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Investigating the role of corticostriatal glutamate signaling in methamphetamine reward and reinforcement

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by

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ABSTRACT

Investigating the role of corticostriatal glutamate signaling in methamphetamine reward and reinforcement

by

Chelsea Nicole Brown

Highly addictive amphetamine-type stimulants, including methamphetamine (MA), pose significant health and socioeconomic issues, with an estimated 27 million users worldwide. Understanding the neurobiological changes underlying various stages of MA abuse is key to developing safe and effective therapies. While the majority of addiction research has implicated the dopamine system, the long-term neuroplasticity that maintains MA Use Disorder also involves drug-induced changes in glutamate signaling in reward regions of the brain like the nucleus accumbens (NAC) and prefrontal cortex (PFC). In order to further characterize the role of glutamate in MA reward and reinforcement, glutamate receptor scaffolding protein Homer2 expression in the NAC and activity in the glutamatergic projection PFC-NAC were manipulated prior to place- and operant-conditioning procedures. Together the results from these studies argue that while research of the functional neuroanatomy of addiction tends to focus on traditional dichotomies between the NAC core and shell or PFC prelimbic and infralimbic sub-regions, the behavioral outcomes of probing these areas were inconsistent with these models across variables measured. Other inputs upstream from our regions of interest may be able to compensate for our manipulations via

neurobiological redundancy. This would suggest that as research technologies advance, targeting functional networks will be crucial for elucidating the neurobiological underpinnings of addiction and discovering potential therapeutics.

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List of Abbreviations

5-HT – serotonin
AAV – adeno-associated virus
AC – anterior commissure
ACC – anterior cingulate cortex
ADHD – attention-deficit/hyperactivity disorder
ANOVA – analysis of variance
B6 – C57BL/6J mice
cDNA – complementary deoxyribonucleic acid
CNO – clozapine-n-oxide
CPA – conditioned place-aversion
CPP – conditioned place-preference
CRF – corticotropin releasing factor
DA – dopamine
DAB – 3,3'-diaminobenzidine
DAT – dopamine transporters
dlPFC – dorsolateral prefrontal cortex
DREADD – designer receptors exclusively activated by designer drugs
EAAT – excitatory amino acid transporters
fMRI – functional magnetic resonance imaging
FR – fixed ratio
GABA – gamma-aminobutyric acid
GFP – green fluorescent protein
GluN – N-methyl D-aspartate receptor subtype
HA – anti-hemagglutinin
IL - infralimbic
IP – intraperitoneal
KO – knock-out
MA – methamphetamine
MAHDR – methamphetamine high drinking mice
MALDR – methamphetamine low drinking mice
MAO – monoamine oxidase
mGluR – metabotropic glutamate receptor
NAC – nucleus accumbens
NE – norepinephrine
NET – norepinephrine transporters
NMDA – N-methyl D-aspartate receptor
OFC – orbitofrontal cortex
PBS – phosphate-buffered saline
PFC – prefrontal cortex
PL – prelimbic
SAL – saline
SEM – standard error of the mean
SERT – serotonin transporters
shRNA – short hairpin ribonucleic acid

TAAR1 – trace amine-associated receptor 1
VMAT-2 – vesicular monoamine transporter-2
vlPFC – ventrolateral prefrontal cortex
vmPFC – ventromedial prefrontal cortex
VTA – ventral tegmental area
WT – wild-type

Chapter 1:

General Introduction

1.1 Scope and significance of methamphetamine use

Methamphetamine (MA) is a powerful central nervous system stimulant, the abuse of which has an increasingly devastating impact on society. Despite its potential utility at low doses as a prescription medication for attention-deficit/hyperactivity disorder (ADHD), narcolepsy, and weight loss, its recreational use can lead to a substance abuse disorder with potentially lethal consequences (NIDA, 2021; Chiu and Schenk, 2012). With 27 million reported MA users worldwide in 2019, the magnitude of this problem has been increasing over the past decade. In 2018, 228 tons of MA were seized globally, up 23% from the previous year, which was greater than the amounts of heroin, morphine, pharmaceutical opioids, and amphetamine. In the category of amphetamine-type stimulants, over 80% of the drugs seized were MA and 95% of the clandestine laboratories dismantled during the years 2014-2018 had been manufacturing MA (World Drug Report, 2020). MA is the drug of abuse that contributes most to violent crime (US Department of Justice DEA, 2016). Most MA in the United States has been smuggled from Mexico, where it can easily be made by violent criminal organizations in clandestine labs using over the counter pharmaceutical ingredients like pseudoephedrine (World Drug Report, 2020; Administration USDoJDE, 2018). Labs can then take smuggled MA in powdered or liquid form and transform it into crystals or pills, processes that have devastating consequences for the environment because of the toxic chemicals used (Administration USDoJDE, 2018).

Also called meth, blue, ice, or crystal, MA has highly addictive properties that vary depending on how it is administered - whether via smoking, snorting, injection, or oral ingestion. Methods such as smoking or injection that put the substance into the bloodstream very quickly immediately cause an intense euphoric rush, termed a flash, that lasts only a few minutes. Experiencing a flash increases the risk of developing a MA use disorder, which

results in a number of other adverse health consequences (NIDA 2021; Substance Abuse and Mental Health Services Administration, 2018).

Short term effects of MA use include wakefulness, increased activity, decreased appetite, euphoria, rapid heartbeat, and hyperthermia. Cardiovascular issues, hyperthermia, and convulsions associated with MA overdose can be lethal (Chomchai and Chomchai, 2015; Panenka et al., 2013). In addition to the risk of developing a MA use disorder, long term misuse can lead to anxiety, confusion, memory loss, insomnia, mood disturbance, aggression, violent behavior, psychosis, paranoia, visual and auditory hallucinations, delusions, weight loss, tooth decay, and skin sores (NIDA, 2021; Akindipe, Wilson, and Stein, 2014; Panenka et al., 2013; Rusyniak, 2013). Cognitive impairments related to MA use affect executive function, learning, episodic memory, information processing, motor skills, working memory, impulse control, and attention. These deficits have been associated with abnormalities in fronto-striatal-thalamo-cortical circuits (NIDA, 2021; Cruickshank and Dyer, 2009; Scott et al., 2007). The changes that occur in the brain due to repeated MA use may underlie the difficulties in overcoming MA use disorder and the high incidence of relapse. Patients suffering with MA use disorder typically experience an escalation in their drug taking and cravings despite persistent attempts to regulate their use, impairment of their social relationships, and physical risk (DSM-V, 2013). Common for misuse of stimulants, users will often fall into a binge and crash pattern of drug-taking, in which the loss of the pleasurable effects of the drug occur prior to a drop in blood concentration that provokes them to take larger and larger amounts of drug. As addiction develops, users often experience a tolerance to the pleasurable effects of MA, so that larger doses are needed more frequently to achieve the desired high. Many with MA use disorder also suffer withdrawal symptoms of depression, anxiety, anhedonia, fatigue, and drug cravings that negatively impact normal

functioning and can drive subsequent drug-taking behavior (NIDA, 2021; Chomchai and Chomchai, 2015; Courtney and Ray, 2014; DSM-V, 2013; Panenka et al., 2013).

1.2 Molecular mechanisms of MA action

MA acts as a potent indirect agonist at monoamine receptors, particularly dopamine (DA) and norepinephrine (NE) receptors. Because of similarities in their chemical structures, MA mimics the catecholamines DA and NE at membrane-bound transporters: dopamine transporters (DAT), and norepinephrine transporters (NET), and vesicular monoamine transporter-2 (VMAT-2) (Han and Gu, 2006). After it enters neurons via DAT or NET, MA reverses the functionality of VMAT-2 by disrupting the pH gradient that drives the catecholamine inside the vesicles. As such, catecholamine levels build up in the cytosol of the terminal, creating a concentration gradient that drives the catecholamine out of the terminal via their transporters into the synaptic cleft. In addition to releasing catecholamines, MA also prevents the reuptake and degradation of DA and NE, prolonging their activity, respectively by blocking DAT and NET and by inhibiting cytosolic degradation by the enzyme monoamine oxidase (MAO) (Cruickshank and Dyer, 2009). MA's ability to interfere with VMAT function and induce DA release is dependent on sigma 1 receptor activation (Hedges et al., 2017; Kaushal and Matsumoto, 2011; Maurice and Su, 2009). MA can also act on serotonin (5-HT) receptors but with a 100-fold lower potency than at DA or NE receptors (Cruickshank and Dyer, 2009). The capacity of MA to augment DA/NE signaling within brain reward pathways is posited to contribute to the positive reinforcing properties of MA that instigates repetitive drug-taking behavior (NIDA, 2021; DSM-V, 2013; Chiu and Schenk, 2012).

Intracellular MA can also interact with trace amine-associated receptor 1 (TAAR1). The endogenous ligands of TAAR1 are trace amines that are structurally related to monoamine

neurotransmitters. TAAR1 activation can modulate dopaminergic signaling (Xie and Miller, 2007) and is also associated with increased sensitivity to the aversive effects of MA (Phillips et al., 2021; Liu, Wu, and Li, 2020). A single nucleotide polymorphism in the TAAR1 gene that renders the receptor non-functional accounts for 60% of the variation in MA intake between mice bred for a high MA drinking phenotype (MAHDR) and those bred for low MA drinking (MALDR) (Phillips et al., 2021; Stafford et al., 2019; Belknap et al., 2013).

Following systemic administration, MA is absorbed more slowly in the brain than in other organs, which means that it accumulates in the body while the user seeks their recreational high and causes toxic sympathomimetic effects. MA clearance in the brain is also slower than in other organs, which prolongs the brain's exposure time to the drug and its opportunity to initiate neurotoxicity by creating reactive oxygen and nitrogen species, increasing cerebral temperature, and releasing calcium ions from internal stores. Neurocognitive symptoms have been connected to these neurotoxic effects (Chiu and Schenk, 2012; Volkow et al., 2010; Cruickshank and Dyer, 2009). MA is metabolized in the liver by the enzyme cytochrome P450, and then it and its metabolites (which likely do not contribute to clinical effects) are excreted in urine (Cruickshank and Dyer, 2009). MA, with a half-life of 12 hours, is a potent stimulant with extended euphoria and increased neurotoxicity compared to other stimulants, such as cocaine which has a half-life of 1 hour. Also in contrast to cocaine, MA and other amphetamines release DA/NE via several mechanisms as outlined above, whereas cocaine acts to block monoamine reuptake by inhibiting DAT, NET and serotonin transporters (SERT). In other words, the effects of cocaine upon synaptic levels of monoamines are impulse-dependent (i.e., depend on neurotransmitter release from the cell), while that of MA is impulse-dependent and results in higher synaptic levels of DA/NE than cocaine (Moszczynska, 2016; Panenka et al., 2013;

Chiu and Schenk, 2012; Gold, 1993). Additionally, MA has stronger effects on DAT, releases more DA, and inhibits reuptake more efficiently than its parent molecule amphetamine (Chiu and Schenk, 2012; Goodwin et al., 2009), and this higher potency to affect catecholamine transmission and long duration of action augment its addiction liability. Research seeking to understand the molecular mechanisms of action of MA aims to expand the potential targets for pharmacological therapies for MA use disorder, but clinically effective options have remained elusive.

Despite the fairly well-established effects of MA on monoaminergic systems, there are few pharmacological options available to treat MA use disorder, and none have yielded clinical results convincing enough to be recommended as an effective therapy. Pharmaceuticals currently under investigation include antidepressants that facilitate release or inhibit reuptake of monoamines, atypical antipsychotics that are partial agonists for dopamine and serotonin, central nervous system stimulants like dexamphetamine or methylphenidate, GABAergic agonists/glutamate antagonists, glutamate regulators, opioid agonists and antagonists, a partial cholinergic nicotinic antagonist, and a CRF antagonist. Though the trials with the most promising results studied some combination of the stimulant agonists, opioid antagonists, and GABA agonist/glutamate antagonists, none of them have advanced past Phase II trials due to their lack of efficacy in preventing cravings and relapse (Paulus and Stewart, 2020; Siefried et al., 2020). Despite the well-characterized effects of MA on the catecholaminergic systems of the brain, neither DA- nor NE-directed therapeutics have proven to be effective clinically. Behavioral interventions, specifically cognitive behavioral therapy and contingency management, have yielded more success, but the benefits rarely persist beyond the cessation of treatment, which is an obstacle because achieving long

term adherence with these therapies is difficult (Paulus and Stewart, 2020; Siefried et al., 2020).

The lack of clinically effective therapies for treating MA use disorder reflects, at least in part, a knowledge chasm related to the neurobiological underpinnings of MA addiction, particularly those involved in a wide range of drug-taking stages, from early social/recreational use to the later compulsive use that characterizes MA use disorder. My dissertation studies seek to help fill this knowledge gap by probing the role for the glutamate-related scaffolding protein Homer2, as well as the glutamatergic projections from the prefrontal cortex (PFC) to the nucleus accumbens (NAC) in murine models of MA reward, reinforcement, and relapse.

1.3 MA use alters the human brain

Clinical studies have implicated frontal cortical and striatal areas as sites of action for MA-induced changes in the brain. Early research on human subjects focused on the dopaminergic areas in the midbrain, the ventral tegmental area (VTA) and substantia nigra, and the targets of their projections, the dorsal and ventral striatum (Goldstein and Volkow, 2011; Everitt, Dickinson, and Robbins, 2001; Wise, 1996; Di Chiara and Imperato, 1988). MA-addicted individuals exhibit changes in the DA system (Paulus and Stewart, 2020; Volkow et al., 2001a; Volkow et al., 2001c; Volkow et al., 2001d; McCann et al., 1998), the 5-HT system (Paulus and Stewart, 2020; Sekine et al., 2006), glucose metabolism (Paulus and Stewart, 2020; Baicy and London, 2007; London et al., 2004; Volkow et al., 2001b), neurometabolite levels (Nordahl et al., 2005; Ernst et al., 2000), grey matter deficits (Paulus and Stewart, 2020; Baicy and London, 2007), and gross structural abnormalities (Chang et al., 2005; Jernigan et al., 2005; Thompson et al., 2004), particularly in the basal ganglia (Chang et al., 2007). They have enlarged striatal volumes, specifically larger NAC volume,

in recent abstinence, but smaller striatal volumes with greater use (Jan, Kydd, and Russell, 2012; Chang et al., 2007; Chang et al., 2005; Jernigan et al., 2005), which may reflect initial inflammatory changes, as well as indicators of reduced neuronal density—potentially due to cell death--- in the basal ganglia associated with the persistence of psychiatric symptoms (Chang et al., 2007; Chang et al., 2005; Sekine et al., 2002; Ernst et al., 2000). Chronic MA use has been associated with decreased D2 receptor density and DAT levels in the NAC and the striatum, an effect that has been tied to motor and memory impairments (Jan, Kydd, and Russell, 2012; Chang et al., 2007; Sekine et al., 2001; Volkow et al., 2001a; Volkow et al., 2001d; McCann et al., 1998). It also leads to low availability of D2-like D3 receptors that play a critical role in reward and motivation in the nucleus accumbens. Lack of this type of receptor correlates with higher impulsivity (Jan, Kydd, and Russell, 2012; Lee et al., 2006; Murray et al., 1994; Sokoloff et al., 1990). The striatum of MA users also shows decreased serotonin transporter density and glucose metabolism (Jan, Kydd, and Russell, 2012; Chang et al., 2007; Sekine et al., 2006; Volkow et al., 2001b; Gibb et al., 1997; Seiden and Sabol, 1996; Preston et al., 1985). Because glucose metabolism in the brains of MA users is increased overall, the reduction in the striatum is thought to reflect the neurotoxic effects of MA (Jan, Kydd, and Russell, 2012; Volkow et al., 2001b), which have been demonstrated to be glutamate-dependent (Tata and Yamamoto, 2007).

More recently, clinical studies have explored MA-induced changes in the activity of frontal cortical areas and their impact on behavior (Goldstein and Volkow, 2011; Volkow and Fowler, 2000). MA-addicted individuals show grey and white matter alterations in the frontostriatal system (Paulus and Stewart, 2020; Lyoo et al., 2015). They have reduced grey matter in the orbitofrontal cortex (OFC) and the dorsolateral PFC (dlPFC), as well as glial activation and inflammation in the frontal grey matter (Jan, Kydd, and Russell, 2012;

Nakama et al., 2011; Chang et al., 2007; Ernst et al., 2000). The reduced glucose metabolism in their frontal white matter indicates attenuated neuronal activity and is associated with altered executive function and decision making (Jan, Kydd, and Russell, 2012; Kim et al., 2005). Concordantly, abnormal patterns of brain connectivity and function have been detected in the OFC-striatal and dlPFC-striatal systems of MA-dependent brains both during cognitive tasks and while at rest (Paulus and Stewart, 2020; London et al., 2015). Functional magnetic resonance imaging (fMRI) demonstrates hypoactivity in the OFC of MA subjects during impaired performance on emotional processing and decision-making tasks (Paulus and Stewart, 2020; Goldstein and Volkow, 2011; Kim et al., 2010). MA-induced decreases in OFC glucose metabolism are correlated with low levels of D2 receptors in the striatum (Chang et al., 2007; Volkow et al., 2001a). fMRI results also indicate that MA use results in a hypoactive ventromedial PFC (vmPFC) during decision-making and hypoactive ventrolateral PFC (vlPFC) during affect matching (Jan, Kydd, and Russell, 2012; Goldstein and Volkow, 2011; Payer, Lieberman, and London, 2010; Paulus et al., 2002). Hypoactivity in the dlPFC and its afferents that provide it with contextual information relates to difficulty with decision-making, inhibitory control, and devaluation of future rewards (Paulus and Stewart, 2020; Harle et al., 2016; Hall et al., 2015; Jan, Kydd, and Russell, 2012; Goldstein and Volkow, 2011; Salo et al., 2009; Aron and Paulus, 2007; Monterosso et al., 2007; Paulus et al., 2003; Paulus et al., 2002). In the MA-experienced anterior cingulate cortex (ACC), hyperactivity during affect matching is associated with self-reported hostility and interpersonal sensitivity, while hypoactivation results in inhibitory control deficits that can be mediated by informative cueing that increases ACC activity (Jan, Kydd, and Russell, 2012; Goldstein and Volkow, 2011; Salo et al., 2009; Leland et al., 2008; Payer et al., 2008). As far as affecting neurotransmitter systems, the PFC of patients with MA use disorder exhibits reduced DAT

levels, SERT levels, and glutamate metabolism (O'Neill et al., 2015; Jan, Kydd, and Russell, 2012; Chang et al., 2007; Sekine et al., 2006; Sekine et al., 2003). Overall, these neurological abnormalities have been connected to cognitive impairments in memory, attention, psychomotor speed, and executive functioning (Chang et al., 2007; Simon et al., 2000). However, none of these measures have been able to aid in diagnosis, prognosis, or treatment (Paulus and Stewart, 2020; Moeller and Paulus, 2018), so continuing to improve our understanding of the neurobiology underlying MA-taking, particularly via more causative experiments, is crucial to improving the lives of those with the disorder.

1.4 The importance of using animal models to study MA use

Clinical studies of drug abuse are both correlational and retrospective, making it almost impossible to untangle behavioral characteristics that are causes from those that are effects. Therefore, using animal models is critical for determining the causal relationships between neural changes and drug administration (Olmstead, 2006). Though measuring complex behavioral pathologies using relatively simple behavioral paradigms with rodents is challenging, it is necessary to illuminate the neural mechanisms underlying substance abuse disorder and other psychiatric disorders (Kuhn, Kalivas, and Bobadilla, 2019). This dissertation work employs both oral self-administration, a contingent model of drug reinforcement in which delivery of the substance depends on the subject's behavior, and conditioned place preference (CPP), a non-contingent model of drug reward in which the substance is delivered regardless of the subject's behavior.

CPP is used to index the subjective rewarding or aversive properties of the drug experience. It consists of pairing the drug with one environmental context and a neutral stimulus with another context. After several pairing sessions for each stimulus, the animal is allowed to roam both environments freely, and if it spends more time in the drug-associated

context, it has developed a conditioned place preference for that substance. Many substances, including morphine, cocaine, amphetamine, MA, nicotine, alcohol, and cannabis can induce a CPP that indicates these drugs have rewarding effects (Garcia Pardo et al., 2017; Scofield et al., 2016; Tzschentke, 2007; Bardo and Bevins, 2000; Beach, 1957). Because the post-conditioning test is conducted in a drug-free state, this model can probe enduring rewarding properties that may reflect long-lasting neuroadaptations (Kuhn, Kalivas, and Bobadilla, 2019), which could be examined in the rodent brain via various molecular techniques. It also allows for the determination of the subjective aversive properties of a drug (Mucha et al., 1982), as indicated when an animal spends less time on the drug-paired side. Brain regions found to be important in CPP for drugs and natural reinforcers include the VTA, NAC, medial PFC, ventral pallidum, amygdala, and pedunculopontine tegmental nucleus (Tzschentke, 1998). Neuropharmacological studies have used CPP paradigms to characterize the role of many neurotransmitter systems, including dopamine and glutamate, in the rewarding effects of drugs of abuse (Tzschentke, 2007). My dissertation studies use CPP to probe the role of glutamatergic signaling mechanisms in the rewarding properties of MA.

Operant conditioning models of drug reinforcement (here on in referred to simply as operant self-administration) are popular models of drug-taking that require animal subjects to perform a behavior to obtain access to the drug and are conducted traditionally in Skinner-type operant-conditioning chambers. Operant self-administration initially involves the coordination of goal-directed behavior, driven by PFC activity, with signals of reward learning in response to reinforcing stimuli, associated with NAC signaling, before the brain transitions to habitual behavior governed by the dorsal striatum (Kuhn, Kalivas, and Bobadilla, 2019; Hopf and Lesscher, 2014; Everitt and Robbins, 2013; Belin and Everitt, 2008). Operant self-administration can involve various routes of drug delivery (i.e.,

intravenous, intragastric, intracranial, or oral). Herein I employ oral operant self-administration because this procedure (almost uniquely) allows for the critical dissociation and simultaneous examination of both the appetitive (drug-seeking/responding) and consummatory (drug-taking/intake) aspects of drug self-administration. Because mice willingly drink MA solution, measurements from self-administration can be separated into drug-seeking behaviors (responding) and drug-taking (MA intake). Operant self-administration can be used to model early recreational drug use and the transition to a drug abuse disorder by examining the rate at which an animal learns the operant-response, while the degree to which an animal stabilizes its responding for, and consumption of, drug can model drug use or habitual drug-taking behavior. Pairing or signaling drug delivery with discrete visual, auditory or olfactory cues engenders a higher level of responding as these cues become secondary or conditioned reinforcers of the operant behavior, garnering the ability to maintain responding even in the absence of the drug, which is generally interpreted as cue-induced or cue-maintained drug-seeking or -craving. My dissertation work employs an oral model of self-administration, as prior work from the Szumlinski laboratory has demonstrated successful acquisition of orally delivered MA self-administration (Shab et al., 2021).

Modeling drug-craving and relapse in animal models is critically important because these factors so often impede treatment success (Kuhn, Kalivas, and Bobadilla, 2019). Permutations of the operant self-administration include “forced abstinence”, in which the animal is removed from the self-administration environment for a period of time (typically the animal remains in its home cage) and then is returned to the self-administration environment, either in the presence or absence of drug, to model relapse to drug-taking or cue/context-elicited craving, respectively. Over the past decade or so, this “forced

abstinence" procedure has become a popular model to examine the mechanism(s) of drug-craving and/or relapse in a drug-abstinent animal (Fredricksson et al., 2021). Another permutation of the operant self-administration model that has been used more traditionally by the addiction field to model relapse is the "extinction-reinstatement" model. In this model, the operant response is first extinguished, over hours or days, in the drug-taking environment in the absence of any drug (quite often also in the absence of discrete drug-associated cues). After the operant response has declined to some prespecified level, the animal is subjected to a manipulation that triggers relapse in the clinical condition and this typically involves the behaviorally non-contingent delivery of the drug (usually via an intraperitoneal or IP injection), the administration of an acute stressor (foot-shock or the anxiogenic drug yohimbine) or the re-presentation of the discrete drug-association cues. All of these manipulations will reinstate or re-instigate the operant response, which is interpreted, respectively, as drug-, stress- or cue-primed relapse. Both "forced abstinence" and "extinction-reinstatement" models have proven effective at clarifying the neurobiological changes underlying the high rates of relapse triggered by drug-associated stimuli (Kuhn, Kalivas, and Bobadilla, 2019; Bossert et al., 2013; Shaham et al., 2003; Meil and See, 1996; de Wit and Stewart, 1981). In my dissertation project, I employed the more traditional extinction-reinstatement model to examine the neurocircuitry of involved in MA cue-primed relapse. This model was selected as prior studies with intravenous cocaine (Fuchs et al., 2004) and unpublished studies in the Szumlinski laboratory using an oral MA self-administration model indicated that cues are more effective at reinstating drug-seeking behavior in mice than are drug primes and at the time of my dissertation, there was no published study examining the efficacy of stressor to reinstate drug-taking behavior.

1.5 The role for the NAC in drug reward and reinforcement

A key region in the brain's reward system is the NAC, which is part of the basal ganglia. The NAC analyzes environmental contingencies to shape adaptive behavioral responding and is well characterized as a regulator of stimulant reinforcement via dopaminergic signaling (Scofield et al., 2016; Wise, 2004). In fact, the incentive salience hypothesis of addiction posits that environmental cues become imbued with incentive salience or motivational importance over the course of repeated drug-taking and come to trigger drug wanting, and this associative conditioning process involves a progressive sensitization of both drug- and cue-elicited DA release within the NAC (Berridge and Robinson, 1998). While such theories implicate changes in DA release within the NAC as a critical mediator of drug-craving that drives relapse, understanding of the role of glutamate circuitry connected to the NAC has also advanced over the past few decades as a major regulator of stimulant addiction-related behavior (Lominac et al., 2016; Quintero, 2013; Kalivas and Volkow, 2011). The process by which environmental, drug-associated cues initiate an immediate urge to use drugs is theorized to rely on rapid, transient increases in synaptic strength in the glutamatergic projections from the PFC to the NAC (Gipson, Kupchik, and Kalivas, 2014).

The NAC is divided into two sub-regions, the core and the shell, which have distinct functions, connectivity, and pharmacology that are still being differentiated (Moorman et al., 2015; Vertes, 2004). Current understanding, derived primarily from animal models of cocaine addiction, is that their distinct circuits regulate different components of addiction-related behavior. Activity in the core is thought to contribute to decision-making functions by signaling the motivational value of expected goals to guide drug-seeking in drug-experienced brains. It is essential for expressing stimulant-taking behavior and cue-induced reinstatement (Scofield et al., 2016). Shell activity is thought to be involved in the initial affective valence of drug-taking during early drug experience, mostly based on studies of cocaine. It is critical

for acquisition and drug-primed reinstatement (Scofield et al., 2016; Everitt and Robbins, 2013; McFarland and Kalivas, 2001; Taylor and Robbins, 1984). Given the sub-region selective effects on cocaine addiction-related behavior, the experiments in this dissertation employ discrete manipulations to the NAC core and shell in an effort to investigate whether or not these subregions play distinct or potentially overlapping roles in different aspects of MA addiction-related behavior.

1.6 PFC-NAC glutamate transmission facilitates neuroplasticity contributing to addiction

The most well-studied mechanism for MA reinforcement is the augmentation of monoaminergic signaling from the VTA to the NAC (Chiu and Schenk, 2012; Cruickshank and Dyer, 2009). However, long-term neuroadaptations necessary for changes in response to MA over time are strongly associated with glutamate transmission (Lominac et al., 2016; Lominac et al., 2012; Koob and Volkow, 2010; Kalivas, 2009), particularly in the PFC-NAC projection. Though the reliance of high MA doses' neurotoxic effects on glutamate has long been well-established (Tata and Yamamoto, 2007), studies have also shown that acute, sub-chronic experience with sub-toxic MA doses affect extracellular glutamate in the terminal regions of excitatory corticostriatal projections implicated in addiction plasticity (Szumlinksi et al., 2017; Lominac et al., 2016; Lominac et al., 2012; Koob and Volkow, 2010; Kalivas, 2009). NMDA glutamate receptor antagonists blunt MA-induced conditioned reward and behavioral sensitization (Kim and Jang, 1997). Increased glutamate transporter activity, which should reduce extracellular glutamate, reduces MA-induced place preference, while inhibiting glutamate transporter activity has the opposite effect (Fujio et al., 2005a; Fujio et al., 2005b; Nakagawa et al., 2005). Repeated MA injections increase basal and drug-primed extracellular glutamate in the NAC of C57BL/6J (B6) mice, and microinjection of an

excitatory amino acid transporter inhibitor, which should elevate extracellular glutamate, to the NAC shell sub-region potentiates MA-CPP. Microinjection of an autoreceptor agonist, which should decrease extracellular glutamate, to the same area results in an MA-induced conditioned place aversion (CPA). The levels of mGluR1 and mGluR5 expression in the NAC core also correlate with MA-CPP score (Szumlinski et al., 2017).

Further, to better understand the genetic basis of variation in sensitivity to MA's neurobiological effects, mice were selectively bred for either high (MAHDR) or low (MALDR) MA intake during 2-bottle choice experiments (Phillips et al., 2021; Shabani et al., 2012a; Shabani et al., 2012b; Shabani et al., 2011; Wheeler et al., 2009; Phillips, Kamens, and Wheeler, 2008). These two groups of mice show differences in basal and drug-primed increases in extracellular glutamate in the NAC (Szumlinski et al., 2017), as well as differences in the expression of glutamate receptors and transporters (Lominac et al., 2012). More specifically, the MAHDR mice have increased basal extracellular glutamate and metabotropic glutamate receptor 5 (mGluR5) expression in the NAC compared to MALDR mice (Szumlinski et al., 2017). Overall, this body of evidence associates MA addiction risk with a hyper-glutamatergic state within the NAC and corroborates the above-mentioned neuropharmacological findings indicating an important role for NAC glutamate in mediating the rewarding properties of this drug.

The PFC is another brain region important for reward signaling and addiction behavior that receives dopaminergic projections from the VTA (Kalivas, Volkow, and Seamans, 2005). As discussed above, the body of evidence implicating glutamatergic signaling in MA addiction-related behavior is growing (Szumlinski et al., 2017; Lominac et al., 2016; Lominac et al., 2012; Koob and Volkow, 2010; Kalivas, 2009). As the primary source for glutamate in the NAC, the PFC is a significant region of interest in examining the role of

glutamatergic signaling in MA reward. In studies of both cocaine (Shin et al., 2015) and MA (Parsegian and See, 2014), cue-elicited drug-seeking is associated with increased extracellular glutamate levels within the vmPFC of rats. In studies of mice, repeated MA injections sensitize drug-induced glutamate release and reduce the expression of GluN1 and GluN2b in the vmPFC. MAHDR mice have increased basal extracellular glutamate and decreased Homer2 and mGluR2/3 expression in the vmPFC than MALDR (Lominac et al., 2016). Finally, increasing glutamate levels in the vmPFC augments the magnitude of a MA-CPP in mice (Lominac et al., 2016), while decreasing glutamate levels within vmPFC inhibits the expression of incubated cocaine-seeking (Shin et al., 2018), as well as cue-elicited MA-seeking in rats (Parsegian and See, 2014).

The vmPFC can be further divided into the prelimbic (PL) and infralimbic (IL) sub-regions. Pharmaceutical lesion studies have shown that the PL is essential for cocaine-, heroin-, and MA-seeking (LaLumiere and Kalivas, 2008; Rogers et al., 2008; Hiranita et al., 2006; McLaughlin and See, 2003). The PL is also necessary for cue-induced reinstatement of cocaine- and MA-seeking (Hiranita et al., 2006; Ciccocioppo et al., 2001). Conversely, IL inactivation does not impact cue-induced cocaine or MA-seeking (Hiranita et al., 2006; McLaughlin and See, 2003) but is critical for driving extinction learning (LaLumiere et al., 2010; Peters et al., 2008). Thus, further research is needed to clarify the specific influences of each sub-region on MA-seeking and -taking. As such, a portion of my dissertation research focuses on distinguishing the relative roles played by PL and IL projections in regulating different aspects of MA addiction-related behavior.

Since the primary source of glutamate in the NAC is from the terminals originating in different aspects of the PFC, PFC-NAC circuits are of particular interest in addiction studies. Traditionally, the PL is thought to project primarily to the NAC core and the IL to the NAC

shell, with these circuits playing opposite roles in addiction-related behavior. Opto- and chemo-genetic studies suggest that glutamate signals from the PL to the NAC core drive drug-taking and -seeking behavior, while glutamate signals from the IL to the NAC shell inhibit these behaviors (Augur et al., 2016; Ma et al., 2014), at least with regards to cocaine. Glutamate projections from the PL to the NAC core are necessary for cocaine- and cue-primed reinstatement of cocaine-seeking (Gipson et al., 2013; McFarland et al., 2003; McFarland and Kalivas, 2001). While glutamate from the IL to the NAC shell is unnecessary for cue-induced reinstatement, it is important for cocaine- and context-induced reinstatement of cocaine-seeking (Scofield et al., 2016; Cruz et al., 2013; Anderson et al., 2003; McFarland and Kalivas, 2001), as well as extinction learning (LaLumiere et al., 2010; Peters et al., 2008). However, some evidence argues against a strict dichotomy in the functional neuroanatomy of PL versus IL projections to specific NAC subregions and supports a role for overlapping projections from PFC sub-regions to NAC sub-regions in regulating food-reinforcement behavior (Moorman et al., 2015). Clarifying how these sub-circuits differ functionally and anatomically is crucial to understanding the roles that PFC and NAC sub-regions may play in regulating MA abuse.

1.7 Homer2 modulates the impact of glutamate on substance abuse

Understanding the molecular basis for glutamate changes is important for understanding its role in MA sensitivity. In this regard, a protein called Homer2 has been implicated in facilitating the long-term neuroplasticity underlying MA addiction (Szumlinski et al., 2017; Lominac et al., 2012; Szumlinski, Ary, and Lominac, 2008). Homer2 is a post-synaptic protein that regulates placement and scaffolding of glutamate receptors relative to the synapse, most notably Group 1 mGlu receptors (mGluR1 and mGluR5), as well as the NMDA receptor (Szumlinski et al., 2017; Szumlinski, Ary, and Lominac, 2008; Szumlinski

et al., 2008). Exposure to drugs of abuse, including MA, alters the expression of Homer2 in reward-related brain regions. In particular, repeated MA injections result in higher levels of Homer2 in the NAC after 3 weeks of abstinence (Szumlinski et al., 2008). This effect is specific to the NAC shell, but not the core, of B6 mice (Szumlinski et al., 2017). Along with higher levels of expression of mGluR5, Homer2 expression is also increased in the NAC core and shell of "addiction-vulnerable" MAHDR mice. In B6 mice, levels of Homer2 expression in both sub-regions of the NAC, core and shell, positively correlate with the magnitude of a MA-induced CPP (Szumlinski et al., 2017). Causal manipulations show that reducing Homer2 expression in the shell via shRNA infusions produces a downward trend in MA-primed CPP and reduces mouse responding and intake for MA at high demand schedules and low MA concentrations (Szumlinski et al., 2017). Further study of the functional impact of Homer2 in reward-related brain regions on MA-induced behavior is needed to elucidate its overall contribution to neurobiological mechanisms. As such, this dissertation project includes a series of studies examining the relative role for Homer2 within the NAC core versus shell in regulating MA-conditioned reward and reinforcement.

1.8 DREADDs as a tool for selectively targeting specific neuronal subcircuits

Precisely targeting sub-circuits between the PFC and the NAC requires anatomically specific manipulation of cells whose soma lie in either the PL or the IL and terminate in the NAC core or shell. Chemogenetic techniques use Designer Receptors Exclusively Activated by Designer Drugs (DREADDs), which are modified muscarinic receptor proteins that modulate cellular activity (Rogan and Roth, 2011) when bound to a synthetic ligand, clozapine-n-oxide (CNO) (Ferguson and Neumaier, 2012; Armbruster et al., 2007). Upon injection of CNO, the DREADDs can either stimulate, if the Gq-type, or inhibit, if the Gi-type, neuron activity. The DREADDs can be expressed via microinfusion of viral vectors,

typically a recombinant adeno-associated viral (AAV) vector (Urban and Roth, 2015), to allow for spatial and temporal control of neuron activity.

A dual virus DREADDs approach enables the targeting of a projection, rather than just a region of cells. By pairing chemogenetic technology with the Cre system, it is possible to affect the activity of only cells that signal from one region to another (Augur et al., 2016; Kerstetter et al., 2016). A retrograde Cre recombinase virus is infused into the target region (e.g., a specific NAC subregion), and then a Cre-dependent DREADDs virus is infused into the area where the projection originates (e.g., a specific PFC subregion), so that the DREADDs are only expressed in cells that terminate in the target destination region from this origin. When the CNO is injected systemically, the only neurons whose activity will be affected are these cells where the DREADDs and Cre recombinase have been expressed. This allows for bidirectional, temporally specific manipulation of a specific sub-circuit. The latter studies of my dissertation project employ this DREADD technology to alter the activity of specific PFC-NAC subcircuits or projections in order to decipher whether or not they play unique or overlapping roles in regulating different aspects of MA addiction-related behavior in my various mouse models.

1.9 Aims

The studies presented in this dissertation characterize the contributions of glutamatergic signals in the functional neuroanatomy of MA reward and reinforcement. First, manipulating glutamate receptor scaffolding protein Homer2 expression levels in the NAC attempted to delineate sub-region specific control of MA-induced behavior. Then, intervening on glutamatergic projections from PFC to NAC sub-regions sought to elucidate the precise role of these sub-circuits in MA-taking and -seeking. The specific aims of this work were to: 1) establish a causal role of Homer2 in regulating MA reward and reinforcement, 2) investigate

the NAC core and shell as suspected neuroanatomical loci for the causal effects of Homer2, and 3) bidirectionally manipulate the prefrontal glutamatergic afferents to the core and shell to determine their anatomical connectivity and functional input in MA reward and reinforcement.

Chapter 2:

Transgenic analyses of Homer2 function within nucleus accumbens subregions in the regulation of methamphetamine reward and reinforcement in mice

2.1 Introduction

Amphetamine-type stimulants, including methamphetamine (MA), are the most highly abused psychostimulants in the world, with an estimated 29 million users worldwide in 2017 (United Nations, 2019). Despite the prevalence and severity of MA Use Disorder, the lack of knowledge regarding the neurobiological substrates underlying risk, development and severity impedes therapeutic progress. MA reinforcement and psychomotor activation involves monoamine release and reuptake inhibition, particularly within dopaminergic neurons from the ventral tegmental area (VTA) to the nucleus accumbens (NAC) (Chiu and Schenk, 2012)., Accumulating evidence supports the role of glutamate transmission, especially glutamatergic projections from the prefrontal cortex (PFC) to the NAC, in both MA addiction vulnerability and the long-term neuroplasticity maintaining the MA-addicted state (Lominac et al., 2012; Lominac et al., 2016; Kalivas, 2009; Koob and Volkow, 2010).

It has been known for decades that binge-like, high-dose (>4 mg/kg) MA exposure induces glutamate-dependent neurotoxicity within the dorsal striatum (Tata and Yamamoto, 2007). However, sub-chronic administration of sub-toxic MA doses (<2 mg/kg) can also elevate extracellular glutamate within the NAC (Lominac et al., 2012; Szumlinski et al., 2017). In addition, such exposure is sufficient to increase the expression/function of mGlu1/5 glutamate receptors and their associated scaffolding protein Homer2 within this region (Szumlinski et al., 2017). Indeed, a survey of the extant literature on animal models of MA abuse supports a correlative link between potentiated indices of glutamate signaling and addiction-related behavior, including self-administration, MA-induced reinstatement of drug-seeking after abstinence or extinction, incubation of MA-craving, and conditioned place-preference (CPP) (Mishra et al., 2016; Schwendt et al., 2012; Parsegian and See, 2014;

Scheyer et al., 2016; Murray et al., 2019; Szumlinski et al., 2017; Kim and Jang, 1997; Fujio et al., 2005a,b; Nakagawa et al., 2005).

Supporting a link between NAC glutamate and MA addiction vulnerability, drug-naïve mice selectively bred for high MA intake (MAHDR) exhibit several glutamate anomalies within the NAC, relative to MALDR mice selectively bred for low MA drinking (Phillips et al., 2008; Shabani et al., 2012a,b; Shabani et al., 2011; Wheeler et al., 2009). These differences include elevated basal and MA-induced increases in extracellular glutamate, increased expression of Homer2 and mGlu5, and decreased expression of the EAAT3 glutamate transporter responsible for clearing synaptic glutamate (Lominac et al., 2012; Szumlinski et al., 2017). Further, NMDA glutamate receptor antagonists attenuate MA-conditioned reward and behavioral sensitization (Kim and Jang, 1997), while pharmacological manipulations of extracellular glutamate in the NAC bidirectionally regulate the expression of MA-conditioned reward in B6 mice (Szumlinski et al., 2017). These results provide causal evidence for a relationship between glutamate and MA-induced behavior. Finally, small hairpin RNA (shRNA)-mediated knockdown of Homer2 expression in the shell subregion of the NAC reduces the magnitude of both a MA CPP and oral MA intake during operant-conditioning procedures (Szumlinski et al., 2017), indicating for the first time a causal role for Homer2, at least within the NAC shell, in regulating the rewarding and reinforcing properties of MA.

The present study sought to extend our earlier results in the NAC shell (Szumlinski et al., 2017) to the NAC core subregion and to probe the bidirectionality of the effects of transgenic manipulations of NAC Homer2 expression on MA addiction-related behaviors. The core and shell subregions of the NAC have distinct functions, connectivity, and pharmacology that are still being characterized within the context of addiction (Everitt and Robbins, 2013). Current

theories argue that the NAC core is embedded within subcircuits involved in decision-making by signaling the motivational value of expected goals to guide drug-seeking in drug-experienced animals. In contrast, the NAC shell appears to be more involved in the initial affective valence of the drug during early drug experience (Everitt and Robbins, 2013). As Homer2 expression within both subregions is correlated with MA addiction vulnerability in mouse models (Szumlinski et al., 2017), we first examined the effects of knocking down Homer2 expression in the NAC core on MA-induced CPP and the acquisition of oral MA self-administration in inbred C57BL/6J (B6) mice. The combined results of our knockdown studies suggest opposing roles for Homer2 within the NAC shell and core in regulating MA reward and reinforcement. To determine whether Homer2 contributes to the development of MA CPP and oral intake, we also determined the effects of up-regulating Homer2 expression in both NAC subregions on the behavior expressed by constitutive *Homer2* knockout (KO) mice and their wild-type (WT) counterparts.

2.2 Materials and methods

2.2.1 Subjects

The knockdown studies employed adult, male C57BL/6J (B6) mice (~8 weeks of age; The Jackson Laboratory, Sacramento, CA). The remaining studies used both male and female adult (6-8 weeks of age) *Homer2* knock-out (KO) and wild-type (WT; on a mixed 129X1/svJ X C57BL/6J background) mice (see Shin et al., 2003) that were bred in-house from the mating of heterozygous breeder pairs in the Psychological and Brain Sciences vivarium at UCSB. Animals were housed in groups of 3-5 mice in standard ventilated polycarbonate cages, under standard, reverse-light, housing conditions in an AAALAC-accredited vivarium (lights on/off: 2200/1000h), with *ad libitum* access to food and water. All behavioral procedures were conducted during the dark phase of the circadian cycle. All procedures were

consistent with NIH guidelines and approved by the Institutional Animal Care and Use Committee of UCSB.

2.2.2 General experimental design

Homer2 within the NAC regulates both cocaine- (Szumlinski et al., 2004) and alcohol-induced (Szumlinski et al., 2005b; Szumlinski et al., 2008a,b; Cozzoli et al., 2009; Cozzoli et al., 2012) changes in behavior in murine models, but the subregional specificity of Homer2's role in MA-related behavior has received relatively little experimental attention (Szumlinski et al., 2017). Thus, two experiments were conducted to further address the role for NAC Homer2 expression in gating the rewarding and reinforcing properties of MA. The first experiment in this report sought to extend the results of a prior study of the NAC shell (Szumlinski et al., 2017) to the NAC core by determining whether or not Homer2 expression within the NAC core is necessary for MA reward/reinforcement. To accomplish this, the first experiment in this report employed a similar experimental design and approach as that described in our previous report (Szumlinski et al., 2017), which involved knocking down Homer2b expression in the NAC core of B6 mice using an adeno-associated viral vector (AAV) carrying a small hairpin RNA (shRNA) against *Homer2b*. Control animals were infused with an AAV carrying green fluorescent protein (GFP). The details of the AAV-shRNA construct and the control AAV are provided in Klugmann and Szumlinski (2008) and Cozzoli et al. (2009) and the details of the specific procedures employed in this shRNA study are provided in the subsections below. A time-line of the procedures is provided in **Fig.1A**.

Combined, the results of our prior shRNA study of the NAC shell (Szumlinski et al., 2017) and those of the present study of the NAC core (see Results below) argued that Homer2 expression within the NAC shell and core plays opposing roles in gating MA reward/reinforcement, with Homer2 in the shell promoting, and Homer2 in the core,

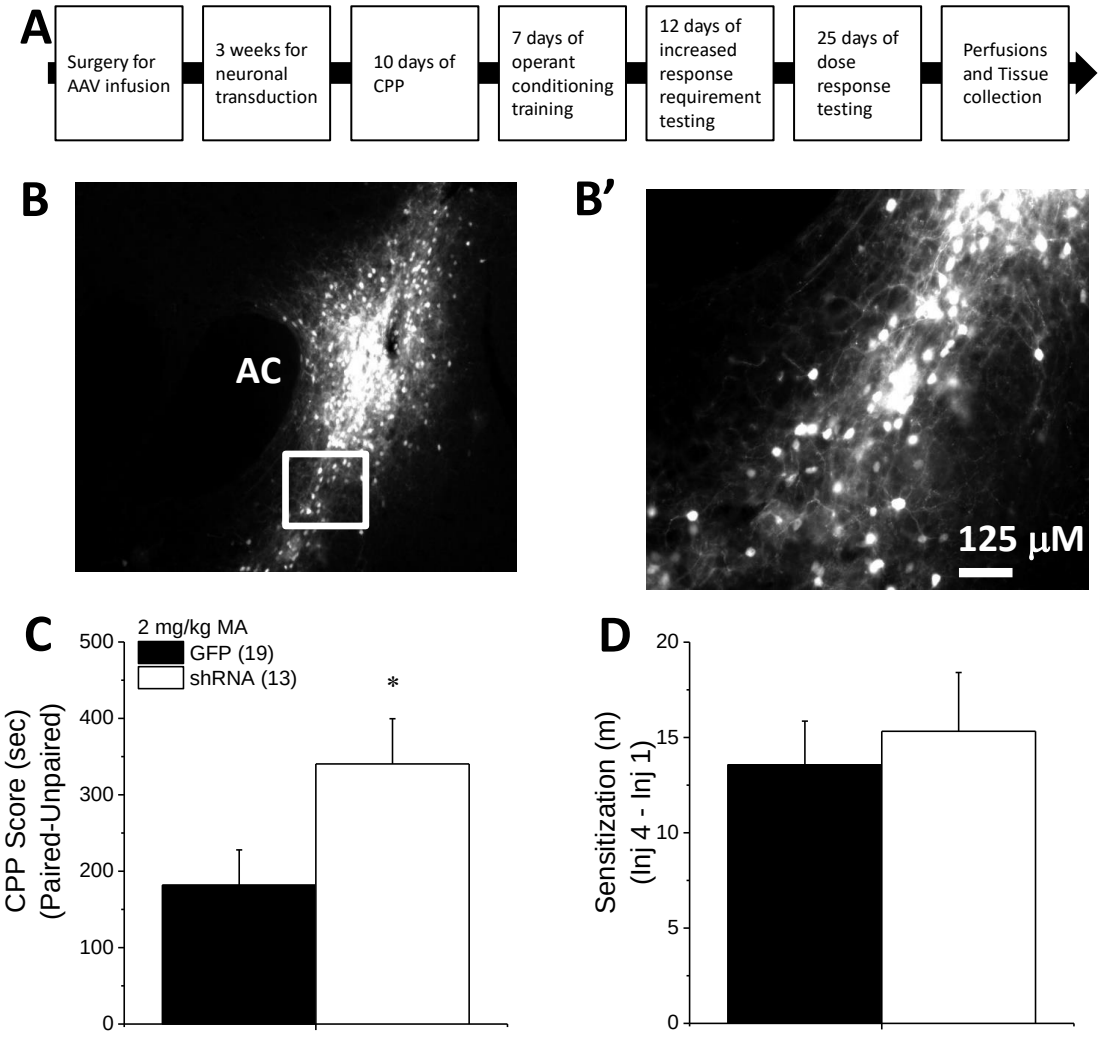


Figure 1. Homer2b knockdown in the NAC core potentiates a MA-induced CPP. (A) The procedural timeline for the study examining the effects of shRNA-mediated knock-down of Homer2b within the NAC core. Representative micrographs of the neuronal transduction within the NAC core by GFP-tagged AAV-shRNA against Homer2b at 10 X magnification **(B)** and 40 X magnification **(B')**. AC=anterior commissure. **(C)** shRNA infusion potentiated MA-induced place conditioning, without altering the magnitude of locomotor sensitization that developed during conditioning **(D)**. The data represent the means ± SEMs of the number of mice indicated in Panel B. *$p<0.05$ vs. GFP.

suppressing MA addiction-related behaviors. Thus, a follow-up experiment was conducted to determine whether or not mimicking a MA-induced increase in Homer2 expression within the NAC shell and core (Szumlinski et al., 2017) would be sufficient to respectively promote and suppress MA-induced place- and operant-conditioning. To address this question, we employed an AAV *Homer2b*-cDNA strategy similar to that used in previous studies from our laboratory (Szumlinski et al, 2004; Szumlinski et al., 2005b; Lominac et al., 2005). As in our earlier work (e.g., Szumlinski et al., 2004), we infused a *Homer2b* AAV-cDNA construct (see Szumlinski et al., 2004 and Klugmann and Szumlinski, 2008 for details of the cDNA construct) into the NAC shell or core of *Homer2* WT and constitutive KO mice, the latter of which enabled determination of an active role for Homer2 within each subregion in gating behavior. As the effects of constitutive *Homer2* deletion upon MA addiction-related behaviors had yet to be characterized, we first compared the MA place- and operant-conditioning phenotypes of *Homer2* KO and WT mice on a mixed B6-129 hybrid genetic background. Then we replicated the experiment in a second cohort of *Homer2* KO and WT mice infused with either the AAV-cDNA or -GFP control. A time-line of procedures is presented in **Fig.5A**.

2.2.3 Surgeries and AAV infusion

The surgical procedures to infuse the AAVs carrying either shRNA-Homer2b, cDNA-Homer2b, or cDNA-GFP were consistent with those previously described by our laboratory (Szumlinski et al., 2017; Goulding et al., 2011; Cozzoli et al., 2009). For B6 mice, we used the following stereotaxic coordinates from Bregma (in mm): for core, AP: +1.3; ML: ±1; DV: -4.3; for shell, AP: +1.3; ML: ±0.5; DV: -4.8. Based on our experience conducting craniotomies on B6-129 hybrid mice (e.g., Szumlinski et al. 2004; Goulding et al., 2011; Cozzoli et al., 2009), the following stereotaxic coordinates were used for *Homer2* KO and

WT mice: for core, AP: +1.4; ML: ±1; DV: -4.3; for shell, AP: +1.4; ML: ±0.5; DV: -4.6. Mice were anesthetized with 1.5% isoflurane and positioned on the stereotaxic apparatus. Thirty-gauge microinjectors (12 mm) were lowered bilaterally, directly into the core or shell. AAVs were infused at a rate of 0.10 μl/min for 5 min (total volume/side = 0.50 μL), and injectors were left in place for an additional 5 min prior to closing the incision site with tissue adhesive. The shRNA and cDNA infusions procedures have been demonstrated previously to reduce and increase, respectively, Homer2b protein expression in mouse brain by approximately 50% (Goulding et al., 2011; Klugmann and Szumlinski, 2008; Ary et al., 2013). Animals were left in their home cages for a minimum of 3 weeks prior to behavioral testing to allow for maximal neuronal transduction (Klugmann and Szumlinski, 2008).

2.2.4 Place-conditioning and locomotor activity

MA place-conditioning procedures also followed those previously employed by our laboratory (Szumlinski et al., 2017) and included 3 main phases: habituation/pre-conditioning test (day 1), MA/saline (SAL) conditioning (days 2-9) and a postconditioning test (day 10, post-test). The apparatus consisted of two distinct compartments –one with black and white marble-patterned walls and a textured floor, and the other with wood-patterned walls and a smooth Plexiglas floor. During the habituation and post-test sessions, mice were allowed free access to both compartments for 15 minutes via a divider with a door. During conditioning, mice received 2 mg/kg MA intraperitoneal (IP) injections and were immediately confined to one of the compartments. On alternating days, mice were injected with an equivalent volume of SAL (10 mL/kg) and confined to the other compartment. Each conditioning session was 15 min in duration and mice received 4 conditioning sessions for each unconditioned stimulus. Overall, mice did not exhibit a strong preference for one compartment vs. the other during the habituation session, so the time spent on the SAL-paired side during the post-test

was subtracted from the time spent on the MA-paired side to calculate a CPP score (Szumlinski et al., 2017; Fultz and Szumlinski, 2018). This CPP score served to index the direction and magnitude of the MA-conditioned reward. During each 15-min session, the locomotor activity of the animals was recorded by digital video cameras, interfaced with a PC-type computer equipped with ANY-Maze software (Stoelting), recorded the distance traveled (in m) during each of the sessions. As in our prior studies (e.g., Szumlinski et al., 2017), MA-induced locomotor sensitization was measured by subtracting the distance travelled during the first 15-min MA-conditioning session from that on the fourth/last MA-conditioning session.

2.2.5 Operant-conditioning

In our prior study of the effects of Homer2 knock-down in the NAC shell (Szumlinski et al., 2017), the generalization of a place-conditioning phenotype to operant-conditioning for MA reinforcement was determined using a within-subjects design. To the best of our knowledge, a parametric analysis of the effects of prior behaviorally non-contingent MA upon subsequent drug-taking has not been performed. Thus, we cannot speak to any potential effects our place-conditioning procedures might have upon the MA self-administration of the mice. However, we do know from our prior study of B6 mice, that a mere history of non-contingent MA treatment (4 injections of 2 mg/kg MA, as employed in the present study) does not necessarily promote subsequent MA reinforcement/intake as MA-injected mice self-segregate into high versus low MA-taking phenotypes when allowed to orally self-administer the drug (Szumlinski et al., 2017). To be consistent with our prior study (Szumlinski et al., 2017), following place-conditioning procedures, mice were trained in daily 1-h sessions to nose-poke for delivery of unadulterated MA solutions (prepared in tap water; reinforcer volume = 20 μl). Standard mouse operant-conditioning chambers (MedAssociates, St Albans,

VT, USA) were used to measure instrumental responding for MA. Operant chambers were fitted with 2 nose-poke holes, with a liquid receptacle located in between and chambers were housed in ventilated, sound-attenuated chambers. Responses in the active (MA-associated) hole resulted in the activation of the infusion pump, delivery of 20 μl MA into the receptacle, and the presentation of a 20-sec light/tone compound stimulus. During the 20-sec MA-delivery period, further responding in the active hole was recorded but had no programmed consequences. Throughout the session, responding in the inactive hole had no programmed consequences but was recorded to assess the selectivity of responding in order to determine reinforcer efficacy. Mice were first trained for 7 days to nose-poke for delivery of a 10 mg/L MA solution under an FR1 schedule of reinforcement. Animals that did not reach the acquisition criteria of at least 10 active nose-pokes during the 1-h session, with greater than 65% of their total nose-pokes directed at the active hole were excluded from the study. Using these criteria, 9/48 mice were excluded from the shRNA study and 20/114 mice were excluded from the cDNA studies. As in our prior study of the NAC shell (Szumlinski et al., 2017), we next progressively increased the number of nose-pokes required for delivery of the 10 mg/L MA reinforcer (maintaining the 20-sec time-out) over subsequent days (4-5 days/schedule). We then conducted a dose-response study of MA reinforcement and intake (5-80 mg/L) under the initial FR1 (20-sec time-out) reinforcement schedule (5 days/dose) as data indicated an inverse relationship between MA intake and reinforcement schedule (see Results). Given the inverse relationship between operant-responding and reinforcement schedule, we opted to forego this phase of testing in the cDNA study and animals proceeded from training directly into dose-response testing. In the operant-conditioning study of *Homer2* WT and KO mice, technical issues interfered with the testing of 13 of the 21 WT

mice at the 80 mg/L concentration. As such, the data from this concentration were analyzed separately from the rest of the dose-response function.

At the end of each 1-h operant session, the volume of solution remaining in the receptacle was determined by pipetting. Mice were returned to the colony room and left undisturbed until the next day. Total MA intake was calculated each day by subtracting the volume of MA remaining in the receptacle from the total volume delivered to determine the total volume of MA consumed. The volume consumed was converted into mg consumed based on the concentration of the solution an then amount of MA intake was expressed as a function of body weight (in mg/kg), which was measured weekly (Szumlinski et al., 2017).

2.2.6 Extinction and reinstatement of the operant response

In the cDNA study, the strength of the conditioned operant response was established by repeatedly testing mice in daily operant sessions in a MA-free state, with no light/tone stimulus, until the number of active nose-pokes in a 1-hr session dropped to 25% of initial MA-free responding (i.e., extinction). Animals that did not reach this extinction criteria within 30 days were excluded from the remainder of the study. Two additional mice from the cDNA studies were excluded for failing to reach extinction criteria. This extinction procedure was conducted immediately upon the completion of dose-response testing (see above). Following extinction, AAV-infused mice were then subjected to a series of reinstatement of MA-seeking tests in which responding in the active hole resulted in the presentation of only the light/tone stimulus previously predictive of MA delivery (i.e., MA reinforcement was withheld during reinstatement testing). For reinstatement testing, mice were administered a once-daily IP injection of 0.0 (SAL), 0.5 or 0.25 mg/kg MA, with doses increased across days, to examine the degree of cue- and MA-induced reinstatement of the conditioned response. Immediately following injection, mice were placed into the operant-

conditioning chamber for a period of 1 h, at which time they were removed and returned to their home cages and the number of active versus inactive nose-pokes were recorded.

2.2.7 Histology

The goal of this study was to determine the subregional specificity of the effects of AAV-mediated Homer2 manipulations within the NAC for MA addiction-related behavior. As such, we deemed it important to determine the neuroanatomical specificity of AAV infusion and thus, employed immunohistochemical, *in lieu* of immunoblotting, procedures to localize neuronal transduction within the NAC shell versus core. For this, animals were euthanized with an overdose of Euthasol (Virbac AH, Fort Worth, TX, USA) and transcardially perfused with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde. Brains were then removed and coldstored in PBS until slicing. Tissue was sectioned (40 μm) along the coronal plane on a vibratome at the level of the nucleus accumbens. As in our recent work (Szumlinski et al., 2017), localization of the transfection of neurons by our shRNA-Homer2b, as well as by our GFP control viruses, was examined using an anti-GFP antibody (Invitrogen, Carlsbad, CA, USA; 1:200 dilution) and fluorescence microscopy. As in our prior work (Szumlinski et al., 2017; Szumlinski et al., 2005b; Szumlinski et al., 2004), tissue from cDNA-Homer2b infused mice was stained with a mouse anti-hemagglutinin (HA) primary antibody (Biolegend, San Diego, CA, USA; 1:1000 dilution) to visualize the viral construct, followed by a biotinylated anti-mouse secondary IgG (Vector Laboratories, Burlingame, CA, USA; 1:2,000 dilution), and visualized with 3,3'-diaminobenzidine (DAB). Post-staining, all tissue was mounted on slides and cover-slipped. Slides were viewed using a Nikon Eclipse E800 microscope equipped with a Hamamatsu CCD camera (model C4742-95) and MetaMorph imaging software (Molecular Devices, Sunnyvale, CA, USA). Only mice

exhibiting localized neuronal transduction within the NAC shell and core subregions were included in the statistical analyses of the results.

2.2.8 Statistical approaches

The effects of Homer2b knockdown in the NAC core upon place-conditioning related measures were analyzed using t-tests. The operant-conditioning data were analyzed using multivariate ANOVAs, with the between subjects factors of Sex and AAV (GFP vs. shRNA or GFP vs. cDNA) and/or Genotype (WT vs. *Homer2* KO) and the within-subjects factors of Day, FR schedule, and Dose, when appropriate. As initial analyses of the data for both place- and operant-conditioning in *Homer2* WT and KO mice indicated no main Sex effects or interactions, the data were collapsed across sex prior to re-analyses. As described above, the data for *Homer2* WT/KO mice tested for the self-administration of 80 mg/L MA were analyzed separately using t-tests. Two-tailed Pearson correlational analyses were also conducted to relate dependent measures with CPP score. α=0.05 for these analyses. The effects of *Homer2* KO on the dose-response function for MA-induced place-conditioning were analyzed using ANOVAs, with the between-subjects factors of Genotype (WT vs. KO) and Dose (0.5-4.0 mg/kg MA, 4 levels). All data was analyzed using SPSS ver 12 (IBM) and for all ANOVAs, the homogeneity of variance was confirmed. Alpha was set at 0.05 for all analyses.

2.3 Results

2.3.1 Homer2 knockdown in the NAC core augments a MA CPP in B6 mice

To extend recent results for the NAC shell (Szumlinski et al., 2017) to the core subregion, B6 mice were infused with an AAV carrying shRNA to knockdown *Homer2b* in the NAC core and then tested for MA-induced CPP. Expression of the AAV was confirmed as confined to the NAC core using fluorescence microscopy (**Fig.1B,B'**). shRNA-infused mice

exhibited higher CPP following 4 pairings of 2 mg/kg MA than GFP-infused controls (**Fig.1C**) [t(30)=2.14, p=0.04]. The shRNA-Homer2b NAC core infusion did not affect the acute locomotor response to MA [data not shown; t(30)=0.39, p=0.70], nor did it alter the magnitude of MA-induced locomotor sensitization that developed over the course of the conditioning (**Fig.1D**) [t(30)=0.47, p=0.64]. These data indicate that Homer2 within the NAC core normally suppresses the positive affective and motivational valence of MA, independent of effects upon without interfering with the locomotor-activating effects of the drug.

2.3.2 Homer2 knockdown in the NAC core augments oral MA reinforcement and intake in B6 mice

During the first 5 days of self-administration training under an FR1 reinforcement schedule, both GFP and shRNA animals exhibited a similar pattern of active nose-pokes (**Fig.2A**) [Day effect: F(1,29)=28.33, p<0.0001; AAV effect, interaction: p's>0.10], ratio of active vs. inactive responding (**Fig.2B**) [Day effect: F(1,29)=2.56, p=0.04; AAV effect and interaction, p's>0.20], and MA intake (**Fig.2C**) [Day effect: F(1, 29)=12.20, p=0.002; AAV effect, interaction: p's>0.10]. When tested under increasing response requirements, responding on the active lever increased (**Fig.2D**) [FR effect: F(1,29)=76.00, p<0.0001], the ratio of active vs. inactive responding increased (**Fig.2E**) [FR effect: F(1,29)=25.32, p<0.0001], and MA intake decreased (**Fig.2F**) [FR effect: F(1,29)=20.17, p<0.0001], but there was no effect of Homer2b knockdown on any of these measures (**Fig.2D-F**; AAV effects and interactions, all p's>0.20). Thus, Homer2 within the NAC core is not necessary for the acquisition of oral MA self-administration or MA demand, at least when behavior is reinforced by a low, 10 mg/L MA concentration.

In contrast, Homer2b knockdown in the NAC core shifted upwards the dose-response function for active nose-poking behavior (**Fig. 2G**) [AAV effect: F(1,29)=5.31, p=0.03; Dose

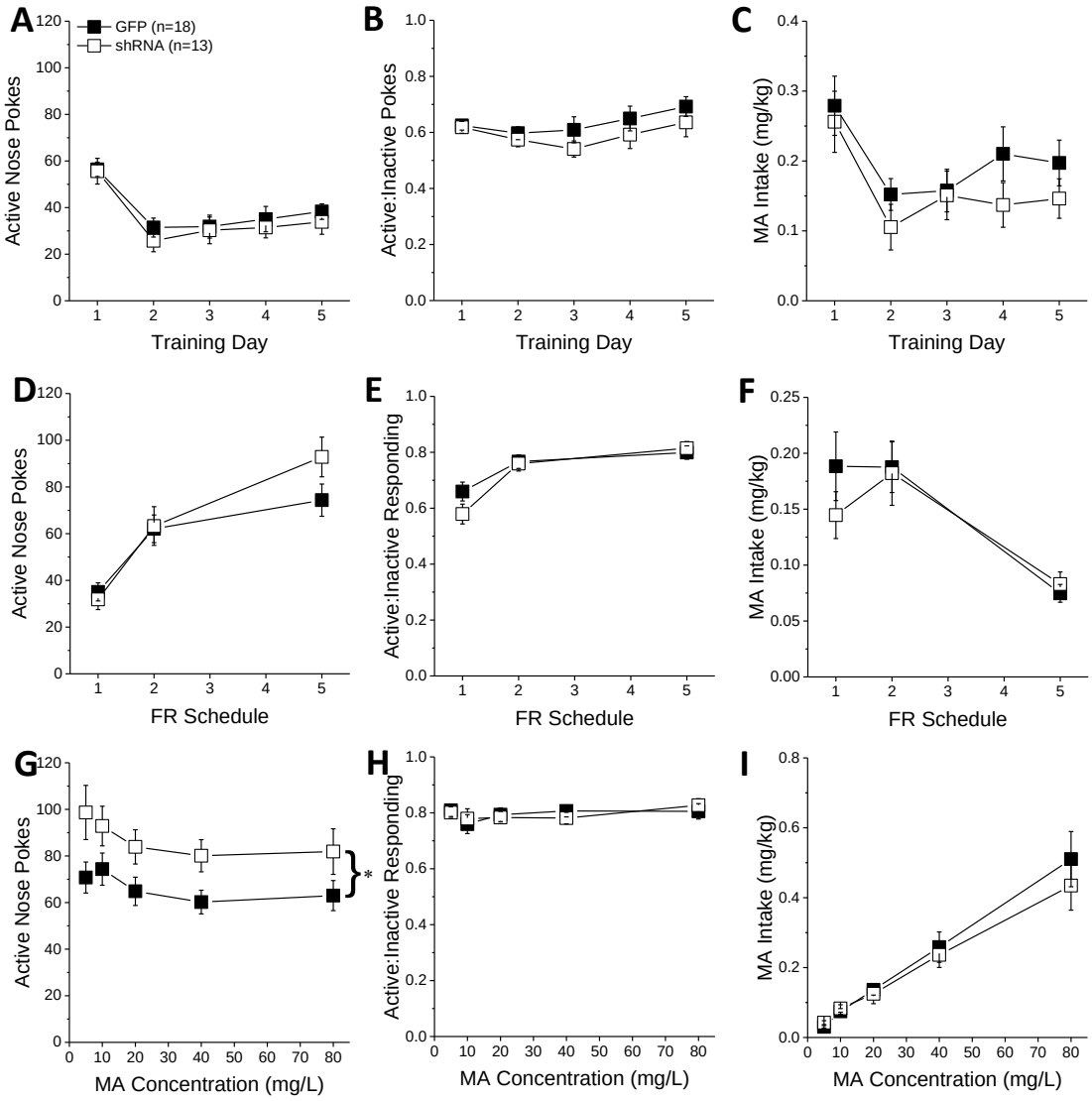


Figure 2. Homer2b knockdown in the NAC core increases MA's reinforcing efficacy without altering MA intake. shRNA against Homer2 did not influence: **(A)** the number of active nose pokes, **(B)** the relative responding on the active versus inactive hole or **(C)** MA intake during the first 5 days of self-administration training (10 mg/L MA as reinforcer). **(D-F)** shRNA infusion also did not alter these measures when mice were tested under increasing response requirements on an FR schedule of reinforcement. **(G)** shRNA infusion shifted the dose-response function for active hole poking upwards of GFP controls but did not affect the dose-response functions for **(H)** response allocation or **(I)** MA intake. The data represent the means ± SEMs of the number of mice indicated in Panel A. *$p<0.05$ vs. GFP (main AAV effect).

effect: F(1,29)=4.65, p=0.002; interaction, p=0.799], without impacting the ratio of active vs. inactive responding (**Fig.2H**; AAV X Dose ANOVA, p's>0.35), or the dose-response function for MA intake (**Fig.2I**) [Dose effect: F(1,29)=77.35, p<0.0001; AAV effect, interaction, p's>0.30). These data indicate that Homer2 within the NAC core normally curbs the reinforcing efficacy of MA in mice with a history of self-administration, but this effect does not translate into a change in MA intake.

2.3.3 Constitutive Homer2 knockout increases MA-induced CPP

The results of our shRNA study above indicate that Homer2 within the NAC core normally suppresses behavioral indices of MA reward and reinforcement, which is a finding opposite to that reported for Homer2 in the NAC shell (Szumlinski et al., 2017). Thus, we employed a complementary AAV-cDNA strategy (Cozzoli et al., 2009; Szumlinski et al., 2005b; Szumlinski et al., 2004) in WT littermates and *Homer2* KO mice to determine whether Homer2 in the NAC shell promotes, while that in the core suppresses, MA place- and operant-conditioning. We know that *Homer2* KO mice exhibit greater sensitivity to the psychomotor-activating effects of MA (Szumlinski et al. 2005a); however, their MA reward/reinforcement phenotype has yet to be characterized. Therefore, we first assayed the effects of a constitutive *Homer2* KO on MA place- and operant-conditioning. A genotypic comparison of the dose-response function for the time spent in the MA-paired vs. -unpaired side during the post-test phase of place-conditioning indicated greater MA-induced CPP, irrespective of MA dose (**Fig.3A**) [Genotype effect: F(1, 86)=14.83, p<0.0001; Genotype X Dose: F(3, 86)=2.54, p=0.06], although the genotypic difference in MA-conditioned behavior was most obvious at lower MA concentrations. Despite exhibiting potentiated MA-conditioned reward, *Homer2* KO mice did not differ significantly from WT littermate controls regarding the acute locomotor stimulatory effects of MA during the first

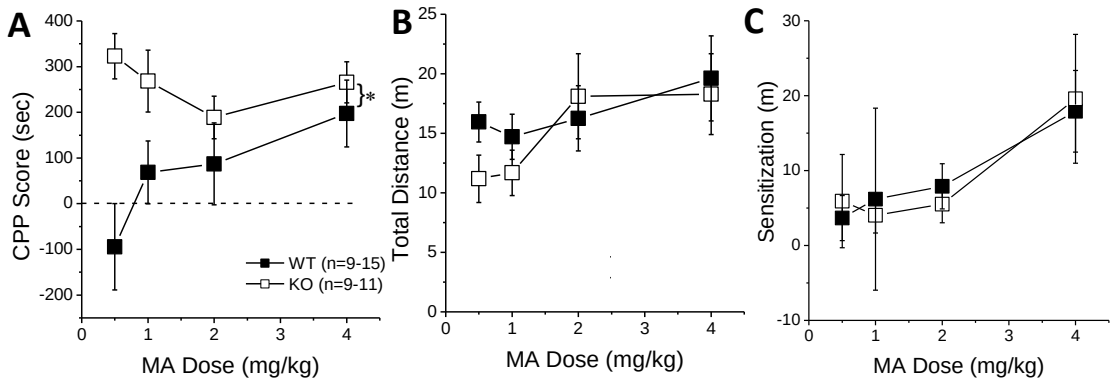


Figure 3. Constitutive *Homer2* deletion augments a MA-induced CPP. When compared to WT mice, *Homer2* KO animals exhibited **(A)** a shift upwards in the dose-response function for a MA-induced CPP. In contrast, gene deletion did not alter the dose-response functions for **(B)** acute MA-induced locomotor activity or **(C)** the increase in locomotor activity from the first to the last MA-conditioning session (sensitization). The data represent the means ± SEMs of the number of mice indicated in Panel A. }*$p<0.05$ vs. WT (main Genotype effect).

conditioning session (**Fig.3B**) (Genotype X Dose ANOVA, all p's>0.14) or in the capacity of the 4 MA injections to elicit a dose-dependent sensitization of locomotion during the conditioning phase of the study, as determined by the difference in the distance traveled from the first to the fourth injection (**Fig.3C**) [Dose effect: $F(1,79)=8.00$, $p<0.0001$; Injection: $F(3,237)=8.94$, $p<0.0001$; Dose X Injection: $F(9,237)=2.47$, $p=0.01$; Genotype X Dose: $F(3,79)=2.23$, $p=0.09$; all other p's>0.30]. While this result contradicts our earlier report, these conditioning sessions were only 15 min long, while in Szumlinski et al. (2005a), the sessions were 1 h so the difference in duration of testing likely mitigated genotypic differences.

2.3.4 Constitutive Homer2 knockout increases MA-reinforcement and intake

The number of active hole pokes emitted by KO mice progressively increased across training days, whereas the responding of WT mice fluctuated during early training (**Fig. 4A**) [Genotype X Day: $F(4,140)=5.71$, $p<0.0001$]. *Post-hoc* analyses indicated greater active hole responding in KO versus WT mice on day 2, 3, and 5 of training (**Fig. 4A**; t-tests, p's<0.03). No genotypic differences were observed for the number of inactive hole pokes (data not shown; Genotype X Day, all p's>0.25). However, KO mice tended to exhibit lower active vs. inactive responding than WT mice during early training (**Fig.4B**) [Genotype X Day, $F(4,140)=3.59$, $p=0.008$], with *post-tests* indicating significantly lower relative responding on Days 3 and 5 of training (t-tests, p's<0.04). Despite the lower active nose-poke responding in KO, only the KO mice escalated their MA intake during early training (**Fig. 4C**) [Genotype X Day: $F(4,140)=5.98$, $p<0.0001$]. While *Homer2* KOs exhibited significantly lower MA intake than WT animals on the first training day [$t(35)=2.05$, $p=0.05$], their MA intake was significantly higher than WT animals by the 5th training day [$t(35)=3.18$, $p=0.003$]. Thus, constitutive *Homer2* deletion increases low-concentration MA reinforcement and intake

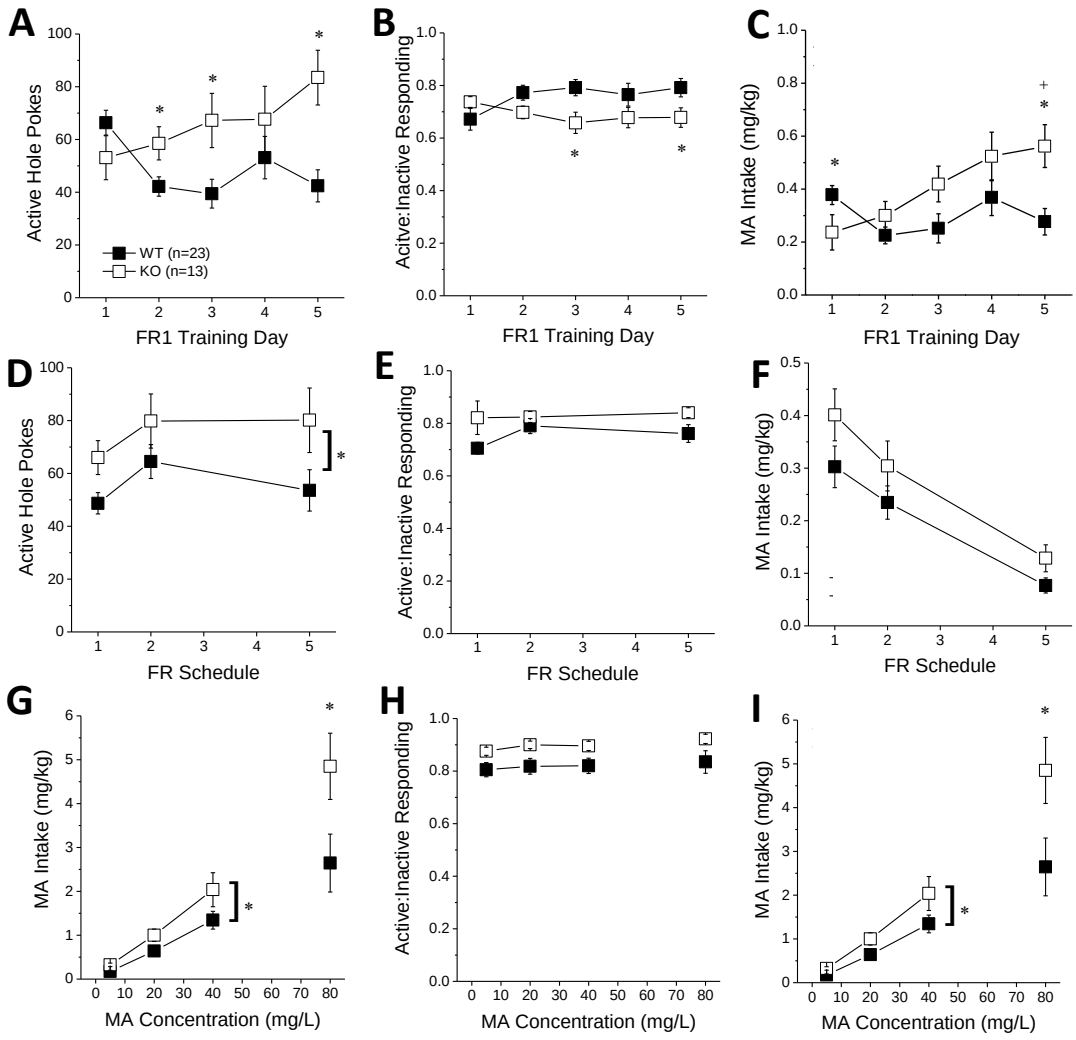


Figure 4. Constitutive Homer2 deletion increases MA reinforcement and intake. When compared to WT mice, *Homer2* KO mice exhibited a greater: **(A)** the number of active nose pokes, **(B)** relative responding on the active versus inactive hole and **(C)** MA intake during the first 5 days of self-administration training (10 mg/L MA as reinforcer). **(D)** *Homer2* KO mice also exhibited more active hole responding under increasing response requirement but did not differ from WT mice regarding **(E)** response allocation or **(F)** MA intake during this phase of testing. Relative to WT mice, the dose-response function for active hole-responding was shifted upwards **(G)**, without a change in that for response allocation in the active hole **(H)**. **(I)** KO mice also consumed more MA than WT mice across the range of doses tested. The data represent the means ± SEMs of the number of mice indicated in Panel A. *p<0.05 vs. WT (main Genotype effect).

during early training in a manner similar to shRNA-mediated knockdown of Homer2b expression within the NAC core.

When the response requirement for reinforcement by 10 mg/L MA progressively increased across days, KO mice exhibited more active hole responding, overall, than WT mice (**Fig. 4D**) [Genotype effect: $F(1,35)=5.27$, $p=0.03$; interaction: $p>0.6$]. The number of inactive hole pokes declined with increasing response requirement (data not shown) [Schedule effect: $F(2,70)=15.85$, $p<0.0001$] but was not influenced by genotype (all p's>0.60). In contrast to the early acquisition phase (**Fig.4B**), KO mice exhibited slightly higher active hole response allocation than WT mice during this phase of testing (**Fig.4D**) [Genotype effect: $F(2,70)=3.63$, $p=0.07$; Day effect and interaction, p's>0.15]. The MA intake of KO mice was also slightly higher than WT controls as response requirement increased (**Fig.4E**) [Genotype effect: $F(1,35)=3.26$, $p=0.08$; FR effect: $F(2,70)=43.48$, $p<0.0001$; interaction: $p=0.68$]. These data provide some limited evidence that constitutive *Homer2* deletion increases demand for a low-concentration MA solution.

The dose-response function (5-40 mg/L MA) for active hole pokes under the original FR1 schedule of reinforcement was shifted upward in KO mice, compared to WT mice [Genotype effect: $F(1,35)=5.57$, $p=0.02$; interaction, $p>0.10$], an effect especially apparent at lower MA doses (**Fig.4F**). KO mice also tended to exhibit higher active hole responding for the 80 mg/L solution (t-test, $p=0.09$). Inactive hole pokes declined as a function of MA concentration, but no genotypic differences were detected (data not shown) [5-40 mg/L MA: Dose effect: $F(2,70)=4.19$, $p=0.02$; Genotype effect and interactions, p's>0.40; 80 mg/L: t-test, $p=0.25$]. KO mice continued to show modestly higher relative responding on the active versus inactive lever during dose-response testing, but genotypic differences were not statistically significant (**Fig.4H**; 5-40 mg/L: Genotype X FR ANOVA, p'>0.10; at 80 mg/L,

WT vs. KO: t-test, p=0.06). Finally, in contrast to Homer2 knockdown (**Fig.2I**), the MA dose-intake function was shifted upwards in KO versus WT mice (**Fig.4I**) [5-40 mg/L: Genotype effect: $F(1,35)=4.70$, $p=0.04$; Dose effect: $F(2,70)=50.73$, $p<0.0001$; Genotype X Dose: $p=0.17$; 80 mg/L: $t(22)=2.16$, $p=0.04$]. These latter data indicate that the potentiation of MA reinforcement and intake by constitutive *Homer2* deletion extends across a relatively broad dose-range.

2.3.5 Homer2b over-expression in the NAC core, but not shell, augments a MA-induced CPP

The final series of experiments examined the effects of Homer2b over-expression within the NAC core and shell of *Homer2* WT and KO mice upon MA-induced place- and operant-conditioning. Immunohistochemical staining for the HA-tag indicated neuronal transduction within the NAC core (**Fig.5B**) that was comparable to that observed in prior reports from our group (e.g., Szumlinski et al., 2004, 2008b). Intriguingly, similar to NAC core knockdown of Homer2b (**Fig.1C**) and constitutive *Homer2* deletion (**Fig.3A**), Homer2b over-expression within the NAC core also potentiated the magnitude of a CPP induced by the repeated pairing of 2 mg/kg MA (**Fig.5D**) [AAV effect: $F(1,33)=7.18$, $p=0.01$]. While the initial dose-response study failed to support genotypic differences in the magnitude of the conditioned response elicited by pairing with 2 mg/kg MA (**Fig.3A**), the CPP elicited by this dose in the cDNA study was lower overall in KO versus WT mice (**Fig.5D**) [Genotype effect: $F(1,33)=6.20$, $p=0.02$; interaction: $p=0.74$]. Homer2b over-expression within the NAC core did not influence the acute locomotor-response to 2 mg/kg MA (**Fig.5E**; Genotype X AAV ANOVA, p's>045) nor did it influence the sensitization of this response during conditioning (**Fig.5F**; Genotype X AAV ANOVA, p's>0.20). Thus, curiously, over-expressing Homer2b within the NAC core produces an effect on MA-conditioned reward akin to that observed

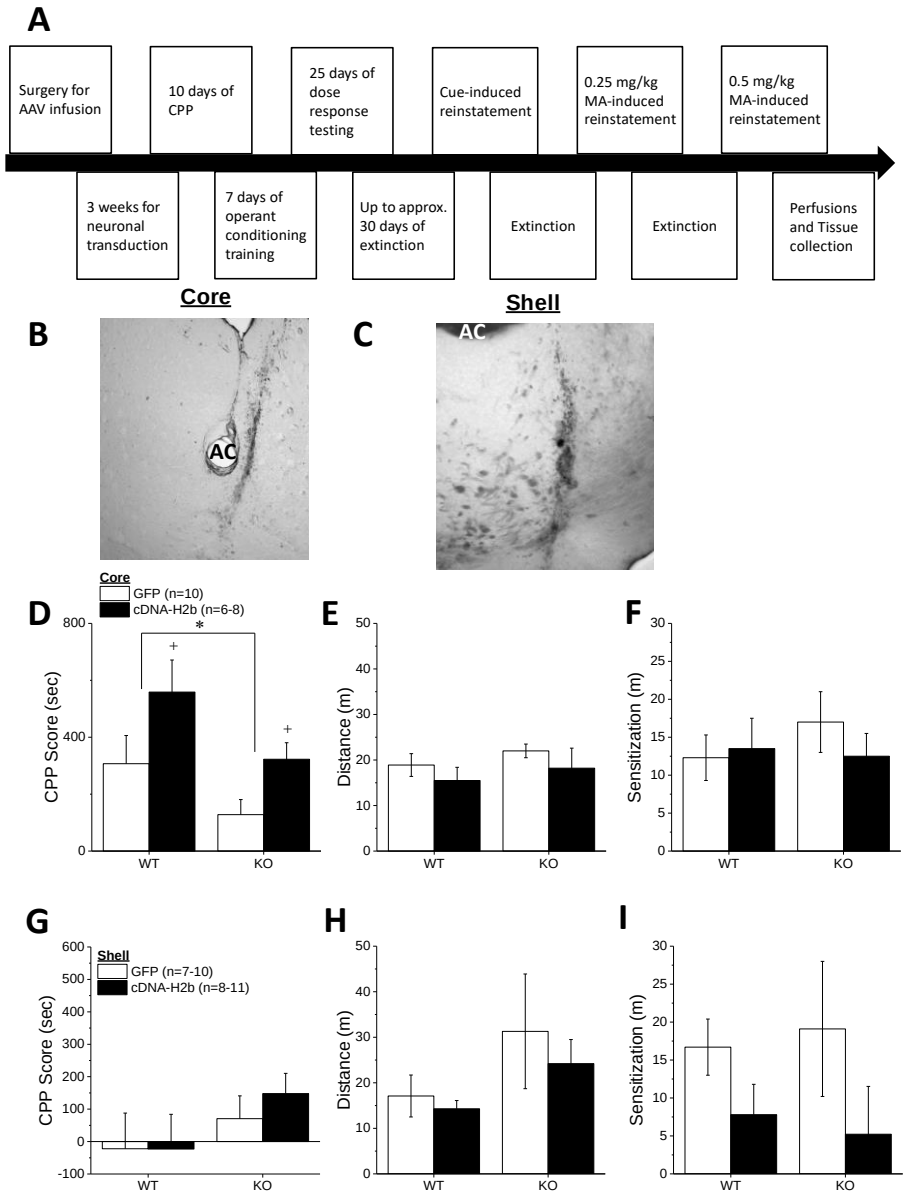


Figure 5. Homer2b over-expression in the NAC core, but not NAC shell, potentiates a MA-induced CPP. (A) The procedural timeline for the study examining the effects of cDNA-mediated over-expression of Homer2b within the NAC core and shell. Representative micrographs of the neuronal transduction within the NAC core **(B)** and NAC shell **(C)** by HA-tagged AAV-cDNA encoding Homer2b (images at 20 X magnification). AC=anterior commissure. **(D)** The magnitude of a MA-induced CPP was lower in *Homer2* KO mice versus WT controls and cDNA infusion into the NAC core potentiated MA-induced place conditioning in both genotypes, without affecting the **(E)** acute or **(F)** sensitized locomotor response to MA. No genotypic difference or cDNA effect were apparent for **(G)** MA-induced CPP, **(H)** the acute locomotor response to MA or **(I)** the magnitude of MA-induced locomotor sensitization, when the cDNA was infused into the NAC shell. The data represent the means ± SEMs of the number of mice indicated in Panel C for NAC core and Panel F for NAC shell. *p<0.05 vs. WT; +p<0.05 vs. GFP.

upon either constitutive gene deletion (**Fig.3A**) or protein knockdown within this region (**Fig.1C**).

Immunohistochemical staining indicated robust neuronal transfection within the NAC shell, with no overt signs of infection or tissue damage (**Fig.5C**). Thus, we were surprised that the level of MA-induced place-conditioning was lower overall in the mice infused with GFP/cDNA into the NAC shell (**Fig.5G**), than that observed for the other place-conditioning experiments in this report. This low level of conditioning may have precluded our ability to detect group differences in the MA-conditioned response (Genotype X AAV, all p's>0.15). Despite lower CPP Scores, the locomotor response to an acute injection of 2 mg/kg MA was comparable to that observed in the other studies herein and was not affected by either *Homer2* deletion or intra-shell cDNA infusion (**Fig.5H**; Genotype X AAV ANOVA, all p's>0.07). Although intra-NAC shell cDNA infusion appeared to augment the difference in MA-induced locomotor activity observed from the first to the fourth conditioning session (sensitization), this effect was not statistically significant (**Fig.5I**; Genotype X AAV ANOVA, all p's>0.06). These data do not support an active role for Homer2b within the NAC shell in gating MA-induced locomotion or -conditioned reward under place-conditioning procedures.

2.3.6 Homer2b over-expression within NAC subregions does not influence the acquisition of oral MA self-administration

Based on the above results, we predicted that the effects of Homer2b over-expression upon place-conditioning would translate to operant-conditioning procedures. However, we found that Homer2b-cDNA infusion into either the NAC core (**Fig.6A-C**) or shell (**Fig.6D-F**) had no significant effect on any measure during the first 5 days of training under operant-conditioning procedures. A significant Genotype X Day interaction was detected for active

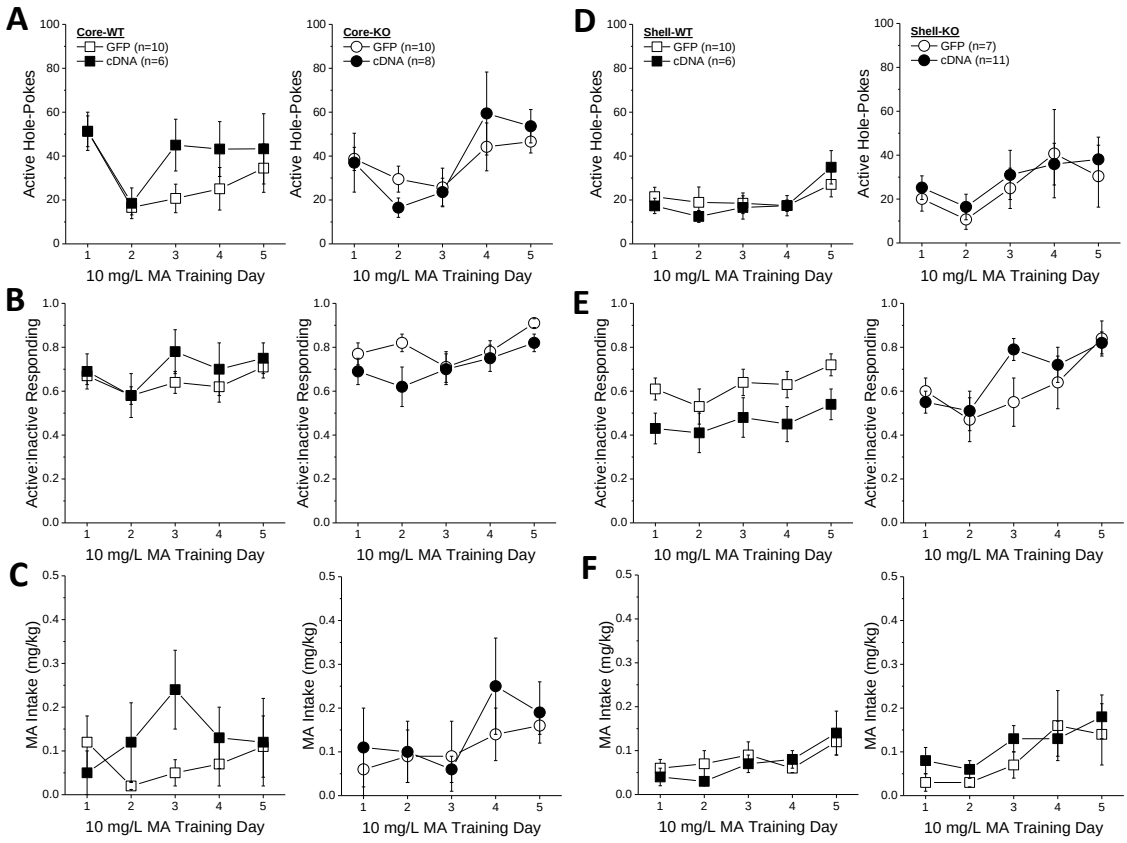


Figure 6. Homer2b over-expression does not alter MA reinforcement and intake during early training for MA self-administration. When compared to WT **(left)** and *Homer2* KO **(right)** mice infused with GFP, cDNA infusion into the NAC core did not alter: **(A)** active hole responding, **(B)** response allocation or **(C)** MA intake during the first 5 days of self-administration training. **(D-F)** Similarly, cDNA infusion into the NAC shell did not affect any measure of self-administration during early training. The data represent the means ± SEMs of the number of mice indicated in Panel A (NAC core) and Panel D (NAC shell).

hole responding in mice infused intra-NAC core (**Fig.6A**) [F(4,120)=2.83, p=0.03] that reflected a differential time-course of acquisition between WT and KO mice as *post-hoc* comparisons between WT and KO mice failed to indicate genotypic differences in responding on any training day (t-tests, p's>0.15). In neither genotype did NAC core Homer2b over-expression alter active hole responding during the first 5 days of self-administration training (AAV effect and interactions, p's>0.20). In mice infused intra-NAC core, response allocation increased progressively during early training (**Fig.6B**) [Day effect: F(4,120)=4.2, p=0.003] and KO mice exhibited overall greater MA-appropriate responding than WT mice during this phase of study [Genotype effect: F(1,30)=4.34, p=0.05]. However, this measure was not altered by Homer2b over-expression within the NAC core (no AAV effect or interactions, p's>0.10). Finally, KO mice tended to consume more MA during the first 5 days of training (**Fig.6C**; Genotype X Day: p=0.09), but there was no effect of intra-NAC core infusion of Homer2b cDNA upon MA intake during early training (AAV effect and interactions, p's>0.25). Taken together, these data do not support an effect of Homer2b over-expression within the NAC core in regulating initial MA reinforcement or intake.

Initial active hole responding was lower in mice infused with Homer2b-cDNA into the NAC shell than that typically observed under our oral MA operant-conditioning procedures (**Fig.6D**) and the Genotype X Day interaction failed to reach statistical significance [Day effect: F(4,128)=4.79, p=0.001; Genotype X Day, p=0.095]. However, as observed for the NAC core, intra-NAC shell cDNA infusion did not alter active hole responding (AAV effect and interactions, p's>0.22). In mice infused intra-NAC shell, response allocation progressively increased across day, irrespective of the genotype or AAV treatment (**Fig.6E**) [Day effect: F(4,128)=8.80, p<0.0001; no interactions with the Day factor, p's>0.25]. However, in contrast to the data for the NAC core (**Fig.6B**), a significant Genotype X AAV

interaction was detected for the ratio of active to inactive hole pokes exhibited by mice infused with AAV into the NAC shell (**Fig.6E**) [Genotype X AAV: $F(1,32)=5.0$, $p=0.03$]. Averaging across the 5 training days, this interaction reflected a cDNA-induced reduction in the response ratio in WT mice [$t(16)=3.29$, $p=0.005$], but no effect in KO animals (t-test, $p=0.83$). Finally, MA intake fluctuated during early training in the mice infused intra-NAC shell (**Fig.6F**) [Day effect: $F(4,128)=8.08$, $p<0.0001$]. However, we detected no effect of gene deletion or intra-NAC shell cDNA infusion during the early training period (Genotype X AAV X Dose ANOVA, other p's>0.25).

2.3.7 Homer2b over-expression within the NAC shell reduces the efficacy of oral MA to serve as a positive reinforcer

Homer2b-cDNA infusion into the NAC core (**Fig.7A-C**) did not influence any self-administration measure as a function of the concentration of the MA reinforcer. The dose-response function for active hole-poking was relatively flat in mice infused intra-NAC core with our AAVs and there was no effect of *Homer2* deletion or AAV infusion upon this measure (**Fig.7A**; Genotype X Dose X AAV ANOVA, all p's>0.12). Although KO mice tended to exhibit a higher ratio of active versus inactive responding during dose-response testing (Genotype effect, $p=0.07$), no significant group differences were detected for this measure at any MA dose tested (**Fig.7B**; Genotype X Dose X AAV ANOVA, other p's>0.15). In this experiment, *Homer2* deletion shifted the dose-response for MA intake (**Fig.7C**) [Genotype X Dose: $F(4,120)=3.04$, $p=0.02$], but *post-hoc* tests failed to confirm genotypic differences at any MA dose (t-tests, p's>0.07) and no AAV effects or interactions were detected (p's>0.40). Taken together, these cDNA data argue against an active role for Homer2b within the NAC core in regulating MA intake or sensitivity to its reinforcing effects.

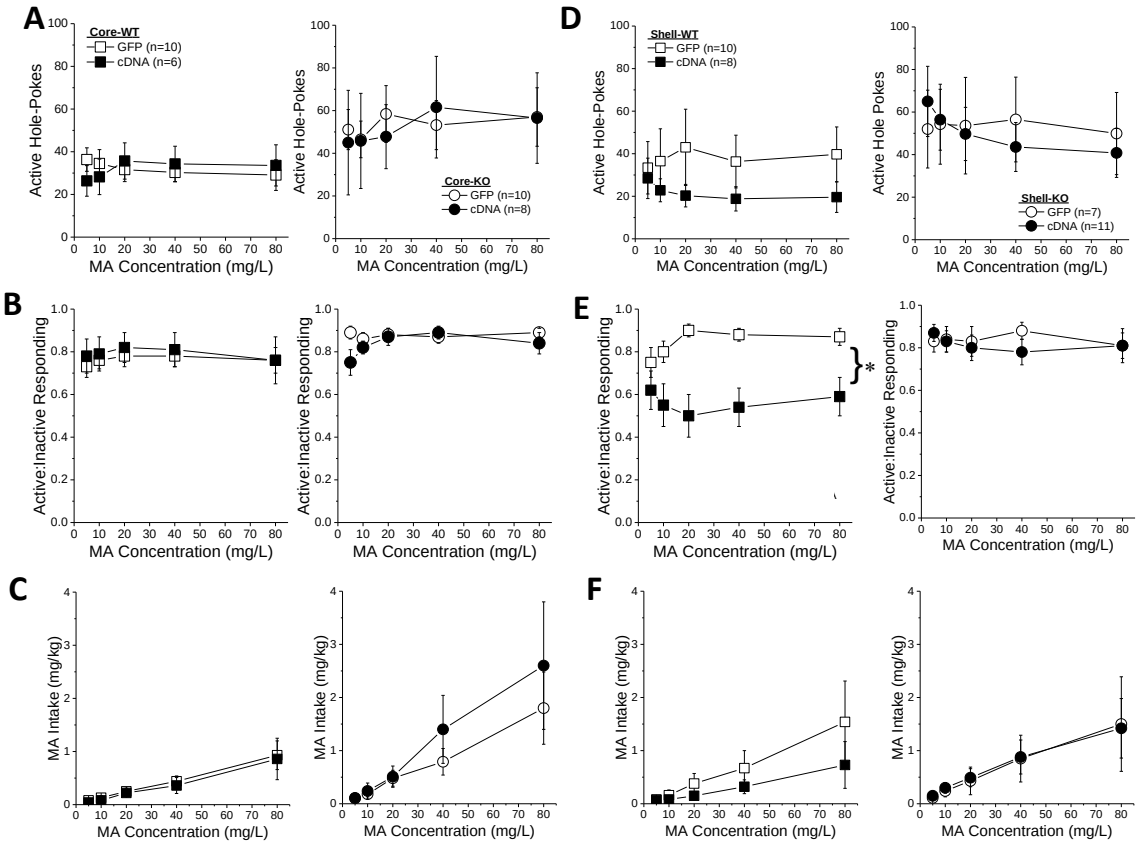


Figure 7. Homer2b over-expression in the NAC shell blunts MA reinforcement only in WT mice. When compared to WT **(left)** and *Homer2* KO **(right)** mice infused with GFP, cDNA infusion into the NAC core did not alter the dose-response functions for: **(A)** active hole responding, **(B)** response allocation or **(C)** MA intake. **(D)** cDNA infusion into the NAC shell caused a declining dose-response function for active hole-responding in both WT and KO mice, **(F)** lowered the dose-response function for response allocation in the active hole in WT mice, but **(G)** did not significantly alter the dose-response function for MA intake. The data represent the means ± SEMs of the number of mice indicated in Panel A (NAC core) and Panel D (NAC shell). *$p<0.05$, main AAV effect. Note: main Genotype effects are not indicated for clarity but are described in the text.

In contrast to the NAC core, Homer2b-cDNA infusion into the NAC shell altered the dose-response function for active nose-poking behavior (**Fig.7D**) [AAV X Dose: $F(4,120)=2.89$, $p=0.03$]. Although inspection of **Fig.7D** suggested that this interaction was driven by the results from the WT mice, there was no genotype effect or interactions with the genotype factor (Genotype effect: $p=0.10$; all interactions with Genotype factor: p's>0.40). Collapsing the data across genotype, *post-hoc* analyses did not indicate any significant GFP-cDNA difference at any of the MA concentrations tested (p's>0.25), arguing that the AAV X Dose interaction reflected the distinct shapes of the dose-response functions for GFP- versus cDNA-infused mice (respectively, flat vs. descending). Homer2b-cDNA into the NAC shell also altered the dose-response function for the ratio of active versus inactive responding (**Fig.7E**) [AAV X Dose: $F(4,120)=2.89$, $p=0.03$] - an effect driven by the shift down-wards in the dose-response response produced by cDNA infusion in the WT mice (**Fig.7E**) [Genotype X AAV: $F(1,30)=4.20$, $p=0.05$]. While it appeared that an intra-NAC shell infusion of Homer2b-cDNA lowered MA intake selectively in WT mice (**Fig.7F**), no group differences were observed with respect to the MA dose-intake function [Dose effect: $F(1,124)=12.73$, $p<0.0001$; all other p's>0.40]. Taken together, these data argue that Homer2b over-expression within the NAC shell lowers the efficacy of MA to serve as a reinforcer, without significantly impacting MA intake.

2.3.8 Homer2b over-expression within NAC subregions does not alter the extinction or reinstatement of MA-seeking

Although Homer2b-cDNA infusion into the NAC core appeared to reduce the number of trials to reach extinction criterion in both WT and KO mice (**Fig.8A**), no group differences were detected for this measure (Genotype X AAV ANOVA, p's>0.09). Likewise, neither *Homer2* deletion nor Homer2b-cDNA infusion into the NAC shell altered the time taken to

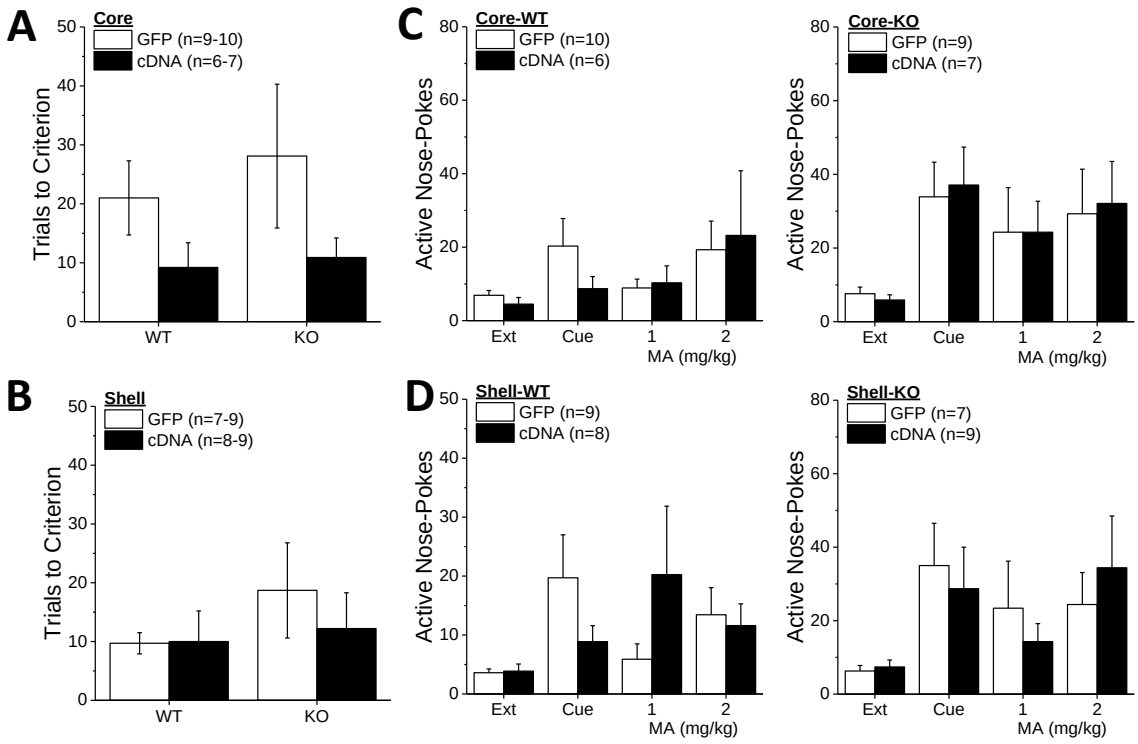


Figure 8. Homer2b over-expression in NAC subregions does not significantly alter genotypic differences in responding under extinction-reinstatement procedures. Although it appeared that cDNA infusion into the NAC core reduced the number of trials taken to reach extinction criterion in both WT and *Homer2* KO mice, no cDNA effect was observed on this measure when infused into either the NAC core **(A)** or the NAC shell **(B)**. **(C)** *Homer2* KO mice exhibited greater cue- and MA-primed reinstatement of active hole responding than WT mice, but NAC core infusion of cDNA did not affect responding in either WT (**left**) or KO animals (**right**). **(D)** cDNA infusion into the NAC shell also did not alter the genotypic difference in reinstatement. The data represent the means ± SEMs of the number of mice indicated in their respective panels. Note: main Genotype effects are not indicated for clarity but are described in the text. The sample sizes in this figure are lower than those indicated in Figs.6 and 7 as some animals were euthanized following MA self-administration procedures due to illness.

extinguish responding in the active hole (**Fig.8B**; Genotype X AAV ANOVA, p's>0.31). A comparison of active hole responding during the last day of extinction with that elicited by presentation of the MA-associated cue or a priming injection of 1 or 2 mg/kg MA indicated greater responding, overall, in *Homer2* KO versus WT mice, irrespective of the AAV infused into the NAC core (**Fig.8C**) [Test effect: $F(3,81)=6.31$, $p=0.001$; Genotype effect: $F(1,27)=5,26$, $p=0.03$; no AAV effect and no interactions, p's>0.25]. Inspection of **Fig.8C** suggested that cDNA into the NAC core differentially affected the magnitude of cue-induced reinstatement (0 mg/kg MA), while exerting no effect on MA-primed responding. However, a direct comparison of responding on the cued reinstatement test and the extinction baseline failed to detect any interaction with the AAV factor [AAV effect and interactions, p's>0.30; Genotype X Test: $F(1,28)=5.91$, $p=0.02$]. Akin to the findings for the NAC core, cDNA infusion into the NAC shell also did not significantly influence active hole responding during the tests for reinstatement of drug-seeking (**Fig.8D**; no AAV effect or interactions, p's>0.17), but again, *Homer2* KO mice exhibited greater responding overall, compared to WT mice [Genotype effect: $F(1,27)=4.40$, $p=0.04$]. These data for the extinction and reinstatement of MA-seeking argue a suppressive role for Homer2 in regulating behavior but do not support either NAC subregion as the active loci of these effects.

2.4 Discussion

Homer2 is a post-synaptic scaffolding protein regulating the localization and function of glutamate receptors (c.f., Castelli et al., 2017; de Bartolomeis and Iasevoli, 2003; Rong et al., 2003; Sala et al., 2001; Shiraishi et al., 2003; Tu et al., 1999; Xiao et al., 2000; Yuan et al., 2003) and its expression within the NAC plays a necessary and active role in behavioral sensitivity to both cocaine and alcohol (c.f., Castelli et al., 2017; Szumlinski et al., 2008a). In more recent work (Szumlinski et al., 2017), idiopathic, genetic, and MA-induced

vulnerability to MA addiction-related behaviors was found to be associated with increased indices of glutamate signaling within the NAC, including elevated Homer2 expression. More specifically, the magnitude of a MA-induced CPP is highly correlated with Homer2 expression within both the shell and core subregions of the NAC, arguing a potential role for Homer2-dependent neuroadaptations within both subregions in the motivational valence of MA. However, in both genetically vulnerable MAHDR and MA-sensitized B6 mice, increased behavioral sensitivity to MA was associated with elevated Homer2 expression within the NAC shell only, suggesting some subregional specificity may exist within the NAC regarding the relationship between Homer2-dependent signaling and MA-induced behaviors. Using an shRNA strategy to selectively knockdown the major rodent isoform of Homer2 (Homer2b; Soloviev et al., 2000) within the NAC shell, we demonstrated previously little to no effect upon the magnitude of a MA-CPP, but a marked reduction in responding for oral MA reinforcement and for MA intake when the same mice were assayed under operant-conditioning procedures. Such data argued a necessary role for Homer2b within the NAC shell for MA reinforcement/intake and suggested that idiopathic or MA-induced increases in NAC shell Homer2b expression promotes a MA addicted phenotype (Szumlinski et al., 2017). Herein, we extended the results of this prior study to the NAC core.

Further, to determine whether Homer2b within either NAC subregion actively regulates MA-induced changes in behavior, we also applied our cDNA-Homer2b strategy (e.g., Ary et al., 2013; Szumlinski et al., 2004, 2005b, 2008b) in both WT and constitutive *Homer2* KO mice to upregulate Homer2b expression. The results show that constitutive *Homer2* deletion potentiated MA-CPP, oral MA reinforcement/intake under operant-conditioning procedures, and the reinstatement of MA-seeking following response extinction. A subset of these KO effects was recapitulated by Homer2b knockdown in the NAC core, providing new evidence

that Homer2b within the NAC shell and core oppositely regulate MA reinforcement. While the present shRNA findings argue a suppressive role for NAC core Homer2b expression in regulating MA reward/reinforcement, cDNA-Homer2b infusion within this subregion also potentiated a MA-CPP, without affecting measures of MA reinforcement/intake/reinstatement. Opposite our expectations (Szumlinski et al., 2017), Homer2b over-expression within the NAC shell reduced indices of MA reinforcement but did not affect other behavioral measures. Below, we discuss these effects of bi-directional manipulations of Homer2b within the NAC in the context of animal models of MA reward and reinforcement. The data collected during this study are summarized in **Table 1**.

2.4.1 Constitutive Homer2 deletion tends to promote MA reward/reinforcement

Constitutive *Homer2* deletion potentiated: MA-conditioned reward (**Fig.3A**), responding for oral MA reinforcement and MA intake under operant-conditioning procedures (**Fig.4,7**), and the number of trials required to extinguish MA-seeking behavior (**Fig.8A**). Additionally, *Homer2* deletion increased the magnitude of both cue- and MA-primed reinstatement of MA-seeking behavior following extinction (**Fig.8C,D**). Further, the increased MA reinforcement and intake observed in *Homer2* KO mice was apparent early during self-administration training (**Fig.4A-C**) and persisted across a range of MA doses in MA-experienced mice (**Fig.4G-I**). Such data argue a suppressive role for Homer2 in both gating vulnerability to early MA abuse and maintaining an addicted phenotype. Although genotypic differences in MA-induced locomotor activity were not observed in the present study (**Fig.3B,C**), we reported previously that the dose-response function for acute MA-induced locomotor activity is shifted upwards in *Homer2* KO mice versus WTcontrols (Szumlinski et al., 2005a). Thus, it is possible that the increased MA reinforcement and intake exhibited by *Homer2* KO mice herein relates to the greater efficacy of the drug to induce psychomotor activation. The

Table 1. Summary of the results of the present experiments. ↑ denotes an increase in behavior relative to control. ↓ denotes a decrease in behavior relative to control. – denotes no effect of manipulation relative to control. N/D denotes not determined.

Behavioral Measure	Core Knockdown	Constitutive KO	Core Over-expression: Effect of KO	Core Over-expression: Effect of cDNA	Shell Over-expression: Effect of KO	Shell Over-expression: Effect of cDNA
CPP	↑	↑	↓	↑	--	--
Locomotor Sensitization	--	--	--	--	--	--
Self-Administration Training	--	↑	--	--	--	--
Increasing Response Requirement	--	↑	N/D	N/D	N/D	N/D
Self-Administration Dose Response Curve	↑	↑	--	--	--	↓
Trials to Extinction	N/D	N/D	--	--	--	--
Cue- & MA-induced reinstatement of self-administration	N/D	N/D	↑	--	--	

precise reason for the present failure to replicate genotypic differences in MA-induced locomotion is not entirely clear but likely reflects procedural differences between the studies. First and foremost, the two studies were conducted in two distinct research institutions (Medical University of South Carolina vs. University of California Santa Barbara); thus a host of environmental differences may have contributed to the differential results to include the fact that the mice in the present study were bred in-house, while those in our earlier study were obtained from the laboratory of Dr. P.F. Worley at Johns Hopkins University School of Medicine. Also, the present experiments employed both a shorter testing period (15 vs. 60 min) and a smaller testing arena than our prior report. Additionally, mice in the present study underwent saline-conditioning sessions on the days intervening between MA injections, while mice in the prior study were injected with MA only (Szumlinski et al., 2005a). Nevertheless, the present data for MA reward/reinforcement in *Homer2* KO mice aligns well with those reported for cocaine reward/reinforcement (Szumlinski et al., 2004), providing new evidence for a generalization of a "pro-addictive" phenotype of *Homer2* KO mice across different psychomotor stimulant drugs of potential relevance for the neurobiology of psychomotor stimulant abuse liability and/or MA-cocaine co-abuse.

Drawbacks of a constitutive knockout approach for studying the neurobiology of behavior relate to the lack of developmental and neuroanatomical specificity of gene deletion. There exist three different Homer isoforms, with Homer1 and Homer2 isoforms expressed in mid- and forebrain regions highly implicated in addiction neurobiology (Soloviev et al., 2000). Further, distinct Homer1 isoforms differentially regulate spontaneous and stimulant-induced changes in behavior (Szumlinski et al., 2005a; Lominac et al., 2005), with imbalances in the relative expression of Homer1 versus Homer2 isoforms within mPFC gating cocaine-conditioned reward (Ary et al., 2013) and the reinstatement of cocaine-

seeking behavior (Gould et al., 2015). Although extant correlative evidence does not support a relationship between Homer1 protein expression within either NAC subregion or within the mPFC and MA behavioral sensitivity (Lominac et al., 2016; Szumlinski et al., 2017), such findings do not preclude the possibility that compensatory changes in Homer1 expression/function may contribute to the "pro-addictive" phenotype of *Homer2* KO mice.

2.4.2 Subregional selectivity in the effects of Homer2b knockdown within NAC upon MA reward/reinforcement

In brain, Homer2 expression is regulated in a regionally selective manner by prior MA experience in inbred B6 mice, with increases in protein expression observed selectively within the NAC shell (Lominac et al., 2016; Szumlinski et al., 2017). Further, increased Homer2 expression within the NAC shell, but not core, is a biochemical correlate of genetic vulnerability to consume MA in mice on a heterogeneous genetic background (Szumlinski et al., 2017). Providing causal evidence that Homer2 functions to alter MA reward/reinforcement in a subregionally-distinct manner, Homer2b knockdown in the shell reduces (Szumlinski et al., 2017), while knockdown in the core increases, both the magnitude of a MA-CPP and responding for a MA reinforcer (**Fig.1,2**). Thus, the effects of constitutive *Homer2* deletion upon MA reward/reinforcement/intake (**Fig.3,4**) are recapitulated, albeit incompletely, by Homer2b knockdown within the NAC core (**Fig.1,2; Table 1**). Given the neuroanatomical nature of our research question, we deemed it more critical to decipher the site of AAV transduction within NAC subregions than quantify the efficiency of our shRNA construct to alter Homer2b protein expression. The fact that the phenotype produced by Homer2b knockdown in the NAC core did not fully recapitulate that of the *Homer2* KO mouse is perhaps not surprising as we know from prior work that our shRNA-Homer2b infusion procedure consistently reduces protein expression by 40-50% *in vivo* (Ary et al.,

2013; Goulding et al., 2011; Haider et al., 2015; Klugmann and Szumlinski, 2008) and does not completely eliminate protein expression as is the case for gene deletion. Further, in humans, MA addiction is associated with anomalies in the function of many brain structures that were not targeted herein (Chang et al., 2002; Kim et al., 2011; London et al., 2005). Indeed, lower Homer2 expression within the mPFC of mice is associated with both genetic and idiopathic vulnerability to express a MA-CPP, and to respond for/consume the drug under operant-conditioning procedures (Lominac et al. 2016). Thus, it is highly likely that Homer2 within other structures embedded within putative addiction neurocircuits functions also to regulate MA-conditioning, -seeking and -taking behavior and contribute to the robust MA phenotype of *Homer2* KO mice. Although Homer2b knock-down in the NAC core does not fully recapitulate the effect of constitutive gene deletion, it is interesting to note that the phenotype produced by Homer2b knock-down in the NAC core predominates in the *Homer2* KO mouse (**Table 1**).

In our limited experience using shRNA to target Homer2 expression within both NAC subregions (Cozzoli et al., 2009) and to the best of our knowledge of the extant Homer2 literature, our shRNAHomer2 findings for MA reward/reinforcement (Szumlinski et al., 2017; present study) are the first to demonstrate opposing roles for Homer2b within NAC subregions in regulating addiction-related behavior. We know through studies of constitutive *Homer2* KO mice and of the effects of intra-cranial shRNA-Homer2b infusion that intact Homer2 expression is important for maintaining basal extracellular glutamate levels within both the NAC and mPFC (Szumlinski et al., 2004; Ary et al., 2013; Haider et al., 2015), cocaine- and alcohol-stimulated glutamate release (Szumlinski et al., 2004, Szumlinski et al., 2005b; Lominac et al., 2005; Goulding et al., 2011), and the expression/function of glutamate receptors, transporters and signaling molecules within these regions (Szumlinski et al., 2004;

Szumlinski et al., 2005b; Ary et al., 2013). However, we are unaware of any study that has directly compared the effects of either *Homer2* deletion or Homer2b knockdown upon any biochemical measure *between* NAC subregions to inform the mechanisms underpinning the opposing MA effects of Homer2b knockdown observed herein. That being said, we do know from studies of the mPFC that shRNA-Homer2b (and cDNA-Homer2b) infusion can produce not only local effects upon basal and drug-stimulated changes in extracellular glutamate, in addition to changes in the expression of Homer2 and glutamate receptor-related proteins, but can also alter these biochemical measures within NAC, intriguingly in a direction sometimes *opposite* that observed at the site of infusion (Ary et al., 2013). As striking examples, intra-mPFC infusion of cDNA-Homer2b elevates basal extracellular glutamate content and Homer2 expression, in addition to blunting drug-stimulated glutamate release at the site of infusion, but lowers the glutamate content and expression of both Homer1/2 and mGlu1/5 and potentiates drug-stimulated glutamate release within the NAC. Further, intra-mPFC infusion of shRNA-Homer2b reduces Homer2 and mGlu5 expression at the infusion site, but elevates markedly the expression of GluN2b, without affecting extracellular glutamate or the expression of Homers or mGlu1/5 within the NAC (Ary et al., 2013). These adaptations within NAC cannot be readily explained by anterograde transport of the AAVs and argue that the opposing effects of shRNA-Homer2b infusion into the NAC shell and core observed herein could reflect yet uncharacterized distinctions in local changes in extracellular glutamate and/or glutamate receptor function/expression that differentially alter the activation of efferents or could reflect yet uncharacterized biochemical alterations within those efferent structures (e.g., ventral pallidum).

2.4.3 Inconsistent effects of increasing Homer2b expression within the NAC shell and core upon reward/reinforcement

The observed effects of intra-NAC core/shell shRNA-Homer2b infusion argued that Homer2b expression within the NAC core suppresses, while that in the NAC shell promotes, certain MA addiction-related behaviors in mice (**Fig.1,2**; Szumlinski et al., 2017). However, when this hypothesis was tested directly using well-established AAV-cDNA approaches that increase local Homer2b expression by approximately 50% (Szumlinski et al., 2004; Szumlinski et al., 2005b; Szumlinski et al., 2008; Ary et al., 2013; Haider et al., 2015), we found no supporting evidence for either notion. If anything, the results from our cDNA study were opposite those predicted from our shRNA experiments. For one, intra-NAC shell infusion of cDNA-Homer2b lowered the dose-response functions for MA-reinforced/appropriate responding in WT mice (**Fig.5F; Fig.6D,E**) –an effect qualitatively similar to (albeit more robust than) that observed upon Homer2b knockdown in this subregion of B6 animals (Szumlinski et al., 2017). Also, an intra-NAC core infusion of either shRNA-Homer2b in B6 mice or cDNA-Homer2b in B6-129 hybrid WT mice produced a quantitatively similar increase in the magnitude of a MA-CPP (**Fig.1D** vs. **Fig.5E**). Despite baseline differences in responding, Homer2b over- and under-expression within NAC core produces similar effects upon the MA-conditioned reward expressed in each experiment. Finally, within the context of operant-conditioning, in no instance did cDNA-Homer2 infusion into either NAC subregion significantly alter, let alone reverse, the MA phenotype of *Homer2* KO mice (**Fig.7, 8**).

These null data are in stark contrast to our earlier reports demonstrating a complete reversal of the behavioral and/or neurochemical phenotype of *Homer2* KO mice by site-directed infusions of our cDNA-Homer2b construct (Szumlinski et al., 2004; Szumlinski et al., 2005b; Ary et al. 2013). In only one instance did the data for cDNA-Homer2b infusion align with our predictions and this was observed within the context of

extinction/reinstatement procedures. Although the results failed to reach statistical significance, cDNA-Homer2b infusion into the NAC core facilitated the extinction of operant-behavior (an effect observed in both WT and KO mice; **Fig.8A**) and blunted the capacity of the MA-associated cues to reinstate responding in WT mice (**Fig.8B**). That being said, cDNA-Homer2b infusion into the NAC shell produced a comparable reduction of cue-induced reinstatement as that observed in mice infused intra-NAC core (**Fig.8A vs. B**). Such null results argue strongly against an active and autonomous role for Homer2 within either NAC subregion in regulating MA-conditioned reward or self-administration. Alternatively, these data could also suggest that any dysregulation in Homer2 expression, be it over- or under-expression, is sufficient to perturb normal glutamate transmission within NAC subregions to affect MA reward/reinforcement. Which, and how, specific signal transduction pathways are affected by increasing versus decreasing Homer2 expression within different NAC subregions remains to be determined and are important research questions for future studies aimed at understanding more precisely the role played by this scaffolding protein in regulating MA addiction-related behaviors.

2.4.4 Additional caveats of the current study

Table 1 summarizes the major findings from this study, which are complicated to interpret to say the least. Adding to the interpretational difficulty is the notable fact that the baseline behavior of the control animals varied considerable across the different experiments. For instance, the baseline CPP behavior of GFP-infused B6 mice in the shRNA study of the NAC core was approximately half that of the WT B6-129 mice in the cDNA study of this region (**Fig.1C** vs. **Fig5D**). These experiments were conducted over a year apart; thus, we cannot decipher from the current experimental design whether or not this difference in baseline CPP reflects environmental factors (e.g., differences in laboratory or animal care

personnel) or strain differences in behavioral sensitivity to MA. Indeed, marked strain differences are reported between C57BL/6J mice and DBA2/J mice with respect to MA intake, with C57BL/6J mice exhibiting significantly lower MA intake than DBA2/J mice (e.g., Harkness et al. 2015; Shi et al., 2016; Reed et al., 2018). To date, we have yet to directly compare MA CPP, reinforcement, or intake between B6 mice and mice on a mixed B6-129 background so we cannot rule out the potential contribution of background strain to our findings. However, arguing more in favor of environmental factors as contributors to the differences in baseline CPP, the 2 mg/kg MA dose elicited negligible CPP in the B6-129 mice infused with cDNA into the NAC shell (**Fig.5G**), despite these animals exhibiting similar acute and sensitized locomotor responses to the drug as those infused with cDNA into the NAC core (**Fig.5E,F** vs. **H,I**). The MA self-administration behavior of the B6-129 mice infused with cDNA into the NAC shell was also lower than that exhibited by their NAC core counterparts, particularly during the training phase of the experiment **(Fig.6)**. Such behavioral differences cannot be attributable to differences in genetic background.

Further, we would like to be forthcoming and report that, unfortunately, during the year we were conducting the NAC shell cDNA study, building renovations were occurring on the level beneath our laboratory. While arrangements were in place to minimize the noise and vibration during the daylight hours when the animals were being tested, we cannot rule out the possibility that the construction conducted during the evening hours affected the behavior of the animals nor did we have any control over, or ability to predict, any construction that took place during the day. For this very reason, the cDNA study of the NAC shell was conducted in 3 distinct cohorts of 21-25 B6-129 mice, spaced 1-3 months apart in accordance with the limited information we were provided regarding heavy construction/demolition. However, despite our best attempts to avoid this confound, we were unsuccessful at eliciting

a CPP in this experiment. Indeed, the number of mice exhibiting a conditioned place-aversion (CPP Score <-100 sec; see Szumlinski et al., 2017) in each cohort of the cDNA study of the NAC shell was higher than that observed in the cDNA study of the NAC core (shell: 3-5/cohort vs. core: 2-3/cohort), with more mice exhibiting place-ambivalence. It is also possible that the AAV-GFP infusion into the NAC shell might have inadvertently affected the behavior of the B6-129 mice, although we observed no overt signs of infection or tissue damage. However, we deem this unlikely as we have conducted numerous experiments in which this AAV was infused into the NAC shell, to include studies of methamphetamine- (Szumlinski et al., 2017), alcohol- (Szumlinski et al., 2005; Szumlinski et al., 2008) and cocaine-induced place-conditioning (Szumlinski et al., 2004) and observed no obvious off-target effects of the AAV upon the expression of the conditioned response. Thus, we surmise that factors related to building renovations likely confounded data interpretation from the cDNA study of the NAC shell.

2.4.5 Concluding remarks and future directions

Considerable neuropharmacological, chemogenetic, and optogenetic work has established that the NAC shell and core are embedded within distinct neural subcircuits that differentially contribute to aspects of drug-conditioning, drug-taking, and drug-seeking behavior, the most well characterized of which are the relatively dense afferents from, respectively, the infralimbic (IL) and prelimbic (PL) subregions of the mPFC (e.g., Moorman et al. 2015; Vertes 2004). The majority of data argue that PL-NAC (core) projections are involved in driving or executing operant behavior in the context of drug self-administration, whereas IL-NAC (shell) projections are more critical for suppressing or inhibiting responding (e.g., Peters et al., 2009; Van den Oever et al., 2010). This being said, there is overlap in the PL and IL projections to specific NAC subregions (Heidbreder and

Groenewegen, 2003; Vertes et al., 2004) that can bear on how specific corticoaccumbens projections might influence responding for drugs and natural reinforcers (see Moorman et al., 2015; Moorman and Aston-Jones, 2015; Shin et al., 2018). Thus, while the available data pertaining to Homer2 regulation of MA addiction-related behavior in mice do not reliably support an active role for Homer2 within NAC subregions for gating MA addiction-related behaviors, our AAV findings do not negate a role for this Homer isoform within NAC afferents, in particular those from the mPFC, in this regard. Although repeated MA does not alter Homer2 expression in samples from the entire PFC (to include PL, IL and anterior cingulate), reduced PFC Homer2 expression is associated with both genetic and idiopathic MA addiction vulnerability in mouse models (Lominac et al., 2016). Given the importance of mPFC-NAC subcircuits for gating drug-taking and -seeking behavior and based on our earlier cocaine studies of Homer2 function within mPFC (Ary et al., 2013), one goal of future work is to characterize the neuroanatomical selectivity of MA-induced changes in Homer2/glutamate signaling within PFC subregions and to interrogate the role played by distinct mPFC-NAC subcircuits and Homer2 expression within these subcircuits in MA-taking and -seeking behavior.

Chapter 3:

Effects of bidirectional manipulation of PFC-NAC subcircuits on methamphetamine reward and reinforcement in mice

3.1 Introduction

Highly addictive amphetamine-type stimulants, including methamphetamine (MA), pose significant, global, health and socioeconomic issues. There are an estimated 27 million MA users worldwide (United Nations, 2020), with MA accounting for 80% of the amphetamine-type stimulants seized by international law enforcement (World Drug Report, 2020). MA is a highly potent psychomotor stimulant with a long (~10-12 h) duration of action. It is the drug of abuse that contributes most to violent crime in the United States (US Department of Justice DEA, 2016), and long term MA misuse can cause anxiety, confusion, memory loss, insomnia, mood disturbance, aggression, violent behavior, psychosis, paranoia, hallucinations, delusions, weight loss, tooth decay, skin sores, and potentially lethal cardiovascular complications (NIDA, 2021; Chomchai and Chomchai, 2015; Akindipe, Wilson, and Stein, 2014; Panenka et al., 2013; Rusyniak, 2013). The high prevalence of MA use disorder is particularly concerning because no clinically approved pharmacotherapy exists for treatment and relapse to drug-taking is common. Developing safe and effective therapies for treating MA use disorder requires that we augment our understanding of the neurobiological changes underlying the various stages of MA abuse.

MA's highly addictive properties are often attributed to its ability to increase dopamine signaling, particularly in the nucleus accumbens (NAC) (Chiu and Schenk, 2012; Suzuki et al., 1997; Arai et al., 1996). In addition to dopamine, the long-term neuroplasticity that maintains MA Use Disorder involves drug-induced changes in glutamate signaling within this region (Szumlinski et al., 2019; Szumlinski et al., 2017; Lominac et al., 2016; Lominac et al., 2012; Koob and Volkow, 2010; Kalivas 2009). Neuropharmacological studies demonstrate that NAC glutamate modulates the direction and magnitude of a MA-induced conditioned place preference (CPP) (Szumlinski et al., 2017; Fujio et al., 2005a; Fujio et al.,

2005b; Nakagawa et al., 2005; Kim and Jang, 1997; Layer et al., 1993), implicating NAC glutamate in the initial rewarding properties of MA that might contribute to the acquisition of drug-taking behavior. In support of this, mice selectively bred for high MA-drinking (MAHDR) exhibit glutamate anomalies in the NAC, including increased basal extracellular glutamate and MA-stimulated glutamate release, that correlate with their increased propensity to exhibit a MA-CPP and to self-administer MA (Szumlinski et al., 2017; Shabani et al., 2011; Wheeler et al., 2009; Phillips et al., 2008).

Repeated high-dose MA exposure (e.g., 4 injections of 10 mg/kg) is well-characterized to produce neurotoxic effects in the striatum via mechanisms involving augmented glutamate-dependent signaling, while sparing the more ventral NAC (Tata and Yamamoto, 2007). In contrast, repeated administration of non-toxic MA doses (e.g., 7 injections of 1-2 mg/kg), or intravenous MA self-administration, increases extracellular glutamate levels within the NAC (Lominac et al., 2012; Szumlinski et al., 2017), and increases the expression of mGlu1/5 receptors and their associated scaffolding protein Homer2 (Szumlinski et al., 2017). Arguing an active role for NAC glutamate in gating behaviors associated with MA use disorder, both elevated expression of glutamate-related proteins in the NAC and their pharmacological stimulation is associated with increased MA reward and reinforcement in laboratory rodents. Conversely, reduced protein expression or function lowers the expression of MA-conditioned behaviors (Brown et al., 2020; Szumlinski et al., 2017; Fujio et al., 2005a; Fujio et al., 2005b; Nakagawa et al., 2005; Kim and Jang, 1997). In addition, glutamate autoreceptor expression is down-regulated within the NAC during drug abstinence following a period of MA self-administration and this deficit can be restored by extinction learning (Schwendt et al., 2012). Extinction may reduce drug-seeking by altering the glutamatergic adaptations that underlie chronic MA abuse. Implicating a role for NAC glutamate in relapse, extracellular NAC

glutamate increases during both cue- and MA-primed reinstatement of MA-seeking in rats with a history of intravenous MA self-administration (Parsegian and See, 2014). Thus, glutamate signaling within the NAC appears to impact the expression, extinction, and reinstatement of MA-conditioned behavior in rodent models.

The NAC has two sub-regions, the core, which signals motivational values of expected goals to guide drug-seeking in drug-experienced brains, and the shell, which is involved in the initial affective valence of drug-taking in early drug experience (Everitt and Robbins, 2013). MA injection regimens induces few subregion-selective changes in certain glutamate-related proteins within the NAC of mice (Mishra et al., 2017; Szumlinski et al., 2017), and a recent study failed to detect overt changes in mGlu5 receptor expression or Homer binding within the NAC core of rats exhibiting incubated MA-craving, although mGlu5-dependent synaptic depression was impaired, indicative of reduced receptor function (Murray et al., 2021). Interestingly, idiopathic and genetic vulnerability to the rewarding and reinforcing properties of MA is associated with subregion-selective changes in glutamate-related proteins (Szumlinski et al., 2017). For examples, both B6 mice spontaneously expressing a MA-conditioned place-preference (CPP) and selectively bred MAHDR mice exhibit elevated expression of mGlu1 or mGlu5 in the NAC core, relative to their low MA-preferring controls. In contrast to the receptors, the expression of the Homer2 scaffolding protein is increased within both NAC subregions in B6 mice expressing a CPP and in MAHDR mice (Szumlinski et al., 2017). However, the results of virus-mediated knock-down and over-expression studies indicate that Homer2 expression within the NAC core and shell (and presumably, its effects upon NAC glutamate receptor function and glutamate release; e.g., Szumlinski et al., 2004) plays distinct, sometimes opposing roles in regulating MA addiction-related behaviors, including MA-taking (Brown et al., 2020; Szumlinski et al., 2017).

The primary source of glutamate projections to the NAC originates from the prefrontal cortex (PFC) - a brain region also highly implicated in drug abuse and substance use disorder (Kalivas, 2009; Vertes, 2004). Sub-chronic, sub-toxic dosing with MA sensitizes drug-induced glutamate release in the ventromedial aspect of the PFC (vmPFC) of mice and reduces both GluN1 and GluN2b expression in this region (Lominac et al., 2016). High MA-consuming MAHDR mice also exhibit increased basal extracellular glutamate levels in the vmPFC, concomitant with decreased Homer2 and mGluR2/3 expression. Supporting an active role for vmPFC glutamate in MA addiction-related behavior, pharmacological studies indicate that increasing vmPFC glutamate levels enhances the magnitude of a MA-CPP in mice (Lominac et al., 2016). Increased PFC glutamate is also observed during cue- and MA-induced reinstatement of MA-seeking behavior (Parsegian and See, 2014). Although such findings suggest that stimulation of glutamate release within the vmPFC drives the conditioned rewarding and reinforcing properties of MA, they cannot inform as to the precise subcircuits involved.

The vmPFC can be divided into the prelimbic (PL) and infralimbic (IL) sub-regions, which differ in connectivity and functionality. Traditionally, the PL is thought to project primarily to the NAC core and the IL to the NAC shell, with these sub-circuits playing opposite roles in addiction-related behavior. We are unaware of any study examining the relative roles of PFC-NAC subcircuits in an animal model of MA-taking or -seeking. However, in rodent models of cocaine addiction, opto- and chemo-genetic studies suggest that glutamate signals from the PL to the NAC core drive drug-seeking behavior, while those from the IL to the shell inhibit drug-seeking (Augur et al., 2016; Ma et al., 2014). However, some evidence challenges this strict dichotomy in the functional neuroanatomy of PL versus IL projections to the NAC sub-regions and their regulation of food- and drug-seeking (Shin

et al., 2018; Moorman et al., 2015). It is notable that the majority of studies examining the PL-core vs. IL-shell dichotomy in cocaine addiction have largely focused on models of late-stage addiction, in which the animals are highly drug-experienced (Augur et al., 2016; Martin-Garcia et al., 2014; Stefanik et al., 2013). An exception is a study in which the temporary pharmacological inactivation of the PL impaired the acquisition of responding for a cocaine-paired reinforcer (Di Ciano et al., 2007). However, in another study, lesioning the PL did not affect the acquisition of a cocaine-induced conditioned place preference (CPP) (Zavala et al., 2003). Thus, the relative role for the different vmPFC subcircuits in the acquisition drug-related learning is neither well-defined nor -investigated. Activity in the PL is thought to drive cue- and MA-primed reinstatement of drug-seeking following extinction learning (Peters et al., 2009; Hiranita et al., 2006; Rocha and Kalivas, 2010; Moorman et al., 2015), but it remains unclear which subcircuits projecting to NAC subregions are involved.

This study aims to elucidate the functional relevance of PFC-NAC subcircuits in the acquisition, expression, extinction, and reinstatement of MA-conditioned responses under place- and operant-conditioning procedures in mouse. In order to achieve this, we used dual virus chemogenetic techniques that allow for anatomically specific manipulation of cells with somas in the PL or IL subregions of the vmPFC and terminals in the core or shell of the NAC (Augur et al., 2016). Artificial receptors called Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) can increase or decrease neuronal activity when bound to the synthetic ligand clozapine-n-oxide (CNO) (Ferguson and Neumaier, 2012; Rogan and Roth, 2011; Armbruster et al., 2007). We utilized these tools to bidirectionally manipulate activity in the PL-core, PL-shell, IL-core, and IL-shell sub-circuits and determine their relative contributions to MA self-administration during the acquisition phase of operant-conditioning and under increasing schedules of reinforcement once the operant response was acquired. We

then bidirectionally manipulated activity in the PL-core and PL-shell sub-circuits and probed their involvement in the expression, extinction, and reinstatement phases of MA-conditioning. Traditional theory would predict that activating projections originating from the PL would increase MA-reinforced behaviors, whereas activating the IL projections and inhibiting PL projections would decrease these behaviors, potentially block the acquisition of self-administration, or facilitate the extinction of conditioned responses (Augur et al., 2016; Moorman et al., 2015; Ma et al., 2014).

3.2 Materials and methods

3.2.1 Subjects

Subjects were adult, male C57BL/6J (B6) mice (~8 weeks of age; The Jackson Laboratory, Sacramento, CA). Animals were housed in groups of 2-4 mice in ventilated polycarbonate cages, under standard, reverse-light housing conditions in an AAALAC-accredited vivarium (lights on/off: 2200/1000 h), with *ad libitum* access to food and water, except during behavioral testing procedures. Conditioned place-preference testing was conducted during the light phase of the circadian cycle, while operant-conditioning paradigms were conducted during the dark phase. All behavioral procedures were consistent with NIH guidelines and approved by the Institutional Animal Care and Use Committee of the University of California Santa Barbara.

3.2.2 Surgeries and AAV infusion

Virus infusions were conducted in two separate surgeries. In the first surgery, a retrograde adeno-associated virus (AAV) carrying Cre and eBFP [AAV pmSyn1-EBFP-Cre was a gift from Hongkui Zeng (Addgene viral prep #51507-AAVrg; http://n2t.net/addgene:51507; RRID: Addgene_51507); Madisen et al., 2015] was microinjected into either the NAC core or shell. The animals recovered from this first surgery

for 5-7 days and then underwent a second surgery to infuse an AAV8 carrying a Cre-dependent Gq-DREADD, Gi-DREADD, or mCherry control [pAAV-hSyn-DIO-hM3D(Gq)-mCherry (Addgene viral prep #44361-AAV8; http://n2t.net/addgene:44361; RRID: Addgene_44361), pAAV-hSyn-DIO-hM4D(Gi)-mCherry (Addgene viral prep #44362-AAV8; http://n2t.net/addgene:44362; RRID: Addgene_44362), and pAAV-hSyn-DIO-mCherry (Addgene viral prep #50459-AAV8; http://n2t.net/addgene:50459; RRID: Addgene_50459) were a gift from Bryan Roth; Krashes et al., 2011] into the PL or IL of the PFC. We used the following stereotaxic coordinates (in mm from Bregma): for NAC core, AP: +1.4; ML: ±1; DV: -4.3; for NAC shell, AP: +1.4; ML: ±0.5; DV: -4.6; for PL, AP: +1.7, ML: ±0.3; DV: -3.0; for IL, AP: +1.6; ML: ±0.3; DV: -3.3. In both surgeries, mice were anesthetized with 1.5% isoflurane and positioned in the stereotaxic apparatus. Thirty-gauge microinjectors (12 mm long) were lowered bilaterally, directly into the PL, IL, NAC core, or NAC shell. AAVs were infused at a rate of 0.10 µL/min for 3 min (total volume/side = 0.30 µL), and injectors were left in place for an additional 5 min prior their retraction and closing of the incision site. Animals were left in their home cages for a minimum of 3 weeks prior to behavioral testing to allow for neuronal transduction (Kerstetter et al., 2016).

3.2.3 Drugs

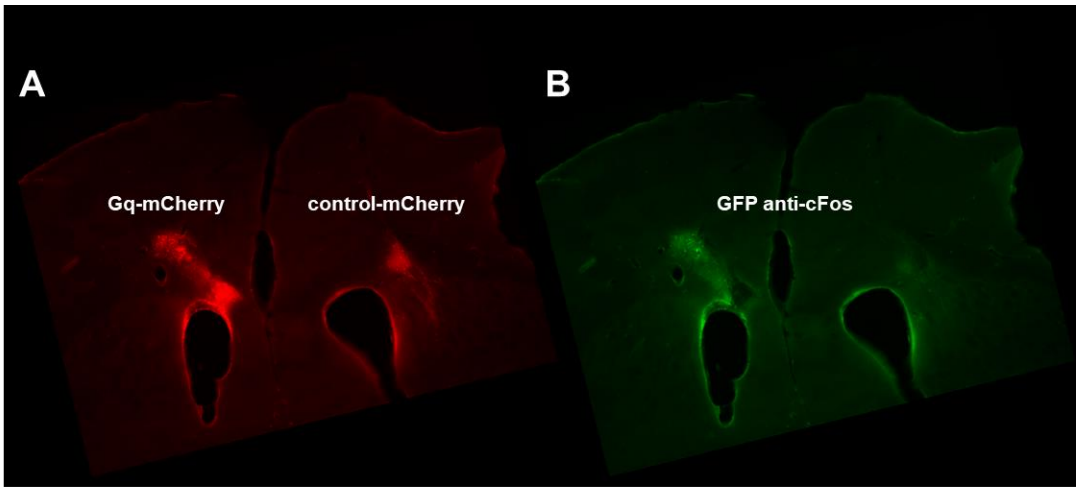
Research published using DREADDs in rodents has employed intraperitoneal (IP) CNO doses from 1-30 mg/kg. Several studies report that 10 mg/kg CNO in rats may produce optimal effects on operant responding (Chiarlone et al., 2014; Mahler et al., 2014), but others have more recently found that lower doses are effective at manipulating amphetamine-induced locomotion (MacLaren et al., 2016). Even less consensus has been reached regarding dosages for mice; some studies use 10 mg/kg CNO IP for mice as well (Gomez et al., 2017), while others use doses as low as 1 mg/kg (Farrell et al., 2013). The group that originated the

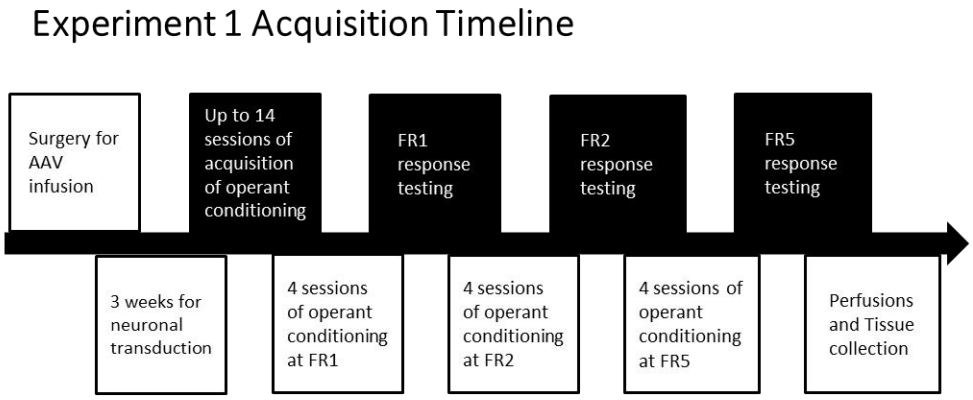
DREADDs technology suggests doses as little as 0.1-3 mg/kg should be sufficient (Rogan and Roth, 2011), but they are rarely used experimentally. Because CNO can get converted to clozapine in the body, which has sedative effects, using the lowest effective dose is important to avoid off-target effects (Gomez et al., 2017; MacLaren et al., 2016). Based on all of this information as well as studies of the time course of CNO's effects on mouse physiology, a dose of 5 mg/kg CNO IP (Abcam, Cambridge, UK) was selected to be administered thirty minutes prior to behavioral testing (Gomez et al., 2017). These parameters were tested during the pilot study as described below.

MA for oral self-administration was prepared in tap water to make an unadulterated solution with a 20 mg/L concentration (Sigma-Aldrich, St. Louis, MO, USA). For place-conditioning procedures, mice received 2 mg/kg IP injections of MA (Sigma-Aldrich, St. Louis, MO, USA) prepared in saline (SAL) or SAL IP injections of equivalent volume (10 mL/kg).

3.2.4 Pilot study

In order to confirm the effects of CNO injection on neuron activity, animals underwent surgery to infuse the PL-core sub-circuit with a Gq-DREADD in one hemisphere and mCherry in the other. After the neuronal transduction period had passed, mice were injected with a 5 mg/kg IP injection of CNO. Thirty minutes later, they were euthanized and perfused as described in the histology section below. Tissue was sectioned 40 µm thin along the coronal plane on a vibratome from the level of the PFC to the NAC. It was then stained with an anti-cFos fluorescent antibody (MilliporeSigma, Burlington, MA, USA; 1:1,000 dilution), mounted on slides, and cover-slipped. Slides were viewed using the 10x objective of a Keyence BZ-X800 All-in-one Fluorescence Microscope and BZ-X800 Viewer software (see **Figure 9** for representative images).





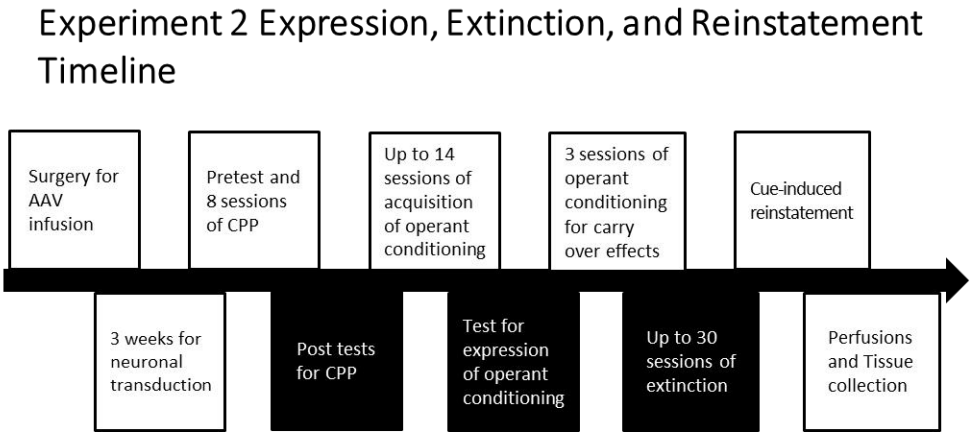


Figure 9. Histological representation and experimental timelines. (A) Representative micrograph of immunofluorescence for the mCherry tag on the AAVs carrying either the Gq-DREADD infused into the right hemisphere and the control vector infused into the left hemisphere. **(B)** cFOS induction in the same animal following IP injection with 5 mg/kg CNO, visualized using a GFP secondary antibody. **(C)** Procedural timeline of Experiment 1. **(D)** Procedural timeline of Experiment 2.

3.2.5 Experimental design

The goal of Experiment 1 was to ascertain the role of PL-core, PL-shell, IL-core, and IL-shell sub-circuits in the acquisition of operant-conditioning for oral MA reinforcement and for MA demand under increasing schedules of MA reinforcement. After surgery and the 3-week transduction period, CNO was administered to the animals prior to each operant-conditioning session during the two-week acquisition phase of the study. Mice that successfully reached the acquisition criteria were then trained to respond for MA under increasing schedules of reinforcement. Mice responded for 5 consecutive days under each new schedule, and CNO pretreatment occurred prior to the 5^{th} session under each schedule.

Based on the results of Experiment 1, Experiment 2 sought to determine the contribution of PL-core and PL-shell sub-circuits to the expression of MA conditioned place-preference and to the expression and extinction of operant-conditioning for MA reinforcement. Following the 3-week transduction period, mice underwent place-conditioning procedures as detailed below. CNO was administered only prior to the test for the conditioned response to examine for effects on conditioned reward. These same animals were then trained under operant-conditioning procedures to orally self-administer MA. Once mice reached acquisition criteria for self-administration and self-administration behavior had stabilized, they were pretreated with CNO prior the operant session and tested immediately for DREADD effects on the expression of the operant response, as well as for any carry-over effects over the next 3 days. To examine for DREADD effects on drug-extinction learning, mice then underwent an extinction procedure, with CNO administered prior to each extinction session. To determine whether subcircuit manipulation during extinction impacted subsequent relapse-like behavior, mice that attained extinction criteria were then tested for cue-induced reinstatement of responding, in the absence of a CNO injection.

3.2.6 Operant-conditioning

Mice were trained to orally self-administer 20 mg/L MA during daily 1-h sessions in standard mouse operant-conditioning chambers (MedAssociates, St Albans, VT, USA). The ventilated, sound-attenuated chambers were equipped with 2 nose-poke holes and a liquid receptacle located between them. Responses in the active (MA-reinforced) hole resulted in the activation of the infusion pump, delivery of 20 µL of an unadulterated MA solution into the receptacle, and the presentation of a 20-sec light/tone compound stimulus. During the 20-sec MA-delivery period, further responding in the active hole was recorded but had no programmed consequences. Throughout the session, responding in the inactive hole had no programmed consequences but was recorded to assess the selectivity of responding as an index of reinforcer efficacy. At the end of each 1-h operant session, the volume of MA solution remaining in the receptacle was determined by pipetting. Mice were returned to the colony room and left undisturbed until the next day. Total MA intake was calculated each day by subtracting the volume of MA remaining in the receptacle from the total volume delivered and then was expressed as a function of body weight (in mg/kg), which was measured weekly (e.g., Brown et al., 2020; Ruan et al., 2020; Szumlinski et al., 2017).

As conducted in previous reports (Brown et al., 2020; Szumlinski et al., 2017), mice were initially trained under an FR1 schedule of reinforcement, with a 20 mg/L MA solution serving as the reinforcer. In Experiment 1, mice were given IP injections of 5 mg/kg CNO 30 minutes prior to each session of the acquisition phase (**Figure 9C**). Mice were trained in this manner until they reached acquisition criteria, which required the mice to emit at least 10 active nose-pokes during the 1-h session, with a minimum of 70% of their total nose-pokes directed at the active hole, across 3 consecutive sessions. Subjects that did not reach these

criteria within 14 sessions were considered to have not acquired the operant-response and were excluded from the rest of the study.

Animals that did successfully meet the acquisition criteria in Experiment 1 were then tested for MA reinforcement under increasing schedules of reinforcement (Brown et al., 2020; Szumlinski et al., 2017). For this, we progressively increased the number of nose-pokes required for delivery of the 20 mg/L MA (maintaining the 20-sec time-out) over subsequent days from 1 to 2 to 5, with 5 sessions conducted under each reinforcement schedule. On the 5th session for each schedule, mice received a CNO injection 30 minutes before the start of the session to examine the effects of subcircuit manipulation upon responding under the increasing response requirements (**Figure 9D**).

In Experiment 2, animals that successfully reached the acquisition criteria for MA self-administration were pretreated IP with 5 mg/kg CNO and tested, 30 minutes later, for MA reinforcement and taking over a 1-hour period. Additional testing sessions were conducted over the next 3 days, in the absence of any further CNO treatment, to examine for potential carry-over effects (**Figure 9D**).

In order to test the contributions of the targeted sub-circuits to the persistence of the conditioned operant response, mice in Experiment 2 then underwent additional 1-hour sessions under extinction conditions (i.e., no MA delivery or light/tone stimulus presentation) in the presence of CNO treatment (30 min prior) until the number of active nose-pokes dropped below 25% of initial MA-free responding across 3 consecutive sessions. Mice that failed to meet these extinction criteria within 30 sessions were excluded from the remainder of the study (<5% of mice were excluded). Mice that reached our extinction criteria within the allotted time were then assayed for the effects of prior CNO treatment upon cue-induced reinstatement of the operant response. For this final phase of the experiment, responding in

the active hole resulted in the presentation of the light/tone stimulus only over the 1-hour session.

3.2.7 Place-conditioning

Consistent with our previous reports (Brown et al., 2020; Szumlinski et al., 2017), MA place-conditioning procedures included three main phases: preconditioning test (session 1), MA/SAL conditioning (sessions 2-9), and postconditioning test (sessions 10-11). The apparatus consisted of two-visually and tactilely differentiated compartments, one with black and white marble patterned walls and a textured floor, and the other with wood-patterned walls and a smooth floor. During the habituation and post-test sessions, mice were allowed to freely explore both compartments for 15 minutes through a divider with a door. During MA-conditioning sessions, mice received 2 mg/kg MA IP injections and then were immediately confined to one compartment. In alternating sessions, mice received SAL injections of equivalent volume (10 mL/kg) and were then immediately confined to the other compartment. Mice underwent four, 15-minute, conditioning sessions for each unconditioned stimulus. Thirty minutes prior to the post-test sessions, animals were given a 5 mg/kg CNO IP injection. The first post-test was conducted in a MA-free state to determine the effect of subcircuit manipulation on the unadulterated MA-conditioned approach response. The second post-test was conducted after a MA priming injection of a 2 mg/kg dose to examine behavior under the drugged state (Kirkpatrick and Bryant, 2015; Mucha and Iverson, 1984). Overall, the mice did not exhibit a strong preference for one compartment vs. the other during the habituation session, so CPP score was calculated by subtracting the time spent on the SAL-paired side during the post-test from the time spent on the MA-paired side. This difference score served as a measure of the direction and magnitude of the MA-conditioned response (Brown et al., 2020; Szumlinski et al., 2017; Fultz and Szumlinski, 2018). During

each 15-minute session, the locomotor activity of the animals was recorded by digital video cameras, interfaced with a PC-type computer equipped with ANY-Maze software (Stoelting, Wood Dale, IL, USA). The distance traveled (in m) was recorded during each session. As in prior studies (Brown et al., 2020; Szumlinski et al., 2017), MA-induced locomotor sensitization was calculated by subtracting the distance traveled during the first MA conditioning session from that on the last MA conditioning session.

3.2.8 Histology

Animals were euthanized with an overdose of Euthasol (Virbac AH, Fort Worth, TX, USA) and transcardially perfused with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde. Brains were then removed and cold-stored in PBS until slicing. Tissue was sectioned 40 µm thin along the coronal plane on a vibratome, from the level of the PFC to the level of the NAC. All tissue was mounted on slides and cover-slipped. Slides were viewed using the 10x objective of a Keyence BZ-X800 All-in-one Fluorescence Microscope and BZ-X800 Viewer software.

3.2.9 Statistical Analyses

The effects of CNO injection on the relative number of subjects in each virus infusion group that reached acquisition criteria were assessed using a Chi-squared test, and number of trials required to reach these criteria was analyzed using a one-way ANOVA with the between subjects factor of Virus (Gq vs. mCherry vs. Gi). As different subcircuits may be recruited very early during drug-related learning and the extinction of that learning versus those engaged later in these learning processes (Everitt and Robbins, 2005), the data from the first 3 and last 3 self-administration sessions were analyzed to index initial responding and the magnitude of the learned response, respectively, in a manner similar to published work (Parsegian and See, 2014). Operant conditioning data were analyzed with ANOVAs, with

the between subjects factor of Virus and the within subjects factor of either Trial or Schedule of Reinforcement (FR1, FR2, or FR5) where appropriate. Place conditioning data were tested via one-way ANOVAs with the between subjects factor of Virus. For all ANOVAs the homogeneity of variance was confirmed, and alpha was set at 0.05 for all analyses. All data were analyzed on SPSS version 25 (IBM).

3.3 Results

3.3.1 Gq-DREADD stimulation of the PL-shell subcircuit blocks the acquisition of MA self-administration

The data pertaining to the relative number of subjects within each virus infusion group that reached acquisition criteria and the average number of trials required to reach acquisition criteria are presented in **Table 2**, along with the results of the statistical analyses of these data. Virus infusion into the PL-core, IL-core or IL-shell sub-circuits had no effect upon either the relative number of subjects that reached acquisition criteria (**Table 2, top**) or the number of trials taken to reach that acquisition criteria (**Table 2, bottom**). In stark contrast, none of the mice infused with Gq-DREADD into the PL-shell circuit reached acquisition criteria by the end of the 14-day training session (see **Table 2**).

3.3.2 Early acquisition of MA self-administration behavior

***Gq-DREADD stimulation of the PL-core subcircuit increases initial responding for MA*.**

The data for the effects of chemogenetic stimulation and inhibition of the four PFC-NAC sub-circuits on the number of responses in the active and inactive holes, as well as MA intake, over the first 3 days of acquisition training are summarized in **Figure 10.** As reported previously by our group (Brown et al., 2020; Ruan et al., 2020; Szumlinski et al., 2017), responding in both the active (**Figure 10A-D**) and inactive (**Figure 10E-H**) holes was relatively high on the first day of training in all sub-experiments and declined over the next

Table 2. PL-shell activity prevents subjects from reaching acquisition criteria. Summary of the effects of chemogenetic manipulation of PL-NAC subcircuits on the percentage of subjects that met the acquisition criteria for oral MA self-administration (**top**) and the number of acquisition trials required to reach those criteria (**bottom**) in Experiment 1. The data represent the means ± SEMs of the number of mice indicated in parentheses. Statistical results for each analysis are also provided. Statistically significant results are bolded.

Study 1 Acquisition				
Percent of Subjects Reaching Criteria				
	mCherry	Gq	Gi	Statistic
PL-Core	90% ± 10 (n=10)	80% ± 13 (n=10)	100% ± 0 (n=8)	X^2(2, N=28)=1.867, p=0.393
PL-Shell	71% ± 18 (n=7)	0% ± 0 (n=9)	44% ± 18 (n=9)	**X^2(2, N=25)=9.155, p=0.010**
IL-Core	77% ± 12 (n=13)	92% ± 8 (n=13)	77% ± 12 (n=13)	X^2(2, N=39)=1.393, p=0.498
IL-Shell	67% ± 17 (n=9)	67% ± 17 (n=9)	78% ± 15 (n=9)	X^2(2, N=27)=0.355, p=0.837
Number of Acquisition Trials				
	mCherry	Gq	Gi	Statistic
PL-Core	9.80 ± 1.13 (n=10)	9.50 ± 1.34 (n=10)	9.38 ± 0.78 (n=8)	F(2,25)=0.035, p=0.965
PL-Shell	11.57 ± 1.65 (n=7)	15.00 ± 0 (n=9)	12.11 ± 1.20 (n=9)	F(2,22)=2.915, p=0.075
IL-Core	7.77 ± 1.25 (n=13)	8.31 ± 1.00 (n=13)	8.69 ± 1.15 (n=13)	F(2,36)=0.167, p=0.847
IL-Shell	9.67 ± 1.52 (n=9)	10.22 ± 1.41 (n=9)	9.56 ± 1.51 (n=9)	F(2,24)=0.058, p=0.944

few days of training [Trial effect for active hole, for PL-core: $F(2,50)=13.836$, $p<0.0001$; for PL-shell: $F(2,44)=37.205$, $p<0.0001$; for IL-core: $F(2,72)=20.197$, $p<0.0001$; for IL-shell: $F(2,48)=9.361$, $p<0.0001$; Trial effect for inactive hole, for PL-core: $F(2,50)=19.653$, $p<0.0001$; for PL-shell: $F(2,44)=49.526$, $p<0.0001$; for IL-core: $F(2,72)=98.594$, $p<0.0001$; for IL-shell: $F(2,48)=18.458$, $p<0.0001$]. Also, the majority of mice directed approximately 60% of their total responses towards the active hole during early testing (**Figure 10I-L**), and this hole-discrimination either not vary over the first 3 days of training in mice in which the PL was targeted [Trial effect, for PL-core: $F(2,50)=1.083$, $p=0.346$; for PL-shell: $F(2,44)=0.186$, $p=0.831$] or gradually increased in mice in which the IL was targeted [for IL-core: $F(2,72)=5.322$, $p=0.007$; for IL-shell: $F(2,48)=6.664$, $p=0.003$] (**Figure 10I-L**).

Mice expressing the Gq-DREADD in the PL-core sub-circuit emitted more active (**Figure 10A**) nose-pokes during the first acquisition trial than either those expressing the Gi-DREADD or mCherry. This observation was supported by a statistical trend towards a significant Virus X Trial interaction [$F(4,50)=2.349$, $p=0.067$], and a follow-up analysis of the active hole-responding on Trial 1 confirmed this observation [one-way ANOVA, $F(2,25)=5.190$, $p=0.013$; LSD post-hoc tests: Gq vs. mCherry, $p=0.013$; Gq vs. Gi, $p=0.009$; one-way ANOVAs for Trials 2 and 3, p's>0.40]. A similar pattern of results were obtained for responding directed at the inactive hole (**Figure 10E**) [Virus X Trial interaction [$F(4,50)=2.946$, $p=0.029$; trial 1 one-way ANOVA, $F(2,25)=3.420$, $p=0.049$; LSD post-hoc tests: Gq vs. mCherry, $p=0.033$; Gq vs. Gi, $p=0.0035$; one-way ANOVAs for Trials 2 and 3, p's>0.30]. As PL-core Gq-DREADD infusion increased responding in both the active and inactive holes, there was no effect of this manipulation or Gi-DREADD infusion on hole discrimination during early testing (**Figure 10I**) [Virus effect: $F(2,25)=0.481$, $p=0.624$; Virus X Trial interaction: $F(4,50)=0.189$, $p=0.943$]. In contrast, neither Gq- nor Gi-DREADD

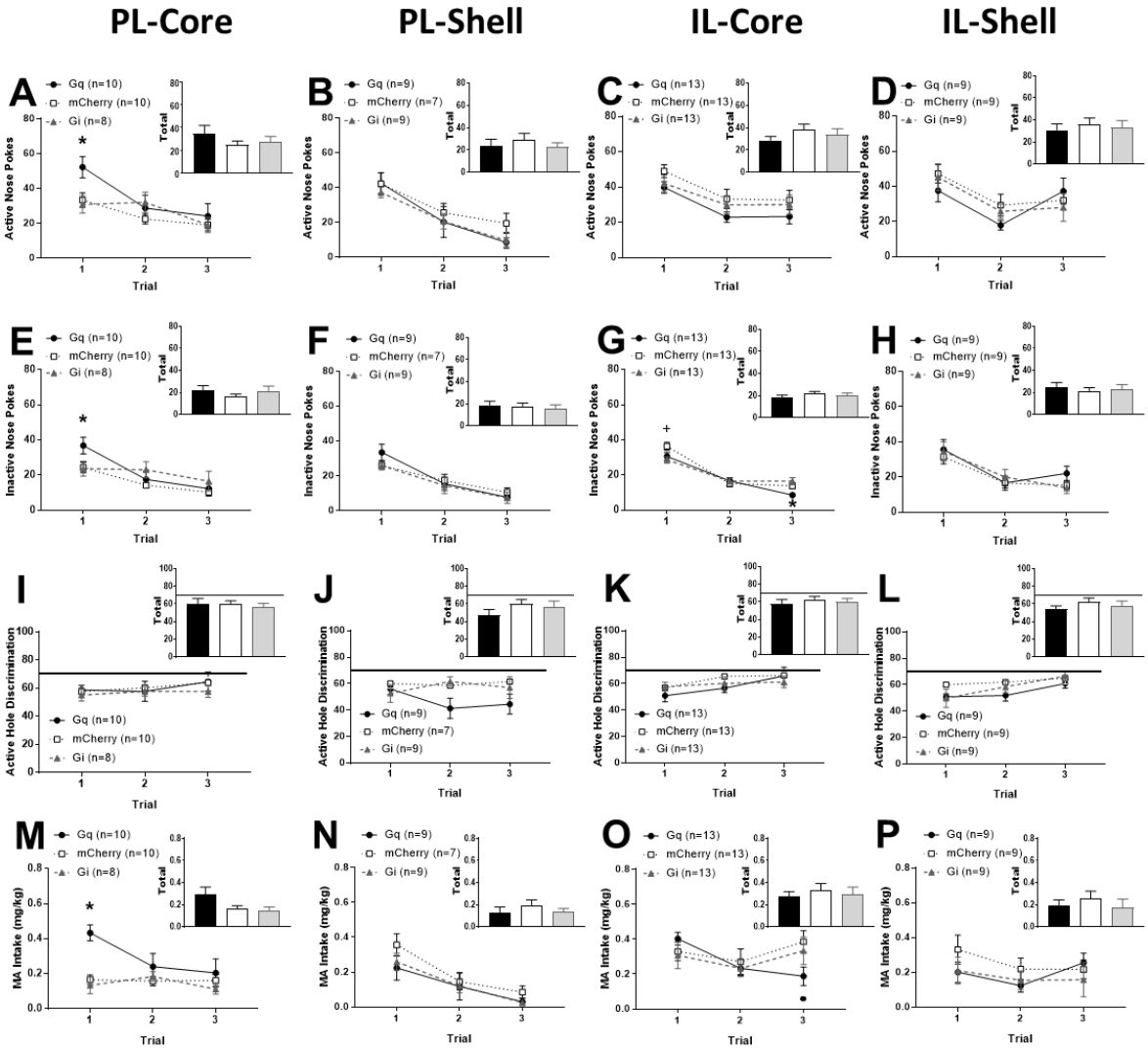


Figure 10. Summary of the effects of chemogenetic manipulations of PFC-NAC sub-circuits on behavior during early acquisition of oral MA self-administration. (A-D) Summary of the data for the total number of nose-pokes into the active, MA-reinforced, hole emitted by mice infused with AAVs carrying a Gq-DREADD (Gq), a Gi-DREADD (Gi) or mCherry control vector (mCherry) over the first 3 days of self-administration training. **(E-H)** Summary of the data for the total number of nose-pokes into the inactive, non-reinforced, hole emitted by the same mice. **(I-L)** Summary of the data for the percentage of total responses directed at the active, MA-reinforced, hole (discrimination). **(M-P)** Summary of the total MA intake (mg/kg body weight) during each of the 1-h sessions. The data represent the means ± SEMs of the number of mice indicated in parentheses. *p<0.05 Gq vs. other groups; •p<0.05 Gq vs. mCherry; +p<0.05 mCherry vs. other groups (post-hoc tests).

infusion into the PL-shell projection affected responses directed at the active or inactive hole during early training (**Figure 10B,10F**) [for active hole, Virus effect: F(2,22)=0.571, p=0.573; Virus X Trial: F(4,44)=0.514, p=0.726; for inactive hole, Virus effect: F(2,22)=0.330, p=0.723; Virus X Trial: F(4,44)=1.442, p=0.236]. Not surprisingly, chemogenetic manipulation of the PL-Shell subciruit also did not affect hole discrimination during early training either (**Figure 10J**) [Virus effect: F(2,22)=2.004, p=0.159; Virus X Trial interaction: F(4,44)=1.640, p=0.181]. Although neither DREADD infusion into the IL-core projection altered responding in the active hole (**Figure 10C**) [Virus effect: F(2,36)=1.696, p=0.198; Virus X Trial interaction: F(4,72)=0.198, p=0.939], both DREADDs lowered the number of responses in the inactive hole on Trial 1, relative to mCherry controls, with the mice infused with the Gq-DREADD also exhibiting lower inactive hole responding on Trial 3 versus both mCherry controls and mice infused with the Gi-DREADD (**Figure 10G**) [Virus effect: F(2,36)=1.728, p=0.192; Virus X Trial interaction: F(4,72)=4.126, p=0.005; one-way ANOVA for trial 1, F(2,36)=3.806, p=0.032 with LSD post-hoc tests: mCherry vs. Gq, p=0.053; mCherry vs. Gi, p=0.012; one-way ANOVA for Trial 2, p>0.40; one-way ANOVA for Trial 3, F(2,36)=5.198, p=0.010 with LSD post-hoc tests: mCherry vs. Gq: p=0.041; Gq vs Gi: p=0.003]. Despite the observed group differences in responding in the inactive hole, chemogenetic manipulation of the IL-core did not alter response discrimination during early training (**Figure 10K**) [Virus effect: F(2,36)=0.665, p=0.520; Virus X Trial interaction: F(4,72)=0.876, p=0.483]. Finally, chemogenetic manipulations of the IL-shell projection did not alter responding in either the active (**Figure 10D**) [Virus effect: F(2,24)=0.390, p=0.681; Virus X Trial interaction: F(4,48)=1.075, p=0.379] or inactive hole (**Figure 10H**) [Virus effect: F(2,24)=0.0.483, p=0.623; Virus X Trial interaction: F(4,48)=0.600, p=0.665], nor did it alter response

discrimination during early training (**Figure 10L**) [Virus effect: $F(2,24)=1.305$, $p=0.290$; Virus X Trial interaction: $F(4,48)=0.804$, $p=0.529$].

Gq-DREADD stimulation of the PL-core subcircuit increases initial MA intake. The data for MA intake across the first 3 days of self-administration training are summarized in **Figure 10M-P**. As reported previously by our group (Brown et al., 2020; Ruan et al., 2020; Szumlinski et al., 2017), MA intake during early self-administration training was relatively high on Trial 1 and generally declined over the next few days of training [Trial effect, for PL-core: $F(2,50)=4.577$, $p=0.015$; for PL-shell: $F(2,44)=0.346$, $p<0.0001$; for IL-core: $F(2,72)=4.248$, $p=0.018$; for IL-shell: $F(2,48)=1.963$, $p=0.152$].

In line with their data for active hole responding (**Figure 10A**), Gq-DREADD infusion in the PL-core increased MA intake, but only during the first self-administration session (**Figure 10M)**. This finding was supported by the results of a Virus X Trial ANOVA [$F(4,50)=4.783$, $p=0.002$], and a follow-up group comparison on Trial 1 [one-way ANOVA, $F(2,25)=16.980$, $p<0.0001$; LSD post-hoc tests: Gq vs. mCherry, $p<0.0001$; Gq vs. Gi, $p<0.0001$; one-way ANOVAs for Trials 2 and 3, p's>0.45]. No virus effect was observed on the MA intake during the first 3 trials of acquisition of animals infused into the PL-shell (**Figure 10N**) [Virus effect: $F(2,22)=1.228$, $p=0.312$; Virus x Trial interaction: $F(4,44)=0.323$, $p=0.861$] or the IL-shell (**Figure 10P**) [Virus effect: $F(2,24)=0.663$, $p=0.524$; Virus X Trial interaction: $F(4,48)=0.888$, $p=0.478$]. However, Gq-DREADD infusion into the IL-core sub-circuit decreased MA intake during the third self-administration session (**Figure 10O**). This result was supported by a significant Virus X Trial interaction [$F(4,72)=3.084$, $p=0.021$] and a follow-up analysis on Trial 3 [one-way ANOVA, $F(2,36)=2.478$, $p=0.098$; LSD post-hoc tests: Gq vs. mCherry, $p=0.039$; Gq vs. Gi, $p=0.122$; one-way ANOVAs for Trials 1 and 2, p's>0.45].

3.3.3 Late acquisition of MA self-administration behavior

Gq-DREADD stimulation of the PL-shell subcircuit reduces MA-directed responding and intake during late acquisition. Initial analyses of the time-course of MA-directed responding and intake failed to indicate any main effect of, or interaction with, the Trial factor (for all analyses, Time effect, $p>0.200$; Virus X Time interactions, $p>0.230$). As such, the data for the average number of active and inactive hole pokes over the last 3 self-administration trials, as well as the average hole discrimination and MA intake during this period, are summarized in **Figure 11**. Neither the Gq- nor the Gi-DREADD affected these indices of MA reinforcement when infused into the PL-Core (**Figure 11A,E,I,M**) [for active hole: $F(2,25)=2.226$, $p=0.129$; for inactive hole: $F(2,25)=0.947$, $p=0.401$; for discrimination: [$F(2,25)=0.311$, $p=0.735$; for intake: $F(2,25)=0.916$, $p=0.413$], the IL-Core (**Figure 11C,G,K,O**) [for active hole: $F(2,36)=0.088$, $p=0.916$; for inactive hole: $F(2,36)=0.468$, $p=0.630$; for discrimination: $F(2,36)=0.688$, $p=0.509$; for intake: $F(2,36)=0.172$, $p=0.842$], or the IL-Shell (**Figure 11D,H,L,P**) [for active hole: $F(2,24)=0.544$, $p=0.587$; for inactive hole: $F(2,24)=1.671$, $p=0.209$; for allocation: $F(2,24)=0.105$, $p=0.901$; for intake: $F(2,24)=0.644$, $p=0.534$]. In contrast, Gq-DREADD infusion into the PL-shell sub-circuit reduced the average number of active hole-pokes during the last 3 days of testing (**Figure 11B**) [$F(2,22)=5.105$, $p=0.015$; LSD post-hoc tests: Gq vs. mCherry, $p=0.005$; Gq vs. Gi, $p=0.052$], without affecting inactive hole responding (**Figure 11F**) [$F(2,22)=0.083$, $p=0.920$]. Accordingly, mice infused with Gq-DREADD into the PL-shell projection also exhibited reduced hole discrimination at this time (**Figure 11J**) [one-way ANOVA: $F(2,22)=3.249$, $p=0.058$; LSD post-hoc tests: Gq vs. mCherry, $p=0.020$; Gq vs. Gi, $p=0.142$] and lower MA intake (**Figure 11M**) [$F(2,22)=5.244$, $p=0.014$; LSD post-hoc tests: Gq vs. mCherry, $p=0.004$; Gq vs. Gi, $p=0.053$].

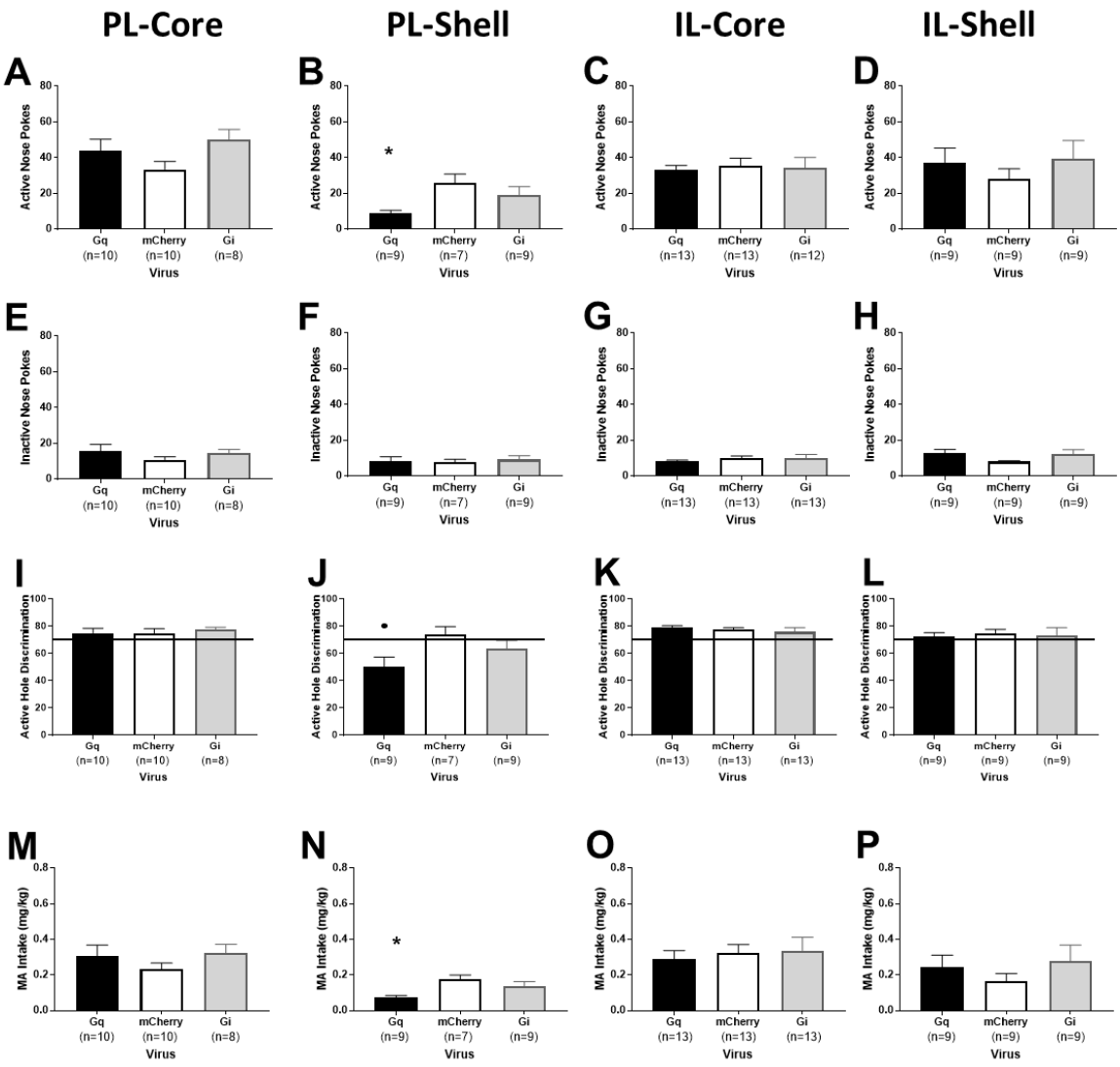


Figure 11. Summary of the effects of chemogenetic manipulations of PFC-NAC sub-circuits on behavior during late acquisition of oral MA self-administration. (A-D) Summary of the average number of nose-pokes into the active, MA-reinforced, hole emitted by mice infused with AAVs carrying a Gq-DREADD (Gq), a Gi-DREADD (Gi) or mCherry control vector (mCherry) over the last 3 days of self-administration training. **(E-H)** Summary of the mean number of nose-pokes into the inactive, non-reinforced, hole emitted by the same mice. **(I-L)** Summary of the data for the percentage of total responses directed at the active, MA-reinforced, hole (discrimination) during late acquisition. **(M-P)** Summary of the mean total MA intake (mg/kg body weight) during the last 3 days of self-administration. The data represent the means ± SEMs of the number of mice indicated in parentheses. *$p<0.05$ Gq vs. other groups. •$p<0.05$ Gq vs. mCherry.

3.3.4 Demand testing

Gq-DREADD stimulation of the PL-core subcircuit reduces MA response allocation, but not MA intake, during demand testing. The data for the effects of CNO injection upon active hole-responding, response allocation and MA intake during demand testing are summarized in **Figure 12**. As reported previously (Brown et al., 2020; Ruan et al., 2020; Szumlinski et al., 2017), the total responding in the active hole was relatively stable as the response requirement increased (**Figure 12A-D**) [FR effect, for PL-core: $F(2,44)=0.583$, $p=0.562$; for PL-shell: effect of FR: $F(2,12)=0.338$, $p=0.720$] or increased modestly with higher response requirement [FR effect, for IL-core: effect of FR: $F(2,56)=2.688$, $p=0.077$; for IL-shell: effect of FR: $F(2,30)=11.460$, $p<0.0001$]. Responding in the inactive hole was uninfluenced by the reinforcement schedule in mice with chemogenetic manipulations of the PL-core, PL-shell and IL-core (**Figure 12E-G**) [FR effect, for PL-core: $F(2,44)=1.520$, $p=0.230$; for PL-shell: $F(2,12)=0.259$, $p=0.776$; for IL-core: $F(2,56)=0.167$, $p=0.847$], while mice in the IL-shell study increased inactive hole responding within increasing schedules of reinforcement (**Figure 12H**) [FR effect: $F(2,30)=12.104$, $p<0.001$]. None of the chemogenetic manipulations altered hole discrimination (**Figure 12I-L**) [FR effect, for PL-core: $F(2,44)=1.934$, $p=0.157$; for PL-shell: $F(2,12)=2.682$, $p=0.109$; for IL-core: $F(2,56)=0.340$, $p=0.713$; for IL-shell: effect of FR: $F(2,30)=1.019$, $p=0.373$]. However, consistent with our prior work, MA intake declined as a function of increasing response requirement in mice well-trained to nose-poke for oral MA (**Figure 12M-P)** [FR effect, for PL-core: $F(2,44)=20.050$, $p<0.0001$; for PL-shell: $F(2,12)=4.384$, $p=0.037$; for IL-core: $F(2,58)=23.800$, $p<0.0001$; for IL-shell: $F(2,30)=6.219$, $p=0.006$].

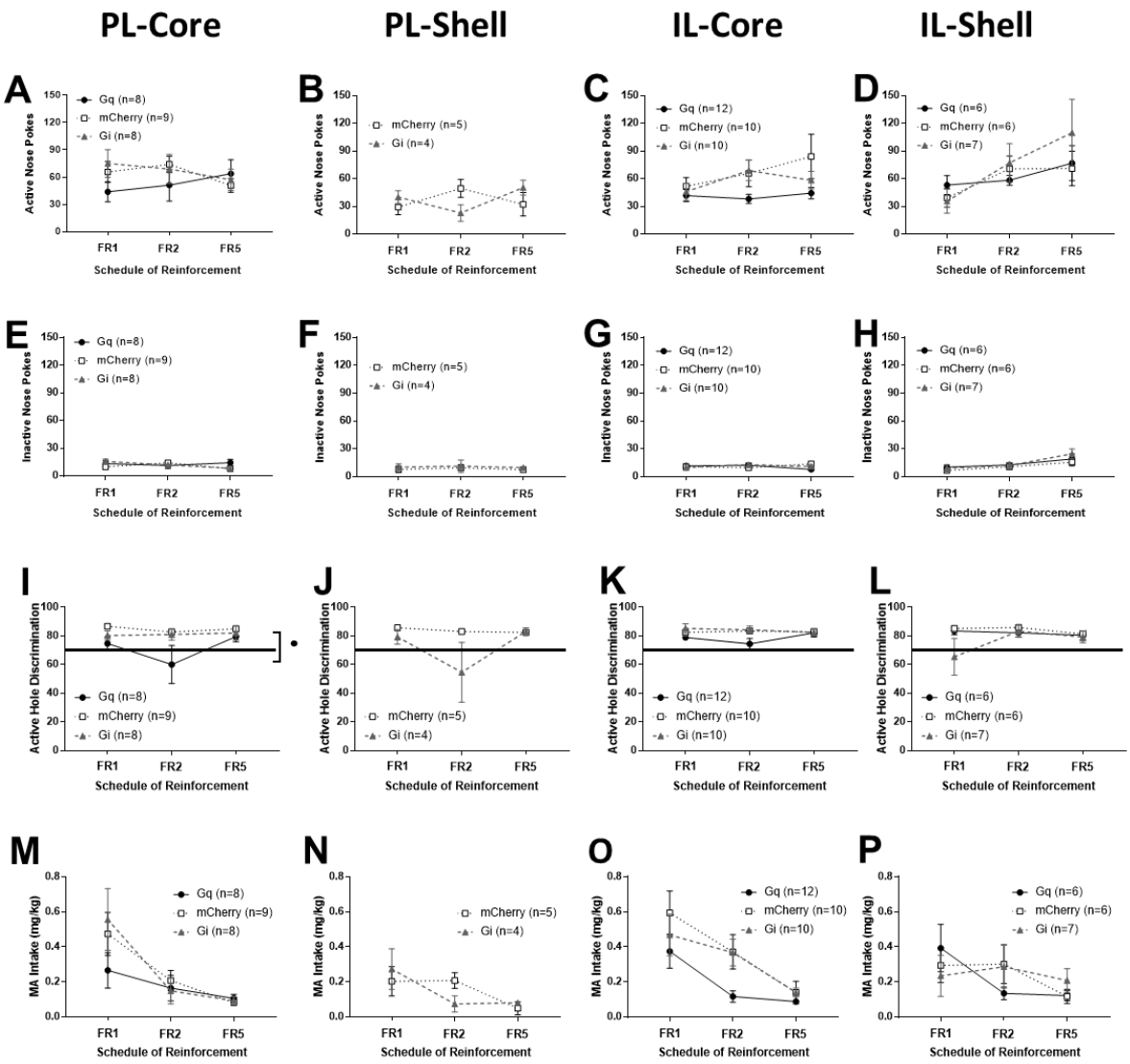


Figure 12. Summary of the effects of chemogenetic manipulations of PFC-NAC sub-circuits on behavior during MA demand testing. (A-D) Summary of the average number of nose-pokes into the active, MA-reinforced, hole emitted by mice infused with AAVs carrying a Gq-DREADD (Gq), a Gi-DREADD (Gi) or mCherry control vector (mCherry) under an FR1, FR2 and FR5 schedule of reinforcement. **(E-H)** Summary of the mean number of nose-pokes into the inactive, non-reinforced, hole emitted by the same mice. **(I-L)** Summary of the data for the percentage of total responses directed at the active, MA-reinforced, hole (discrimination) during demand testing. **(M-P)** Summary of the mean total MA intake (mg/kg body weight) with increasing MA demand. The data represent the means ± SEMs of the number of mice indicated in parentheses. •$p<0.05$ Gq vs. mCherry.

Gq-DREADD infusion into the PL-core sub-circuit reduced hole discrimination during demand testing (**Figure 12I**) [Virus effect: F(2,22)=3.687, p=0.042; LSD post-hoc tests: Gq vs. mCherry, p=0.015; Gq vs. Gi, p=0.075; Virus x FR interaction: F(4,44)=1.183, p=0.325]. This Gq-DREADD effect on hole discrimination reflected an increase in inactive hole responding under the FR5 reinforcement schedule as indicated by a trend towards a Virus x FR interaction for inactive hole responding (**Figure 12E**) [Virus effect: F(2,22)=0.441, p=0.649; Virus x FR interaction: F(4,44)=2.389, p=0.065], with no change detected for either active hole responding (**Figure 12A**) [Virus effect: F(2,22)=0.438, p=0.651; Virus x FR interaction: F(4,44)=1.920, p=0.124] or MA intake (**Figure 12M**) [Virus effect: F(2,22)=0.458, p=0.638; Virus x FR interaction: F(4,44)=1.736, p=0.159].

As mice infused with a Gq-DREADD into the PL-shell projection did not acquire MA self-administration behavior (**Table 2**), they were not tested in the demand-testing phase of the study. Gi-DREADD infusion into the PL-shell projection did not affect total or relative responding, nor did it alter MA intake, during demand testing (**Figure 12B,F,J,N**) [for active nose-pokes, Virus effect: F(1,6)=0.168, p=0.696; Virus x FR interaction: F(2,12)=2.666, p=0.112; for inactive hole pokes: Virus effect: F(1,6)=0.666, p=0.446; Virus x FR interaction: F(2,12)=0.050, p=0.951; for response allocation, Virus effect: F(1,6)=3.441, p=0.113; Virus x FR interaction: F(2,12)=2.576, p=0.117; for intake: [Virus effect: F(1,6)=0.001, p=0.971; Virus x FR interaction: F(2,12)=2.451, p=0.128]. Neither DREADD infused into the IL-core affected behavior during demand-testing (**Figure 12C,G,K,O**) [for active nose-pokes, Virus effect: F(2,28)=1.963, p=0.159; Virus x FR interaction: F(4,56)=1.418, p=0.220; for inactive nose-pokes, Virus effect: F(2,28)=0.483, p=0.623; Virus x FR interaction: F(4,56)=2.040, p=0.101; for response allocation, Virus effect: F(2,28)=2.276, p=0.121; Virus x FR interaction: F(4,56)=1.021, p=0.405; for intake: Virus

effect: F(2,28)=2.375, p=0.111; Virus x FR interaction: F(4,56)=1.196, p=0.322], or the IL-shell (**Figures 12D,H,L,P**) [for active nose-pokes, Virus effect: F(2,15)=0.274, p=0.764; Virus x FR interaction: F(4,30)=1.623, p=0.194; for response allocation, Virus effect: F(2,15)=0.489, p=0.622; Virus x FR interaction: F(4,30)=0.666, p=0.620; for intake: Virus effect: F(2,15)=0.060, p=0.942; Virus x FR interaction: F(4,30)=1.983, p=0.123].

3.3.5 Manipulations of PL-NAC subcircuits do not affect the expression of behavior under a MA-conditioned place-conditioning paradigm

The data and statistical results pertaining to the MA-conditioned place-preference paradigm of Study 2 are summarized in **Table 3**. We were only able to detect a conditioned place-preference in animals infused with Gi-DREADD in the PL-shell under a MA-primed state. All other groups expressed place-ambivalence following place-conditioning procedures. Viral infusion into the PL-core or PL-shell sub-circuits did not affect either the time spent in the MA-conditioned compartment nor did it affect locomotor activity of the mice during either conditioning test (**Table 3**).

3.3.6 Manipulations of PL-NAC subcircuits do not affect the maintenance of MA self-administration behavior in well-trained mice

The percentage of animals in Experiment 2 that successfully reached the acquisition criteria and the data for the average number of hole pokes, response allocation, and MA intake over the last 3 acquisition trials of Experiment 2 are presented in **Table 4**. Prior to any CNO pretreatment, we detected no group differences for any of our measures in mice infused with DREADDs into the PL-core projection (**Table 4, top**). Inexplicably, mice infused with either DREADD into the PL-shell projection emitted more nose-pokes into the inactive hole, relative to mCherry controls, but did not differ with respect to active hole-poking or MA intake (**Table 4, bottom**).

Table 3. DREADDs infusion did not alter MA place-conditioning. Summary of the results of the MA-induced place-conditioning study in which mice were infused with either Gq- or Gi-DREADDS into the PL-NAC subcircuits and injected with 5 mg/kg CNO prior to the tests for a conditioned place-preference (CPP) in a MA-free and –primed state. The data represent the means ± SEMs of the number of mice indicated in parentheses. Statistical results for each analysis are also provided.

Conditioned Place Preference				
PL-Core				
	mCherry (n=12)	Gq (n=13)	Gi (n=12)	Statistic
Acute Locomotion	32.89 ± 2.21	29.77 ± 2.14	36.78 ± 3.59	F(2,34)=1.710, p=0.196
Sensitization	2.37 ± 2.55	5.05 ± 2.64	3.50 ± 3.25	F(2,34)=0.232, p=0.794
CPP	81.37 ± 82.23	-52.22 ± 65.17	4.88 ± 53.19	F(2,34)=0.986, p=0.384
CPP Locomotion	28.54 ± 1.55	26.88 ± 1.63	27.79 ± 2.43	F(2,34)=0.194, p=0.825
MA-prime CPP	70.567 ± 40.68	24.95 ± 63.86	-51.00 ± 85.40	F(2,34)=0.848, p=0.437
MA-primed Locomotion	65.11 ± 4.07	58.90 ± 3.09	67.22 ± 4.76	F(2,34)=1.203, p=0.313
PL-Shell				
	mCherry (n=12)	Gq (n=13)	Gi (n=11)	Statistic
Acute Locomotion	28.75 ± 2.67	27.87 ± 1.51	33.74 ± 3.81	F(2,33)=1.320, p=0.281
Sensitization	9.41 ± 2.32	9.85 ± 2.22	11.81 ± 3.46	F(2,33)=0.220, p=0.804
CPP	-7.17 ± 95.24	89.59 ± 89.23	78.91 ± 91.53	F(2,33)=0.335, p=0.718
CPP Locomotion	28.40 ± 1.68	26.48 ± 1.23	30.73 ± 1.18	F(2,33)=2.335, p=0.113
MA-primed CPP	12.37 ± 102.44	27.63 ± 109.71	137.02 ± 81.89	F(2,33)=0.435, p=0.651
MA-primed Locomotion	60.80 ± 5.53	50.81 ± 4.80	62.99 ± 4.23	F(2,33)=1.787, p=0.183

Table 4. Summary of the results for the acquisition phase of oral MA self-administration of Experiment 2. Prior to any CNO pretreatment, no group differences were detected for any variable in mice in which the PL-Core projection was targeted (**top**). However, mice infused with the Gq- or Gi-DREADD into the PL-Shell subcircuit exhibited higher inactive hole-poking behavior prior to CNO injection, with no other group difference detected (**bottom**). The data represent the means ± SEMs of the number of mice indicated in parentheses. Statistical results for each analysis are also provided. +p<0.015 mCherry vs. other groups (LSD post-hoc tests).

Study 2 Acquisition of Self-Administration Prior to Introduction of CNO				
PL-Core				
	mCherry (n=12)	Gq (n=13)	Gi (n=12)	Statistic
Subjects Reaching Criteria	92% ± 8	85% ± 10	83% ± 11	X^2(2, N=37)=0.417, p=0.812
Active Pokes	48.81 ± 4.15	50.67 ± 3.72	54.50 ± 4.91	F(2,34)=0.452, p=0.640
Inactive Pokes	13.72 ± 2.17	17.05 ± 3.94	15.89 ± 2.14	F(2,34)=0.330, p=0.721
MA Intake (mg/kg)	0.45 ± 0.05	0.40 ± 0.05	0.47 ± 0.06	F(2,34)=0.544, p=0.585
PL-Shell				
	mCherry (n=12)	Gq (n=13)	Gi (n=11)	Statistic
Subjects Reaching Criteria	100% ± 0	69% ± 13	73% ± 14	X^2(2, N=36)=4.391, p=0.111
Active Pokes	44.17 ± 5.99	54.38 ± 8.04	49.24 ± 5.80	F(2,33)=0.581, p=0.565
Inactive Pokes	**9.64 ± 1.47**	**18.82 ± 2.44+**	**17.15 ± 1.68+**	**F(2,33)=6.316, p=0.005**
MA Intake (mg/kg)	0.39 ± 0.06	0.46 ± 0.07	0.42 ± 0.06	F(2,33)=0.289, p=0.751

The subjects in Experiment 2 that successfully reached the acquisition criteria were then tested for the expression of MA self-administration 30 minutes after CNO injection. The results are presented in **Figure 13** and show no effect of viral infusion into the PL-core or PL-shell on active responses **(Figure 13A,B)** [PL-core: $F(2,29)=0.167$, $p=0.847$; PL-shell: $F(2,26)=0.884$, $p=0.425$], inactive responses (**Figure 13C,D**) [PL-core: $F(2,29)=0.278$, $p=0.759$; PL-shell: $F(2,26)=0482$, $p=0.623$], hole discrimination **(Figure 13E,F)** [PL-core: $F(2,29)=0.923$, $p=0.409$; PL-shell: $F(2,26)=0.414$, $p=0.665$], or MA intake **(Figure 13G,H)** [PL-core: $F(2,29)=0.087$, $p=0.917$; PL-shell: $F(2,26)=0.897$, $p=0.418$] during the expression test.

3.3.7 Gq-DREADD stimulation of both PL-NAC subcircuits facilitated initial extinction of a MA-conditioned operant-response

Following 2 additional days of MA self-administration, we next determined the effects of chemogenetic manipulations of the PL-core and PL-shell projections on extinction learning. As conducted for the acquisition of operant-conditioning in Experiment 1, we examine the effects of DREADD activation during both early (first 3 days) and late (last 3 days) of extinction learning and the data for these different phases are summarized in **Figure 14** and **Figure 15**, respectively. As expected, active responding declined over the course of the first 3 extinction trials (**Figure 14A,B**) [Trial effect, for PL-core: $F(2,58)=12.729$, $p<0.0001$; for PL-shell: $F(2,52)=3.885$, $p=0.027$]. Responding in the inactive hole showed a moderate decline over the first 3 days of extinction training (**Figure 14C,D**) [Trial effect, for PL-core: $F(2,58)=3.142$, $p=0.051$; for PL-shell: $F(2,52)=2.058$, $p=0.138$]. Consequently, hole discrimination remained fairly stable, with animals continuing to direct approximately 70% of total responses towards the active hole (**Figure 14E,F**) [Trial effect, for PL-core: $F(2,58)=0.085$, $p=0.919$; for PL-shell: $F(2,52)=2.023$, $p=0.143$].

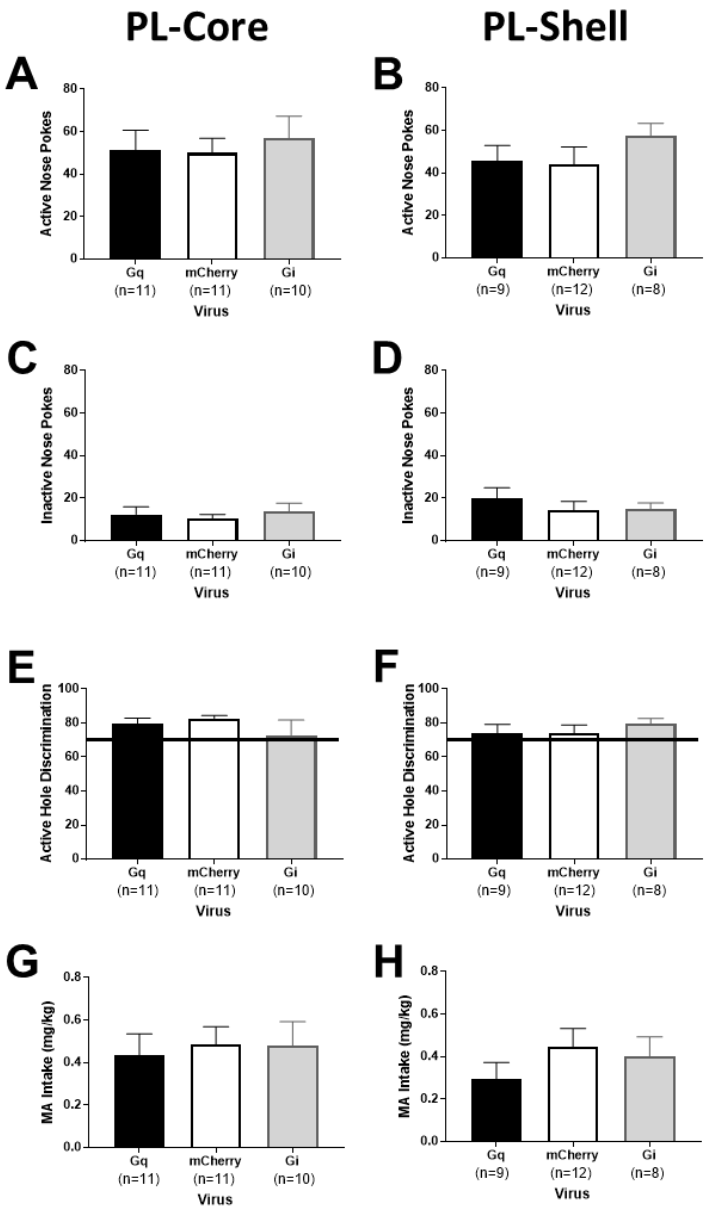


Figure 13. Summary of the effects of chemogenetic manipulations of PL-NAC sub-circuits on behavior during established MA self-administration behavior. (A,B) Summary of the average number of nose-pokes into the active, MA-reinforced, hole emitted by mice infused with AAVs carrying a Gq-DREADD (Gq), a Gi-DREADD (Gi) or mCherry control vector (mCherry) during a 1-h self-administration session. **(C,D)** Summary of the mean number of nose-pokes into the inactive, non-reinforced, hole emitted by the same mice. **(E,F)** Summary of the data for the percentage of total responses directed at the active, MA-reinforced, hole (discrimination) during established self-administration behavior. **(G,H)** Summary of the mean total MA intake (mg/kg body weight) during established self-administration behavior. The data represent the means ± SEMs of the number of mice indicated in parentheses.

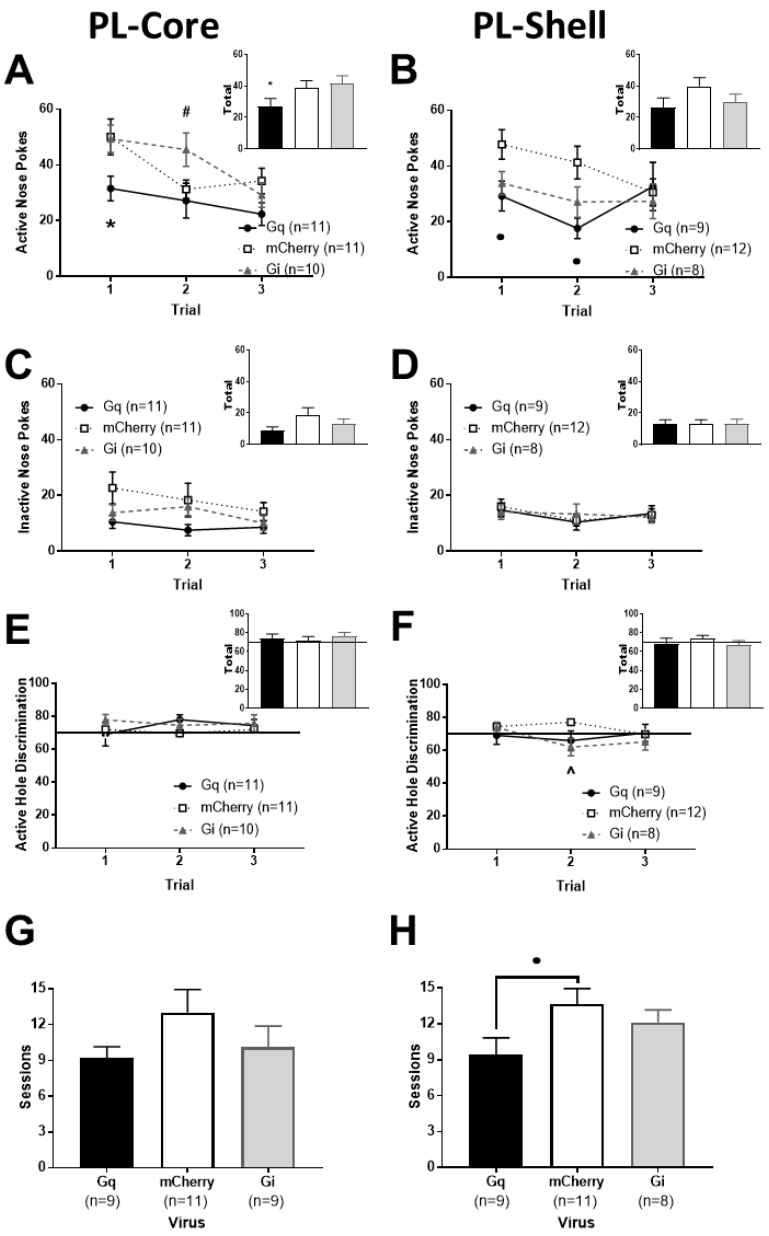


Figure 14. Summary of the effects of chemogenetic manipulations of PL-NAC sub-circuits on behavior during early extinction learning. (A,B) Summary of the average number of nose-pokes into the active, MA-reinforced, hole emitted by mice infused with AAVs carrying a Gq-DREADD (Gq), a Gi-DREADD (Gi) or mCherry control vector (mCherry) during the first 3 days of extinction learning. **(C,D)** Summary of the mean number of nose-pokes into the inactive, non-reinforced, hole emitted by the same mice. **(E,F)** Summary of the data for the percentage of total responses directed at the active, MA-reinforced, hole (discrimination) during early extinction training. **(G,H)** Summary of the mean total MA intake (mg/kg body weight) during early extinction. The data represent the means ± SEMs of the number of mice indicated in parentheses. *p<0.05 Gq vs. other groups. •p<0.05 Gq vs. mCherry. #p<0.08 Gi vs. other groups. ^p<0.05 Gi vs. mCherry.

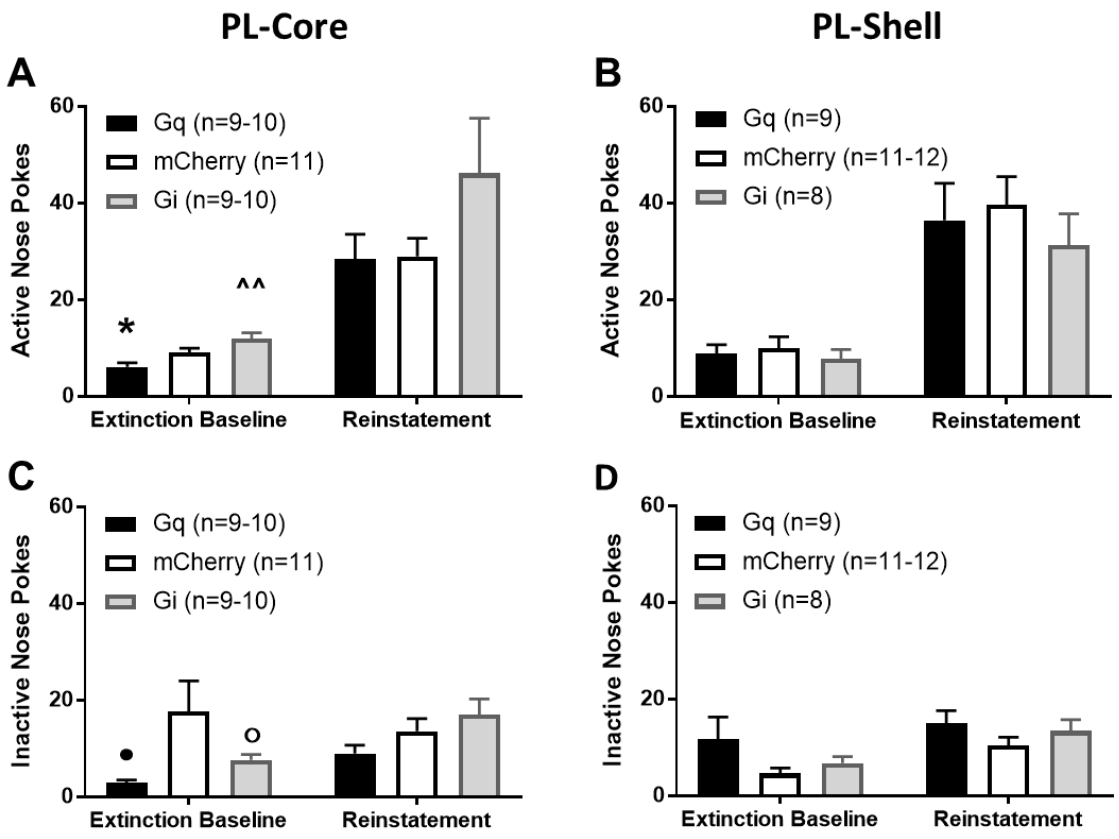


Figure 15. Summary of the effects of chemogenetic manipulations of PL-NAC sub-circuits on behavior at extinction baseline and during a test for cue-induced reinstatement of MA-seeking. (A,B) Summary of the average number of nose-pokes into the active, MA-reinforced, hole emitted by mice infused with AAVs carrying a Gq-DREADD (Gq), a Gi-DREADD (Gi) or mCherry control vector (mCherry) during the last 3 days of extinction learning (extinction baseline) and on the test for cue-induced reinstatement of MA-seeking. **(C,D)** Summary of the mean number of nose-pokes into the inactive, non-reinforced, hole emitted by the same mice. The data represent the means ± SEMs of the number of mice indicated in parentheses. *$p<0.05$ Gq vs. other groups. •$p<0.05$ Gq vs. mCherry. ^^$p<0.05$ Gi vs. other groups. ◦$p<0.10$ Gi vs. mCherry.

Gq-DREADD infusion into the PL-core lowered the number of active hole pokes early in the extinction training phase, versus mCherry- and Gi-DREADD infusion (**Figure 14A**). This finding was supported by a statistical trend toward a significant Virus X Trial interaction [$F(4,58)=2.409$, $p=0.059$], and a follow-up analysis of active responses revealed decreased active responses in Gq-infused animals during Trial 1 [one-way ANOVA, $F(2,29)=3.917$, $p=0.031$; LSD post-hoc tests: Gq vs. mCherry, $p=0.019$; Gq vs. Gi, $p=0.027$] and a trend toward increased active responses in Gi-infused animals during Trial 2 [one-way ANOVA, $F(2,29)=3.167$, $p=0.057$; LSD post-hoc tests: Gi vs. mCherry, $p=0.071$; Gi vs. Gq, $p=0.022$; one-way ANOVA for Trial 3, $p>0.15$]. This effect was selective for the active hole as no effect of chemogenetic manipulation of the PL-core was detected for inactive nose-pokes (**Figure 14C**) [Virus effect: $F(2,29)=2.367$, $p=0.112$; Virus x Trial interaction: $F(4,58)=1.137$, $p=0.348$]. However, neither chemogenetic manipulation of the PL-core projection altered hole discrimination during early extinction learning (**Figure 14E**) [Virus effect: $F(2,29)=0.425$, $p=0.658$; Virus X Trial interaction: $F(4,58)=0.973$, $p=0.429$].

Similar to the effect of Gq-DREADD expression in the PL-core sub-circuit, infusion of this DREADD into the PL-shell sub-circuit also decreased active responses in the early trials of the extinction phase, compared to mCherry and Gi-DREADD infusion (**Figure 14B**). Statistical analysis revealed a significant Virus X Trial interaction [$F(4,52)=3.411$, $p=0.015$], and follow-up tests show decreased responses from Gq-DREADD infused animals on Trial 1 [one-way ANOVA, $F(2,26)=3.797$, $p=0.036$; LSD post-hoc tests: Gq vs. mCherry, $p=0.015$; Gq vs. Gi, $p=0.557$] and on Trial 2 [one-way ANOVA, $F(2,26)=5.315$, $p=0.012$; LSD post-hoc tests: Gq vs. mCherry, $p=0.004$; Gq vs. Gi, $p=0.254$; one-way ANOVA for Trial 3, $p>0.80$]. The effect of chemogenetic stimulation of the PL-shell subcircuit was selective for the active hole as no DREADD effects were detected for inactive hole-poking during early

extinction (**Figure 14D**) [Virus effect: F(2,26)=0.015, p=0.985; Virus x Trial interaction: F(4,52)=0.408, p=0.802]. An examination of hole discrimination over the first 3 extinction trails revealed a significant Virus x Trial interaction (**Figure 14F**) [F(4,52)=2.578, p=0.048], which reflected a slight decrease in discrimination by Gi-DREADD infused mice on Trial 2 [one-way ANOVA, F(2,26)=2.741, p=0.083; LSD post-hoc tests: Gi vs. mCherry, p=0.038; Gi vs. Gq, p=0.605; one-way ANOVAs for Trials 1 and 3, p's>0.60].

Although it appeared that Gq-DREADD activation within both the PL-core and the PL-shell projections reduced the number of trials required to reach extinction criteria to a similar extent **(Figure 14G,H)**, we detected only a statistical trend for the data from the mice in which the PL-shell was targeted [one-way ANOVA, for PL-core: F(2,26)=1.470, p=0.248; for PL-shell: F(2,25)=2.845; p=0.077; LSD post-hoc tests: Gq vs. mCherry, p=0.026; Gq vs. Gi, p=0.172].

3.3.8 Gq-DREADD stimulation of only the PL-core subcircuit facilitated late extinction of a MA-conditioned operant-response, without altering subsequent cue-elicited reinstatement of responding

The data for the last 3 extinction trials under CNO pretreatment (extinction baseline) and for the subsequent test for cue-elicited reinstatement of responding (sans CNO) are presented in **Figure 15**. An examination of the extinction baseline of animals infused with DREADDs into the PL-core sub-circuit indicated a non-selective reduction in responding in both the active (**Figure 15A**) and inactive (**Figure 15C**) hole in mice infused with Gq-DREADD, while those infused with the Gi-DREADD into the PL-core subcircuit exhibited higher responding in the active hole, but lower responding in the inactive hole [one-way ANOVAs, for active hole: F(2,28)=7.937, p=0.002; LSD post-hoc tests: Gq vs. mCherry, p=0.051; Gq vs. Gi, p<0.0001; Gi vs. mCherry, p=0.051; for inactive hole: F(2,28)=3.685, p=0.038; LSD

post-hoc tests: Gq vs. mCherry, p=0.013; Gq vs. Gi, p=0.413; Gi vs. mCherry, p=0.083]. Despite these effects of chemogenetic manipulation of the PL-core projection on baseline responding during late extinction, neither DREADD altered responding on the test for cue-induced reinstatement of MA-seeking (**Figure 15A,C**) [for active hole: F(2,26)=1.899, p=0.170; for inactive hole: F(2,26)=2.392, p=0.111].

In contrast to the PL-core study, viral infusions into the PL-shell sub-circuit did not affect responding in either the active or inactive hole during extinction baseline (**Figure 15B,D**) [for active hole: F(2,26)=0.260, p=0.773; for inactive hole: F(2,26)=1.989, p=0.157] or the subsequent test for cue-elicit reinstatement of responding [for active hole: F(2,25)=0.394, p=0.678; for inactive hole: F(2,25)=1.246, p=0.305].

3.4 Discussion

Currently, the neurocircuitry mediating the initial reinforcing properties of MA and the establishment of drug-taking and -seeking behavior is under-characterized. MA can induce glutamate release within both the medial PFC and NAC (Szumlinski et al., 2017; Lominac et al., 2016; Parsegian and See, 2014; Lominac et al., 2012), and extracellular glutamate levels within both the soma and terminal regions of corticoaccumbens projections are biochemical correlates of a genetic vulnerability to prefer and self-administer MA (Szumlinski et al., 2017). However, to the best of our knowledge, the functional relevance of specific PFC projections to NAC sub-regions in MA-seeking and -taking behavior have not been examined directly for either intravenous or other routes of MA self-administration. Thus, we employed chemogenetic techniques to manipulate the activity of specific PFC-NAC sub-circuits, previously implicated in mediating different aspects of addiction-related behavior, to determine whether or not they similarly modulate MA-seeking and MA-taking in a procedurally facile, non-invasive, mouse model of oral MA self-administration, as well as in

a model of MA-conditioned reward. We detected relatively few effects of our chemogenetic manipulations on MA-directed responding and oral MA intake, many of which were unexpected and call to question a dichotomous role for the PL-core and IL-shell projections in regulating oral MA-seeking and -taking behavior as discussed below.

3.4.1 Unexpected effects of chemogenetic stimulation of the PL-core projection on early MA reinforcement

The PL subregion of the PFC sends a dense glutamatergic projection to the NAC core core (Vertes, 2004; Heidbreder and Groenewegen, 2003), which has been theorized to promote or drive drug-seeking and -taking in animal models (c.f., Everitt and Robbins, 2013). For examples, site-directed pharmacological inhibition of the PL is reported to decrease both active cocaine-taking following acquisition of the operant-response (Di Pietro et al., 2006), as well as to block the reinstatement of cocaine-seeking behavior induced by stress (McFarland et al., 2004; Capriles et al., 2003), context (Fuchs et al., 2005), cues (Gipson et al., 2013; Di Pietro et al., 2006; McLaughlin and See, 2003), and a drug priming injection (Vassoler et al., 2013; Di Pietro et al., 2006; Capriles et al., 2003; McFarland and Kalivas, 2001). While fewer studies focus on the role for the PL in MA reinforcement, similar results were reported for both cue- and drug prime-induced reinstatement of MA-seeking in rats with a history of intravenous MA self-administration (Rocha and Kalivas, 2010; Hiranita et al., 2006). In line with these neuropharmacological studies targeting the PL, optogenetic inhibition of the PL-core sub-circuit is reported also to reduce cocaine-induced reinstatement of drug-seeking in rats (Stefanik et al., 2013). Consistent with these prior studies, we showed herein that the chemogenetic activation of the PL-core sub-circuit increased MA-directed responding and intake, but only on the very first acquisition trial of our operant-conditioning procedure (**Figure 10A,2M**). Such an interpretation aligns with the

results of a much earlier neuropharmacological study examining the effects of an intra-PL infusion of a baclofen-muscimol cocktail on the acquisition of intravenous cocaine self-administration by rats, which detected a reduction in active lever-pressing behavior only on day 1 of the 8-day acquisition phase of the study (Di Ciano et al., 2007). Together, such findings suggest that activation of the PL-core sub-circuit might facilitate the initial reinforcing effects of MA.

Alternatively, chemogenetic stimulation of the PL-core projection may merely produce some generalized hyper-reactivity to the novel operant-conditioning environment, augmenting nose-poking behavior and the exploration of the MA-delivery receptacle. Although MA intake in our oral paradigm is voluntary, increased exploration of the MA-delivery receptacle increases the likelihood that mice will encounter and sample the MA solution, resulting in higher initial MA intake. Indeed, we have reported consistently that both responding for an oral MA reinforcer and MA intake follows a triphasic pattern in mice, with high levels of responding/intake detected on the first day of self-administration training, followed by a precipitous drop in behavior over the next 2-4 days and then a progressive escalation of responding and intake over the following 7-9 days, which typically stabilizes following 10-14 days of training (e.g., Brown et al., 2020; Fultz et al., 2017, 2021; Ruan et al., 2020; Sern et al., 2020; Shab et al., 2021; Szumlinski et al., 2017). Consistent with this prior work, the mice in all sub-experiments of Experiment 1 exhibited what we interpret as being novelty-induced responding on day 1 of training; it is non-selective as indicated by similar pattern of responding in both the active and inactive holes and a hole-discrimination of 45-60% (**Figure 10**) and thus, does not likely reflect a high level of MA-reinforcement per se. The observation that PL-core activation potentiated responding in both the active and inactive holes on day 1, without affecting hole discrimination, argues moreso in favor of this

manipulation producing a general increase in behavioral reactivity to the novel, self-administration, environment rather than a potentiation of the reinforcing properties of MA. Further supporting this suggestion, PL-core activation did not potentiate responding on any of the subsequent training days in Experiment 1 (**Figure 11**), nor did it potentiate MA reinforcement or intake in mice with an established history of MA self-administration in Experiment 2 (**Figure 13**), as discussed in more detail below. Finally, the fact that chemogenetic manipulations of the PL-shell or the IL-core did not impact MA self-administration behavior on day 1 of training (**Figure 10**) points to the neuroanatomical specificity of this potentiation, implicating the glutamatergic projections from the PL to the NAC core as a key driver of novelty-induced behavioral reactivity that can influence initial MA-taking behavior.

To the best of our knowledge, this study is the first to examine the effects of selectively targeting any PFC-NAC subcircuit during the early acquisition phase of operant-conditioning for oral MA reinforcement and thus, there is no direct basis for comparison in the addiction literature. This being said, in line with the present results, an earlier neuropharmacological study examining the effects of transient PL inhibition on the acquisition of intravenous cocaine self-administration by rats (using an intra-PL infusion of baclofen/muscimol) detected a reduction in active lever-pressing behavior only on day 1 of the 8-day acquisition phase of the study. Moreover, PL inhibition did not alter conditioned responding for cocaine-associated cues, in the absence of any further cocaine delivery (Di Ciano et al., 2007). However, in this same study, PL inactivation also inhibited the re-acquisition of the conditioned operant-response following its extinction, leading to the suggestion that the PL activity mediates the ability to acquire an operant response, but that habitual responding for either drug or a drug-conditioned reinforcer is independent of PL activity (Di Ciano et al.,

2007; Killcross and Coutureau, 2003). Our findings regarding the effects of chemogenic activation of the PL-core projection upon MA-directed responding and intake during the acquisition phase of Experiment 1 align with the results of Di Ciano et al. (2007) and point to PL projections specifically to the NAC core as mediating the initial reinforcing properties of oral MA, as neither chemogenetic activation or inhibition of the PL-shell projection influenced behavior during the first few days of operant-conditioning (**Figure 10**).

3.4.2 Unexpected effects of chemogenetic stimulation of the PL-core and PL-shell projections on established MA self-administration behavior

Some inconsistencies exist in the intravenous cocaine self-administration literature regarding the involvement of PL activity in established drug-taking behavior, with some neuropharmacological studies failing to detect an effect of PL inhibition upon responding for drug or a drug-conditioned reinforcer once established (e.g., Di Ciano et al., 2007), and other studies reporting reduced cocaine-taking in rats that previously acquired the operant response (Di Pietro et al. 2006). As mentioned above, neither chemogenetic activation nor inhibition of the PL-core affected MA-directed responding or intake during either the late acquisition phase of Experiment 1 (**Figure 11**) or the maintenance phase of self-administration in Experiment 2 (**Figure 13**). Rather unexpectedly, chemogenetic activation of the PL-core projection *decreased* hole discrimination during demand testing in Experiment 1 (**Figure 12I**). Although we failed to detect statistically significant differences between our three PL-core groups for either active or inactive hole pokes during demand testing, visual inspection of the data argue that the reduction in hole discrimination reflects lower active hole poking in mice infused with the Gq-DREADD, rather than a change in inactive hole poking behavior (**Figure 12A vs. 12E**). As this Gq-DREADD-mediated reduction in hole discrimination was not accompanied by any statistically significant change in MA intake (**Figure 12M**), we

interpret this finding as indicating that activation of the PL-core may devalue or blunt the demand for MA, without affecting the propensity to consume drug that is available.

Interestingly, a similar pattern of hole discrimination effect was detected in mice infused with the Gq-DREADD into the IL-core projection, albeit to a lesser degree than mice in which the PL-core projection was targeted (**Figure 12K vs. 12I**). In the case of the IL-core projection, the Gq-DREADD effect on active hole poking and MA intake appeared to be more robust than that within the PL-core projection, particularly under the FR2 schedule of reinforcement. However, despite the relatively large sample sizes employed in our IL-core sub-experiment (n-10-12), the effect of IL-core Gq-DREADD infusion was statistically unreliable. While replication is required to increase the statistical power of study, these data for the IL-core projection suggest that this glutamate pathway may also gate MA demand, but may do so via effects on the unconditional rewarding properties of MA.

3.4.3 Unexpected effect of chemogenetic stimulation of the PL-shell projection on MA reinforcement during later acquisition

Although chemogenetic manipulation of the PL-shell sub-circuit did not affect behavior during early acquisition (i.e., across the first 3 days of training), activation of this projection *decreased* both MA-directed responding and intake later during the acquisition phase (**Figure 11B**). Consequently, none of the mice with chemogenetic stimulation of the PL-shell sub-circuit reached the acquisition criteria by the end of the 14-day training period (**Table 2**). The robust effect of PL-shell stimulation was rather unexpected given the less dense glutamate projection from the PL to the NAC shell versus that to the core (Vertes, 2006; Vertes, 2004; Sesack et al., 1989; Berendse et al., 1992) and the PL's putative role in driving drug-seeking and reinforcement, discussed above. The lower MA self-administration upon chemogenetic activation of the PL-shell projection argues that neuronal activity within the

PL-shell sub-circuit normally serves to suppress oral MA self-administration behavior, while the latency of the effect (i.e., the fact that the effects of chemogenetic activation of the PL-shell projection manifested during later self-administration training) argue that this sub-circuit may be progressively recruited by MA experience, by learning, or both to suppress MA reinforcement.

Whether or not the observed reduction in MA reinforcement and intake produced by PL-shell activation reflects a learning impairment, a memory impairment, and/or a reduction in the primary reinforcing properties of MA cannot be ascertained directly from the experimental design of Experiment 1 as their failure to acquire the operant-response precluded them from further study. However, no effect of PL-shell stimulation was detected for inactive nose-poking during late acquisition training in Experiment 1 (**Figure 11F**), arguing against off-target motor effects to account for the reduced MA self-administration behavior during later acquisition. Furthering arguing this point, none of our chemogenetic manipulations altered the locomotor activity of MA-experienced mice under place-conditioning procedures (**Table 3**) and acute chemogenetic activation of the PL-shell sub-circuit failed to alter nose-poking behavior or MA intake in Experiment 2 mice that successfully acquired the operant-response (**Figure 13**). Thus, it is not likely that the stimulation of the PL-shell projection facilitated the onset of some gross motor impairment or induced stereotypic behaviors that might be incompatible with operant-responding in MA-experienced animals. Arguing along these same lines, if activation of the PL-shell sub-circuit reduced the reinforcing properties of MA and/or impaired recall of the operant response or the conditioned reinforcing properties of the drug-associated context/cues, then chemogenetic stimulation of this circuit should have lowered MA-directed responding and intake also in

Experiment 2, unless this subcircuit is only recruited and engaged during the active operant learning process and not during its expression.

The majority, if not all, studies focused on the functional neuroanatomy of addiction-related behavior tend to dissect PFC-NAC projections according to the density of their glutamate projections, with the majority of studies functionally delineating between PL-core vs. IL-shell sub-circuits. While our results for the PL-shell may be the first chemogenetic demonstration that this projection may function to suppress MA reinforcement and intake, they are not the first to support an inhibitory role for the PL in drug self-administration behavior. For examples, PL lesions were reported to enhance intravenous cocaine self-administration (Weissenborn et al., 1997), while transient neuropharmacological inactivation of the PL enhanced cue-elicited heroin-seeking (Schmidt et al., 2005), in drug-experienced rats. More recently, optogenetic inhibition of the PL was found to increase cocaine-taking by rats exhibiting high frequency self-administration (Martin-Garcia et al., 2014) and by punishment-resistant rats (Chen et al., 2013). Further, and in line with the present data, Chen et al. (2013) also reported a decrease in cocaine-taking by punishment-resistant rats upon optogenetic stimulation of the PL. Although our Gi-DREADD infusion procedures were relatively ineffective at altering behavior throughout our course of study, our findings for PL-shell activation by a Gq-DREADD point to the presumably glutamatergic projections from the PL to the NAC shell as at least one sub-circuit driving response inhibition in the context of drug self-administration.

3.4.4 Chemogenetic stimulation of both PL projections facilitate extinction learning

Consistent, in part with the notion that the PL may play a role in response inhibition (c.f., Moorman et al., 2015), chemogenetic stimulation of both the PL-core and the PL-shell subcircuits reduced, by approximately 25%, the number of sessions required by mice to reach

the criterion for extinction of their drug-seeking response in Experiment 2 (**Figure 15G,H**). Notably, Gq-DREADD-mediated activation of both subcircuits selectively reduced responding in the formerly MA-reinforced hole during the first few days of extinction learning (**Figure 14A,B**), suggesting that the activation of either the PL-core or PL-shell projection is sufficient to drive initial inhibitory learning in the absence of drug and response-contingent presentation of drug-associated cues. It should also be noted that chemogenetic inhibition of the PL-core, but not the PL-shell, projection increased responding in the active hole under extinction conditions; this effect was apparent during both the early and late phases of extinction learning (**Figure 14A, 15A**), with no corresponding increase in inactive hole responding. In fact, although chemogenetic inhibition of the PL-core sub-circuit did not alter responding in the inactive hole early during extinction training (**Figure 14C**), it reduced inactive hole-poking by extinction baseline (**Figure 15C**). In contrast, chemogenetic inhibition of the PL-shell projection lowered active hole responding during early extinction learning to a similar extent as that observed with chemogenetic stimulation (**Figure 14B**) and did not affect responding in either hole at extinction baseline (**Figure 15B,D**). Thus, while PL-shell activity may be sufficient to facilitate or even initiate inhibitory learning during early drug abstinence, this projection does not play an active role in inhibitory learning and/or may come "off-line" once the inhibitory learning process is engaged, no longer limiting drug-directed behavior.

Taken all together, these findings from Gi-DREADD infused mice indicate that our Gi-DREADD/CNO approaches are effective for influencing behavior, which mitigates concerns that poor neuronal transduction by the AAV carrying the Gi-DREADD or insufficient CNO dosing contributed to the many null effects of chemogenetic inhibition observed in this report. Why we detected behavioral effects of chemogenetic inhibition of the PL-core sub-

circuit only during drug abstinence and not during any phase of MA self-administration is not clear at the present time, but raises the possibility that a higher CNO dose may be required to more effectively engage the Gi-DREADD when animals are engaged in active drug-taking and this possibility will be explored in future studies. Nevertheless, our Gi-DREADD findings regarding behavior during extinction learning are consistent with the prior report by Schmidt et al. (2005) indicating that PL inhibition augments heroin-seeking during drug withdrawal, as well as earlier indications that the activation of Gq/o-coupled mGlu5 glutamate receptors within the PL is required for extinction learning in cocaine-experienced animals (Ben-Shahar et al., 2013). Together, these results point to a necessary and sufficient role for the PL-core projection in the suppression of drug-seeking behavior during drug abstinence.

As reported previously by our group, the response-contingent presentation of cues previously associated with low-dose MA delivery during drug abstinence selectively reinstates nose-poking behavior in the MA-appropriate, active, hole following extinction of responding (Brown et al., 2020; Ruan et al., 2020). We had hypothesized at the outset of this study that chemogenetic manipulations that facilitated extinction learning would perhaps blunt the capacity of drug-associated cues to reinstate nose-poking behavior. However, despite facilitating extinction learning (albeit to different degrees), chemogenetic manipulations of neither the PL-core or the PL-shell projection altered the subsequent cue-induced reinstatement of MA-seeking in the absence of any further CNO pretreatment (**Figure 15**). Such a finding indicates that the capacity of MA-associated cues to reinstate drug-seeking behavior does not appear to depend upon the extent to which animals learn to suppress their behavior during drug/cue abstinence, which aligns with the notion that the memory of the drug-cue association persists during extinction learning and reflects a distinct

set of neuroadaptations (Torregrossa and Taylor, 2013; Lasseter et al., 2009). Given the extensive CNO history of the mice from Experiment 2, we opted not to pretreat the mice prior to the reinstatement test in the present study. Thus, it remains to be determined how chemogenetic manipulation of the PL-NAC sub-circuits alters MA-seeking behavior in this model of relapse. Given the extensive body of work employing single-site approaches to distinguish between the roles for the IL versus the PL in animal models of drug relapse (Gipson et al., 2013; Vassoler et al., 2013; Ball and Slane, 2012; LaLumiere et al., 2012; Peters et al., 2008; Di Pietro et al., 2006; Hiranita et al., 2006; Fuchs et al., 2005, McFarland et al., 2004; Capriles et al., 2003; McLaughlin and See, 2003; McFarland and Kalivas, 2001), such a study is duly warranted and is a focus of future work.

3.4.5 Limitations, pitfalls, and future directions

It is concerning that manipulations of IL sub-circuits did not affect any of the variables measured in this study. Because the pilot study targeted only the PL-core sub-circuit to minimize the number of animals used, the transduction and placement of the viruses into the IL was not confirmed. It is possible that the IL manipulations had no behavioral effects because the placements were inaccurate, and the virus did not successfully reach the IL neurons that terminate in the NAC sub-regions where Cre was being expressed. However, there have been studies that fail to find an association between IL activity and cocaine-seeking (Moorman et al., 2015; Mihindou et al., 2013). Additionally, the IL sub-circuits were only tested during the acquisition and demand testing phases of the study. Previous research characterizes the IL as particularly important for extinction learning and reinstatement (Van den Oever et al., 2013; LaLumiere et al., 2010; Peters et al., 2009; Peters et al., 2008; Maren and Quirk, 2004), so future experiments should be conducted in which the IL-core and IL-shell activity is bidirectionally manipulated during the extinction and reinstatement testing.

Manipulation of neither PL-NAC sub-circuit altered the expression of a MA-induced CPP or the maintenance of MA self-administration. However, these data are difficult to interpret because all but one group failed to establish a MA-conditioned place preference and instead expressed place-ambivalence (Brown et al., 2020; Szumlinski et al., 2017). This may be due to the dose of MA used during the place conditioning, as since commencing this study, it has become apparent that 1 mg/kg MA elicits the most reliable CPP compared to the 2 mg/kg MA used in this study (Fultz et al., 2021; Brown et al., 2020; Ruan et al., 2020; Sern et al., 2020). Though the PL sub-circuits did not affect the expression of MA self-administration after successful acquisition, given the issue with the dose of MA, it would be worth running the CPP phase of the experiment again to see whether the role of these projections depends on the behavioral paradigm tested. In addition to that, the functional relevance of the IL sub-circuits to CPP expression should be examined.

As discussed above, the only effects of the Gi-DREADD virus were observed during the extinction phase of experiment 2, calling the efficacy of the inhibitory virus into question. It has been observed that high titers of the Gi-DREADD virus causes neuroinflammation and significant neuronal loss (Goossens et al., 2021), which could confound the behavioral results. However, the 0.3 µL infusion of viral vector appears to be low enough to avoid these side effects. On the other hand, perhaps it was too small an infusion to transfect a majority of the targeted neurons. Additionally, we have heard anecdotal reports that higher CNO doses, up to 10 mg/kg, are required to see behavioral effects from the inhibitory DREADD than the excitatory DREADD. Though the 5 mg/kg CNO dose was tested during the pilot study, that was conducted with only the Gq DREADD so its impact on the Gi DREADD was not confirmed. However, some evidence demonstrates that a CNO dose of 3-5 mg/kg IP suffices (Jendryka et al., 2019). Still, it would be useful to perform dose-response experiments with

the CNO to determine which dose provides the optimal impact. Furthermore, it would be helpful to verify bidirectional manipulation of neuron activity using electrophysiological techniques. Given the increasing utility of chemogenetics in neuroscience research, it would be helpful to optimize the parameters of these procedures.

Chapter 4:

General Discussion

4.1 Summary of findings

The data presented in this dissertation illuminate the contributions of glutamate signaling in the NAC core and shell to the regulation of MA reward and reinforcement and are summarized in **Table 5**. The first study, described in Chapter 2, focused on how the glutamate receptor scaffolding protein Homer2, especially in the NAC, impacts MA CPP and self-administration. In experiment 1, mice bred for a constitutive KO of the glutamate receptor scaffolding protein Homer2 were tested under place-conditioning and operant-conditioning procedures to obtain causative evidence to bolster the already established correlation between NAC Homer2 expression and MA reward. Homer2 KO mice exhibited increased CPP, active responding for, and intake of, MA, compared to WT controls, arguing for a globally inhibitory role of Homer2 in MA-induced behavior. To test neuroanatomical candidates for the loci of Homer2's effects, shRNA viral vectors were infused into the NAC core and shell to knock down Homer2 expression specifically in those sub-regions (experiment 2). Homer2 knockdown in the core increased, while knockdown in the shell decreased, magnitude of a MA-CPP and responding for a MA reinforcer. The results of experiment 2 support opposing roles for core and shell Homer2 in the expression of both MA reward and reinforcement. A third experiment assessed whether Homer2 actively regulates MA reward and reinforcement by expressing a cDNA viral vector to over-express Homer2 in the NAC core and shell of Homer2 KO and WT mice. The effects of this manipulation on MA-CPP and self-administration were, if anything, the same as those of knocking down Homer2. Overall, the data from study 1 suggest that either dysregulating Homer2 expression in either direction can perturb NAC glutamate and influence MA reward and reinforcement in similar ways, or more likely, that Homer2's regulation of MA behavior is not localized to the NAC core or shell, implicating other regions of interest. Though globally inhibitory,

Table 5. Summary of the results of the experiments presented herein. ↑ denotes an increase in behavior relative to control. ↓ denotes a decrease in behavior relative to control. – denotes no effect of manipulation relative to control. N/D denotes not determined.

	CPP	Self-Administration Acquisition	Increasing Response Requirement	Self-Administration Dose Response	Extinction	Reinstatement
Homer2 KO	↑	↑	↑	↑	N/D	N/D
Homer2-shRNA core	↑	--	--	↑	N/D	N/D
Homer2-shRNA shell	--	--	↓	↓	N/D	N/D
Homer2-cDNA core	↑	--	N/D	--	--	--
Homer2-cDNA shell	--	--	N/D	↓	--	--
PL-core activation	--	↑	↓	N/D	↑	--
PL-shell activation	--	↓	--	N/D	↑	--
IL-core activation	N/D	--	--	N/D	N/D	N/D
IL-shell activation	N/D	--	--	N/D	N/D	N/D

Homer2 in NAC sub-regions does not appear to have an active or autonomous role in regulating MA reward and reinforcement. These results prompted me to change my research direction towards characterizing the source of glutamate within the NAC core and shell in regulating MA-induced behavior.

Because the best-characterized glutamatergic input to the NAC originates in the PFC, study 2 used chemogenetic technology to bidirectionally manipulate neuronal activity in sub-circuits between the PL and IL PFC and NAC core and shell to investigate their functional relevance to MA-seeking and -taking. Stimulating the PL-shell circuit prevented animals from acquiring MA self-administration and attenuated responding and intake for MA, while stimulating the PL-core augmented initial MA responding and intake. These outcomes indicate that the PL-core drives initial MA reinforcement, and the PL-shell inhibits MA-related operant learning, which argues against a strict dichotomy between PL-core and IL-shell projections and may reflect properties of MA as a specific reinforcer. PL-NAC sub-circuit activity did not determine the maintenance of MA self-administration or the expression of a MA-CPP, but as these animals did not express a CPP overall, the results from that phase of the experiment are inconclusive. Stimulating both PL-NAC projections facilitated extinction learning but did not affect subsequent cue-elicited reinstatement. These findings demonstrate that both of these sub-circuits are sufficient, but not necessary, to drive extinction learning, which corroborates the body of literature implicating PL glutamate in extinction learning. These chemogenetic studies are the first to examine the role for specific PFC-NAC subcircuits in gating MA reward and reinforcement and indicate that glutamatergic innervation in the NAC core and shell by the PFC has a complex but influential role in MA-taking and -seeking.

4.2 Glutamatergic contributions to MA response

As mentioned in the Introduction, the functional relevance of PFC glutamate to addiction plasticity is emerging as more research is conducted (Szumlinski et al., 2017; Lominac et al., 2016; Quintero, 2013; Lominac et al., 2012; Kalivas and Volkow, 2011). Stimulating glutamate transmission, particularly in the NAC, has been associated with increasing the rewarding properties of MA, and some evidence argues for a hyperglutamatergic NAC state underlying MA addiction vulnerability (Szumlinski et al., 2017; Fujio et al., 2005a; Fujio et al., 2005b; Nakagawa et al., 2005). Understanding the molecular basis for that relationship has provided a challenge.

Despite correlational studies linking NAC core and shell Homer2 to positive MA valence (Szumlinski et al., 2017), the causal data from Chapter 2 of this dissertation did not consistently support an active or autonomous role for Homer2 in NAC glutamate's regulation of MA reward and reinforcement. Investigating other glutamate-related molecules of interest, like TAAR1, in order to elucidate the neurobiological mechanisms underlying glutamate's addiction-related functions, should be a priority. TAAR1 is expressed in reward-related brain regions, including the VTA, NAC, and vmPFC and is under investigation as a promising target of interest in understanding cocaine and MA abuse (Phillips et al., 2021; Liu et al., 2017). Mice bred for high MA drinking that show increased extracellular glutamate and glutamate receptor expression in the NAC also have a hypofunctional TAAR1 gene that has a SNP accounting for 60% of the variation between them and low MA drinking mice (Phillips et al., 2021; Stafford et al., 2020; Szumlinski et al., 2017; Belknap et al., 2013; Shabani et al., 2011), and TAAR1 signaling has been shown to modulate glutamate receptor function in the PFC (Espinoza et al., 2015).

Results from Chapter 3 of this dissertation found effects of altering PL-NAC activity to support the importance of these glutamatergic projections in stimulant reinforcement, but not

necessarily in the ways hypothesized by literature. Specifically, these results do not align perfectly with prior indications that the PL is necessary for cocaine and MA reinforcement and reinstatement (Gipson et al., 2013; Stefanik et al., 2013; Vassoler et al., 2013; Di Pietro et al., 2006; McLaughlin and See, 2003; McFarland and Kalivas, 2001). Additionally, manipulating the IL-NAC sub-circuits failed to affect responses to MA during the acquisition of MA reinforcement and increasing demand testing. To the best of our knowledge, no other studies have examined the role of IL signaling in the acquisition phase of drug self-administration, but prior evidence implicates this region in extinction learning and reinstatement (Van den Oever et al., 2013; LaLumiere et al., 2010; Peters et al., 2009; Peters et al., 2008; Maren and Quirk, 2004). Though chemogenetic activation of the IL-shell projection reduced cue-induced reinstatement of cocaine-seeking (Augur et al., 2016), in keeping with the traditional model of the IL as an inhibitor of drug-seeking behavior, IL inactivation did not affect cue- or drug-induced reinstatement of MA-seeking (Hiranita et al., 2006). Viral expression and placements must be confirmed to confidently interpret the outcome of the experiments in Chapter 3, but given the previously reported inconsistencies in the role of the IL in drug-seeking (Moorman et al., 2015; Mihindou et al., 2013), bidirectionally manipulating the IL-NAC sub-circuits during extinction and reinstatement procedures will be an important direction for future work.

Though these dissertation studies aimed to target glutamatergic mechanisms implicated in MA abuse, neither study directly manipulated or measured glutamate levels in the PFC or NAC. We know that the viral vectors used in study 1 alter Homer2 expression (Ary et al., 2013; Goulding et al., 2011; Klugmann and Szumlinski, 2008), but we have not measured the effect that has on the post-synaptic response to glutamate in the NAC. Using electrophysiology or microdialysis downstream to index the impact of manipulating Homer2

expression on neurotransmission could elucidate the functional consequences of this technique. Study 2 targeted the neurons in circuits known to be glutamatergic but did not specifically transfect glutamatergic neurons or probe glutamate release. Expressing DREADDs with a glutamate-specific promoter may help to clarify the functional relevance of this mechanism in the various stages of MA addiction behavior. Measuring glutamate release in the destination of the projection via microdialysis during chemogenetic manipulation could also implicate a neurotransmitter-specific effect.

The efficacy of utilizing DREADDs as a tool to manipulate glutamate could be questioned due to the rapid regulation of glutamate levels compared to neuromodulators like dopamine or serotonin. However, chemogenetic manipulations of glutamatergic projections have successfully altered cocaine-seeking behavior in drug-experienced rats (Augur et al., 2016). Of note, cocaine-experienced rats exhibit low extracellular glutamate levels in the NAC (Baker et al., 2003), due to a host of molecular adaptations (to include, changes in the function of system Xc- and the excitatory amino acid transporters) that disrupt the normally tight regulation of extracellular glutamate in this region. This dysregulation of extracellular glutamate in cocaine-experienced animals may allow for more efficacious, or at least detectable impacts of DREADD approaches for increasing glutamatergic neuron activity in the NAC. However, opposite cocaine, withdrawal from repeated MA exposure elevates basal extracellular glutamate in the NAC (Lominac et al., 2016) - a scenario in which DREADD activation may be predicted to be less likely to produce an observable behavioral effect. Interestingly, there is no change in NAC basal extracellular glutamate during early withdrawal in MA-experienced animals (Lominac et al., 2016), which may explain why I detected effects of the Gq DREADDs during acquisition or during the early phase of extinction training. The behavioral effects of DREADD activation observed during extinction

could also reflect differences in the neuroadaptations that occur during extinction learning versus forced abstinence. Indeed, in the cocaine literature, evidence indicates distinct changes in glutamate receptor-related signaling molecules between cocaine-withdrawn rats that underwent extinction learning versus those left alone in the home cage (Ghasemzadeh et al., 2009). Whether or not extinction learning alters NAC glutamate signaling in a manner different from forced abstinence has not yet been examined. Thus, it is possible that the Gi DREADDs might be more effective at altering MA-seeking in animals undergoing forced abstinence, which was not examined herein.

4.3 Properties of MA as a reinforcer

Previous studies have posited that Homer2 expression is part of a hyperglutamatergic state in the NAC associated with increased drive for MA-seeking and -taking (Szumlinski et al., 2017). This correlation would make MA more like alcohol (Szumlinski et al., 2008b) than cocaine (Ary and Szumlinski, 2007) with regards to Homer2-related mechanisms driving addiction-related behavior. The data from Chapter 2 of this dissertation call into question the straightforwardness of increased NAC Homer2 as a sole driver of MA use, emphasizing the importance of probing MA as an individual reinforcer rather than considering it part of a general category of stimulants that are largely represented by cocaine in the literature, especially given the dire need for more treatment options for patients suffering with MA abuse disorder.

The results in this dissertation found little evidence to support a strict neuroanatomical dichotomy between PL-core and IL-shell projections with respect to MA-taking or -seeking behavior. Although the majority of the literature argues that the PL, projecting primarily to the core, drives drug-seeking while the IL, connected primarily with the shell, inhibits drug-seeking (Augur et al., 2016; Ma et al., 2014), some findings are not consistent with this

dichotomy (Martin-Garcia et al., 2014; Chen et al., 2013; Pelloux et al., 2013; Vassoler et al., 2013; Jonkman et al., 2009). There is evidence that circuits between the PFC and the NAC sub-regions form more of a gradient than a strict dichotomy, especially with regards to some PL fibers terminating in the shell (Moorman et al., 2015), which could explain why we found so many effects of manipulating the PL-shell circuit. This finding contributes to the justification for testing understudied projections. Some studies have found that PL activity can block stimulant reinforcement (Martin-Garcia et al., 2014; Chen et al., 2013; Jonkman et al., 2009; Miller and Marshall, 2004; Weissenborn et al., 1997), which relates to our findings that stimulating PL sub-circuits inhibit acquisition for MA reinforcement. Much of the research promoting the PL as a driver of reinforcement stems from studies of cocaine, while many of the studies calling that model into question focus on food reinforcement (Calu et al., 2013; Capriles et al., 2003; McFarland et al., 2003; McFarland and Kalivas, 2001). This begs the question of whether the functional relevance of these projections depends on the individual reinforcer under investigation. Could it be that the subcircuit(s) governing the reinforcing of MA are more similar to food than cocaine?

4.4 Elucidating the functional neuroanatomy in early drug use

When designing studies 1 and 2, I prioritized maximizing the amount of behavioral data collected from each subject to make the most of each animal sacrificed as well as each viral infusion surgery performed. This is why these experiments had several behavioral phases and tended to involve several weeks of behavioral testing. However, had I streamlined the behavioral model used, I may have been able to understand more deeply about how a specific protein or sub-circuit regulates a particular aspect of addiction-related behavior that would be easier to contextualize. Focusing solely on the neurobiology of drug-taking acquisition, would have allowed me to test other parameters to probe factors that contribute to addiction

risk and optimize our behavioral testing procedures. Given glutamate's impact on neuroplasticity, it seems crucial to determine the glutamatergic contributions to drug-induced neural adaptations that underlie the transition from drug use to abuse in certain people.

After starting the experiments for Chapter 3, in which the first behavioral tests focused on the role of PFC-NAC sub-circuits in the acquisition of oral MA self-administration, I realized that most addiction research does not examine the functional neuroanatomy of the acquisition phase of drug use. Most of the material I found on the molecular mechanisms or circuitry of addiction studied extinction and reinstatement of drug-seeking as a model of relapse, probably due to the huge challenge that relapse poses to successful substance use disorder treatment. However, there has not been much success in developing treatments for addiction thus far. Perhaps it would be helpful to intervene during early stages of drug-taking to prevent people from getting to the point when their drug use has become a substance abuse disorder? Given that the majority of people who use recreational drugs do not develop a disorder, early intervention will require determining which factors make some people more vulnerable to severe disease. We already know some important factors, including age, sex, environment, stress, genetics, and comorbidities (particularly mental illnesses such as anxiety, depression, PTSD, and ADHD), can put someone at higher risk of developing a substance use disorder, but the neurobiological mechanisms of how these factors increase risk are not yet well understood. In order to design treatments that could be effective during the transition from drug use to abuse to addiction, understanding these mechanisms will be paramount. I hope that this dissertation work will help to inspire further studies of acquisition, but also acknowledge that focusing more on this behavioral phase could have provided a more comprehensive view of the neurobiological mechanisms and circuitry

underlying early drug-taking, which may be distinct from those that have been relatively well-characterized to underpin drug-seeking.

4.5 Caveats

So far, the addiction field has been relatively reliant on the results from studies in which a single brain region is manipulated (a.k.a. single-site studies), such as the experiments described in Chapter 2 of this dissertation. However, we know that when manipulating a single brain region, particularly with a pharmacological agent or a viral manipulation, we don't only affect that one brain region, we affect cellular and molecular activity within inter-connected brain regions. An example of this can be found in Ary et al. (2013), in which AAV-mediated changes in Homer2 expression within the mPFC resulted in changes in Homer and glutamate receptor protein expression within the NAC, which lies far outside the targeted site. When we fail to detect behavioral differences, it could be because the region of interest is not critical for the behavior, or it could be because downstream areas can compensate for the manipulation. Comparing results from circuit manipulations to single site studies is difficult because brain regions don't function in isolation. My dissertation underlines the limitations of probing one brain region at a time. The closer we can get to modeling network functions with our experimental techniques, the more valid they are.

Compensatory mechanisms may be obscuring the role that our target protein or circuit has in MA-related behavior. In study 1, Homer1-dependent scaffolding may be compensating for our manipulations on Homer2 expression. Manipulating Homer2 expression in the mPFC yielded changes in basal glutamate and cocaine-induced glutamate release, but no effects on cocaine-induced behavior until the Homer2/Homer1 ratio was increased, which shifted the dose response curve for cocaine-CPP to the left (Ary et al., 2013). These results could indicate that the ratio of Homer2 to Homer1 expression affects drug-induced behavior more

than Homer2 levels alone. Alternatively, we could have increased the post-synaptic response to glutamate by over-expressing Homer2, but downstream targets have compensated, especially considering the reciprocal GABAergic projection the NAC has with the VTA. Presumably this could also occur when stimulating PFC-NAC neurons. Although DREADDs don't seem to desensitize in the target neurons (Roth, 2016; Krashes et al., 2011; Alexander et al., 2009), we could have affected expression or internalization of post-synaptic receptors or autoreceptors secondary to the stimulation of the DREADD. Targeting a projection could ameliorate some of these off-target effects, but we are still not at the level of controlling an entire network and far from understanding how DREADD activation impacts the normal function or expression of the many different kinds of receptors, transporters, and intracellular signaling molecules within a neuron, which may compensate for the effects of DREADD stimulation.

4.6 Limitations and future directions

Although this dissertation work sought to determine the role of corticostriatal glutamate signaling in MA reward and reinforcement, there are still many aspects of these complex behaviors that were not addressed. The following proposed experiments would attempt to investigate some of these outstanding issues:

1) Locate the neuroanatomical loci for the effect of Homer2 on MA addiction behavior. Homer2 had a global effect on MA responding, but the NAC did not appear to be the locus. Investigating the effects of manipulating Homer2 expression in other reward-related brain regions on MA reward and reinforcement may help to understand its globally inhibitory role. Potential sites of action include the PFC, VTA, and amygdala, in which Homer2 function has been implicated in mediating the rewarding/reinforcing properties of other drugs of abuse (Szumlinski et al., 2008).

2) Focusing future experiments on a single phase of behavioral testing, like acquisition, would allow us to test more of the factors that could impact early drug-taking behaviors. We could optimize different aspects of our behavioral models of both CPP and operant conditioning, including short access vs long access self-administration, the training dose of MA, oral vs IV or other routes of administration, olfactory cues rather than or in addition to audio and visual, stress exposure, and sex differences. These all reflect factors known to influence addiction risk in humans.
3) Future work with DREADDs could take advantage of what we learned during the experiments reported in Chapter 3. First, I would conduct more extensive pilot studies in which I would confirm the placements and expression of all viruses in every region of interest. I would also do more thorough dose response testing of CNO to ensure that we could successfully target both the Gq- and the Gi-DREADD, and collaborating with an electrophysiologist to measure activity in the target neurons could ensure that administering CNO has the intended effect. In addition, using microdialysis or some kind of glutamate/neurotransmitter-specific probe to determine the effect of activating DREADDs on neurotransmission could help to confirm the efficacy of the DREADD manipulation. Finally, administering CNO prior to euthanizing the animal would provide for an opportunity to use immediate early gene immunostaining to confirm not only the placement of the viral expression but also the efficacy of the CNO for altering neuronal activity.
4) Although we found no effect of manipulating IL-NAC activity on the acquisition and demand testing of MA self-administration, the contribution of these sub-circuits to MA extinction and reinstatement was not investigated. Given the evidence that the IL regulates extinction learning (Van den Oever et al., 2013; LaLumiere et al., 2010;

Peters et al., 2009; Peters et al., 2008; Maren and Quirk, 2004) and that the IL-shell governs cue-induced reinstatement of cocaine-seeking (Augur et al., 2016), it will be important to study how altering IL-core and IL-shell activity might impact the extinction of MA-seeking behavior.

5) Study 2 measured the effect of sub-circuit manipulation during extinction on cue-induced reinstatement but did not probe the effects of altering sub-circuit activity during the reinstatement test itself due to the animals' extensive experience with CNO during the extinction phase. Especially because the PL has been shown to be important for cue-, stress-, and cocaine-primed reinstatement (Gipson et al., 2013; LaLumiere et al., 2012; Di Pietro et al., 2006; Hiranita et al., 2006; Fuchs et al., 2005; McFarland et al., 2004; Capriles et al., 2003), and the IL-shell has an observed role in cue-induced cocaine reinstatement (Augur et al., 2016), future studies should allow animals to extinguish their MA responding without administering CNO, and then inject it prior to the reinstatement test only. It would also be useful to induce reinstatement in other ways, like a drug-primed reinstatement, especially since evidence suggests the role of the NAC in cue- vs. drug-primed reinstatement is sub-region specific (Scofield et al., 2016).
6) We did not obtain the expected results in our study the PL-core sub-circuit in the maintenance of MA self-administration. Given the evidence for NAC core involvement in the maintenance of drug-taking and reinstatement of drug-seeking, it would be worth investigating other afferents to the NAC core related to learning and reward. Some candidates for origins of projections terminating in the core that could be important for drug-taking include the hippocampus, amygdala, more dorsal areas of the PFC, and more dorsal regions of the striatum. In a similar vein, dorsal areas of

the PFC have also been implicated in cue- and drug-induced reinstatement, which would make the ACC a region of interest.

4.7 Conclusion

In conclusion, this dissertation contributes to the body of knowledge on addiction-related glutamate signaling and challenges the practicality of modeling the functions of different brain regions or projections as dichotomies. Though it may seem straightforward to directly compare two sub-regions of interest like the core and shell or PL and IL, we found that whether studying a single protein like Homer2 or a sub-circuit between the PFC and NAC, viewing the data in a simple dichotomy fails to recognize the complex, understudied nature of the functional neuroanatomy of MA addiction-related behaviors. There are likely overlapping projections and connectivity gradients that prevent the behavioral relevance of these regions from being neatly divided into opposing roles. This dissertation work highlights the importance of researching understudied substances like MA and developing advanced techniques that allow us to probe complex behaviors in increasingly sophisticated ways.

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