessions, rats were provided 15-30 g of chow per day with free access to drinking water in their respective home cage. Eight days after rats were killed for endpoint measures of electrophysiology.

Brain Slice Preparation and Electrophysiology Solutions

Saline, cocaine or methamphetamine-treated rats were deeply anesthetized with isoflurane, brains were removed, and coronal PFC slices (300 µm) were cut on a vibratome (Leica, VT1200S, Nussloch, Germany) in ice-cold sucrose-containing ACSF (in mM): sucrose, 200; KCl, 1.9; Na_2HPO_4, 1.2; $NaHCO_3$, 33; $MgCl_2$, 6; $CaCl_2$, 0.5; D-Glucose, 10; ascorbic acid, 0.4. Slices were incubated at 32ºC for at least 1 h in a solution consisting of (mM): NaCl, 120; KCl, 2.5; NaH_2PO_4, 1.25; $NaHCO_3$, 25; $MgCl_2$, 4; $CaCl_2$, 1; D-Glucose, 10; ascorbic acid, 0.4. and then transfer to a recording chamber. Recordings were performed at room temperature using a recording ACSF consisting of (in mM): NaCl, 126; KCl, 2.5; NaH_2PO_4, 1.4; $NaHCO_3$, 25; $CaCl_2$, 2.0; $MgCl_2$, 1.3; D-Glucose, 10; ascorbic acid, 0.4 at a rate of 2-3 ml/min. All ACSF solutions were constantly aerated with a mixture of 95% O_2–5% CO_2 (pH 7.2, 300-310 mOsm).

Whole-cell voltage-clamp recordings were obtained from visually identified pyramidal neurons in layers V/VI of the PL-mPFC and IL-mPFC (Chapters 4, 5 and 6) or nucleus accumbens medium spiny neurons (Chapter 6) using differential interference contrast optics (Axioskop 2, Zeiss, Thornwood, NY) attached to a camera (Dage-MTI, Michigan City, IN, USA). Recordings electrodes (2.5-3 MOhm pipette resistance) were filled with (in mM): CsCl, 130; HEPES, 10; $MgCl_2$, 2; EGTA, 0.5; Na_2ATP, 2; Na-GTP, 0.3; QX-314, 2; phosphocreatine, 10; at 290 mOsmols. Spermine, 0.1 was added to the internal solution in experiments described in Chapters 3 and 4.

Data Collection and Analysis

Recordings were obtained with a Multiclamp 700B amplifier (Molecular Devices, Mississauga, ON, Canada). Signals were low-pass filtered at 3 kHz and digitized at 10 kHz. Data were stored on PC for off-line analysis. Data acquisition was performed using Axograph-X software (J. Clements, Sydney, Australia) and analysis of sEPSCs and eEPSC peak amplitude data was done in MiniAnalysis (v6.0.7; Synaptosoft). Each parameter was measured in the following order: A) sEPSCs and B) AMPA-eEPSC Rectification Index (RI). A) Briefly, membrane potential was held at −70 mV and glutamate-mediated events were pharmacologically isolated by adding 50µM picrotoxin to the bath. Series resistance (Rs) was continuously monitored by applying a small hyperpolarizing voltage step (-5mV, 50ms) and recordings that exceeded Rs>30MΩ were discarded. Spontaneous excitatory postsynaptic current (sEPSC) recordings consisted of 5 sweeps/10s-long recordings that were analyzed for amplitude and frequency of detected events. For sEPSC kinetics, all detected events per cell were used to obtain average: rise (ms), decay (ms) and area (pA*ms) in all experimental groups. B) The membrane potential was slowly shifted to +40mV, 50m µM D-APV, an NMDA receptor blocker, was added for at least 5 minutes to isolate AMPA-mediated responses. 8-10 isolated AMPA responses were recorded at +40mV, then the

membrane potential was slowly shifted to -70mV where 8-10 responses were recorded. Rectification index (RI) was calculated as the average eEPSC at -70mV over the average eEPSC at +40mV.

Chapter 4 and 5:

Picrotoxin (50 µM) was included in the perfusion solution to block $GABA_A$ receptors. For voltage-clamp recordings, electrodes (2.5-3.5 MΩ resistance in situ) were filled with a solution containing (in mM): CsCl, 130; HEPES, 10; $MgCl_2$, 2; EGTA, 0.5; Na_2ATP, 2; Na-GTP, 0.3; QX-314, 2; phosphocreatine, 10; 285 mOsmols. Series resistances (10-25 MΩ), and input resistances were continually monitored throughout the experiment via a -5 mV (50 ms) hyperpolarizing pulse. Neurons were clamped at -70 mV and spontaneous excitatory postsynaptic currents (sEPSCs) and paired-pulse responses were recorded. For AMPA/NMDA experiments, EPSCs were evoked using a concentric bipolar electrode placed within 300-400 µm of the recording electrode. Neurons were clamped at +40 mV and currents were recorded for 5 min at 0.033 Hz then the NMDA receptor antagonist AP-5 (D(-)-2-amino-5-phosphonovaleric acid (50 µM) was applied to isolate the AMPA currents. After 5 minutes, eEPSCs were recorded again for 5 minutes. The NMDA current was obtained by digital subtraction (I_{Total}- I_{AMPA}) and the ratio was calculated with the following formula: I_{AMPA} / I_{Total} – I_{AMPA}. Paired-pulse ratio (PPR) experiments were performed using an ISI of 50 msec, at 0.033 Hz. Recordings were made using a Multiclamp 700B amplifier (Axon Instruments, CA), connected to a computer running Windows XP and Axograph X software.

For statistical comparisons, all electrophysiological measurements for PL, IL-mPFC and NAc cells were compared under saline control conditions. Comparisons were performed using two-tailed, unpaired *t*-tests or analyses of variance (ANOVAs) followed by Dunnets post-hoc test when appropriate. For cocaine-induced place preference results, two-tailed paired t-tests were used to compare preference ratios with saline controls as a measure

of side preference. (Chapter 3) Individual two-sample Kolmogorov-Smirnov tests were used to detect shifts between cocaine-treated groups and saline control sEPSC amplitude and inter-event interval distributions. Differences of alpha ≤ 0.05 were considered statistically significant. All data are presented as mean ± SEM.

Drugs

Cocaine HCl was obtained from the National Institutes of Health and dissolved in 0.9% sterile saline. Methamphetamine HCl (Sigma, St. Louis, MO, USA) was dissolved in 0.9% sterile saline. QX 314 chloride was purchased from Tocris. Spermine and Picrotoxin were purchased from Sigma-Aldrich. D/L-APV was purchased from Abcam. All drugs used in electrophysiological recordings were dissolved in recording ACSF and bath applied except for QX 314 and spermine which were dissolved into internal ACSF and stocks were further diluted by adding to the internal solution the day of recording.

Chapter 3:

Abstinence from cocaine-induced conditioned place preference produces discrete changes in glutamatergic synapses onto deep layer 5/6 neurons from prelimbic and infralimbic cortices.

Introduction

Addiction treatment studies have demonstrated the need for novel biomarkers that target the symptoms associated with abstinence from drug use (Sinha, 2011). The prefrontal cortex (PFC) processes information relevant for the regulation of addiction-related behaviors, such as: impulse inhibition, behavioral monitoring, and decision making; among other behaviors (Mansouri et al., 2009; Goldstein and Volkow, 2011; Kim and Lee, 2011; Coutlee and Huettel, 2012). In addicted individuals, the loss of control over drug-taking despite negative consequences is one of the most significant symptoms and suggests cognitive deficits associated with abnormal PFC activity; for a review, see:(Goldstein and Volkow, 2011). Similarly, preclinical studies in rodents have demonstrated that cognitive dysfunction following prolonged cocaine exposure is mediated by altered PFC activity (Briand et al., 2008; George et al., 2008; Ghasemzadeh et al., 2011).

The role of the glutamatergic projections from medial PFC (mPFC) to the nucleus accumbens (NAc) in mediating cocaine-seeking behavior has been extensively studied (McFarland et al., 2003; Stefanik et al., 2013; Gipson et al., 2014; McGlinchey et al., 2016; Stefanik et al., 2016). Indeed, in rats trained to self-administer cocaine followed by extinction of the operant response, activity in the prelimbic mPFC (PL-mPFC) has been shown to drive cocaine-seeking behavior, while the infralimbic mPFC (IL-mPFC) plays an opposite role (McLaughlin and See, 2003; Peters et al., 2008; Van den Oever et al.,

2010; LaLumiere et al., 2012). These discrete PFC subregions send glutamatergic projections to specific targets in the ventral striatum. PL-mPFC projections innervate mainly the core of the NAc while IL-mPFC projections target the shell of the NAc (Heidbreder and Groenewegen, 2003; Vertes, 2004).

Whereas much of the research on cocaine addiction has focused on the NAc, less is known about long-term synaptic changes occurring in mPFC pyramidal projection neurons as a consequence of the reinforcing properties of a cocaine-associated context. We hypothesize that neurons within the PL-mPFC will express a strengthening of synaptic transmission that promotes the retention of the cocaine-context associations. In contrast, IL-mPFC neurons will show no change in glutamatergic synaptic markers, suggesting a mPFC subregion-specific enhancement of synaptic glutamate transmission in cocaine-context associations. The following study used whole-cell electrophysiological recordings from deep layer 5/6 pyramidal neurons in rats that underwent cocaine-induced conditioned place preference (CPP), followed by different abstinence intervals, to understand the differences in synaptic glutamate neuroadaptations between PL- and IL-mPFC neurons. These findings will inform the differential impact cocaine has in the mPFC and the particular role each region plays in mediating the reinforcing effects of a cocaine-conditioned context.

Results

We used the cocaine CPP paradigm to assess the cocaine-associated contextual cue effects on glutamatergic synaptic transmission in mPFC. Whole-cell voltage-clamp recordings were performed from layer 5/6 pyramidal neurons of PL- or IL- mPFC in brain slices of adult male rats (Fig. 1A and B).

Behavior: Cocaine-induced conditioned place preference

During the preconditioning test, rats spent similar amounts of time in the black and white compartments confirming the unbiased construction of the testing apparatus (Fig1 C). Saline control rats did not show a conditioned response, given that preference ratios did not differ from chance performance on the initial preference test ($t[15] = 1.103$, $p>0.05$ or the short abstinence test ($t[11] = 0.71$, $p>0.05$) (Fig.1 D). Rats conditioned with 20 mg/kg i.p. cocaine displayed a preference for the cocaine-paired compartment as indicated by a preference ratio greater than chance performance on the initial test for place conditioning ($t[38] = 4.194$ $p = 0.0002$) the short abstinence test (SA(+), $t[32] = 4.835$, $p< 0.0001$) and the prolonged abstinence test (PA(+), $t[12] = 2.255$, $p = 0.0436$) (Fig.1 D). In summary, these results suggest that in the course of abstinence, when cocaine-treated rats are re-exposed to the cocaine-associated context under drug-free conditions, the difference in preference ratios from the respective saline control group becomes higher with longer abstinence. These results could be interpreted as a disruption of the rewarding memory of the initial cocaine experience, leading to a slight decrease in cocaine-seeking behavior.

Electrophysiology: Spontaneous EPSCs

We measured frequency and amplitude of sEPSCs in our experimental and control groups in order to investigate the basal differences between PL- and IL-mPFC deep layer 5/6 pyramidal neurons and to assess the changes in synaptic glutamate transmission after two different abstinence time points (SA, 8 days of abstinence; and PA, 30 days of abstinence) from cocaine-induced CPP.

When comparing amplitude and frequency of sEPSCs between PL- and IL-mPFC in saline animals, we found no statistical differences in the amplitude of spontaneous events but we found that IL-mPFC neurons exhibit a significantly lower frequency of sEPSCs than the PL-mPFC (Ctrl: $t[11] = 4.676$, $p = 0.0007$ Fig.2 A and B).

Figure 1

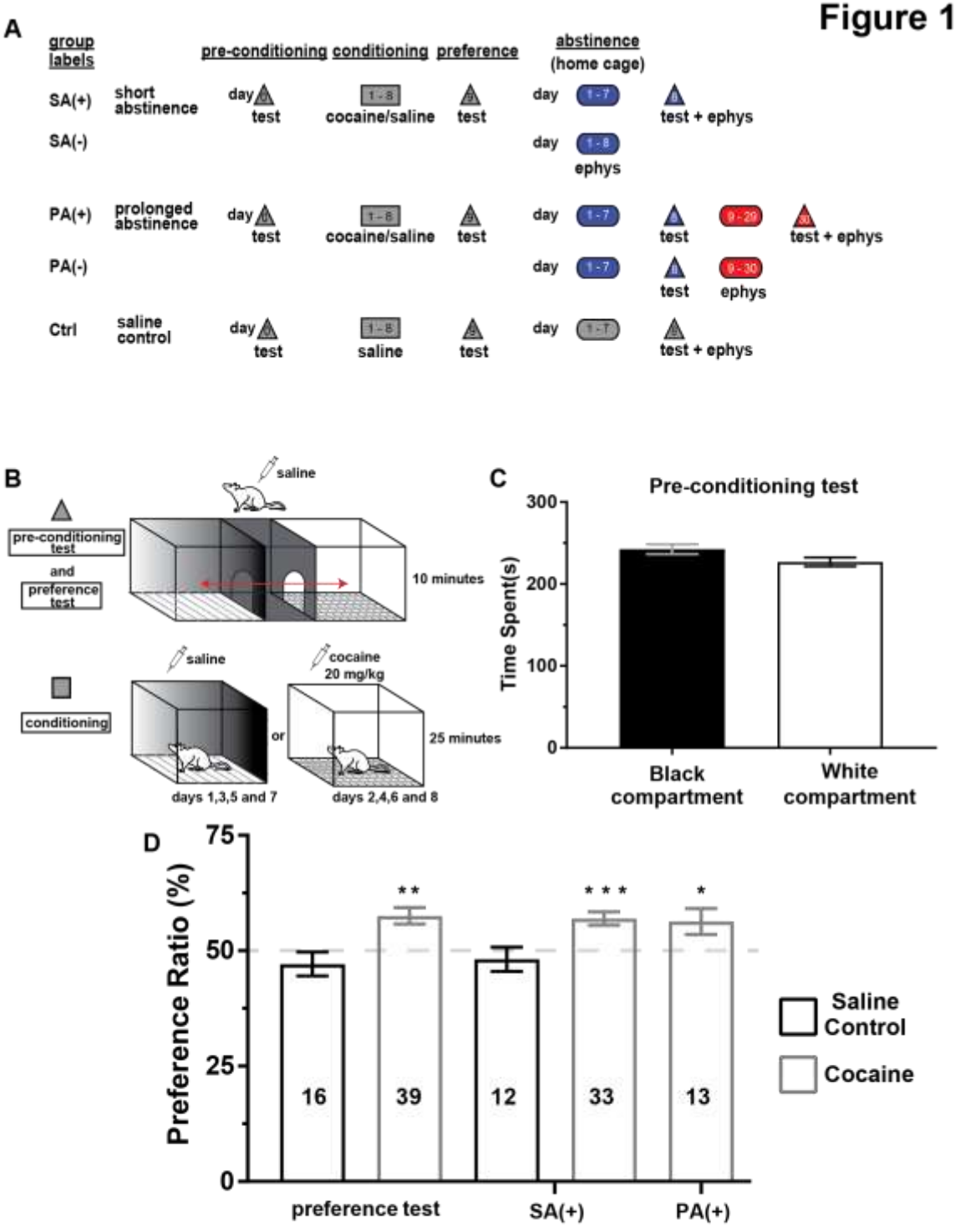

Figure 1. Cocaine conditioned place preference is retained following short and prolonged abstinence from cocaine experience. **A.** Timeline of experiments with labels for each experimental group. **B.** Place preference apparatus diagram with details for each stage of the cocaine-induced place conditioning procedure. **C.** Time spent in the black and white compartments during the pre-conditioning test confirms the unbiased construction of the testing apparatus. **D.** Preference ratio ((time in cocaine paired compartment/ time spent in both compartments) x 100). Saline treated (black border) rats and cocaine-conditioned (grey border) rats tested 24 hours after the last day of conditioning, after 8 days of abstinence (SA(+)) and after 30 days of abstinence (PA(+)). *p = 0.0436, **p = 0.0002 and ***p < 0.0001 indicate significantly above 50%.

Cocaine-induced CPP after short or prolonged abstinence: sEPSCs Amplitude

In the PL-mPFC, we found a significant reduction in the amplitude of sEPSCs relative to Ctrl levels exclusively in neurons from SA(-) rats that were not re-exposed to the cocaine-associated context (SA(-): $t[10] = 2.914$, $p = 0.031$; Fig.2 D, inset). Similarly, in the IL-mPFC, we observed a reduction in the amplitude of sEPSCs only in SA(-) rats ($t[9] = 3.086$, $p = 0.026$; Fig 2.G inset).

Cocaine-induced CPP after short or prolonged abstinence: sEPSCs Frequency

Our results showed that PL-mPFC deep layer pyramidal neurons exhibit a significant reduction in the frequency of sEPSCs in all cocaine CPP groups relative to saline values, except for the PA(-) group: (SA(-): $t[10] = 6.498$, $p = 0.0001$; (SA+): $t[16] = 3.618$, $p = 0.0046$; PA(+): $t[12] = 4.815$. $p = 0.0008$; Fig.2 E inset). In contrast to the findings in the PL-mPFC, cocaine-induced CPP followed by abstinence did not elicit significant changes in the frequency of sEPSCs in IL-mPFC neurons (Fig.2 H inset).

Our findings suggest that under basal conditions, pyramidal neurons located in layers 5/6 of the PL-mPFC receive higher levels of glutamate input compared to IL-mPFC neurons. Moreover, cocaine CPP followed by abstinence elicits different cortical adaptations in glutamate synaptic transmission in mPFC neurons after short and long abstinence periods. Short abstinence elicited decreases in the amplitude of glutamate-mediated currents, suggesting a postsynaptic decrease in receptor levels in PL- and IL-mPFC neurons in rats that were not re-exposed to the cocaine-associated context (were not tested for CPP). In contrast, we found a decrease in the frequency of excitatory synaptic inputs only in PL-mPFC neurons, in both, short and long abstinence groups. This decrease, perhaps suggesting alternations in presynaptic mechanisms, was independent of re-exposure or deprivation of the cocaine-associated context and suggests a maintained pharmacological mechanism perhaps originating from the repeated cocaine exposure.

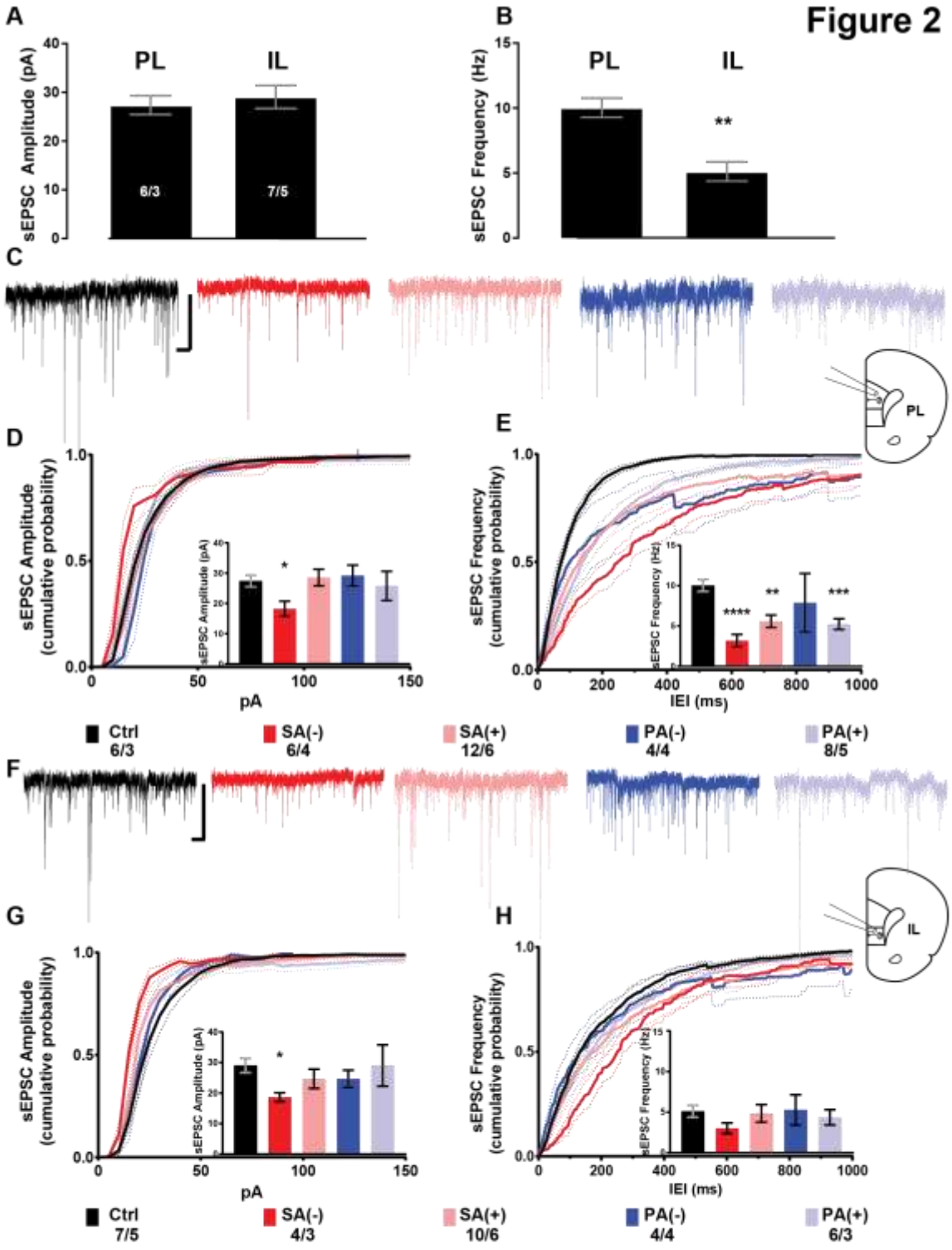
Figure 2
A
sEPSC Amplitude (pA)
PL
IL
6/3
7/5
B
sEPSC Frequency (Hz)
PL
IL
**
C
PL
D
sEPSC Amplitude (cumulative probability)
pA
sEPSC Amplitude (pA)
*
E
sEPSC Frequency (cumulative probability)
IEI (ms)
sEPSC Frequency (Hz)
****
**
***
Ctrl 6/3
SA(-) 6/4
SA(+) 12/6
PA(-) 4/4
PA(+) 8/5
F
IL
G
sEPSC Amplitude (cumulative probability)
pA
sEPSC Amplitude (pA)
*
H
sEPSC Frequency (cumulative probability)
IEI (ms)
sEPSC Frequency (Hz)
Ctrl 7/5
SA(-) 4/3
SA(+) 10/6
PA(-) 4/4
PA(+) 6/3

Figure 2. Cocaine-associated context experience-dependent alterations in spontaneous excitatory postsynaptic current (sEPSCs) properties in deep layer 5/6 pyramidal neurons of PL -and IL-mPFC. **A and B.** Comparison of sEPSC amplitude and frequency (mean ± SEM pA and Hz, respectively) from PL-mPFC versus IL-mPFC deep layer 5/6 pyramidal neurons in saline treated control rats. **C.** Representative traces of sEPSC recordings from PL-mPFC pyramidal neurons for each experimental group with a diagram for recording location on brain slice. **D and E.** Comparison of sEPSC amplitude and frequency cumulative probability distributions from PL-mPFC pyramidal neurons for each experimental group. Insets show average data for sEPSC amplitude and frequency (mean ± SEM pA and Hz, respectively). **F.** Representative traces of sEPSC recordings from IL-mPFC pyramidal neurons for each experimental group with a diagram for recording location on brain slice. **G and H.** Comparison of sEPSC amplitude and frequency cumulative probability distributions from PL-mPFC pyramidal neurons for each experimental group. Insets show average data for sEPSC amplitude and frequency (mean ± SEM pA and Hz, respectively). Statistics on bar graphs represent adjusted p values calculated from multiple t-tests against saline control measurements corrected for multiple comparisons (Bonferroni-Dunn method). Cumulative probability distributions were tested individually against saline control distributions for each sEPSC measurement. *p<0.05, **p<0.005, ***p<0.001, ****p<0.0005. Numbers represent cells/rats. Scale bars: 100ms horizontal, 10pA vertical.

sEPSC kinetics

To further address cocaine CPP changes in spontaneous glutamate transmission, we used the average sEPSC event kinetics as an indirect measure of glutamate receptor dynamics (Hollmann et al., 1989; Keller et al., 1991; Tomita, 2010; Traynelis et al., 2010). Analysis of individual events from sEPSC recordings in PL-mPFC layer 5/6 pyramidal neurons revealed a significant decrease in the area under the curve in sEPSC events from SA(-) PL-mPFC neurons ($t[10] = 2.942$, $p = 0.029$). Consistent with the difference between mPFC subregions, in the IL-mPFC we found no difference in the rise time and decay time for the average of all detected sEPSC events (Fig 3 F-H).

In summary, our results showed that the activation and inactivation rate of sEPSCs are, for the most part, unaltered in PL- and IL-mPFC neurons of saline and cocaine treated rats. The significant decrease in area under the curve observed in 8 days abstinent rats that were not re-exposed to the cocaine-conditioned context (SA(-)) follows the decrease in sEPSC amplitude and is similarly reversed upon context re-exposure. Since changes in sEPSC area under the curve are thought to represent the net charge transfer during an ionotropic glutamate receptor-mediated event, this result suggests that short abstinence after cocaine conditioning decreases the glutamatergic excitatory strength in PL-mPFC deep layer 5/6 pyramidal neurons (Keller et al., 1991).

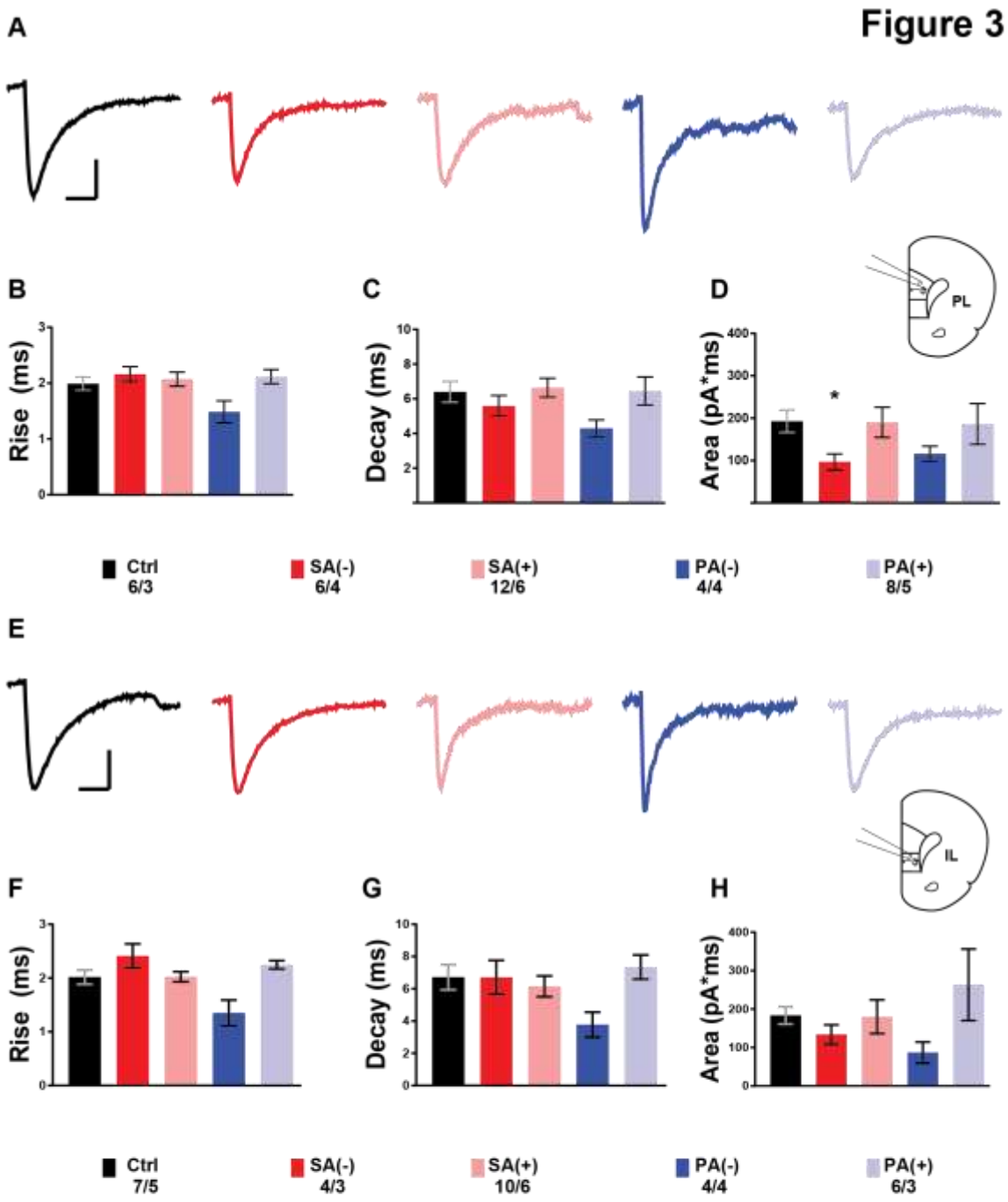

Figure 3. Cocaine-associated context experience-dependent alterations in the kinetics of sEPSCs in deep layer 5/6 pyramidal neurons of PL- and IL-mPFC. **A.** Average traces from single events in representative sEPSC recordings from PL-mPFC pyramidal neurons for each experimental group with diagram for recording location on brain slice. **B. and F.** Average rise time from all events detected in sEPSC recordings for PL- and IL-mPFC pyramidal neurons respectively (mean ± SEM ms). **C. and G.** Average decay time from all events detected in sEPSC recordings for PL- and IL-mPFC pyramidal neurons respectively (mean ± SEM ms). **D. and H.** Average area under the curve from all events detected in sEPSC recordings for PL- and IL-mPFC pyramidal neurons respectively (mean ± SEM pA*ms). **E.** Average traces from single events in representative sEPSC recordings from PL-mPFC pyramidal neurons for each experimental group with diagram for recording location on brain slice. Statistics on bar graphs represent adjusted p values calculated from multiple t-tests against saline control measurements corrected for multiple comparisons (Bonferroni-Dunn method).; $p<0.05$. Numbers represent cells/rats. Scale bars: 10ms horizontal, 10pA vertical.

Rectification Index

Previous studies have shown that protracted abstinence increases Ca^{2+}-permeable AMPARs (CP-ARs) in the NAc of cocaine self-administering rats (Conrad et al., 2008; McCutcheon et al., 2011) and alterations in AMPA receptor Ca^{2+} permeability has been shown to increase neuronal excitability (Li et al., 2012). In order to explore if similar neuroadaptations occur in PL- and IL-mPFC, we assessed the rectification index (RI) in our cocaine CPP rats as an indirect measure of CP-AR levels, where an increase in RI suggests a higher contribution of CP-ARs and a decrease in RI suggests less contribution of CP-ARs. Our results show a significant increase in the RI in PL-mPFC from PA(-) neurons compared to saline control values ($t[7] = 0.0117$, $p = 0.0207$ Fig.4 B). This data suggests that only protracted abstinence (30 days after the initial CPP test) from cocaine treatment produces an increase in RI, similar to what has been previously reported in NAc MSNs from long-access cocaine self-administering rats. When RI was assessed in IL-mPFC neurons, there were no significant differences from saline controls in any of the cocaine-treated groups (Fig.4 D).

In summary, our results showed for the first time that layer 5/6 pyramidal neurons from the PL- and IL-mPFC are intrinsically different in their excitatory synaptic activity (see differences in basal sEPSCs frequency). Furthermore, we showed that cocaine CPP induces differential effects between PL-mPFC and IL-mPFC neurons that are dependent on the length of abstinence. Experiments showed general mPFC decreases in excitatory synaptic inputs to pyramidal cells following short abstinence (8 days after the initial CPP test). Moreover, after a period of prolonged abstinence, (30 days after the initial CPP test) we found a PL-specific increase in the contribution of CP-AR's in response to electrical stimulation of glutamate terminals innervating layer 5/6 pyramidal neurons.

Figure 4

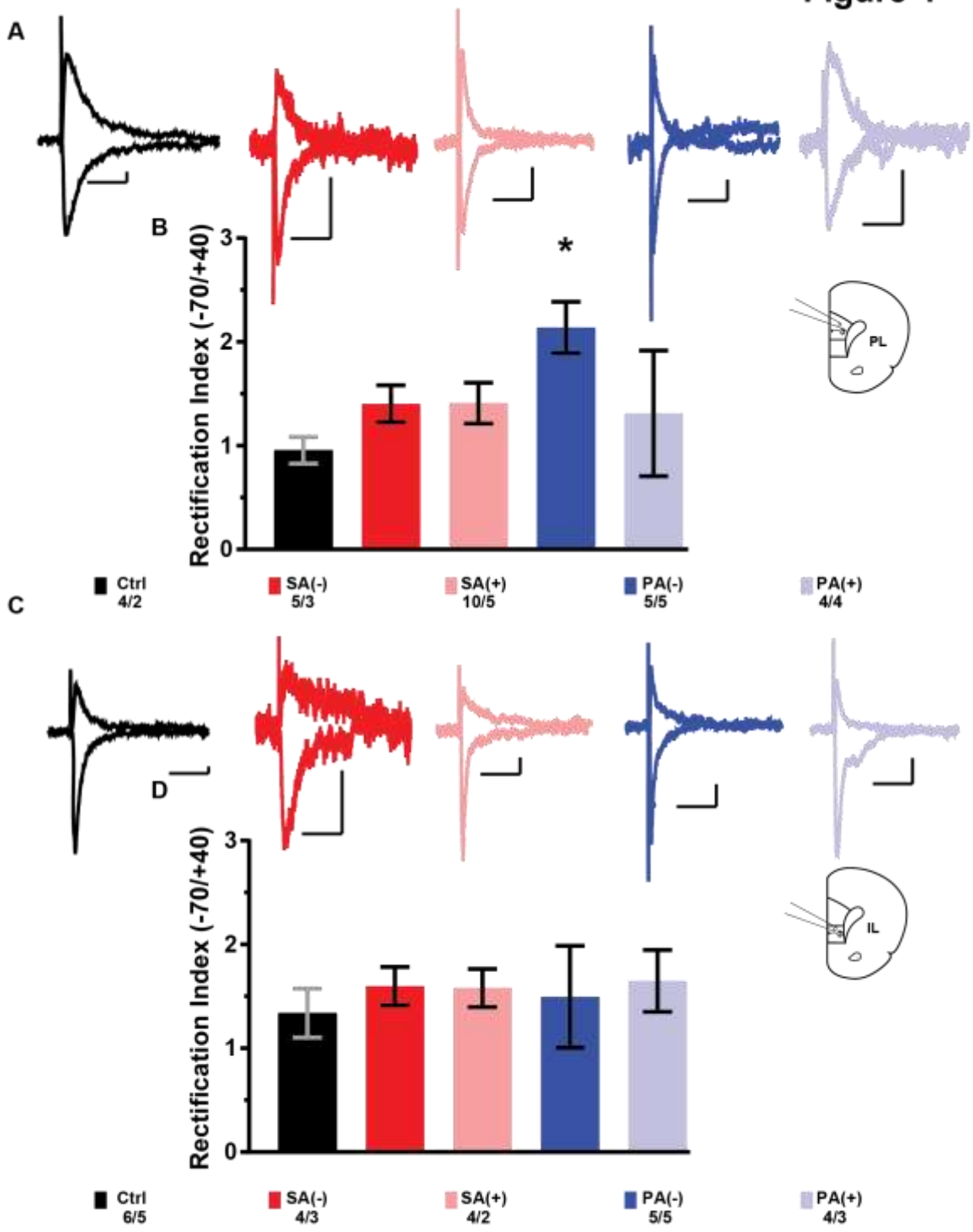
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Figure 4. Cocaine-associated context experience-dependent alterations in the rectification index in deep layer 5/6 pyramidal neurons of PL and IL-mPFC. **A. and B.** Representative traces from single evoked EPSC recordings from PL- and IL-mPFC pyramidal neurons (respectively) at +40mV (outward) and -70mV (inward) for each experimental group with diagram for recording location on brain slice. **B. and D.** Average rectification index values calculated as evoked EPSC amplitude at -70mV over +40mV membrane potentials for PL- and IL-mPFC pyramidal neurons respectively. Statistics on bar graphs represent adjusted p values calculated from multiple t-tests against saline control measurements corrected for multiple comparisons (Bonferroni-Dunn method).; $p<0.05$. Numbers represent cells/rats. Scale bars: 100 ms horizontal, 10pA vertical.

Discussion

Our results are the first to show that deep layer pyramidal neurons from PL- and IL-mPFC differ in the frequency of their excitatory synaptic inputs, with the PL-mPFC exhibiting higher frequency of excitatory inputs than the IL-mPFC. Furthermore, our results showed that cocaine conditioning followed by abstinence elicits differential effects on PL- and IL-mPFC pyramidal neurons and that these glutamatergic changes could represent synaptic alterations that mediate the retention of the cocaine-associated rewarding memory.

Presynaptic markers

It has been proposed that changes in frequency of sEPSCs reflect modifications at the presynaptic level (Engelman and MacDermott, 2004; Costa et al., 2017). Interestingly, our results showed that PL-mPFC neurons exhibit higher basal frequency of sEPSCs compared to IL-mPFC neurons. This reduced basal frequency of synaptic glutamate currents in IL-mPFC neurons may be a phenotype of this distinct neuronal population arising from differences in dendritic cytoarchitecture and glutamate afferents innervating this region (Van Eden and Uylings, 1985; Hoover and Vertes, 2007). Also, the reduced basal frequency of sEPSCs in IL-mPFC neurons could preclude any further reduction in this neuronal population, thus supporting the lack of effects observed following cocaine experience, abstinence and cocaine-context re-exposure. It is tempting to speculate that higher excitatory drive onto PL-mPFC neurons plays a role in the retention of the rewarding effects of cocaine after abstinence.

We show that cocaine-induced CPP elicits a significant reduction in the frequency of sEPSCs in PL-mPFC in all groups except the PA(-) group. In contrast, we did not find changes in sEPSC frequency in IL-mPFC neurons. Based on these results, we propose that abstinence from cocaine experience selectively alters excitatory inputs to the PL-mPFC. A recent study showed that cocaine CPP produced an increase in sEPSC frequency in PL-mPFC neurons (Otis and Mueller, 2017). The discrepancy with our results could be explained by the length between the last conditioning session and the time of recording but was not tested in our experimental conditions. Work from Hoover and Vertes, (2007) show distinct patterns of excitatory afferents from multiple limbic, thalamic and cortical nuclei innervating PL- and IL-mPFC neurons, and thus future studies should selectively target each excitatory input in order to investigate which inputs are relevant to the induction of these specific changes in glutamate transmission after abstinence from cocaine CPP (Hoover and Vertes, 2007). Further experiments should focus on elucidating the origin of our reported synaptic changes by implementing a combination of electrophysiological assays that target presynaptic analysis with postsynaptic measures (Graziane and Dong, 2016).

Postsynaptic markers

Changes in amplitude of sEPSCs are generally associated with differences in the levels of postsynaptic receptors and changes in the dynamics of the receptors (Engelman and MacDermott, 2004; Costa et al., 2017). A comparison of the average sEPSC amplitude found that cocaine CPP elicited only a reduction in the amplitude of sEPSCs in PL- and IL-mPFC pyramidal cells after short abstinence without re-exposure to the cocaine-associated context (SA(-)) and this decrease was not maintained long-term. A recent study (Otis and Mueller, 2017) showed an increase in sEPSC amplitude in PL-mPFC neurons following cocaine CPP. The difference with our results could be explained by a memory reconsolidation mechanism that was not activated in our study since our rats

remained in the home cage prior to the recordings. The presence of the reduction in amplitude of sEPSCs in both mPFC subregions suggests a common short-term neuroadaptation by which cocaine experience, in combination with deprivation from the cocaine-associated context, decreases the levels of postsynaptic glutamate receptors. After short abstinence, retrieval of the cocaine-context association memory could be enough to disrupt this adaptation. Moreover, because our rats were tested at multiple intervals for the retention of cocaine CPP, it is possible that at longer periods of abstinence, this modification is either not necessary for the expression of cocaine-induced CPP or has been disrupted by the emergence of an extinction memory.

Changes in the kinetics of sEPSC events can be indicative of alterations in receptor subunit composition and their interactions with auxiliary subunits as well as alternative RNA splicing and post-translational modifications (Dingledine et al., 1999; Tomita, 2010; Granger et al., 2011; Stincic and Frerking, 2015). Using the CPP protocol with cocaine exposure followed by different periods of abstinence, we found that only PL- mPFC neurons exhibited a decrease in the area under the curve in the SA (-) group. Given the low contribution of NMDA receptors at -70mV, we interpreted these changes in sEPSC area as alterations in AMPA glutamate receptor dynamics and a putative byproduct of the combination of the factors mentioned above. This decrease in area under the curve for the SA (-) group is complemented by a decrease in sEPSC amplitude. Moreover, these changes appear to be dependent on short abstinence and deprivation from the cocaine-conditioned context.

It is important to state that a portion of our experimental groups have small samples sizes; therefore, in future studies we will attempt to expand these experiments to parse out any underpowered effects that might have gone undetected.

Rectification Index

Previous reports have shown that long-access cocaine SA followed by prolonged abstinence elicits an increase in the number of calcium-permeable AMPA receptors (CP-AR's) in NAc medium spiny neurons (MSN) (Conrad et al., 2008), thus providing a synaptic marker of the incubation of cocaine craving in this brain region. Based on these studies and the influence that PFC projection neurons have within the drug-seeking circuit, we assessed changes in rectification index (RI; as a relative measure of CP-AR's contribution) in our different experimental groups. We found that cocaine CPP followed by abstinence elicited a significant increase in RI only in the PL-mPFC (PA(-)). This result suggests that longer periods of withdrawal from the cocaine experience are required to produce an increase in RI, similar to what has been previously reported in NAc MSNs from long-access cocaine self-administering rats (Conrad et al., 2008). An increase in surface level expression of CP-AR's, with enhanced single-channel conductance, could increase the excitability of mPFC pyramidal neurons in similar fashion to the effects of GluR2-lacking AMPARs reported in PVN neurons in spontaneously hypertensive rats (Li et al., 2012). Our results suggest that prolonged abstinence alters the activity of PL-mPFC neurons via an unknown mechanism leading to a compensatory increase in AMPA receptor RI that can potentially drive the retention of cocaine-induced conditioned place preference.

Silent synapses, synapses devoid of AMPA receptors, were initially reported in hippocampal CA1 neurons in studies demonstrating that their "unsilencing" required the insertion of AMPA receptors upon induction of LTP (Isaac et al., 1995; Liao et al., 1995). Huang and colleagues established an association between silent synapses and in vivo cocaine experience, where the salience attributed to the drug experience is sufficient to generate de novo silent synapses (Huang et al., 2009).

Figure 5

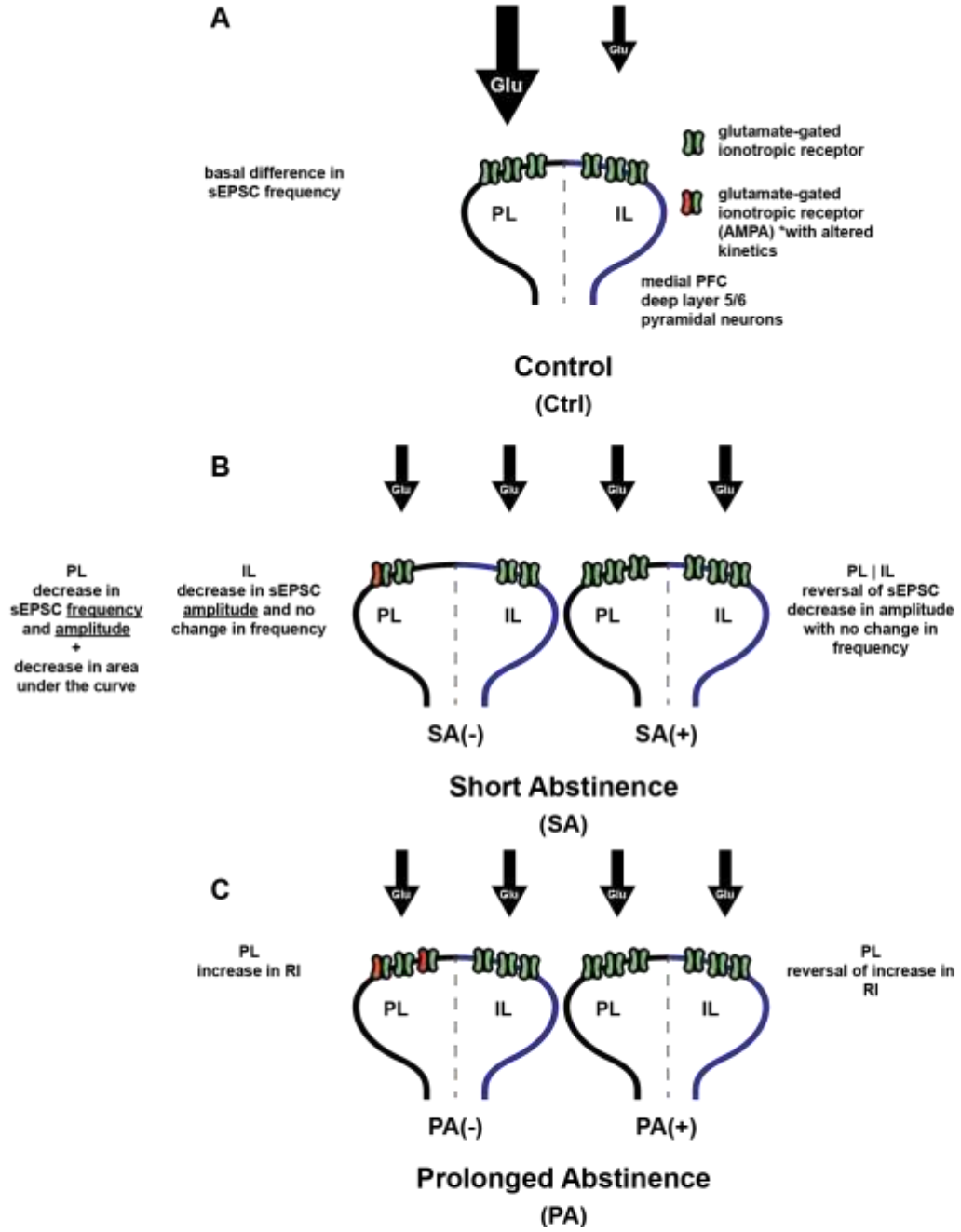

Figure.5 Summary diagram: Targeted effects of cocaine-CPP on PL- versus IL-mPFC deep layer 5/6 pyramidal neurons after short or prolonged abstinence. **A.** Basal PL/IL difference in sEPSC frequency in cocaine naïve control rats: higher frequency of sEPSC in PL- neurons suggest more glutamate inputs to PL- versus IL-mPFC neurons, while no difference in the amplitude of sEPSC suggest no difference in postsynaptic receptor levels. **B.** Short Abstinence from cocaine-CPP (left); SA(-): decreases sEPSC amplitude in PL- and IL –mPFC neurons implying a decrease in postsynaptic receptor levels. SA also produces a decrease in sEPSC amplitude and a decrease in the area under the curve of the average sEPSC. Re-exposure to the conditioning context after SA (right); SA(+): reversal of changes to PL- and IL-mPFC neurons sEPSC amplitude to control levels. **C.** Prolonged Abstinence from cocaine-CPP(left); PA(-): PL- neurons show an increase in AMPA receptor rectification index (RI) suggesting higher levels of GluA2-lacking AMPARs. Re-exposure to the conditioning context after PA(right); PA(+): changes in average sEPSC kinetics are reversed in PL- and IL-mPFC neurons. Increase in AMPAR RI in PL-mPFC neurons is reversed to control levels.

Cocaine-induced silent synapse formation has been shown to generate a permissive state for remodeling of the NAc neurocircuits in several cocaine-related behaviors including conditioned place preference, locomotor sensitization and cue-induced reinstatement of cocaine self-administration (Brown et al., 2011; Lee et al., 2013; Ma et al., 2014; Dong, 2015; Shukla et al., 2017). Particularly relevant to our study is the evidence of silent synapse-based circuit remodeling in the mPFC-NAc pathway during cocaine craving, showing that maturation of the IL-mPFC-NAc pathway requires the recruitment of CP-AMPARs and that maturation of the PL-mPFC-NAc pathway requires insertion of non-CP-AMPARs. Reversing excitatory synapse remodeling with optogenetic stimulation of the IL-mPFC to NAc shell and the PL-mPFC to NAc core pathways can potentiate or inhibit incubation of cocaine craving, respectively (Ma et al., 2014). It remains to be studied whether similar forms of circuit remodeling occur at the level of mPFC afferents. The following questions remain to be answered: do cocaine-context associations require the generation of de novo silent synapses in deep layer mPFC pyramidal neurons, and does this PL- vs IL-mPFC dichotomy prevail upstream from the NAc?

Conclusion:

PL- and IL-mPFC deep layer 5/6 pyramidal neurons differ in their excitatory inputs, with PL-mPFC exhibiting higher basal frequency of sEPSCs, which suggests an important role of this subcortical region for the neuroplasticity of addiction. Cocaine CPP elicits different neuroadaptations in mPFC neurons depending on the length of abstinence, and the specific changes are detailed in the summary diagram (Fig.5). General adaptations appeared after short abstinence (8 days after the initial CPP test) in both PL- and IL-mPFC neurons, suggesting the maintenance of the pharmacological

effects of cocaine, whereas alterations in frequency of glutamate inputs after short abstinence were specific to PL-mPFC neurons. In both cases, these effects were only present in rats deprived from context re-exposure and were not present after prolonged abstinence (30 days after the initial CPP test). Prolonged abstinence produces PL-mPFC specific changes in CP-AMPARs, suggesting a time-sensitivity to the effects of cocaine-induced CPP in mPFC synaptic glutamate transmission.

Chapter 4:

Experience surrounding cue-induced reinstatement of cocaine seeking produces minimal markers of plasticity in excitatory synapses innervating prelimbic and infralimbic deep layer 5/6 pyramidal neurons.

Introduction

Relapse to psychostimulant use (e.g., cocaine and amphetamines) is a chronic health problem that lacks legitimate pharmacological treatments. Therefore there is a need for novel therapeutics in relapse prevention. The prefrontal cortex (PFC) processes information that gaits behaviors such as impulse inhibition, behavioral monitoring, and decision making among others (Everitt and Robbins, 2005; Goldstein and Volkow, 2011). In addicted individuals, symptoms such as loss of control over drug-taking despite negative consequences suggests cognitive deficits associated with abnormal PFC activity; for a review, see (Goldstein and Volkow, 2011). Early studies in cocaine addicts show a correlation between self-reported craving and activation of the PFC and limbic structures in individuals exposed to drug-related stimuli (Grant et al., 1996). Similarly, preclinical studies in rodents have demonstrated that cognitive dysfunction following prolonged cocaine exposure is dependent on altered PFC activity (Briand et al., 2008; George et al., 2008; Ghasemzadeh et al., 2011). Similar cognitive deficiencies are modeled in rat self-administration paradigms that pair a light and tone with an infusion of cocaine. This model of operant conditioning creates a behavioral response that is generated from associative learning mechanisms between the rewarding effects of the drug and the environmental stimuli associated with the drug experience (de Wit and Stewart, 1981; Fuchs et al., 1998; Shaham et al., 2003). Given the relevant role of the PFC in relapse to drugs use, it is of vital importance to understand the mechanisms

behind deep layer 5/6 pyramidal neuron ($PNs^{5/6}$) processing of psychostimulant-associated environmental cues in the PFC.

Previous studies have highlighted the role of excitatory drive in the medial prefrontal cortex (mPFC) to nucleus accumbens (NAc) pathway. Specifically, the prelimbic cortex (PL) to NAc projections enhance goal-directed behaviors and cocaine-seeking (McLaughlin and See, 2003), whereas the infralimbic cortex (IL) to NAc shell path suppresses cocaine seeking under extinction conditions (Peters et al., 2008). This circuit level distinction between mPFC subregions and their projections to the ventral striatum support a model in which PL- and IL-mPFC excitatory drive exerts opposing control over drug seeking behavior. Within this context, the role of the NAc in drug-seeking behavior has been extensively evaluated (Gipson et al., 2014; Loweth et al., 2014); in contrast, there is a dearth of information on the synaptic alterations occurring in synaptic inputs to PL- and IL-mPFC $PNs^{5/6}$ following exposure to drug-related cues and the expression of drug-seeking behavior. Moreover, the underlying influence of synaptic iGluR dynamics in PL- and IL-mPFC $PNs^{5/6}$ within the model of opposing control over drug-conditioned behavioral responses has not been characterized to the same extent as in NAc medium spiny neurons.

We hypothesize, based on the functionally opposed roles between PL- and IL-mPFC subregions, that PL $PNs^{5/6}$ will express enhanced synaptic markers of iGluR transmission, reflecting strengthened excitatory transmission and promoting cocaine-seeking behavior; IL $PNs^{5/6}$ neurons, on the contrary, will show no change in iGluR synaptic transmission due to the lack of engagement of these neurons during active drug seeking in response to exposure to cocaine-associated cues.

Here we used whole-cell electrophysiological recordings in mPFC-containing coronal brain slices from visually identified PL and IL $PNs^{5/6}$ to characterize the synaptic

changes in spontaneous and electrically evoked transmission in response to cue-induced reinstatement of cocaine seeking. The analysis of multiple glutamate synaptic parameters allows us to compare: 1) The basal characteristics of spontaneous and electrically-evoked excitatory postsynaptic currents (EPSCs) between PL and IL $PNs^{5/6}$ in saline-yoked rats; 2) Disruption of iGluR synaptic inputs to $PNs^{5/6}$ within mPFC subregions (PL or IL) for saline control versus cue reinstated (cocaine-associated cue experienced) or extinction only (cocaine-associated cue deprived) rats. Lastly, we asked 3) whether iGluR inputs to PL and IL $PNs^{5/6}$ respond differently between rats that underwent extinction training versus rats that remained abstinent in their home cages for 8 days after cocaine self-administration training. Among the collected parameters were recordings of amplitude and frequency of spontaneous EPSCs to assess the synaptic origin of the iGluR adaptations (Engelman and MacDermott, 2004; Costa et al., 2017), AMPA to NMDA ratio as a measure of synaptic strength(Nicoll, 2017), and rectification index of electrically evoked-AMPA responses as an indirect measure of the prevalence of synaptic calcium-permeable AMPA receptors (CP-AMPARs)(Cull-Candy et al., 2006).

Results

Briefly, adult male Sprague Dawley rats were trained to self-administer cocaine in daily 2-hour sessions for 10 days (Fig. 1 A and B). Saline-yoked rats were used as controls (**Ctrl**) for learning a self-administration operant behavior. After meeting stable cocaine self-administration intake criteria (more than 10 infusions for 10 consecutive sessions), rats entered the extinction phase. During extinction training, rats received at least 14 days of 2-hour sessions, were subsequently placed in the self-administration boxes and allowed to interact with the lever for a saline infusion with no cue pairing (Fig. 1A and B).

Figure 1

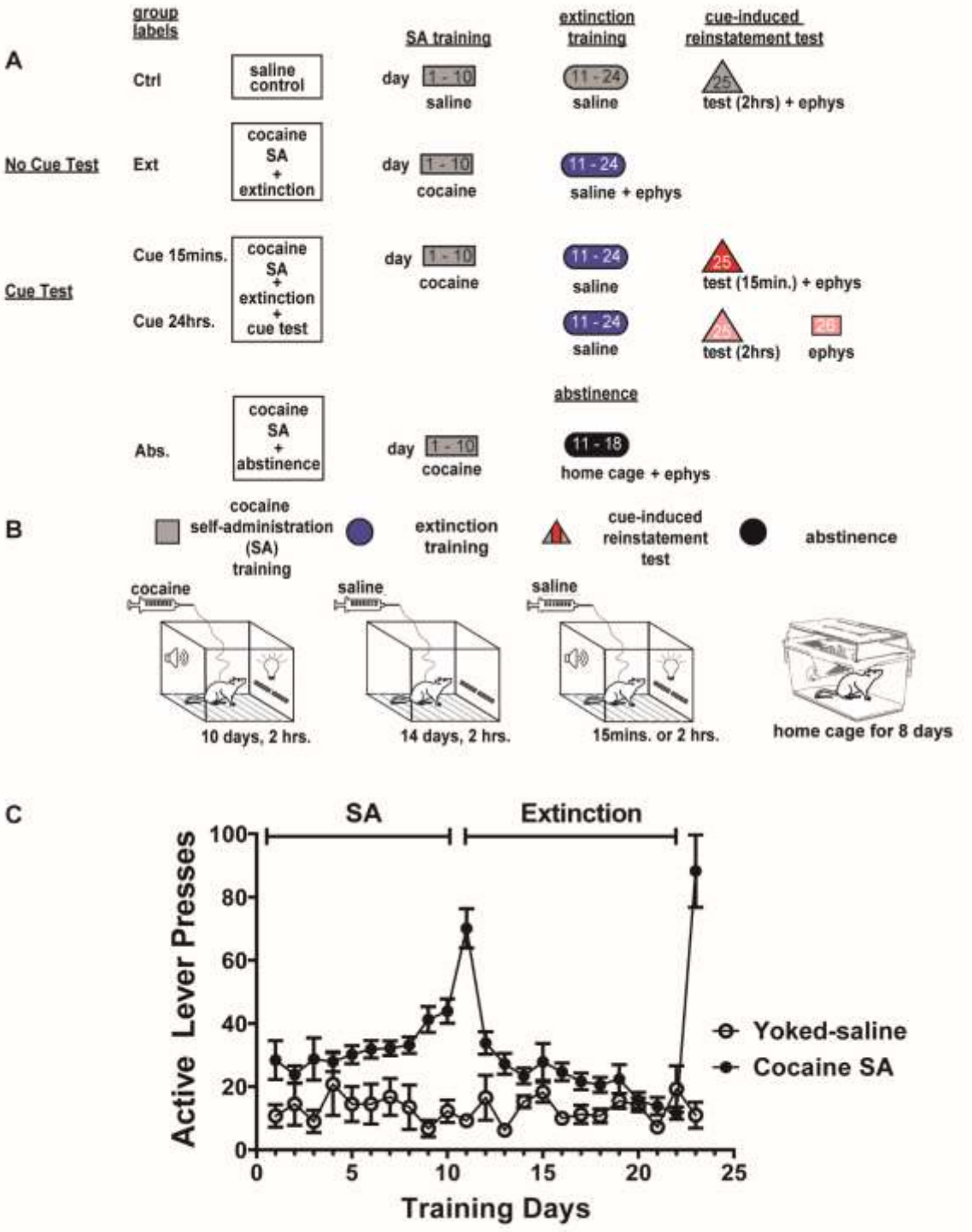

Figure 1. Description of experimental groups using a cue-induced reinstatement model of cocaine seeking. **A.** Timeline of experiments with labels for each experimental group. **B.** Operant training apparatus diagram with details for each stage of the self-administration procedure. **C.** Representative example of active lever responding from saline-yoked and cocaine-treated rats.

A group of rats (**Ext**) were recorded from after meeting the extinction criteria (two consecutive days with less than 25 lever presses) and were not tested for cue-induced reinstatement of cocaine-seeking behavior. The Ext groups serves as the control for no cue test or deprivation from cocaine-associated cue re-exposure. A separate group of rats remained in their home cages (**Abs**) for 8 days after cocaine self-administration training (Fig. 1 A and B). The Abs group was used to compare the effects of extinction versus no extinction (Abs). Finally, two groups of rats received a cue-induced reinstatement test: **Cue 15mins.**, recordings were done after a 15-minute reinstatement session or **Cue 24hrs.**; recordings were done 24 hours after a 2-hour cue-induced reinstatement session. These two time points after a cue-induced reinstatement test, or experience of a cocaine-associated compound cue, allowed us to characterize the time course of the experience-dependent changes in iGluR synaptic transmission. All collected electrophysiological data are presented and analyzed by mPFC subregion (PL and IL) and as pooled data (insets) due to the limited differences between subregions for the parameter analyzed (Fig. 2).

Spontaneous EPSCs

To investigate whether basal differences exist between PL and IL PNs$^{5/6}$ that could explain a difference in function between these two populations of neurons, we evaluated whether combined action potential dependent and independent spontaneous glutamate release is different between PL and IL in saline control rats (Fig. 2A red dashed rectangle, inset). This comparison revealed a trend towards a decrease in the sEPSC frequency in IL versus PL PNs$^{5/6}$ ($t(41) = 1.954$; $p = 0.0575$). To test whether a cue-induced reinstatement test, extinction alone, or abstinence after cocaine-SA, we recorded sEPSC amplitude and frequency from PL and IL PNs$^{5/6.}$ Our results show a significant increase in sEPSC amplitude between PL PNs$^{5/6}$ from saline controls versus abstinent rats and a lack of effects in both mPFC regions when comparing saline-treated

rats to cocaine self-administering rats that went through extinction training alone or two different time points after a cue-induced reinstatement test (15 minutes versus 24 hours) ($F(2, 26) = 3.235$, $p = 0.0485$; Holm-Sidak's multiple comparisons test, $p = 0.0442$; Fig. 2A). These results suggest that synaptic iGluR adaptations in PL- and IL-mPFC $PNs^{5/6}$ inputs are not altered by cocaine treatment, extinction training or cue-induced reinstatement. Because we did not detect any iGluR adaptations at the time the recordings were performed, we believe that the expression of cocaine-seeking behavior involves other synaptic adaptations within these inputs (upstream from the mPFC to NAc pathway) or these adaptations develop transiently such that by the time of the recordings no changes are detected.

AMPA to NMDA ration and Rectification Index

We did not find any change in AMPA receptor RI or AMPA to NMDA ratio between the experimental groups compared to saline control rats (Fig. 2C and D). These results suggest that cocaine treatment, abstinence from cocaine experience, extinction training, nor experiencing a cocaine-associated cue, can alter the relative contribution of calcium-permeable versus calcium-impermeable AMPA receptor synaptic transmission in PL and IL $PNs^{5/6}$. Also, these results suggest that glutamate inputs to $PNs^{5/6}$ are not remodeled or strengthened by these experimental conditions.

Discussion

Our results are the first to show a trend towards a decrease in the frequency of their excitatory synaptic inputs between PL- and IL-mPFC $PNs^{5/6}$, with the PL-mPFC exhibiting higher frequency of excitatory inputs than the IL-mPFC. These results are different from our previous studies.

Figure 2

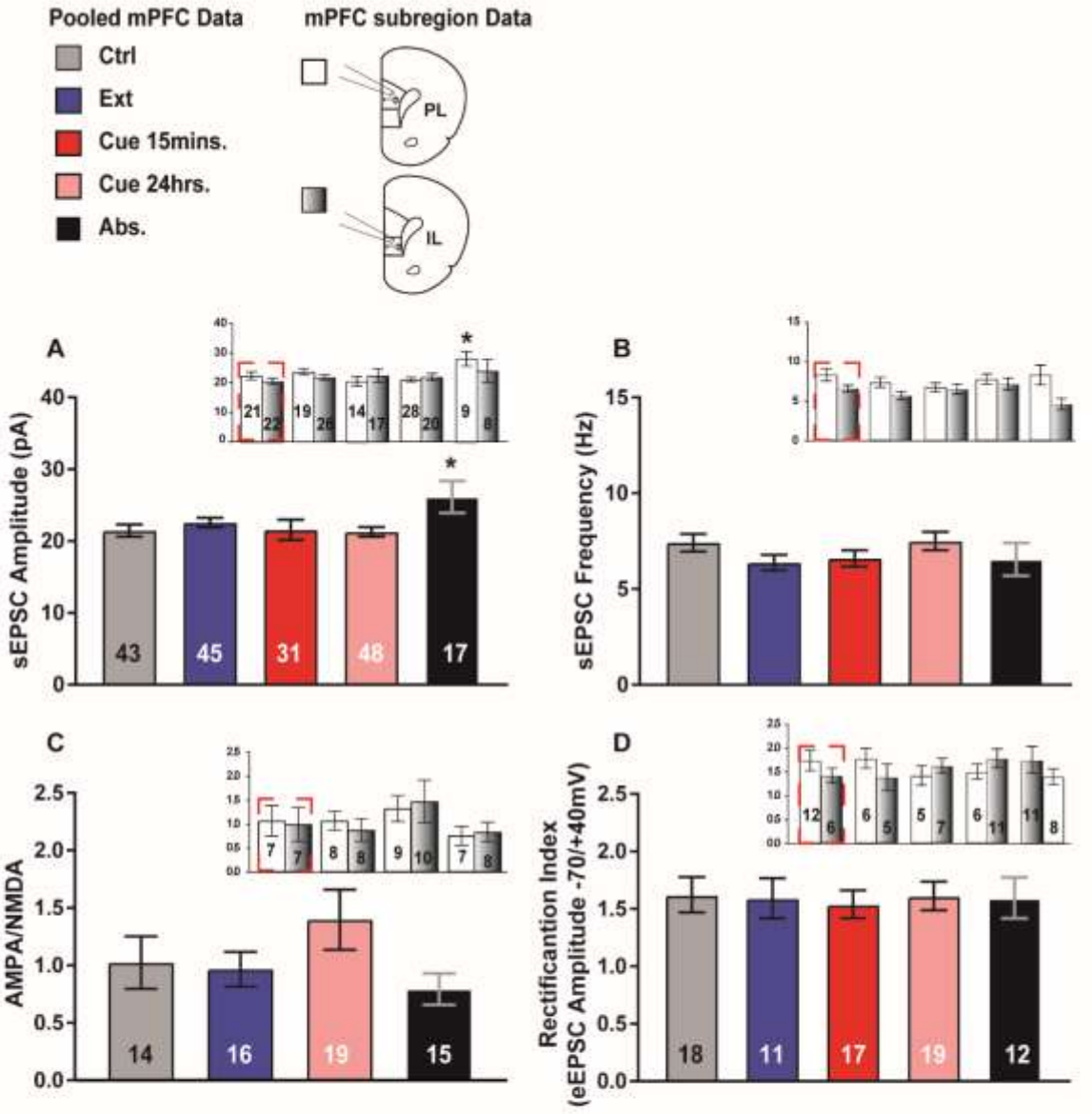

Figure 2. Limited differences in parameters of glutamate synaptic transmission in a cue-induced reinstatement model of cocaine seeking. **Top.** Figure legend for pooled (colored) and mPFC subregion data (white bars, PL and gradient, IL). **A.** Significant increase in sEPSC Amplitude in Abs rats. **B.** No change in sEPSC amplitude between saline-treated and cocaine-treated rats. **C.** No change in AMPA to NMDA ratio between saline-treated and cocaine-treated rats. **D.** No change in AMPAR rectification index between saline-treated and cocaine-treated rats.

Presynaptic Markers:

It has been proposed that changes in frequency of sEPSCs reflect modifications at the presynaptic level. In this context, it was surprising that we did not find any changes in frequency of sEPSCs in the PL- or IL-PFC in any of our cocaine SA groups. A possible interpretation of the lack of change in sEPSC frequency could be that discrete cue association memories are not mediated mechanistically by this presynaptic modification. An alternative interpretation is that the association memory formed with instrumental conditioning does not engage cortical presynaptic mechanisms.

Postsynaptic Markers:

Changes in amplitude of sEPSCs have been related to changes in the number of postsynaptic receptors and changes in the affinity of the receptors (Engelman and MacDermott, 2004; Costa et al., 2017). In our cocaine SA experiments we did not find changes in amplitude of sEPSCs in the PL- or IL PFC in any of the animal groups studied. Additionally, changes in the kinetics of sEPSC events can be indicative of changes in the subunit composition and their interactions with auxiliary subunits (Dingledine et al., 1999; Tomita, 2010; Granger et al., 2011; Stincic and Frerking, 2015); however, in our cocaine SA animals, independently of the experimental groups, we did not find any changes in the kinetics of spontaneous events (data not shown).

Rectification Index

Previous reports have shown that long-access cocaine SA followed by prolonged abstinence elicits an increase in the number of calcium-permeable AMPA receptors (CP-AMPAR) in NAc medium spiny neurons (MSN) (Conrad et al., 2008), thus providing a synaptic marker of the incubation of cocaine craving in this brain region. Based on these studies and the influence that PFC projection neurons have within the drug-seeking

circuit, we assessed changes in rectification index (RI; as a relative measure of CP-AMPAR contribution) in our different experimental groups.

To our surprise, cocaine SA did not elicit changes in cortical synaptic glutamatergic indices. We suggest that the lack of statistical changes in the different synaptic measures may be due to the variability on the samples. Moreover, the variability may be due to the lack of input and output specificity for the neuronal population recorded. Perhaps, using retrograde tracer to identify the outputs of the pyramidal cells and thus grouping the synaptic results according to connectivity could increase the consistency in the data and possibly unmask an effect.

Another intriguing possibility is that synaptic changes occur early, during the cocaine acquisition phase or early withdrawn, but by the time that recordings were performed homeostatic process have occurred to compensate for that early changes.

Conclusion:

Cue-induced reinstatement after cocaine self-administration followed by extinction, extinction plus two different time points after a cue-induced reinstatement session or a short withdrawal did not elicit synaptic changes in glutamatergic indices in the PL- or IL-mPFC PNs[5/6]. Alternatively, other mechanisms that increase glutamate output through the mPFC to NAc pathway are responsible for the expression of drug-seeking behavior

Chapter 5:

Methamphetamine self-administration modulates glutamate neurophysiology.

Introduction

Methamphetamine (meth) is a highly abused psychostimulant (United Nations Office on Drug and Crime report, 2014) and repeated cycles of meth use leads to chronic relapse that is characteristic of addiction. While the underlying pathology of addiction and relapse remains poorly understood, it is clear that a change in synaptic plasticity within the reward circuit is one of the fundamental mechanisms underlying this disorder. The reward circuitry consists mainly of the nucleus accumbens (NAc) and its inputs from the prefrontal cortex (PFC) and it is hypothesized that addictive drugs hijack this circuit (Hyman et al., 2006). Moreover, glutamatergic signaling in the NAc controls drug-seeking behaviors in animal models of addiction (Self, 2004; Kalivas, 2009), as this nucleus has a large population of glutamatergic synapses that impinge upon medium spiny neurons and are subject to lasting molecular and cellular changes in response to addictive drugs (Nestler, 2001). Furthermore, NAc glutamatergic projections originate in the PFC (as well as the hippocampus and basal amygdale) and it is dysfunction of the PFC that is credited with the loss of control over drug-taking behavior (Goldstein and Volkow, 2011).

Importantly, meth changes PFC glutamatergic transmission and causes cognitive deficits in both humans and animals (Dean et al., 2012); Bernheim et al., under review). While considerable efforts have been made to understand the mechanisms underlying meth addiction, relapse, and cognitive dysfunction, very little is known about the synaptic and cellular mechanisms in play. Synaptic physiology data from meth experienced rats is scarce, but it has been reported that experimenter delivered meth (1 mg/kg, 7days)

decreases calcium currents and excitatory transmission in the PFC four days after drug treatment (González et al., 2015). Additionally, a sensitizing regimen of amphetamine increases AMPA/NMDA ratios and frequency and amplitude of miniature excitatory postsynaptic currents (mEPSCs) in the shell of the nucleus accumbens (Jedynak et al., 2016). These examples begin to shed light on cortico-accumbal synaptic function following meth in mice; however, these studies do not relate physiological changes to an addiction model.

Currently, self-administration models that rely on contingent drug delivery have more face and construct validity relative to experimenter delivered drug models. In recent years, self-administration models that employ longer, extended access to drug have received growing attention, likely owing to the noted differences between long- and short-access models (Ahmed and Koob, 1998; Reichel et al., 2012; Peters et al., 2015). Long-access, but not short-access, meth leads to a highly reproducible escalation of meth intake that is thought to reflect a more “addicted” state compared to short-access models reflecting “recreational” drug use (Ahmed and Koob, 1998). Rats given extended access to meth self-administration had elevated basal glutamate levels in the NAc during abstinence from the drug (Lominac et al., 2012). And, we have shown that long-access meth self-administration (6 hrs per day) increased the proportion of burst firing pyramidal cells compared to tonic firing cells in PFC (Parsegian et al., 2011a), however, it is not known if this increased burst firing is a result of presynaptic and/or postsynaptic changes. To test whether meth self-administration impacts pre- and/or postsynaptic plasticity we recorded from pyramidal cells in the PFC and medium spiny neurons (MSN)s in the NAc core in brain slice preparations of the same rats using whole-cell and field recordings. Based on our findings in the PFC we also quantified surface expression of NMDA receptors.

We found that long-access meth self-administration decreased AMPA/NMDA ratio in the PFC, accompanied by an increase in NMDA receptor currents. Concurrently, in the NAc, there was a significant increase in glutamate release probability. Together, these results show that meth elicits different long-term synaptic changes in the reward associated brain regions; suggesting altered synaptic function as one of the fundamental mechanisms affected by drugs of addiction.

Results

Escalation of methamphetamine self-administration during long access conditions

Meth self-administration escalated over time, indexed as an increase in active lever presses during the 6 hour sessions on days 10-14 compared to long access day 1 [day x lever interaction $F(13, 65)=3.07$, $p<0.01$, Dunnett's multiple comparisons, $p<0.05$]. Meth infusions also increased over time with the number of infusions on days 7-10 of long access relative to day 1 [Fig 1, $F(13, 65)=10.24$, $p<0.001$, Dunnett's multiple comparisons, $p<0.05$].

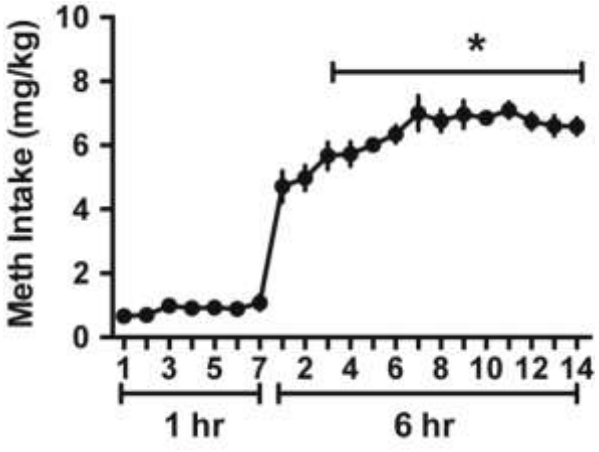

Figure 1. Meth intake (mg/kg) increased over the long access (6h) period.
*Significant difference from day one of long access

Meth decreased AMPA/NMDA ratio in the PFC

Using whole-cell recordings in the PFC we assessed standard markers of pre and post synaptic function: paired-pulse responses (PPR), AMPA/NMDA ratio, and frequency and amplitude of spontaneous (s) EPSCs. PPRs were used to provide an indication of neurotransmitter release probability (Debanne et al., 1996; Bonci and Williams, 1997; Regehr, 2012). The PPR ratio is calculated by dividing the amplitude of the second postsynaptic response by the first. PPR goes down when the probability of release is increased (Zucker and Regehr, 2002). In the mPFC, there were no changes in the PPR [Fig 2a, mean ± SEM: saline 1.33 ± 0.169, meth 1.15±0.209]. We also recorded sEPSCs (a change in frequency and amplitude of spontaneous events suggests a change in pre- and post-synaptic transmission, respectively). There were no changes in frequency [Fig 2b, mean ± SEM: saline 7.20 ± 0.71, meth 7.73±1.30] or amplitude [Fig 2c, mean ± SEM: saline 22.56 ± 1.61, meth 22.38±1.89] of sEPSCs in the PFC.

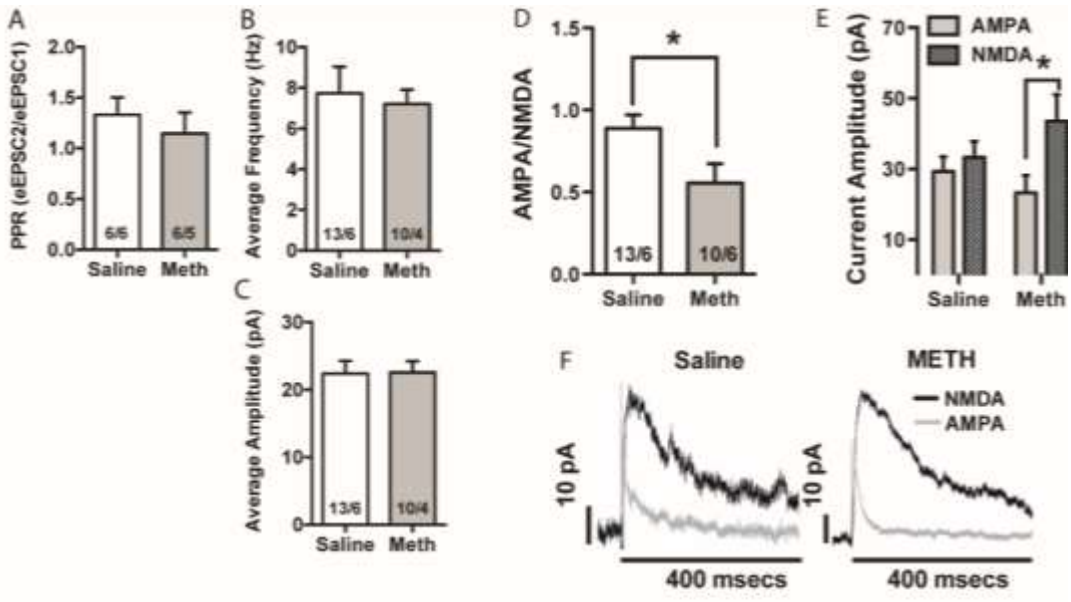

Figure 2. In the mPFC, meth self-administration altered post-synaptic physiology. In the mPFC, long access meth did not have an effect on whole cell recordings of PPR (a) and frequency (b) or on amplitude (c) of sEPSCs. Meth reduced AMPA/NMDA ratio (d, *significant difference from saline), and the decrease was driven by an increase in NMDA current (e, *significant difference between subtype). Representative traces of AMPA/NMDA currents are depicted (f). The number of cells/animal is indicated in the bar graphs, which represent the mean; the errors bars represent the SEM

Synaptic strength can be determined by the ratio of the amplitude of AMPA to NMDA currents and is regularly used for investigating the effects of various psychostimulants on glutamate transmission (Gao and Wolf, 2007). AMPA/NMDA ratio was significantly decreased in meth rats relative to saline [Fig 2d, $t(21)=2.408$, $p<0.05$, mean ± SEM: saline 0.89 ± 0.082, meth 0.55±0.117]. This difference was driven by a significant increase in NMDA receptor currents in the PFC of meth rats (Fig 2e, group X receptor interaction, $F(1,21)=7.28$, $p<0.02$]. Specifically, NMDA currents in meth rats were elevated relative to saline (Sidak's multiple comparisons test, $p<0.05$).

Meth decreased PPR and increased frequency of sEPSCs in the NAcc

Using whole-cell recordings in the NAc we assessed synaptic function. Meth-induced depression of PPR was observed in whole-cell voltage clamp recordings [Fig 3a, $t(14)=2.57$, $p<0.05$; mean ± SEM: saline 1.12 ± 0.173, meth 0.69±0.078]. The frequency of sEPSCs was increased in meth rats compared to yoked saline controls [Fig 3a, unpaired t with Welch's correction, $t(11)=2.56$, $p<0.05$; mean ± SEM: saline 3.766 ± 0.25]. The amplitude of sEPSCs (Fig 3c, mean ± SEM: saline 18.13 ± 1.06, meth 20.37±0.94) and AMPA/NMDA ratio (not shown, mean ± SEM: saline 0.92 ± 0.132, meth 0.93 ± 0.076) did not differ between saline and meth rats.

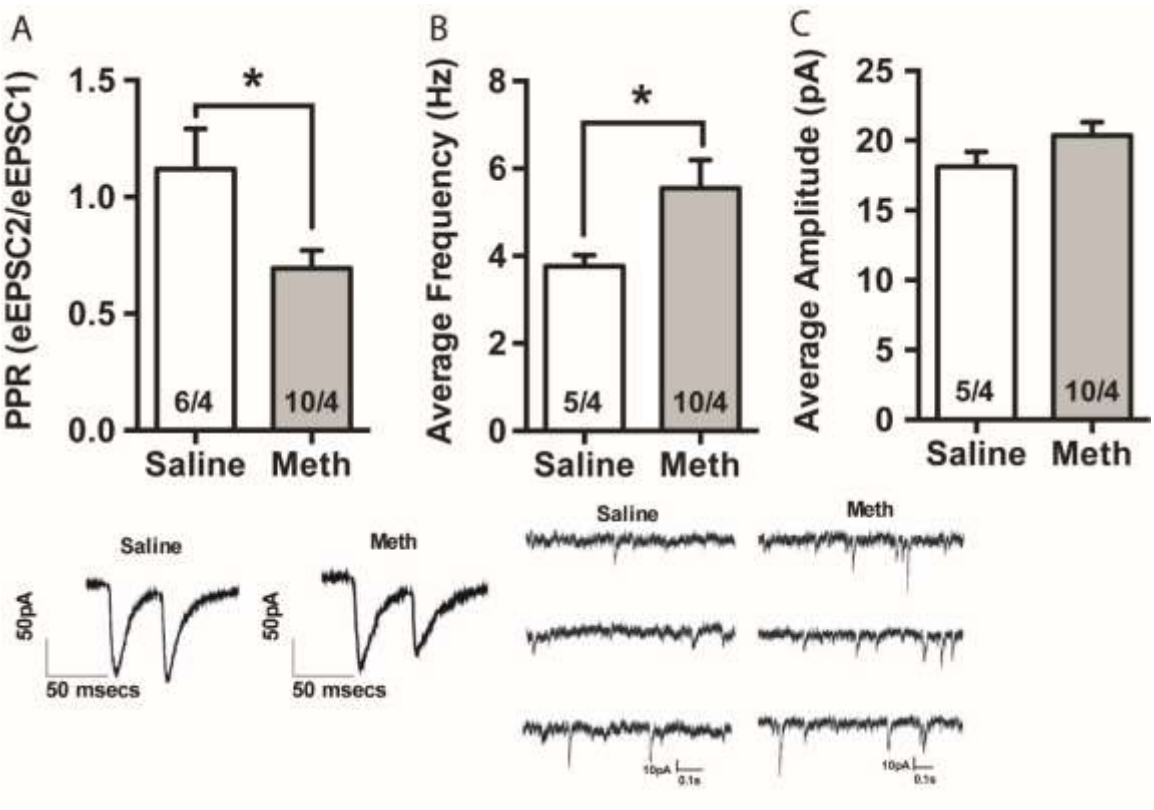

Figure 3. In the NAc, meth self-administration altered presynaptic NAc physiology. In the NAc, long access meth decreased PPR on whole cell recordings (a). Representative traces for significant findings are depicted below. Also, meth increased the frequency (b) of sEPSCs but not the amplitude (c). Representative traces of spontaneous events from three cells of each group are depicted. The number of cells/animal is indicated in the bar graphs, which represent the mean; the errors bars represent the SEM. *Significant difference from saline

Discussion

Here we show that a translationally relevant model of meth self-administration altered pre- and postsynaptic plasticity in the NAc and PFC, respectively. In the PFC meth changed synaptic strength indexed as a decrease in the AMPA/NMDA ratio and this plasticity reflected an increase in NMDA signaling. In the NAc meth increased glutamate release probability, shifted the I/O function, and increased the frequency of sEPSCs. These cortical-accumbens changes in glutamatergic neurotransmission demonstrate alterations in distinct regions that drive addictive behavior.

The long access meth regimen is associated with cognitive impairments mediated by the prefrontal and perirhinal cortices (see Bernheim et al., under review). In our lab, extended access to meth also impairs plasticity of the perirhinal cortex through a GluN2B mechanism (Scofield et al., 2015a). In the perirhinal cortex, however, GluN2B neural adaptions are in the opposite direction, that is GluN2B is down-regulated following meth self-administration (Reichel et al., 2014; Scofield et al., 2015a). This difference in receptor expression may be due to an overall loss of receptors in the perirhinal cortex because meth also causes a decrease in serotonin transporter (Marshall et al., 2007; Reichel et al., 2011b) and mGluR5 (Reichel et al., 2010b). Since these proteins are also membrane-bound, it is possible that meth may cause an overall loss of terminals in the perirhinal cortex that has yet to be documented.

The glutamatergic projection from the PFC to the NAc is subject to synaptic changes following abused drugs. We report an increase in frequency of sEPSCs, suggesting alterations in glutamatergic presynaptic sites. Furthermore, paired-pulse recordings show decreases in the PPR, suggesting an increase in glutamate release. This increase in glutamate release may be the result of an increase in the number of synaptic vesicles in the ready releasable pool (Stevens and Tsujimoto, 1995). Meth increases striatal vesicular glutamate transporter 1 protein expression and vesicular glutamate uptake (Mark et al., 2007). Alternatively, we have shown meth self-administration decreased the number and/or function of presynaptic release regulating group 2 metabotropic glutamate receptors (mGluR2/3) (Schwendt et al., 2012), and down-regulation of mGluR2/3 disinhibits presynaptic neurotransmitter release.

We also found meth self-administration increased I/O function in the NAc, which is consistent with increased glutamate release probability and firing. However, increased I/O function may also reflect increased excitability of MSNs or post-synaptic receptor population changes. We did not observe changes in AMPA/NMDA ratio or amplitude of

sEPSCs to indicate altered postsynaptic function. While puzzling, this lack of postsynaptic plasticity in the NAc is consistent with reports showing no changes in AMPA/NMDA ratio or amplitude in mice following repeated amphetamine injections (Jedynak et al., 2016). These results combined with our finding of increased accumbal glutamate release and increased cortical NMDA currents suggest that in addition to synaptic changes, intrinsic factors strongly influence neuronal activity as discussed in a recent review (Kourrich et al., 2015).

In the PFC we found no indication of a change in neurotransmitter release probability, frequency, or amplitude of sEPSCs. This contrast with reports that mice withdrawn from repeated experimenter delivered meth resulted in a pronounced paired-pulse facilitation mediated by dopamine D1 receptors (González et al., 2015). The most obvious methodological differences between studies are species and the behavioral contingency. Using the same long access meth protocol and rats, Parsegian et al., found an increase in the proportion of burst firing pyramidal cells compared to tonic firing cells in PFC (Parsegian et al., 2011a). A decrease in cortical glutamate signaling would predict reduced presynaptic plasticity in projection targets. But, meth increased NAc glutamate release together with an increase in I/O function without changes in postsynaptic measures. These seemingly paradoxical results can be interpreted two ways based on a recent review by (Kourrich et al., 2015). First, the synaptic changes induced by meth (i.e., up-regulation of GluN2B, increased NMDA current) do not necessarily translate into changes in neuronal activity (i.e., firing frequency). Second, the permissive function hypothesis (Kourrich et al., 2015) suggests cocaine-induced transient increases in the NAc firing is linked to delayed increases in NAc synaptic strength. Applying this hypothesis to our PFC data, the meth-induced changes in intrinsic activity (Parsegian et al., 2011a) may precede our reported differences in

synaptic plasticity. Furthermore, the increase in cortical intrinsic excitability may mediate the increase in glutamate release in the NAc.

Alternatively, since our reported decrease in AMPA/NMDA ratio was mediated by an increase in NMDA currents, our findings may actually reflect more efficient synaptic integration. Support for this notion comes from reports showing that GluN2B containing synapses have slow decay time when cortico-cortical inputs to layer 5 pyramidal neurons in the frontal cortex are stimulated (Kumar and Huguenard, 2003), which facilitates summation of the synaptic inputs (Cull-Candy and Leszkiewicz, 2004). This enhanced integration allows more efficient synaptic function by bringing the neurons close to spike-firing threshold (Kumar and Huguenard, 2003). It is possible that the meth-induced selective increase in GluN2B containing NMDA receptors and increased NMDA receptor current amplitude in the PFC contributed to the decreased PPR and increased frequency of sEPSCs in the NAc.

A strength of our experimental design stems from recording in the PFC and NAc of the same rats. This design allowed us to make the assumption that the changes on glutamatergic synaptic transmission observed on these two regions are occurring simultaneously even though we cannot conclude whether the onset of the changes is also occurring concurrently. In summary, we report meth self-administration alters synaptic plasticity in the corticostriatal circuit. These findings add to a growing body of literature focused on identifying the underlying mechanisms of meth addiction. It remains to be determined how these physiological processes relate to documented cognitive sequel in addicts, but these results show that meth elicits different long-term synaptic changes in the reward circuit, suggesting altered synaptic function is one of the fundamental mechanisms affected by drugs of addiction.

Chapter 6:

Discussion

Although the importance of the medial prefrontal cortex (mPFC) in psychostimulant addiction is well established, the contribution of adaptations in iGluR synaptic transmission to relapse and drug-related memory retention in mPFC PL and IL neurons remains to be fully characterized. Our findings, considering the methodological differences between psychostimulants (cocaine and methamphetamine) and the behavioral paradigms (reinstatement model of SA, escalated drug intake (SA) model and conditioned place preference) utilized, begin to shed light on the differentiable mPFC subregion-specific iGluR transmission dynamics that can aid in the discovery of novel mechanisms and therapeutic targets for relapse prevention.

The combination of iGluR synaptic transmission dynamics sustains the opposing behavioral control of the mPFC subregions over drug-seeking behavior and retention of a cocaine reward memory. There are unknown adaptations in mPFC synaptic iGluR transmission that predispose abstinent rats to relapse into drug seeking and taking. These adaptations in excitatory synaptic transmission, produced by the experience of psychostimulant-associated stimuli during a relapse event, represent a form of plasticity that is experience-dependent, and requires learning and maintenance of drug-stimulus association memories. Preclinically, we can study these forms of experience-dependent plasticity by exposing conditioned rats to drug-associated stimuli and performing acute brain slice whole-cell recordings. Two conditioning models were used in our studies to generate a psychostimulant-conditioned behavioral response: conditioned place preference (CPP) and i.v. self-administration (SA). The first experiment used a cocaine-

induced CPP model to assess the retention of cocaine-related contextual memories via adaptations in synaptic iGluR transmission. The significance of these synaptic adaptations was evaluated at three levels: after short or prolonged abstinence intervals, with and without experience of the cocaine-associated context, and from PL and IL-mPFC deep layer 5/6 pyramidal neurons ($PNs^{5/6}$) recordings. In detail, I recorded from rats that were exposed to a cocaine-associated context and rats that were deprived from that experience. This way, I could measure how a cocaine-associated context experience alters iGluR synaptic transmission in the mPFC. Under these conditions, if the PL and IL-mPFC subregions have opposing roles in the control over drug-conditioned behaviors, our model states that a selective change in iGluR synaptic transmission dynamics is expected in PL-mPFC $PNs^{5/6}$, the region involved with promoting the expression of drug seeking and reward-related behaviors.

All electrophysiological measurements analyzed in this study were taken after short and prolonged abstinence from cocaine-experienced rats to ensure that expression of conditioned behavioral responses were due to the context and not to acute cocaine exposure(Hotsenpiller and Wolf, 2002). Saline-treated rats were used to differentiate the alterations in iGluR transmission that were dependent on cocaine from the context experience-dependent alterations. Therefore, cocaine-induced adaptations in iGluR transmission should be present in context-experienced and -deprived rats, but not in saline-treated rats.

The second study (Chapter 4) used the reinstatement paradigm of SA to compare the effects of repeated cocaine SA, extinction learning and experience of a discrete compound cue (light + tone) on iGluR synaptic transmission between PL- and IL-mPFC neurons. Recordings were made at 15 mins or 24hrs after the cue experience (cue-induced reinstatement test) to test if iGluR adaptations are maintained short-term.

Following our "experienced vs deprived" model, recordings from rats that underwent extinction training and a cue experience were compared to extinction training and saline yoked rats. With this comparison, I can differentiate the alterations in iGluR transmission that depend on cocaine from the cocaine-cue experience alterations.

The third study (Chapter 5) used an extended access model of methamphetamine (meth) self-administration followed by abstinence. Recordings from PL-mPFC neurons from meth- and saline-treated rats were compared for alterations in iGluR synaptic transmission induced by abstinence from meth experience.

Equal measures of iGluR synaptic transmission were acquired in all the studies. All recordings were done in a subregion and layer-specific manner: PL- or IL-mPFC deep layer 5/6 pyramidal neurons. Additionally, all the recordings were performed under drug-free conditions at different intervals after the last drug-experience. This leaves the comparisons drawn between the results to be the most intricate portion of the dissertation. Considering the procedural differences between the behavioral models and psychostimulants used in each of the studies; my aim was to interpret the changes as previously uncharacterized mPFC subregion-specific alterations in iGluR synaptic transmission. These synaptic alterations, despite being generated under different experimental conditions, are hypothesized to be produced by three differentiable phenomena: the experience of psychostimulant-associated stimuli and subsequent execution of a drug-conditioned behavioral response; the long-term effects of repeated psychostimulant experience; and the dorsoventral functional distinction of mPFC subregions. Therefore, any overlapping results could be interpreted as general psychostimulant-associated, experience-dependent alterations in iGluR-mediated retention of drug seeking and reward.

The results from our studies suggest that cocaine and methamphetamine produce different effects in mPFC neurons. Importantly, these changes are dependent on the behavioral paradigm being used. Specifically, cocaine self-administration followed by extinction alone or cue-induced reinstatement of cocaine seeking did not produce any measurable differences in glutamate transmission compared to saline-yoked rats. Cocaine-CPP, on the other hand, produced several changes in glutamate transmission in both PL and IL neurons. These neuroadaptations were dependent on the length of abstinence and were reversed by context re-exposure. Lastly, contrary to the effects of cocaine self-administration, methamphetamine self-administration followed by 8 days of abstinence produced pre- and postsynaptic changes in glutamate transmission in mPFC neurons. In summary, these results provide evidence that general changes in mPFC synaptic glutamate transmission account for aspects of drug-seeking behavior that are not responsive to exposure to drug-associated cues or context, while other alterations in synaptic transmission that meet the functional distinction between mPFC subregions are sensitive to drug cue- or context-associations.

Cocaine and methamphetamine: acute behavioral effects and mechanisms of action

Cocaine and methamphetamine are both categorized as psychostimulants, encompassing drugs that promote wakefulness and alertness, and have mood-boosting properties. Both drugs have different mechanisms of action: cocaine blocks the dopamine transporter (DAT) and other monoamine transporters, increasing the levels of dopamine (DA) in the synapse; methamphetamine depletes DA containing vesicles in the nerve terminals and causes the reverse transport of DA to the synaptic cleft, increasing the levels of DA in the synapse and in the cytoplasm, where it is neurotoxic (Halpin et al., 2014). Previous studies have shown higher concentrations of extracellular

DA in the neostriatum with a methamphetamine injection compared to an injection of cocaine (Zhang et al., 2001). Additionally, methamphetamine in humans produces a long-lasting high, due to the slower pharmacokinetics of the drug compared to the relatively fast metabolism and excretion of cocaine (Fowler et al., 2008; Heal et al., 2013). Interestingly, the cocaine- and methamphetamine-mediated locomotor behavioral responses in rodents are analogous and both are modulated by the dopamine system. These similarities reinforce the importance of studying the glutamatergic system and establishing the difference between the acute effects of drug exposure from the long-lasting changes that endure after abstinence. As an example of similar mechanisms of synaptic plasticity between psychostimulants, a recent study found overlapping changes in glutamatergic signaling following repeated amphetamine or cocaine treatment in NAc medium spiny neurons(MSNs) (Jedynak et al., 2016). The main overlapping alteration produced by both psychostimulants was a synaptic enhancement of AMPA-mediated synaptic transmission (Jedynak et al., 2016). Interestingly, MSN's in the NAc core were exclusively altered by acute amphetamine exposure, while repeated amphetamine specifically potentiated NAc shell neurons (Jedynak et al., 2016). Moreover, repeated exposure to cocaine alone enhanced synaptic AMPA transmission in NAc shell neurons (Jedynak et al., 2016). These and other examples of long-lasting changes in glutamate transmission in the NAc (Scofield et al., 2016) provide a general model for psychostimulant-induced synaptic plasticity and underlie the importance of characterizing the role of glutamate transmission in the dorsal and ventral mPFC regions in maintaining drug-conditioned behavioral responses.

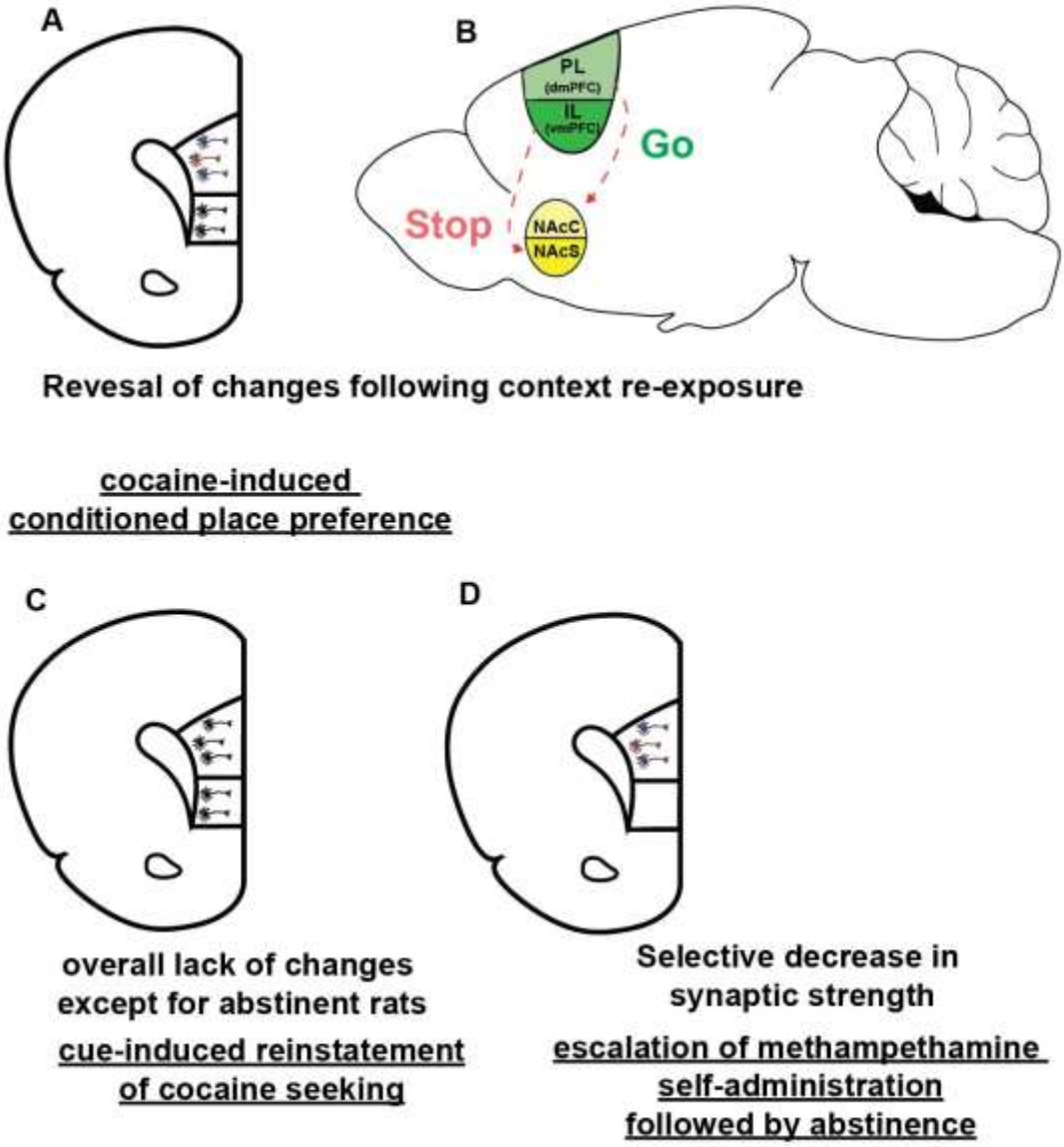

Figure 1

Figure 1. Overall Summary. A. Aim 1; cocaine-induced conditioned place preference followed by short of prolonged abstinence produces distinct changes in synaptic glutamate transmission that are reversed by context re-exposure. B. Putative circuit underlying retention of cocaine conditioned place preference and drug seeking behavior. C. Aim 2; Minimal changes following cue-induced reinstatement of cocaine seeking limited to abstinent rats. D. Aim 3; Escalation of methamphetamine seeking produces a selective decrease in synaptic glutamatergic strength.

Effects of psychostimulants exposure followed by abstinence or extinction on PFC glutamate synaptic transmission.

The role of extinction training in cocaine CPP and cocaine SA

An extinction procedure in the CPP paradigm requires rats to receive a cocaine prime before exposure to the extinguished context for reinstatement of cocaine-induced place preference to occur. Therefore, since our experiments required drug-free conditions, it remains unknown whether recordings from CPP rats undergoing abstinence express different iGluR dynamics than rats undergoing extinction training. A priming injection of cocaine, the acute pharmacological effects of cocaine, would contaminate the extinction-related neuroadaptations. Previous studies show that an acute injection of cocaine produces direct effects in several brain regions that are dependent on cocaine and are independent of cocaine-conditioned context (CS) re-exposure. Acute cocaine administration has been shown to produce several effects on cortical activity specific to the acute-blockade of dopamine (DA) transport and increases in DA in the synaptic cleft: acute administration of cocaine in vivo disrupts cortical membrane bistability and decreases spontaneous firing in cortical cells (Trantham-Davidson and Lavin, 2004). In addition to the effects on DAergic signaling in the mPFC, acute cocaine also causes neuronal release of nitric oxide (Sammut and West, 2008).

The question that comes up when using extinction as an element of interest is, how does extinction influence the changes observed in our coke SA results?
This question addresses the hypothesis that a cocaine experience or experiencing drug-conditioned stimuli, following abstinence or extinction, produces different changes in glutamate transmission. This question was in part addressed by the experiments in which rats were given 8 days of abstinence, the same length of abstinence as the SA group in the CPP studies. Interestingly, abstinence (but not extinction training) produces

differences in sEPSCs in mPFC PL and IL neurons. Delving deeper in the meaning of this difference, one could speculate that in the case of extinction, the changes produced by this procedure could be due to the formation of an extinction memory.

This "new memory" could interfere with (or add to) the direct drug-effects.
B/M inactivation of dmPFC did not block reinstatement after abstinence (dlCPu did). (specifically - not paraphrased: inactivation of these structures failed to alter cocaine seeking in a cocaine-paired environment after a 14-d drug-free abstinence period and no extinction training.) This suggests that there is limited overlap in the substrates of cocaine seeking after abstinence versus extinction, and that habit learning exerts greater control over drug seeking than regions implicated in stimulus-reward associations.

Response-contingent and response-noncontingent psychostimulant administration (and experimenter administration of psychostimulants)

Our results show that repeated cocaine injections (in the CPP paradigm) produce distinct changes in PL and IL -mPFC while cocaine self-administration does not. This discrepancy can be attributed to the different experimental procedures required for the two behavioral paradigms. An alternative view is that the cocaine-evoked adaptation in glutamate transmission in the mPFC neurons, at the time when the recordings were performed, are present in non-contingent drug administration conditions, suggesting that the drug-context associations require this changes in CPP, but response-contingent administration of cocaine, and the drug-CS associations created in the SA paradigm do not rely on adaptations in glutamate transmission.

Rats that underwent abstinence or extinction after heroin self-administration training expressed a strong cue-induced reinstatement (Van den Oever et al., 2008). In this study, the researchers also address the experience-dependent effects of heroin-

associated stimuli, showing a reduction in the AMPA to NMDA ratio in rats that were cue-reinstated after extinction versus extinction only or saline rats.

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