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The role of the prefrontal cortex in cocaine and heroin seeking following extinction training

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THE ROLE OF THE PREFRONTAL CORTEX IN COCAINE AND HEROIN SEEKING FOLLOWING EXTINCTION TRAINING

by

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CERTIFICATE OF APPROVAL

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PHD THESIS

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This is to certify that the Ph.D. thesis of

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To my parents, Ed and Linda, who not only gave me the world, but with it the confidence to know my place in it could never be limited simply because I am a woman. To my husband, Devin, whose unfaltering love and support is reflected in the existence of every page of this document. And to my daughter, Rosalind, whose entrance into this world forever changed who I am as a woman, a citizen, and a scientist. For it's not enough to do good science, we must do science that does good for its people.

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ABSTRACT

The prefrontal cortex (PFC) is considered a critical node in the neural circuitry underlying drug-seeking behaviors. However, the mechanisms by which this region influences drug seeking and whether or not the lateral PFC mediates cocaine or heroin seeking are questions that have yet to be answered. To expand on the role of the PFC in drug seeking, rats were trained on either heroin or cocaine self-administration for a minimum of 12 days before undergoing extinction training and subsequent reinstatement tests (cued and drug-prime). All pharmacological manipulations were delivered immediately prior to reinstatement testing and were targeted at either the ventral region of the medial PFC, the infralimbic cortex (IL), the anterior portion of the medial PFC, the medial orbitofrontal cortex (mOFC), the anterior region of the insular cortex, the dorsal agranular insular cortex (AId), or the posterior region of the insular cortex, the posterior insular cortex (PIc).

In chapter 1, D1 and D2 antagonists were administered into the IL and mOFC prior to cued and cocaine-prime reinstatement. Although previous studies found that the IL inhibits cocaine seeking, blocking D1 receptor activity in this region reduced cued reinstatement and had no effect on cocaine-prime reinstatement, indicating that the IL can promote cocaine seeking under certain circumstances. In contrast, blocking D1 receptors in the mOFC reduced all forms of reinstatement that were examined. Blocking D2 receptors in either region had no effect on cocaine seeking. Our data are the first to demonstrate a role for the mOFC in cocaine seeking and suggest that although the IL and mOFC lie immediately adjacent to one another, they play distinct roles in mediating cocaine seeking.

In chapter 2, we pharmacologically inactivated the AId and PIc via a GABA agonist administered immediately prior to both cocaine and food seeking. Reversible inactivation of the AId reduced cued reinstatement but had no effect on cocaine-prime reinstatement. In contrast, inactivating the PIc had no effect on any form of cocaine seeking. Additionally, blocking the AId during cued and food-prime reinstatement had no effect on food seeking, indicating the role of the AId in reinstatement is specific to cocaine seeking and not general motivated behavior. Additionally, blocking CRF1 receptors in the AId blocked cued reinstatement, suggesting a possible mechanism whereby the AId is influencing cocaine seeking. These data are the first to establish a role for the AId in cocaine seeking and demonstrate that although the PIc influences alcohol and nicotine seeking, it does not mediate cocaine seeking.

Chapter 3 further examined the role of the AId in cocaine seeking and expanded the influence of the insular cortex in drug seeking to heroin. AId D1 receptor blockade reduced both cued and cocaine-prime reinstatement following extinction training, whereas D2 receptor blockade had no effect on cocaine seeking. These results establish a role for the AId in cocaine-prime reinstatement, as pharmacological inactivation showed no role for the AId in cocaine-induced drug seeking. Additionally, blocking the AId during heroin seeking *potentiated* cued reinstatement whereas blocking the PIc during heroin seeking *reduced* cued reinstatement. These results demonstrate a role for the insular cortex in heroin seeking that has never been shown before and further explain how the AId may be influencing cocaine seeking.

PUBLIC ABSTRACT

Understanding the neural circuitry underlying addiction has been one of the main focuses of neurobiological research for decades, with the prefrontal cortex emerging as a region of particular interest in mediating the reinstatement of drug seeking. However, several questions remain as to how the prefrontal cortex exerts influence over drug-seeking behavior and how distinct subregions of this area differentially effect reinstatement behaviors. Therefore, we pharmacologically manipulated several regions of the prefrontal cortex, including the infralimbic cortex, medial orbitofrontal cortex, agranular dorsal insular cortex, and posterior insular cortex, to determine how activity in these regions influences drug seeking.

Our results show that dopaminergic signaling within the infralimbic cortex is essential for cue induced cocaine seeking, whereas dopaminergic signaling within an adjacent structure, the medial orbitofrontal cortex is necessary for numerous types of reinstatement to cocaine seeking. Additionally, the dorsal agranular insular cortex mediates cocaine seeking, as blocking this region reduces cued reinstatement. It appears dopaminergic signaling may mediate this effect as blocking dopamine receptors in this region similarly reduced cued reinstatement to cocaine seeking as well as cocaine-prime reinstatement. Finally, although the dorsal agranular insular cortex drives cocaine seeking, this same region inhibits heroin seeking, whereas the more posterior region of the insular cortex has no influence over cocaine seeking and drives heroin seeking.

Our data demonstrate that although the infralimbic cortex inhibits cocaine seeking, it can also drive cocaine seeking under certain circumstances. Notably, our data also establish a role for the insular cortex in mediating cocaine seeking and suggest

dopamine may mediate this influence. Finally, these data show that distinct regions of the insular cortex independently influence drug seeking depending on the drug as well as the type of reinstatement being examined.

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LIST OF ABBREVIATIONS

AId: Dorsal agranular insular cortex
BLA: Basolateral amygdala
BM: Baclfofen/Muscimol
EXT: Extinction baseline
IL: Infralimbic cortex
lOFC: Lateral orbitofrontal cortex
mPFC: Medial prefrontal cortex
NAcore: Nucleus accumbens core
NAshell: Nucleus accumbens shell
PFC: Prefrontal cortex
PIc: Posterior insular cortex
PL: Prelimbic cortex
SCH: SCH 23390
VP: Ventral pallidum

CHAPTER 1: INTRODUCTION

Understanding addiction and its related behaviors has been a goal of neurobiological research for decades. However, despite major advancements in determining the neural mechanisms underlying these behaviors, addiction remains a debilitating and costly disease, costing the United States an estimated $740 billion in crime, lost productivity, and health care annually (Abuse, 2017). One factor contributing to the persistence of this disease is the long-term risk of relapse present towards all classes of drugs of abuse. Thus, considering that relapse acts as both a hallmark of, and a barrier in the successful treatment of, drug addiction, elucidating the neurobiology underlying this recurring stage of drug abuse is essential in developing successful treatments.

To determine the neural circuitry underlying relapse, researchers have utilized several behavioral paradigms to study addiction related behaviors in rodents. Although both experimenter-administered drugs of abuse and conditioned place preference (CPP) models have yielded significant insight into the neural mechanisms driving recurring drug abuse (Martin-Fardon and Weiss, 2013; Napier et al., 2013; Nader, 2016), self-administration remains the gold standard for investigating the neurobiology of addiction in rodents. In this behavioral paradigm, animals are outfitted with an intravenous catheter to allow for behavior-driven drug delivery and are subsequently trained to make an operant response in order to initiate the delivery of a drug infusion. With this paradigm, self-administration of drugs of abuse can be followed by either abstinence or extinction training and relapse to drug seeking can later be investigated via the presentation of common relapse triggers (cues, drug-prime, stress, etc.), in a test referred to as reinstatement (Haney and Spealman, 2008; Bossert

et al., 2013). Through experiments employing self-administration, our understanding of the neural circuitry underlying reinstatement has grown considerably. However, several questions regarding the precise neural circuitry underlying reinstatement behaviors remain.

The Medial Prefrontal Cortex

One region known to be essential for mediating drug-seeking behaviors is the medial prefrontal cortex (mPFC) (Millan et al., 2011; Jasinska et al., 2015). Defined as the portion of cortex forming the medial wall anterior and dorsal to the corpus callosum; this region is further subdivided into four distinct regions: medial precentral, anterior cingulate, prelimbic (PL), and infralimbic (IL) cortices, (Heidbreder and Groenewegen, 2003; Hoover and Vertes, 2007), with the PL and IL demonstrating the most control over drug taking and drug-seeking behaviors. Research distinguishing these two adjacent regions has found that the PL is phylogenetically more recent with a distinct laminar structure, whereas the IL has very little lamination suggesting it is a phylogenetically older cortical structure (Jinks and McGregor, 1997). Additionally, several studies have demonstrated divergent connectivity surrounding these two mPFC subregions. Tracing studies have found that the PL heavily projects to the agranular insular cortex, nucleus accumbens core (NAcore), basolateral amygdala, and the paraventricular as well as the mediodorsal subregions of the thalamus. The IL, however, maintains dense projections to the bed nucleus of the stria terminalis, posterior insular cortex, hypothalamus, and the nucleus accumbens shell (NAshell), along with several subregions of the amygdala (Sesack et al., 1989; Vertes, 2004; Peters et al., 2009; Moorman et al., 2015). Given these distinctions, the PL is categorized as a limbic oriented structure involved in cognition and emotion-related processing, whereas the IL is thought of as an area with

significant influence over autonomic and visceral activities (Vertes, 2004). With these differences in mind, it is perhaps not surprising that the PL and IL play opposing roles in the regulation of drug-seeking behaviors.

Role of the mPFC in Drug-seeking Behavior

The mPFC is classically associated with decision-making, behavioral control, cognitive functioning, and working memory in both humans and rodents (Daffner et al., 2003; Walton et al., 2015; Ranganath and Jacob, 2016; Funahashi, 2017), making it particularly important in the study of addiction, given that this disease is characterized by low levels of behavioral inhibition and dysregulated motivational processing (Moeller et al., 2014). In fact, previous neuroimaging research has found that mPFC dysfunction in drug addicts is associated with increased drug use and higher relapse rates (Goldstein and Volkow, 2011). Complimenting these findings, research using rodents has successfully observed and manipulated activity throughout the mPFC to better delineate how various mPFC subregions influence discrete stages of addiction, including reinstatement. Along with increased *Arc* and *c-fos* expression throughout the prefrontal cortex during cocaine self-administration and reinstatement (Neisewander et al., 2000; Fumagalli et al., 2009; Moorman et al., 2015), structural differences have also been discovered following cocaine use, as chronic cocaine self-administration reduces dendritic branching within the PL and leads to working memory deficits in a T-maze task (Radley et al., 2015); suggesting that changes in mPFC structural plasticity may underlie an addict's persistent vulnerability to recurring drug use.

Interestingly, rodent research has found that the PL and IL, although immediately adjacent to one another, play distinct roles in drug-seeking behaviors. Pharmacological inactivation of the PL reduces cocaine seeking during a maintenance test and blocks reinstatement to a variety of reinstatement triggers including cocaine-associated cues, a cocaine prime, context, and stress (McFarland and Kalivas, 2001; Capriles et al., 2003; McLaughlin and See, 2003; Fuchs et al., 2005; Di Pietro et al., 2006). In contrast, pharmacological inactivation of the IL induces cocaine seeking and activating this area reduces cued and cocaine-prime reinstatement (Peters et al., 2008; Ebsworth and Lalumiere, 2012). Additionally, the IL has been shown to mediate the inhibition of cocaine seeking that occurs during extinction training, a stage that precedes reinstatement testing in numerous paradigms, as optogenetically inhibiting the IL immediately following an unreinforced lever press increased cocaine seeking during shortened extinction sessions, suggesting that the IL is necessary for the inhibition of cocaine seeking (LaLumiere et al., 2010; Gutman et al., 2017).

However, this simplified dichotomy as go/stop structures does not consistently hold true. Following 21 days of self-administration and subsequent extinction training, IL inactivation via the GABA agonists baclofen and muscimol reduced cocaine-prime reinstatement, directly contrasting findings in which TTX-induced inactivation of the IL had no effect on cocaine-prime reinstatement and activation of the IL reduced cocaine-prime reinstatement (Capriles et al., 2003; Vassoler et al., 2013). Further, IL inactivation following heroin self-administration and extinction training reduced cued and heroin-prime reinstatement, implying that the IL only acts as an inhibitory structure under specific circumstances (Rogers et al., 2008; Bossert et al., 2011b; Peters et al., 2013). One explanation for these

discrepancies may be methodological differences such as the method of inactivation or the behavioral paradigm. Indeed, chemogenetically activating the IL reduces cued-reinstatement to cocaine seeking following extinction training, but has no effect following abstinence (Augur et al., 2016). To probe the mechanisms underlying the role of the IL in cocaine seeking we investigated how dopamine within this region regulates reinstatement to better determine if these chemical signals account for the discrepancies observed within the IL literature.

The Final Common Pathway

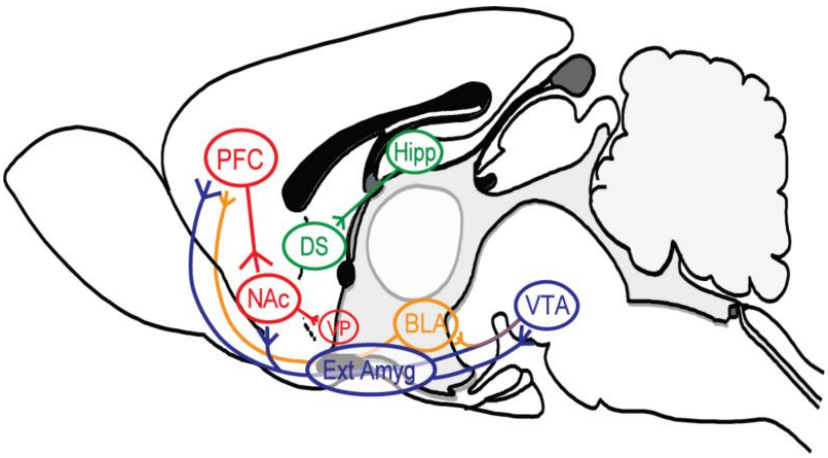

Figure 1. Diagram of the neural circuitry underlying cocaine seeking. The final common pathway (indicated in red) consists of the projection from the mPFC to the NAcore and the NAcore to the VP, and is required for all forms of reinstatement. Conversely, the BLA, extended amygdala, and hippocampus play discrete roles in mediating specific types of reinstatement. Figure adapted from Moussawi and Kalivas, 2010.

Although regions such as the basolateral amygdala (BLA) seem to be exclusively involved in one type of reinstatement, cued in the case of the BLA, the glutamatergic projection from the mPFC to the nucleus accumbens core (NAcore) is critical for all forms of reinstatement (cued, stress, cocaine-prime, contextual). Therefore this pathway, along with the GABAergic projection from the NAcore to the ventral pallidum (VP), is referred to as the final common pathway in the generation of motivated drug-seeking behaviors (Figure 1) (McLaughlin and See, 2003; Kalivas and Volkow, 2005; Fuchs et al., 2006; Knackstedt et al., 2010). Recent studies using optogenetic inhibition have

further confirmed the necessity of these pathways, as silencing PL afferents to the NAcore blocks cued reinstatement. Additionally, inhibition of the indirect projection from the NAcore to the dorsolateral subregion of the VP blocks cue + cocaine-prime reinstatement, whereas inhibition of the direct pathway from the NAcore to the substantia nigra has no effect on cocaine seeking (Stefanik et al., 2013a; Stefanik et al., 2016). Considering the pervasive influence of the final common pathway on cocaine seeking, it is difficult to predict how regions that are highly integrated with this pathway will regulate drug seeking. For instance, the extended amygdala, which integrates with the final common pathway through projections to the mPFC, is exclusively involved in the mediation of stress-induced reinstatement, making it distinct from the more dorsal portion of the amygdala, the BLA, which is known for its role in cued reinstatement (McFarland et al., 2004). Another region that is highly integrated with the final common pathway is **the insular cortex (IC)**. This lateral prefrontal structure receives a dopaminergic innervation from the VTA, similar to the mPFC, and also projects to the nucleus accumbens core (Montaron et al., 1996; Hurd et al., 2001; Naqvi and Bechara, 2009). Additionally, its subregions differentially project to the mPFC, with the anterior portion of the IC projecting to the PL, and the posterior regions of the IC projecting to the IL (Vertes, 2004). This connectivity with the final common pathway, along with several other lines of evidence that will be described below, suggest that the IC is an unestablished part of the circuitry underlying cocaine seeking.

The Insular Cortex

Despite the potential importance of the IC in relapse-related behavior, little work has specifically investigated the role of the IC or its subregions in the relapse to cocaine and

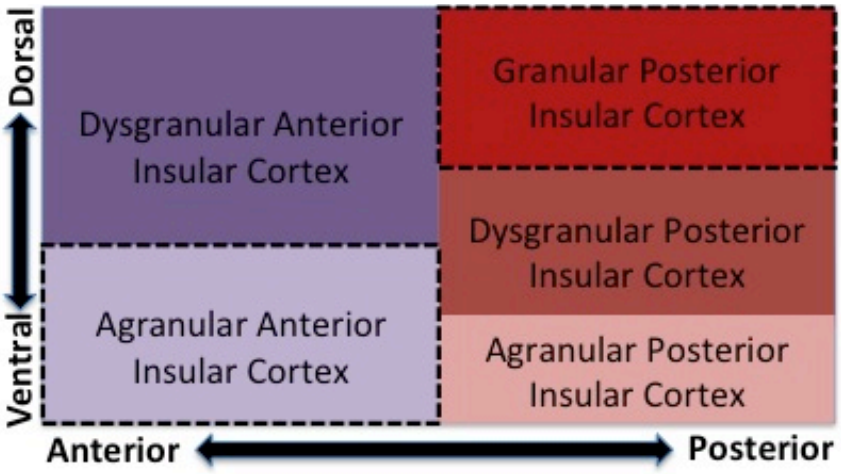

Figure 2. Diagram of the subregions of the insular cortex. Regions targeted in this proposal are highlighted within the dashed boxes agranular anterior insular cortex = AId; Granular Posterior Insular Cortex = Pic; Left to right = Anterior to Posterior axis; Top to bottom = Dorsal to Ventral

heroin seeking in rats. The IC is a heterogeneous structure in the cortex that consists of numerous subregions that are divided based on connectivity, cytoarchitecture, and varying functions. One of the major delineations throughout the IC is the presence or absence of granule cells, which are defined by their small cell bodies, resulting in granular (presence of granule cells), agranular (lack of granule cells), and dysgranular regions (transition between granular and agranular regions). The major divisions within the IC include an anterior portion that consists of both a dysgranular and agranular region, and a posterior region which is made up of a granular, dysgranular, and agranular region (Figure 2) (Li et al., 1998). Throughout all the experiments presented here I either targeted the dorsal agranular insular cortex (AId) in the anterior insular or the granular posterior insular cortex (PIc) in the posterior insular cortex (highlighted with dashed lines in Figure 2).

It is important to note that these regions are also divided based on their connectivity; the anterior IC is highly interconnected with limbic regions (PFC, amygdala, and NAcore) with its major outputs directed at motor regions of the thalamus and frontal cortex. In contrast, the posterior portion receives inputs from the parietal, occipital, and temporal cortices and

maintains its primary connection with the gustatory thalamic nucleus. Given these associations, the anterior IC is commonly thought of as a limbic region and the posterior segment is characterized as non-limbic (Naqvi and Bechara, 2009). Despite the differences between the anterior and posterior regions of the IC these two regions appear to work together, as sensory information from the thalamus initially passes through the granular and dysgranular insular cortices before being processed in the agranular insular cortex (Li et al., 1998); given this flow of information into the anterior IC, it has been suggested that this region integrates the somatosensory, gustatory, and viscerosenosry information fed to it from the posterior IC, and projects this information to the motor cortex to generate a variety of behavioral outcomes (Li et al., 1998).

Considering that drug abuse involves synthesizing internal and external cues to produce a motivated behavior, the IC may lie at the center of a novel pathway influencing drug seeking. Indeed, the distinct connections of the AId and PIc provide valuable insight into the potential functions of the IC in drug addiction, as the anterior region of the IC integrates autonomic and visceral information, such as changes in cardiovascular function or airway stimulation, into motivationally salient information (Naqvi and Bechara, 2009). In contrast, the posterior IC processes somatosensory, vestibular, and motor information that may be essential to the production of motivated behaviors like drug seeking. Further, the AId and PIc maintain distinct connections with several cortical regions already known to be involved in cocaine seeking, including the PL and IL cortices, along with projections to the NAcore and most regions of the amygdala. However, despite the fact that the IC is highly integrated with the existing circuitry believed to mediate drug seeking, its role in reinstatement has yet to be

fully explored (Li et al., 1998; Vertes, 2004; Voorn et al., 2004; Hoover and Vertes, 2007, 2011).

The Insular Cortex in Cognition

Upon its discovery, the IC was functionally categorized as part of the gustatory cortex (Shipley and Geinisman, 1984; Kosar et al., 1986). However, since these early studies, the role of the IC has significantly expanded to include involvement in cardiovascular function, taste reactivity, pain modification, and vestibular responses (Ogawa et al., 1992; Mazzola et al., 2014; Ronchi et al., 2015; Li et al., 2016; Lu et al., 2016; Oppenheimer and Cechetto, 2016). Despite the diversity in these autonomic functions, it is the role of the IC in cognition and interoceptive processing that helped to establish it as a potential node in the circuitry driving drug seeking. Initially, the IC processes interoceptive and exteroceptive cues, or the signals regarding changes to the body's homeostatic state, which are not only vital for survival but are also considered a driving factor in reinstatement. Indeed, each drug of abuse evokes its own unique set of homeostatic changes, which are then internally represented to influence motivated behavior. For example the feeling of smoke in ones airway or an increased heart rate are both interoceptive cues that may act as internal "reminders" to drive drug-seeking behaviors (Naqvi and Bechara, 2010; Gowin et al., 2014).

Beyond interoceptive processing, additional studies have expanded the influence of the IC to other cognitive traits related to drug abuse including impulsive and compulsive behaviors, as well as risky decision-making. Lesions of the anterior IC reduce both impulsivity and compulsive behavior in highly impulsive rodents as measured by a five-choice serial reaction

time task and schedule-induced polydipsia procedure, which is especially pertinent considering that highly impulsive rodents are predisposed to compulsively self-administer cocaine (Belin et al., 2008; Belin-Rauscent et al., 2016). Highly impulsive animals also express reduced zif268 mRNA levels in the anterior IC, and thinning in this same region correlates with motor impulsivity (Belin-Rauscent et al., 2016). Further, studies examining the role of the IC in cognitive functioning have found a link between the anterior IC and risky decision making as pharmacological AId inactivation shifts behavior toward choices with greater reward frequency and fewer punishments in a rodent gambling task (Ishii et al., 2012; Pushparaj et al., 2015b). However, although these studies detail the nature of the IC in several of the phenotypes associated with addiction, they fail to demonstrate how the IC regulates drug-seeking behaviors.

The Insular Cortex in Drug Addiction

Evidence directly linking the IC to drug addiction came when researchers found that IC damage sustained via injury or stroke resulted in almost immediate and complete smoking cessation in chronic nicotine users (Naqvi et al., 2007). This finding has since been confirmed with evidence showing that patients with damage to both the IC and the basal ganglia show significantly higher rates of smoking cessation than those patients whose brain damage is limited to the basal ganglia (Gaznick et al., 2014). Imaging studies have also found activation throughout the IC in response to drug-associated cues across numerous types of drug addiction, making this region especially relevant to the neurobiology underlying cued reinstatement (Wexler et al., 2001; Brody et al., 2002; Kilts et al., 2004; Myrick et al., 2004; McBride et al., 2006). However, research looking at the IC in human drug users has not

informed us on how distinct subregions of the IC differentially influence drug seeking. Rather, animal studies using a variety of reversible manipulations have successfully begun parsing out the distinct functions of IC subregions in drug addiction.

Posterior Insular Cortex

The PIc, or the non-limbic region of the IC, has been implicated in drug seeking for several drugs of abuse including amphetamine, alcohol, and nicotine. Using a conditioned place preference model, researchers determined that amphetamine seeking in control animals induced Fos expression in the PIc, whereas pharmacological PIc inactivation following condition place preference training blocked amphetamine seeking (Contreras et al., 2007). Additionally, PIc inactivation reduced alcohol intake and operant responding during alcohol self-administration; however this study did not evaluate whether disruption of PIc activity influences reinstatement to alcohol seeking (Pushparaj and Le Foll, 2015). In line with previously described human studies, PIc inactivation reduces nicotine self-administration under both a fixed and progressive ratio program, and blocks cued and nicotine-primed reinstatement (Forget et al., 2010). However, PIc inactivation has no effect on food seeking, suggesting a drug specific role for the IC in motivated behavior. Interestingly, electrical stimulation of the PIc produced identical results to those observed with pharmacological inactivation, by reducing nicotine self-administration and reinstatement (Pushparaj et al., 2013). Despite the involvement of the PIc in mediating behavior towards the drugs of abuse described above, the role of this structure in either cocaine or heroin seeking has not been examined.

Agranular Insular Cortex

Similar to the PIc, inactivation of the AId reduces nicotine self-administration as well as cued reinstatement following extinction training in rodents (Pushparaj et al., 2015a). However in contrast to the PIc, there is evidence to suggest that the AId is involved in mediating cocaine seeking. Initially, imaging studies have found that cocaine addicts exhibit agranular cortex abnormalities, including decreased gray matter density and overall volume (Ersche et al., 2011). Additionally, reversible pharmacological inactivation of the AId via lidocaine reduced odor context-dependent cue-induced reinstatement of cocaine-seeking but had no effect on sound context-dependent cue-induced reinstatement, establishing a role for this structure in the reinstatement to cocaine-seeking (Di Pietro et al., 2006). Subsequent studies have also shown that AId inactivation blocks contextual reinstatement, in which animals are trained to self-administer in one context, extinguished in a separate context, and then returned to the cocaine-paired context for a 2 h reinstatement session (Arguello et al., 2017). Yet, this structure has not been examined during any of the other commonly studied forms of cocaine-seeking reinstatement including cued and cocaine-prime reinstatement tests. In contrast to studies demonstrating the AId promotes cocaine seeking, lesions of the anterior regions of the IC, including the AId, potentiate cocaine-seeking behavior following a period of abstinence in a self-administration model (Pelloux et al., 2013). Intriguingly, although not the focus of the work, Di Pietro et al (2006) also found an apparent trend toward increased cocaine-seeking with greater AId inactivation during the presentation of cocaine-prime + sound contextual cues. Thus, given that AId inactivation seems to produce both increased and attenuated drug-seeking behaviors, the precise nature of how the IC regulates the

reinstatement of cocaine-seeking behavior and how various IC subregions are involved remains unclear.

Cocaine versus Heroin Seeking

Although the net result following both cocaine and heroin use is an increased dopamine concentration within the mesocorticoloimbic pathway (Badiani et al., 2011), several neural differences have emerged indicating that these two drugs of abuse cannot be treated as identical compounds. Thus, while establishing the neurobiology underlying cocaine seeking can be informative for heroin addiction, several regions known to influence cocaine seeking perform differently during the reinstatement to heroin seeking. For instance, the IL, which is classically known as an "off" switch for cocaine-seeking behaviors, acts as an "on" switch by driving cued and contextual reinstatement to heroin seeking (Rogers et al., 2008; Bossert et al., 2011b; Peters et al., 2013). Further, heroin-prime reinstatement seems to involve several brain regions that do not effect cocaine-prime reinstatement including the BLA, central nucleus of the amygdala, and the substania nigra (McFarland and Kalivas, 2001; Rogers et al., 2008; Badiani et al., 2011). Even in regions that are involved in both heroin and cocaine seeking, there appears to be separate neuronal populations mediating these influences as in vivo extracellular recordings in the mPFC and NAc found that only ~20% of the neurons examined responded similarly to both cocaine and heroin self-administration (Chang et al., 1998). Interestingly, although the IC has been studied in relation to cocaine, amphetamine, nicotine, and alcohol use there are no studies, to our knowledge, examining this structure in relation to heroin addiction. Thus, given the established differences that exist between the circuitry underlying heroin and cocaine seeking it is unclear how the IC will regulate

reinstatement to these two distinct drugs of abuse. Thus the current studies investigated how the AId and PIc contribute to the regulation of both cocaine and heroin seeking.

Summary

Below is a brief summary of each question addressed by these experiments.

- How does dopamine in the IL and mOFC mediate cued and cocaine-prime reinstatement? **Chapter 2**
- What role does the IC play in mediating reinstatement to cued and drug-prime reinstatement following either cocaine or heroin self-administration? **Chapters 3 and 4**
- Does the IC mediate reinstatement to natural rewards such as food seeking? **Chapter 3**
- What mechanisms influence the role of the AId in cocaine seeking? **Chapter 4**

CHAPTER 2. D1, BUT NOT D2, RECEPTOR BLOCKADE WITHIN THE INFRALIMBIC AND MEDIAL ORBTIOFRONTAL CORTEX IMPAIRS COCAINE SEEKING IN A REGION-SPECFIC MANNER

Increasing evidence has pointed to a critical role for the ventromedial prefrontal cortex (vmPFC), and especially the infralimbic (IL) region of the vmPFC, in regulating drug seeking (Peters et al., 2008; for a larger review of PFC involvement in drug seeking, see Van den Oever et al., 2010; LaLumiere et al., 2012b). Prior work indicates that IL *inactivation* induces cocaine seeking during an extinction session, whereas IL *activation* reduces cocaine seeking during cocaine-prime or cued reinstatement (Peters et al., 2008; LaLumiere et al., 2012b). Together with other findings (LaLumiere et al., 2010), these results suggest that IL activity suppresses cocaine seeking following self-administration and extinction training and is involved in the consolidation of extinction learning for cocaine-seeking behavior.

However, prior studies have not investigated the role of IL dopamine receptors in cocaine seeking, despite known dopaminergic projections to the vmPFC (Van Eden et al., 1987; Smiley et al., 1992; Hitchcott et al., 2007). Previous work examining this region during non-drug related motivated behaviors indicates that intra-IL infusions of dopamine or a D1 receptor antagonist produce a shift from habitual to goal-oriented instrumental behaviors (Hitchcott et al., 2007). Additionally, D1 receptor antagonism and D2 receptor agonism within the IL reduce compulsive sucrose seeking (Barker et al., 2013). Given that addiction is conceptualized as a shift from goal-oriented behaviors to a habitual response (Koob and Volkow, 2010), such findings suggest that dopamine activity within the IL may be critical for the expression of relapse behaviors. Indeed, addressing this issue may be of importance to related controversial questions raised about the role of the IL in inhibiting drug seeking in

general, as previous studies have shown blocking 5-HT_{2A} receptors in the vmPFC decreases both cue and cocaine-primed reinstatement (Pockros et al., 2011). Moreover, IL/vmPFC activity drives, rather than inhibits, the reinstatement of heroin seeking (Rogers et al., 2008; Bossert et al., 2011a), leading Peters and colleagues (2013) to suggest that dopamine activity within the IL may account for these discrepant results regarding the ability of the IL to regulate drug seeking but note that no data exist to address this question.

In addition to the IL, the vmPFC also includes a more anterior subregion known as the medial orbitofrontal cortex (mOFC). Previous research has found that identical manipulations in different vmPFC regions along the rostrocaudal axis produce distinct behavioral effects (Smith and Berridge, 2005). Indeed, IL activation appears to suppress feeding behavior that is induced via glutamate receptor blockade in the nucleus accumbens shell, whereas mOFC activation enhances this feeding behavior (Richard and Berridge, 2013). However, akin to prior work on the IL, mOFC inactivation using a $GABA_{A/B}$ agonist cocktail has no effect on the reinstatement of cocaine-seeking behavior (Fuchs et al., 2004). Nevertheless, previous research on the nucleus accumbens shell has found conflicting results using GABA receptor activation vs. dopamine receptor manipulations in terms of the reinstatement of cocaine seeking (McFarland and Kalivas, 2001; Anderson et al., 2006). Given that the mOFC receives dopaminergic innervation from the VTA (Swanson, 1982) and maintains efferent projections to the amygdala and nucleus accumbens (Brog et al., 1993; Ishikawa and Nakamura, 2003; Malkusz et al., 2015), we considered the possibility that dopaminergic manipulations in the mOFC would alter reinstatement. As D1 receptor blockade in the lateral OFC reduces context-induced cocaine seeking (Lasseter et al., 2014),

we hypothesized that blocking these receptors in the medial region of the OFC would similarly result in a disruption of cocaine seeking. To examine these issues, the current study investigated the effects of D1 and D2 receptor blockade in the IL and mOFC during the reinstatement of cocaine seeking.

Materials and Methods

Subjects

Male Sprague-Dawley rats (250-275 g at time of arrival; Charles River Laboratories; n = 98) were single-housed on a 12-hour reverse light cycle (and kept at constant temperature) with food and water ad libitum in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved vivarium. All animals were allowed to acclimate to the vivarium for a minimum of 5 days before undergoing surgery. All procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by University of Iowa Institutional Animal Care and Use Committee.

Surgery

Rats were anaesthetized using ketamine (100 mg/kg, i.m.) and xylazine (6 mg/kg, i.m.). Additionally, ketorolac was given (3 mg/kg, i.p.) as an analgesic on both the day of surgery as well as the day immediately following surgery. Catheter implantation was performed, as described previously (Cosme et al., 2015). Rats were then placed in a small animal stereotaxic instrument (Kopf Instruments, Tujunga, CA, USA). Jewelers' screws were affixed to the skull surface to serve as anchors. Double-barreled cannulae with a 1.2 mm center-to-center distance (Plastics One, Roanoke, VA, USA) were implanted, aimed at either the IL or mOFC and then secured using dental cement. Coordinates were as follows: IL: 2.8

mm anterior to bregma and 2.1 mm ventral from the skull surface (cannula aimed 3 mm above IL); mOFC: 3.5 mm anterior to bregma and 2.8 mm ventral from skull surface (cannula aimed 3 mm above mOFC). These coordinates were chosen based on previous work (LaLumiere et al., 2010) and refined in our laboratory.

After surgery, animals received 3 ml subcutaneous saline and a topical application of the anesthetic bupivacaine to the cranial and chest incision sites. Dummy injectors were placed in each cannula along with a tight fitting cap. Rats were then returned to their home cages where they were allowed to recover for 5-7 days. During recovery, catheters were flushed daily with 0.1 ml of heparinized saline (100 USP) to ensure catheter patency.

Cocaine Self-administration and Extinction

Self-administration was conducted in standard operant chambers (Med Associates, Fairfield, VT) with two retractable levers, a house light, a cue light, and a tone-generator (4500 Hz). Rats had all food removed 24 h prior to a single 15-h overnight food training session. During this session, a press on the active lever resulted in a single food pellet (45 mg) on a fixed-ratio 1 (FR1) schedule. Following the single food training session, rats were fed 10 g of rat chow and began self-administration training the following day. All rats received ~20 g of rat chow daily after each cocaine self-administration, extinction, or reinstatement session. After food training, rats' catheters were assessed for patency using 0.1 ml of sodium brevital (10 mg/ml).

One day after food training, cocaine self-administration began. Rats were placed in operant chambers for 2 h/d for a minimum of 12 d, during which active lever presses produced an infusion of cocaine (50 µl infusion of 150 µg cocaine dissolved in sterile saline, given over 2.18 s; cocaine kindly provided by the National Institute on Drug Abuse) along with a 5 s light and tone cue on an FR1 schedule. A 20 s timeout period followed each infusion. Inactive lever presses had no consequence. Animals began extinction training if they received a minimum of 15 infusions of cocaine per day for at least 10 d, including the last 3 d of self-administration, and demonstrated discrimination between the active and inactive lever. During extinction training, active lever presses did not produce cocaine infusions or the light and tone cues. Each rat underwent extinction for a minimum of 7 d and entered reinstatement testing when it had 25 or fewer active lever presses for at least 2 consecutive days immediately prior to the reinstatement session. The final 2 d of extinction training prior to each reinstatement test served as the extinction baseline.

Microinjections

Immediately prior to each reinstatement test, rats received intra-IL or intra-mOFC microinjections. Microinjectors (with 3 mm projections beyond the end of the respective cannula) were connected to PE20 tubing, which was attached to 10-µl Hamilton syringes controlled by an infusion pump. Microinjections (0.3 µl/side) were given over 1 min, and injectors were left in place for an additional 2 min to permit drug diffusion. Following the microinjection, rats were immediately placed into the operant chamber for the appropriate reinstatement test. Microinjected drugs consisted of the D1 receptor antagonist SCH 23390 (0.1 µg/side) and the D2 receptor antagonist sulpiride (30 ng/side), each dissolved in artificial

cerebral spinal fluid (aCSF) as the vehicle. Doses were chosen based on prior research (Lalumiere et al., 2004). Although some previous studies have infused higher concentrations of sulpiride (1-3 μg/side) into the mPFC (Pirronti et al., 2000; Winter et al., 2009), others have shown that smaller concentrations of sulpiride are equally as effective (Chitkara et al., 2000), and, in some cases, lower doses of sulpiride in the mPFC and nucleus accumbens have proven to be more effective at revealing behavioral differences (Setlow and McGaugh, 1998; Cheshenko et al., 2007).

Reinstatement Testing

During each reinstatement test (2 h), active lever presses never produced a cocaine infusion. Following each reinstatement test, rats had their lever pressing re-extinguished to baseline levels for a minimum of 3 days using the same criteria described previously. Each rat underwent three reinstatement tests (cued, cocaine-prime, and cue + cocaine-prime) and completed each test twice, once following a vehicle microinjection and once following a drug microinjection in a counterbalanced design, resulting in a total of 6 reinstatement sessions per rat. For cued reinstatement, active lever presses produced the light and tone cues that were previously paired with the drug infusion during self-administration. Cocaine-prime reinstatement consisted of an injection of cocaine (10 mg/kg, i.p.) immediately before the reinstatement session. Cued + cocaine-prime reinstatement involved an injection of cocaine (10 mg/kg, i.p.) immediately prior to a cued reinstatement session. As cue + cocaine-prime reinstatement typically produces higher levels of cocaine-seeking behavior than cue or cocaine-prime reinstatement alone, we included this test in our experimental design to

provide us with the optimal opportunity to observe behavioral differences following dopamine receptor blockade.

Experiment 1. Prior to reinstatement testing rats received intra-IL microinjections of the D1 receptor antagonist SCH 23390 (n = 11). All rats underwent reinstatement testing in the following order: cued, cocaine-prime, and cued + cocaine-prime as described above. Due to clogged cannula and illness, three rats were removed from the experiment after the cued reinstatement and one more was removed following the cocaine-prime test.

Experiment 2. Prior to reinstatement testing rats received intra-IL microinjections of the D2 receptor antagonist sulpiride (n = 14). All rats underwent reinstatement testing in the following order: cued, cocaine-prime, and cued + cocaine-prime, as described above.

Experiment 3. Prior to reinstatement testing rats received intra-mOFC microinjections of the D1 receptor antagonist SCH 23390 (n = 12). Rats underwent reinstatement testing in the following order: cued, cocaine-prime, and cued + cocaine-prime, except for a subset of rats that, unintentionally, received exposure to the cues during extinction training. Therefore, the data for this subset (n = 5) for the cued reinstatement were not included in the analysis due to concern about the robustness of the reinstatement levels. The data for this subset for the cued + cocaine-prime reinstatement were included, as the levels of this reinstatement were robust and the results were not different from those observed with the rest of the rats in this experiment. Due to clogged cannula and illness, two rats were removed from the experiment after the cued reinstatement and four more was removed following the cocaine-prime test.

Experiment 4. Prior to reinstatement testing rats received intra-mOFC microinjections of the D2 receptor antagonist sulpiride (n = 6). All rats underwent reinstatement testing in the following order: cued, cocaine-prime, and cued + cocaine-prime, as previously described. Due to a lack of significant cocaine-prime reinstatement, an additional group of rats underwent only cocaine-prime reinstatement with no prior reinstatement tests (n = 6).

Histological Analysis

To verify cannula placement, rats were overdosed with sodium pentobarbital (100mg/kg, i.p.) and intracardially perfused using phosphate-buffered saline. Brains were then placed in 3.7% formaldehyde for a minimum of 24 h before being sectioned. Coronal slices were 75 μm thick and were mounted directly onto a gelatin-coated slide. Sections were stained using Cresyl violet and analyzed for the correct termination site of the microinjectors. Any rat with an injector termination point outside the borders of the IL or mOFC was excluded from further analysis.

Data Analysis

Two-way analyses of variance (ANOVA) were used to analyze reinstatement lever pressing data with both comparisons as within-subjects (extinction vs. reinstatement; vehicle vs. drug). Post-hoc analysis was completed using Holm-Sidak's multiple comparison tests. p-values of less than 0.05 were considered significant. All measures were expressed as mean $\pm$ SEM. Each group's n is indicated in the figure.

Results

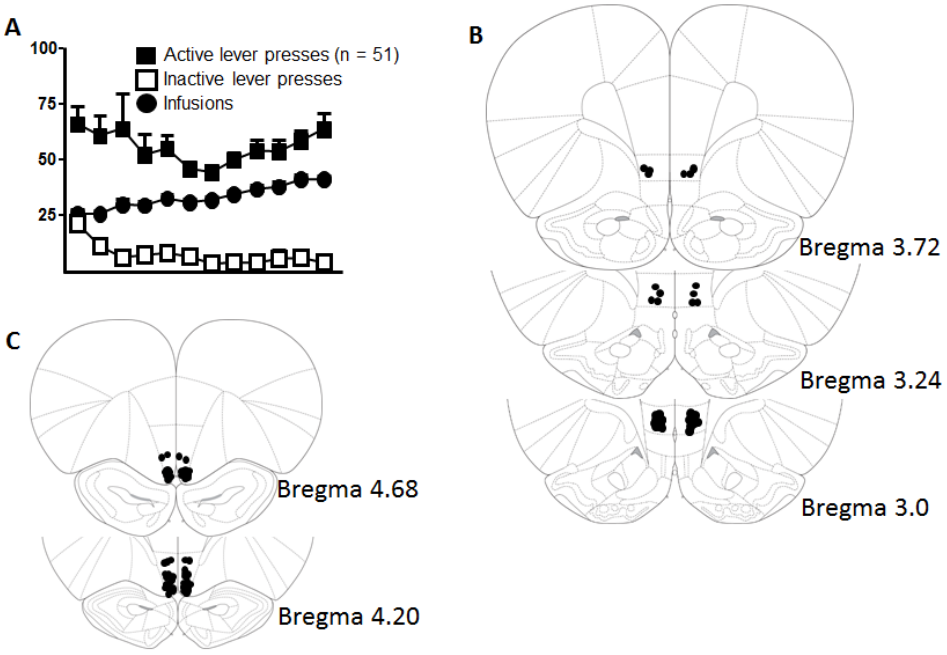

Figure 3. Self-administration data and histological representations. **A,** Number of active and inactive lever presses and cocaine infusions for the last 12 days of cocaine self-administration for all rats included in the final analysis. **B and C**, Diagrams showing the termination of needle tracks for microinjections aimed at the IL and mOFC, respectively. Figures are adapted from Paxinos and Watson (2007), and A/P coordinates (in mm) are given relative to Bregma.

Out of a total of 96 rats used in the present experiments, 51 rats were included in the final data. Rats were excluded due to misplaced (18 rats) or unverifiable (15 rats) microinjection termination locations, failure to acquire self-administration (1 rat), loss of cannula patency (7 rats), and loss of catheter patency (4 rats). Figure 3A shows the number of active and inactive lever presses and cocaine infusions over the final 12 days of cocaine self-administration. Active lever pressing was significantly higher than inactive lever pressing over the last 3 days of self-administration ($t(50) = 9.55$, $p < 0.05$). Figures 3B and C show the termination site of the microinjector tips in the IL and mOFC, respectively.

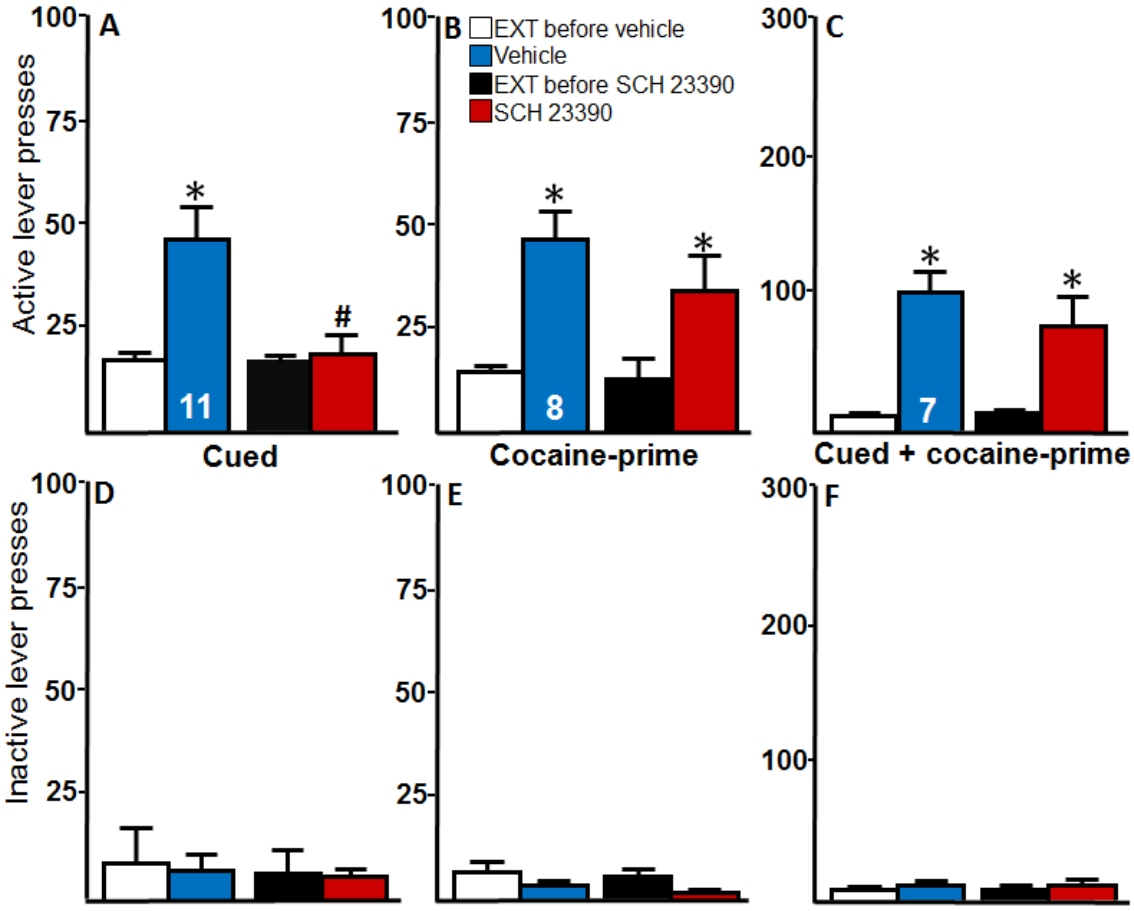

Figure 4. Intra-IL D1 receptor blockade via SCH 23390 (SCH) mediates cued reinstatement. (Note that the y-axis maximum is either 100 or 300 across the different experiments to accommodate the large differences in values.) **A-C,** The active lever presses and, **D-F,** the inactive lever presses for each reinstatement test for Experiment 1 are shown. **A,** Intra-IL microinjections of the D1 receptor antagonist SCH significantly reduced active lever pressing during cued reinstatement compared to vehicle controls (n = 11). **B,** Intra-IL microinjections of SCH had no effect on active lever presses during cocaine-prime reinstatement (n = 8). **C,** Intra-IL microinjections of SCH had no effect on active lever pressing during cued + cocaine-prime reinstatement (n = 7). **D-F,** There were no effects of reinstatement or microinjections on inactive lever presses for any type of reinstatement test. *$p < 0.05$ compared with extinction baseline. #$p < 0.05$ compared to vehicle-control group. EXT, extinction baseline.

Experiment 1. D1 Receptor Blockade in the IL Attenuates Cued Reinstatement

D1 receptor blockade within the IL significantly reduced active lever presses for cued reinstatement (Figure 4A). A two-way repeated-measures ANOVA of active lever presses indicated a significant effect of reinstatement ($F_{(1, 10)} = 7.78$, $p < 0.05$), a significant effect of microinjection ($F_{(1,10)} = 7.23$, $p < 0.05$) and a significant interaction ($F_{(1,10)} = 7.017$, $p < 0.05$).

Post hoc tests revealed that, although active lever pressing during reinstatement with vehicle treatment was significantly higher than the extinction baseline ($p < 0.05$), active lever pressing during reinstatement following SCH treatment was no different from extinction baseline and was significantly lower than what was observed with the vehicle treatment ($p < 0.05$). Intra-IL D1 receptor blockade had no effect on active lever pressing during cocaine-prime reinstatement (Figure 4B). A two-way repeated measures ANOVA of active lever presses indicated a significant effect of reinstatement ($F_{(1,7)} = 17.37$, $p < 0.01$), no effect of microinjection ($F_{(1,7)} = 2.28$, $p > 0.05$), and no interaction ($F_{(1,7)} = 0.96$, $p > 0.05$). Post hoc tests revealed that active lever pressing during reinstatement was significantly higher than extinction for both treatments ($p < 0.05$). Intra-IL D1 receptor blockade had no effect on cue + cocaine-prime reinstatement (Figure 4C). A two-way repeated measures ANOVA of active lever presses indicated a significant effect of reinstatement ($F_{(1,6)} = 27.53$, $p < 0.01$), no effect of microinjection ($F_{(1,6)} = 1.07$, $p > 0.05$), and no interaction ($F_{(1,6)} = 1.48$, $p > 0.05$). Post hoc tests revealed that active lever pressing during reinstatement was significantly higher than extinction for both treatments ($p < 0.05$). There were no significant differences in inactive lever presses across any type of reinstatement (Figures 4D-F).

Experiment 2. D2 Receptor Blockade in the IL has no Effect on Cocaine-seeking Behavior

Intra-IL D2 receptor blockade had no effect on active lever pressing during cued reinstatement (Figure 5A). A two-way repeated measures ANOVA indicated a significant effect of reinstatement ($F_{(1,13)} = 13.60$, $p < 0.05$), no effect of microinjections ($F_{(1,13)} = 1.37$, $p > 0.05$), and no interaction ($F_{(1,13)} = 1.13$, $p > 0.05$). Post hoc tests revealed that active lever pressing during reinstatement was significantly higher than extinction for both treatments

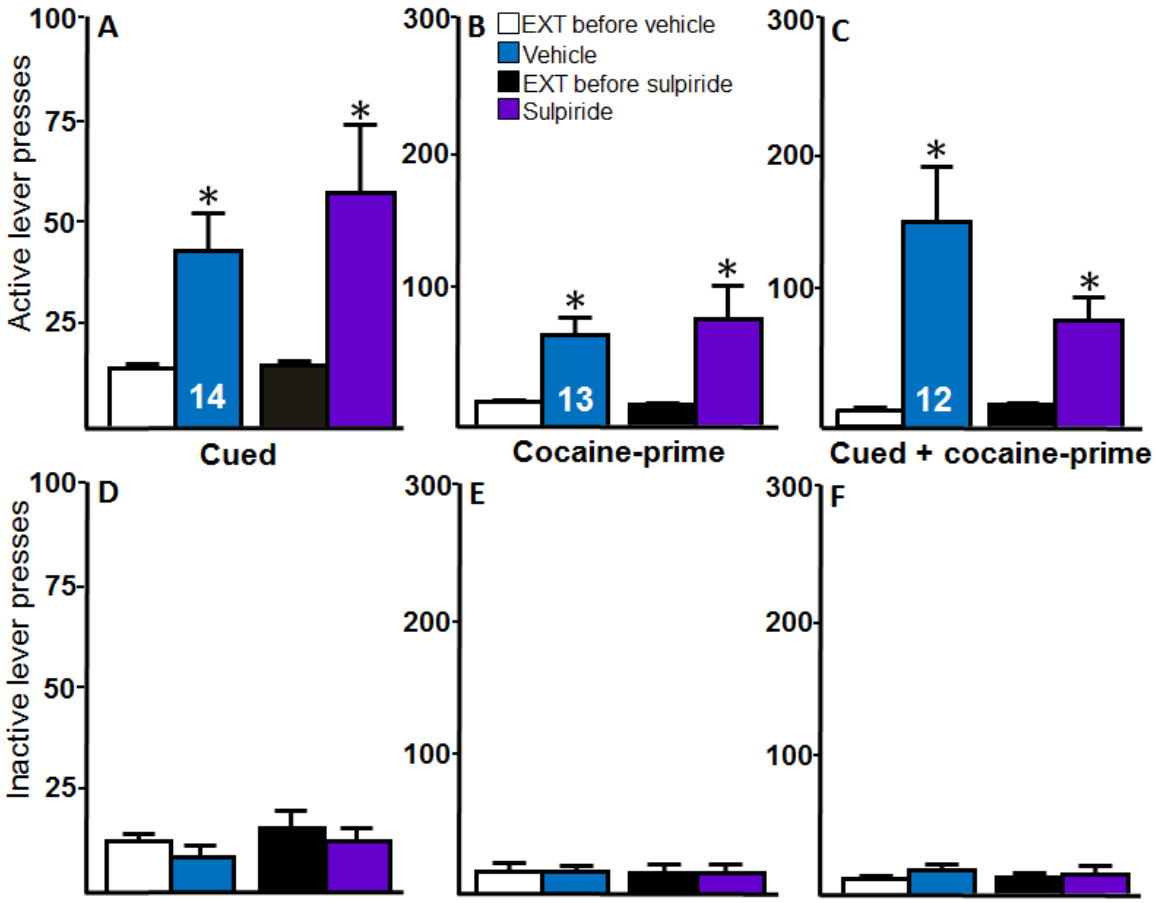

Figure 5. Intra-IL D2 receptor blockade via sulpiride does not alter cued or cocaine-prime reinstatement. **A-C,** The active lever presses and, **D-F,** inactive lever presses for each reinstatement test for Experiment 2 are shown. **A,** Intra-IL microinjections of the D2 receptor antagonist sulpiride had no effect on cued reinstatement (n = 14). **B**, Intra-IL microinjections of sulpiride had no effect on cocaine-prime reinstatement (n = 13). **C,** Intra-IL microinjections of sulpiride had no effect on cued + cocaine-prime reinstatement (n = 12). **D-F,** There were no effects of reinstatement or microinjections on inactive lever presses for any type of reinstatement. *$p <$ 0.05 compared with extinction baseline. EXT, extinction baseline.

(vehicle vs. sulpiride; $p < 0.05$). Intra-IL D2 receptor blockade had no effect on active lever pressing during cocaine-prime reinstatement (Figure 5B). A two-way repeated measures ANOVA indicated a significant effect of reinstatement ($F_{(1,12)} = 12.54$, $p < 0.05$), no effect of microinjections ($F_{(1,12)} = 0.06$, $p > 0.05$), and no significant interaction ($F_{(1,12)} = 0.23$, $p > 0.05$). Post hoc tests revealed that active lever pressing during reinstatement was significantly higher than extinction for both treatments ($p < 0.05$). Intra-IL D2 receptor blockade had no effect on active lever pressing during cued + cocaine-prime reinstatement (Figure 5C). A

two-way repeated measures ANOVA revealed a significant effect of reinstatement ($F_{(1,11)}$ = 17.18, $p < 0.05$) but no effect of microinjections ($F_{(1,11)} = 3.12$, $p > 0.05$) or significant interaction ($F_{(1,11)} = 3.90$, $p > 0.05$). Post hoc tests revealed that active lever pressing during reinstatement was significantly higher than extinction for both treatments ($p < 0.05$). There were no significant differences in inactive lever presses across any type of reinstatement (Figure 5D-F).

Experiment 3. D1 Receptor Blockade in the mOFC Reduces Cocaine-seeking Behavior

Intra-mOFC D1 receptor blockade significantly reduced active lever pressing during cued-reinstatement (Figure 6A). A two-way repeated measures ANOVA indicated a significant effect of reinstatement ($F_{(1,8)} = 24.00$, $p < 0.05$), a significant effect of microinjections ($F_{(1,8)}$ = 5.12, $p < 0.05$), and a significant interaction ($F_{(1,8)} = 7.51$, $p < 0.05$). A post-hoc analysis revealed that, although vehicle-treated animals had significantly increased active lever pressing during reinstatement compared to the extinction baseline, active lever pressing during reinstatement following SCH treatment did not significantly differ from extinction baseline and was significantly lower than that observed with vehicle treatment ($p < 0.05$). Intra-mOFC D1 receptor blockade also resulted in a significant reduction of active lever pressing during cocaine-prime reinstatement (Figure 6B). A two-way repeated measures ANOVA indicated a significant effect of reinstatement ($F_{(1,11)} = 20.72$, $p < 0.05$), a significant effect of microinjections ($F_{(1,11)} = 8.91$, $p < 0.05$), and a significant interaction ($F_{(1,11)} = 10.07$, $p < 0.05$). Post hoc analysis revealed the vehicle-treated rats demonstrated significantly increased active lever pressing during reinstatement compared to extinction baseline ($p < 0.05$). In contrast, SCH-treated rats showed no difference in active lever presses

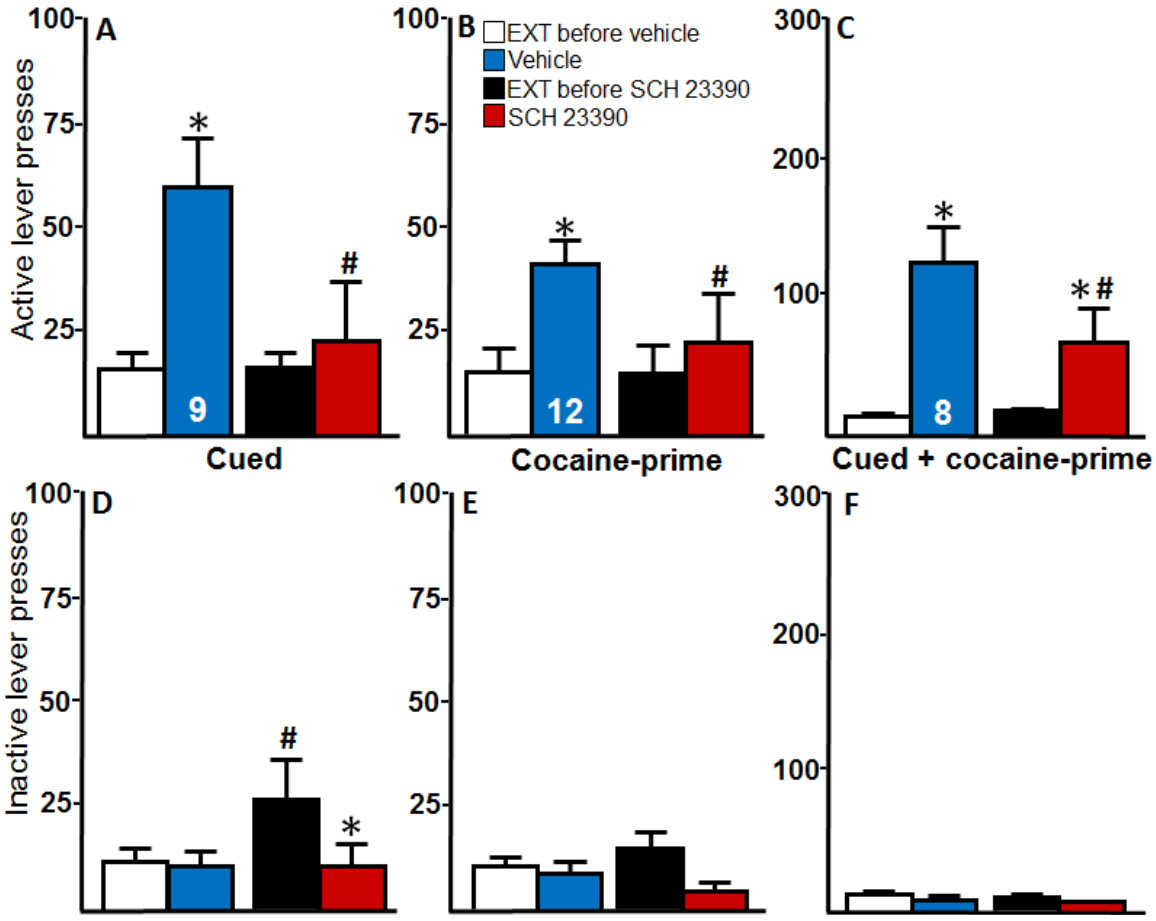

Figure 6. Intra-mOFC D1 receptor blockade via SCH 23390 (SCH) mediates cocaine-seeking behaviors. **A-C,** The active lever presses and, **D-F,** the inactive lever presses for each reinstatement test for Experiment 2 are shown. **A,** Intra-mOFC microinjections of SCH significantly reduced active lever pressing during cued reinstatement compared to vehicle controls (n = 9). **B,** Intra-mOFC microinjections of SCH significantly reduced active lever pressing during cocaine-prime reinstatement (n = 12). **C,** Intra-mOFC microinjections of SCH significantly reduced active lever pressing during cued + cocaine-prime reinstatement (n = 8). **D,** Intra-mOFC microinjections significantly reduced inactive lever presses compared to extinction baseline, though the extinction baseline itself was elevated. **E and F,** There were no effects of reinstatement or microinjections on inactive lever presses for cocaine-prime or cued + cocaine-prime reinstatement. *$p < 0.05$ compared with extinction baseline. #$p < 0.05$ compared to vehicle-control group. EXT, extinction baseline.

during reinstatement compared to extinction baseline and had significantly fewer active lever presses compared to the vehicle-treated group ($p < 0.01$). Intra-mOFC D1 receptor blockade significantly attenuated active lever pressing during cued + cocaine-prime reinstatement (Figure 6C). A two-way repeated measures ANOVA revealed a significant effect of reinstatement ($F_{(1,7)} = 11.87$, $p < 0.05$), a significant effect of microinjections ($F_{(1,7)} = 9.90$, $p < 0.05$), and a significant interaction ($F_{(1,7)} = 14.78$, $p < 0.05$). Post hoc analysis showed that,

although both the vehicle- and SCH- treated groups demonstrated significantly higher lever pressing during reinstatement compared to extinction baseline, the SCH-treated animals had significantly fewer active lever presses compared to the vehicle-treated group ($p < 0.05$).

A two-way repeated measures ANOVA of inactive lever presses during cued reinstatement (Figure 6D) revealed a significant effect of reinstatement ($F_{(1,8)} = 6.24$, $p < 0.05$), no effect of microinjections ($F_{(1,8)} = 2.04$, $p > 0.05$), and a significant interaction ($F_{(1,8)} = 8.86$, $p < 0.05$). Post hoc analysis indicated that the SCH-treated animals had significantly higher inactive lever presses during extinction baseline when compared to both the extinction baseline for the vehicle group and the SCH-treated animals during reinstatement testing. However, analyses of inactive lever pressing for the cocaine-prime and cued + cocaine-prime reinstatement showed no significant differences (Figures 6E-F). Because the statistically significant increase in inactive lever pressing shown in Figure 6D occurred during the extinction baseline when no manipulations were given, because there was no significant effect in the active lever presses during the same extinction baseline, and because there were no other statistically significant effects of inactive lever pressing either in the baseline or reinstatement tests in this experiment or across all the experiments, it is difficult to conclude that the observed effect is anything but a random statistical artifact.

Experiment 4. D2 receptor Blockade in the mOFC has no Effect on Cocaine-seeking Behavior

Intra-mOFC D2 receptor blockade had no effect on active lever presses during cued reinstatement (Figure 7A). A two-way repeated measures ANOVA of active lever presses

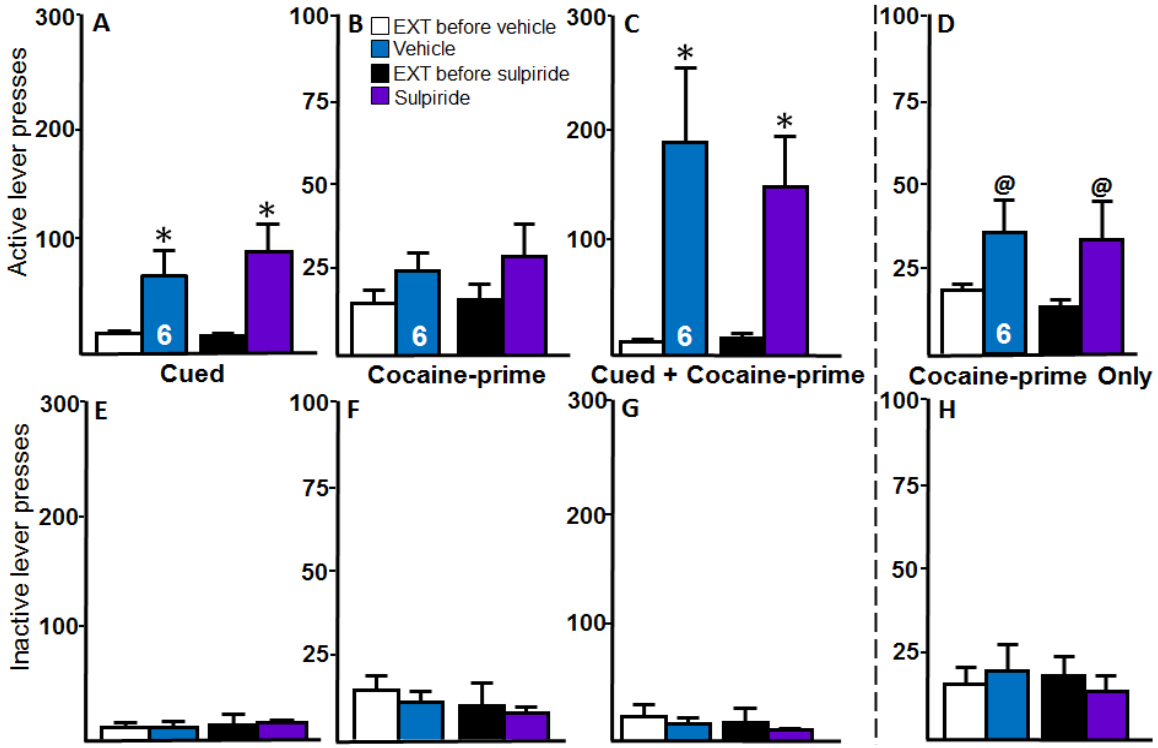

Figure 7. Intra-mOFC D2 receptor blockade via sulpiride has no effect on cocaine-seeking behaviors. **A-D,** The active lever presses and, **E-H,** inactive lever presses for each reinstatement test for Experiment 4 are shown. **A,** Intra-mOFC microinjections of sulpiride had no effect on active lever pressing during cued reinstatement compared to vehicle controls (n = 6). **B,** Although intra-mOFC microinjections of sulpiride had no effect on active lever pressing during cocaine-prime reinstatement, cocaine-prime reinstatement levels were not significantly above the extinction baseline (n = 6). **C,** Intra-mOFC microinjections of sulpiride had no effect on active lever pressing during cued + cocaine – prime reinstatement (n = 6). **D,** Due to the low levels of cocaine-prime reinstatement, a separate group of rats underwent only a cocaine-prime reinstatement (n = 6). Intra-mOFC microinjections of sulpiride did not affect active lever pressing when only a cocaine-prime reinstatement test was conducted. **E-H,** There were no effects of reinstatement or microinjections on inactive lever presses for any type of reinstatement. *$p < 0.05$ compared with extinction baseline. @ $p < 0.09$ compared with extinction baseline. EXT, extinction baseline.

during cued reinstatement revealed a significant effect of reinstatement ($F_{(1,5)} = 10.55$, $p < 0.05$), no effect of microinjection ($F_{(1,5)} = 0.65$, $p > 0.05$), and no interaction ($F_{(1,5)} = 0.89$, $p > 0.05$). Post hoc tests revealed that active lever pressing during reinstatement was significantly higher than extinction for both treatments. Figure 7B shows the active lever presses during cocaine-prime reinstatement following intra-mOFC D2 receptor blockade. A two-way repeated measures ANOVA of active lever pressing during cocaine-prime reinstatement revealed no effect of reinstatement ($F_{(1,5)} = 5.87$, $p > 0.05$), no effect of microinjection ($F_{(1,5)}$

= 0.17, $p > 0.05$), and no interaction ($F_{(1,5)} = 0.10$, $p > 0.05$). Intra-mOFC D2 receptor blockade had no effect on active lever pressing during cued + cocaine-prime reinstatement (Figure 7C). A two-way repeated measures ANOVA of active lever presses revealed a significant effect of reinstatement ($F_{(1,5)} = 15.01$, $p < 0.05$), no effect of microinjections ($F_{(1,5)} = 0.20$, $p > 0.05$), and no significant interaction ($F_{(1,5)} = 0.31$, $p > 0.05$). Post hoc tests revealed that active lever pressing during reinstatement was significantly higher than extinction for both treatments ($p < 0.05$).

Because the cocaine-prime reinstatement did not produce significantly higher lever pressing compared to the extinction baseline, we conducted a separate experiment in which rats underwent only cocaine-prime reinstatement with the goal of achieving more robust levels of this form of reinstatement. Figure 7D shows the active lever presses for those rats that only underwent the cocaine-prime reinstatement. A two-way repeated measures ANOVA of active lever pressing during this cocaine-prime reinstatement test revealed no effect of microinjections ($F_{(1,5)} = 0.72$, $p > 0.05$), no interaction ($F_{(1,5)} = 0.06$, $p > 0.05$), and only a marginal effect of reinstatement ($F_{(1,5)} = 2.96$, $p < 0.15$). Post hoc tests revealed that active lever pressing during reinstatement was marginally higher than extinction for both treatments ($p < 0.09$) and was not different between treatment groups. There were no significant differences in inactive lever presses across any type of reinstatement.

Discussion

In contrast to prior work using GABA receptor activation (Peters et al., 2008), the current findings indicate that D1 receptor activation in the IL and mOFC is involved in the

reinstatement of cocaine seeking, though to different extents. Blocking D1 receptors in the IL reduced cued reinstatement but did not affect cocaine-prime or cued + cocaine-prime reinstatement. Similar blockade in the mOFC reduced cocaine seeking for all forms of reinstatement tested. In contrast, the present results suggest that D2 receptor activation in either structure played little role in the reinstatement of cocaine seeking as blocking D2 receptors in the IL and mOFC had no effect on reinstatement. Although it is possible that the sulpiride dose used in the present experiments was too low to produce any effects on drug seeking, sulpiride doses given in the mPFC within this range have revealed behavioral differences in the past (Chitkara et al., 2000; Cheshenko et al., 2007). Nonetheless, we cannot rule out the possibility that higher doses may be effective at altering drug seeking, though non-specific "off-target" effects may become a concern at higher doses. These findings suggest that activation of dopamine receptors within the vmPFC, particularly the D1 receptors, is involved in the reinstatement of cocaine seeking, though the precise role appears to depend on an interaction of the type of reinstatement and the vmPFC subregion.

Infralimbic Results

Intra-IL administration of the D1 receptor antagonist reduced cue-induced cocaine seeking but did not alter reinstatement when a cocaine prime was given. D2 receptor blockade in the IL had no effect on any form of reinstatement. The results with IL dopamine receptor blockade show partial consistency with prior work, as IL inactivation via GABA receptor activation has no effect on cocaine-prime reinstatement (McFarland and Kalivas, 2001) and blocking $5HT_{2A}$ receptors in the IL decreases cued and cocaine-prime reinstatement (Pockros et al., 2011). Moreover, several studies, including work from our laboratory, suggest that the

IL inhibits cocaine seeking following self-administration and extinction and is also involved in the consolidation of the extinction of cocaine-seeking behavior (Peters et al., 2008; LaLumiere et al., 2010; LaLumiere et al., 2012b). However, those studies also suggest that IL inactivation and activation have no effect and reduce, respectively, cued reinstatement of cocaine seeking.

The reasons underlying this discrepancy regarding the IL remain unclear, though to some degree, they parallel the discrepancies with the mOFC results. However, such conflicting findings are not unprecedented. Previous work investigating the nucleus accumbens shell has found that inactivation via GABA receptor activation does not alter the reinstatement of cocaine seeking and, akin to the IL, induces cocaine seeking during an extinction session (McFarland and Kalivas, 2001; Peters et al., 2008). In contrast, dopamine receptor blockade within the accumbens shell impairs the reinstatement of cocaine seeking (Anderson et al., 2006). Together with the present findings, this work suggests that conclusions drawn based on one type of pharmacological manipulation may not always predict the outcomes for a similar set of studies using a different pharmacological manipulation. Previous work indicates that dopamine in the accumbens shell appears to override excitatory inputs from the IL that would normally suppress cocaine seeking (LaLumiere et al., 2012b). A similar process may occur within the IL itself. More speculatively, this process may also involve different neuronal ensembles. Indeed, recent findings suggest that a small minority of IL neurons is responsible for the suppression of alcohol seeking (Pfarr et al., 2015). However, there may also be IL neurons that promote cocaine seeking (e.g., Koya et al., 2009) and it is possible that D1 receptor blockade on these specific neurons may inhibit the neuronal

ensembles that promote cocaine seeking, resulting in a decrease in the reinstatement of cocaine seeking observed in the present study. These neuronal ensembles that drive cocaine seeking, however, may typically be masked by the prepotent inhibitory drive within the structure with regard to cocaine seeking. Unfortunately, it is not clear whether this would explain the discrepancy for the mOFC, as prior work has not identified a role for this region in inhibiting cocaine seeking.

Alternatively, the actions of dopamine within the IL (and possibly mOFC) may have subtler effects on the type of behavior in which an animal is engaged and may explain the differences between the effects of D1 receptor blockade in the IL during cued and cocaine-prime reinstatement. Past studies have suggested that IL dopamine is critically involved in how the IL influences goal-seeking vs. habit-based behavior (Hitchcott et al., 2007). Cued reinstatement takes advantage of the ability of the cues to serve as conditioned reinforcers that increase cocaine-seeking behavior even when the reinforcement of cocaine itself is removed. Blockade of dopamine receptors in the IL may, therefore, reduce any reinforcing properties of the cues, thus preventing the goal-seeking behavior. In contrast, cocaine-prime reinstatement involves a single cocaine injection given prior to the reinstatement session with no reinforcer given during the session itself. Thus, cocaine-prime reinstatement likely does not involve the same type of goal-seeking behavior as cued reinstatement and, therefore, may not depend on IL dopamine receptors. Nonetheless, it may be a combination of type of reinstatement, and therefore goal-seeking behavior vs. habitual behavior, and the selective activation of specific ensembles within the IL that are responsible for the current results. Additionally, and perhaps in concert with the speculation above, the lack of a role for IL

dopamine receptors during cocaine-prime reinstatement may be due to the direct actions of cocaine on PL dopamine, thereby directly activating the PL-nucleus accumbens core pathway that is necessary for reinstatement (Stefanik et al., 2013b). Indeed, prior studies indicate that PL inactivation, dopamine receptor blockade in the PL, and optical inhibition of the PL inputs to the accumbens core attenuate cocaine-prime reinstatement (McFarland and Kalivas, 2001; Stefanik et al., 2013b).

Medial Orbitofrontal Results

Blocking D1 receptors within the mOFC impaired all forms of reinstatement tested (cued, cocaine-prime, and cued + cocaine-prime reinstatement), whereas D2 receptor blockade in the mOFC did not alter any form of reinstatement. Previous work indicates mOFC activation potentiates the feeding behavior that is induced by AMPA receptor blockade in the nucleus accumbens shell which lies downstream from the mOFC (Hoover and Vertes, 2011; Richard and Berridge, 2013). Given that prior work indicates that inactivation of the accumbens shell induces cocaine-seeking behavior (Peters et al., 2008), it may be possible that activation of the mOFC would potentiate cocaine seeking in the same way that it potentiates feeding. Thus, the present results indicating a role for the mOFC in promoting cocaine seeking may be consistent with those examining feeding. Nonetheless, prior studies directly examining the role of the mOFC in cocaine seeking found no effect of mOFC inactivation on cued reinstatement or of mOFC lesions on cocaine-prime reinstatement (Fuchs et al., 2004), making the present study the first, to our knowledge, to show a role for this region in the reinstatement of cocaine seeking. Indeed, although much work has focused on the lateral OFC and medial PFC in general (PL and IL specifically) in drug seeking and reward-related

behavior, there has been less attention directed toward examining the role of the mOFC in such behaviors. It is worth noting, however, that our results are consistent with previous studies indicating that D1 receptor blockade within the lateral OFC disrupts cocaine-seeking behavior (Lasseter et al., 2009), suggesting that these two distinct regions may be acting similarly to influence reinstatement. Moreover, relatively few studies consider differences along the rostrocaudal axis within the mPFC, which has likely obscured functional differences among these subregions. Thus, with a relative paucity of studies in the literature, it is difficult to reconcile the current results using dopamine receptor blockade with those examining lesions and inactivation of the mOFC. Nonetheless, the present study, along with previous work, provides a critical basis for future work to examine this vmPFC subregion as well as consider functional differences along the rostrocaudal axis during motivated behaviors including drug seeking and feeding.

D1 V. D2 Receptors in the mPFC

Although previous work indicates that systemic and intra-mPFC D2 receptor blockade alters drug-seeking behaviors (Woolverton and Virus, 1989; Sun and Rebec, 2005; Liu et al., 2010), the present results suggest that D2 receptor blockade in the IL and mOFC did not affect cocaine seeking. Given that D1 and D2 receptors typically have excitatory and inhibitory influences, respectively, via opposing actions on adenylyl cyclase activity and cAMP production (Paul et al., 1992; Gonon, 1997), differential localization of these receptors on IL and mOFC neurons may account for our results. Work using *in situ* hybridization indicates that both D1 and D2 receptor mRNAs are found in efferent cortical populations (Gaspar et al., 1995). However, the cortical populations expressing D1 and D2 receptors

appear to differ significantly depending on the receptor type expressed. Indeed, Gaspar et al. estimated that D2 receptor mRNA-containing neurons were 3-4 times less numerous than those containing D1 receptor mRNA in the PFC and noted that, although 25% of corticothalamic neurons express D1 receptors, these projection neurons do not appear to express D2 receptors. Other work has suggested that D1 and D2 receptors within the mPFC are located in minimally overlapping neuronal populations (Vincent et al., 1995). Thus, the present results may reflect mPFC differences in D1 vs. D2 receptor expression level and/or localization on different neuronal populations or even on different parts of the neurons (dendrites vs. axon terminals). Consistent with the importance of the D1 receptor in particular in the mPFC, prior research indicates that mPFC functions such as working memory and mental flexibility depends on dopamine activity at D1 receptors (Sawaguchi and Goldman-Rakic, 1991; Williams and Goldman-Rakic, 1995; Okubo et al., 1997; Goldman-Rakic et al., 2000).

Conclusion: Examining the role of D1 and D2 receptors in the IL and mOFC during the reinstatement of cocaine seeking, the present study found that D1 receptor blockade in the mOFC reduced all forms of reinstatement tested but, in the IL, only reduced cued reinstatement. D2 receptor blockade had no effect in either structure. Together with previous work showing a role for the IL in inhibiting cocaine seeking, the present study suggests that the actions of dopamine within the IL may be quite different from those of the structure as a whole and introduces an additional layer of complexity in our attempt to understand how the IL regulates drug-seeking behavior. Moreover, the current results are among the first to identify the mOFC as a region driving the reinstatement of cocaine seeking and also confirm

the existence of critical functional differences along the rostrocaudal axis within the vmPFC. Future studies examining the vmPFC should carefully consider such differences in drawing functional conclusions about this region.

CHAPTER 3. THE DORSAL AGRANULAR INSULAR CORTEX REGUALTES THE CUED REINSTATEMENT OF COCAINE-SEEKING, BUT NOT FOOD-SEEKING, BEHAVIOR IN RATS

Studies examining the reinstatement of cocaine-seeking behavior have found that the medial prefrontal cortex (PFC) is a critical driver of such behavior (LaLumiere *et al*, 2012; McFarland *et al*, 2003), yet considerably less attention has focused on the roles of the lateral PFC in regulating cocaine seeking. However, recent work suggests that the insular cortex (IC), a region in the lateral PFC, may be critically involved in craving and relapse (Naqvi and Bechara, 2010). Human neuroimaging studies have consistently found that drug-associated cues elicit IC activity in participants across multiple types of drug addiction (Brody *et al*, 2002; Kilts *et al*, 2004; Myrick *et al*, 2004). These observations led to a study demonstrating that insula lesions in humans produce significant disruption in nicotine addiction (Naqvi *et al*, 2007), a finding that has been confirmed in subsequent research (Gaznick *et al*, 2014) and has led to increased attention to this region with regard to its role in addiction.

Experiments using rodent models indicate that reversible inactivation of an IC subregion known as the posterior IC (PIc, also known as the granular insular cortex), as well as electrical stimulation of the IC, reduces both nicotine self-administration and reinstatement in rats (Forget *et al*, 2010; Pushparaj *et al*, 2013). In contrast, the more anterior subregions of the IC, including the dorsal agranular insular cortex (AId), appear to drive amphetamine place preference (Contreras *et al*, 2012). Although the role of the IC has not been extensively investigated with regard to cocaine-seeking behavior, prior work has found that cocaine self-administration increases expression levels of the plasticity-associated gene *Arc*, notably, in

the AId (Zavala *et al*, 2008). Moreover, the AId innervates the nucleus accumbens (NA) core, a structure known to regulate cocaine seeking in rats, supporting a potential role for the AId in cocaine-seeking behavior (McFarland *et al*, 2003; Voorn *et al*, 2004). Indeed, previous work found that AId inactivation reduces cocaine seeking during a reinstatement test in which a contextual odor stimulus associated with cocaine was presented with a conditioned light cue (Di Pietro *et al*, 2006). In contrast, recent work found that lesions of the anterior portion of the IC, including the AId, potentiated cocaine-seeking behaviors when rats underwent forced abstinence and were then reintroduced to the cocaine-seeking context (Pelloux *et al*, 2013), leaving the role of the IC in the reinstatement of cocaine seeking unclear.

It has been argued that the IC regulates relapse to drug use due to its role in mediating interoceptive cues (Naqvi *et al*, 2014). A potential key mediator of these interoceptive cues within the IC is corticotropin-releasing factor (CRF) (Naqvi and Bechara, 2009), which is expressed throughout the cortex and at relatively high levels in the IC (Sanchez *et al*, 1999; Van Pett *et al*, 2000). Indeed, evidence suggests that the central CRF system plays a critical role in driving drug addiction and relapse (Koob, 2013; Zorrilla *et al*, 2014). Nonetheless, despite the potential significance of this issue, the role of the IC, including its different subregions and CRF1 receptors, has not been extensively examined in the reinstatement of cocaine-seeking behavior. Therefore, the present study investigated whether these two subregions of the IC, the AId and PIc, regulate cue-induced reinstatement, as well as whether blocking CRF1 receptors in the AId influences cocaine-seeking behavior during reinstatement.

Methods and Materials

Subjects

Male Sprague-Dawley rats (250-275 g at time of arrival; Charles River Laboratories; n = 82) were single-housed on a 12-hour reverse light cycle (and kept at a constant temperature) with food and water *ad libitum* in an AAALAC-approved vivarium. All animals were given at least 5 days of acclimation before undergoing surgery. All procedures were in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and approved by University of Iowa Institutional Animal Care and Use Committee.

Surgery

Rats were anesthetized with ketamine (100 mg/kg, i.m.) and xylazine (6 mg/kg, i.m.). Ketorolac (3 mg/kg) was given as an analgesic on the day of surgery and the day immediately following surgery. For catheter implantation for cocaine self-administration, a 13 cm piece of Silastic tubing was threaded under the skin from the back to the ventral side of the rat and inserted into the right jugular vein. A silicone ball affixed 4 cm from the end of the catheter served as a stopping point for insertion. Catheters were secured using silk sutures. The opposite end of the catheter was externalized through a small hole in the skin between the animal's shoulder blades. The externalized end was connected to a 22 gauge guide cannula that was secured in the middle of a harness which was looped around the rat's forelimbs.

For both cocaine and food self-administration experiments, the rats were then placed in a small animal stereotaxic instrument (Kopf Instruments, Tujunga, CA, USA). Jewelers screws were affixed to the skull surface. Bilateral cannulae (Plastics One, Roanoke, VA, USA) were implanted and secured with dental cement, aimed at the PIc and AId with coordinates as follows: AId: 3.1 mm anterior to and 4.2 mm lateral from Bregma and 4.4 mm ventral from the skull surface (cannula aimed 2 mm above the AId); PIc: 0.5 mm posterior to and 6.0 mm lateral from Bregma and 3.9 mm ventral from skull surface (cannula aimed 3 mm above the PIc). The coordinates were developed based on prior work (Contreras *et al*, 2012; Di Pietro *et al*, 2006) and refined in our laboratory. Following surgery, all animals received 3 ml sterile saline subcutaneously and a topical application of the local anesthetic bupivacaine to both the animal's head and chest. Obdurators were placed in all cannulae and maintained throughout reinstatement testing. Rats were then returned to their home cages and permitted to recover for 5-7 days. During this time, catheters were flushed daily with 0.1 ml of heparinized saline (100 USP) to ensure catheter patency and 0.1 ml of cefazolin (100 mg/ml) to reduce the opportunity for infection.

Cocaine Self-administration and Extinction

All self-administration experiments occurred in standard operant chambers (Med Associates, Fairfield, VT) that contained two retractable levers, a house light, a cue light, and a tone-generator (4500 Hz). Rats were food deprived 24 h prior to a 15-h overnight food-training session, during which each active lever press resulted in a single food pellet (45 mg) on a fixed-ratio 1 (FR1) schedule. After food training, rats were given ~20 g of food daily, which

was maintained throughout all training and testing. Prior to the start of self-administration all animals had their catheters checked for patency using 0.1 ml of sodium brevital (1 mg/ml).

One day after food training, self-administration began. Rats underwent 2-h self-administration sessions where presses on the active lever produced a single infusion of cocaine (50 µl infusion of 200 µg cocaine dissolved in sterile saline, given over 2.18 s; cocaine kindly provided by NIDA) and a 5 s light and tone cue on an FR1 schedule. A 20 s timeout period followed each infusion. Inactive lever presses had no consequence. Rats underwent daily self-administration 6 days per week for a minimum of 12 days. In order to move into extinction, rats were required to take at least 10 infusions of cocaine per day for at least 10 days, including the last 3 days of self-administration, and demonstrate discrimination between the active and inactive lever. During extinction, active lever presses did not produce cocaine infusions or the light and tone cues. Rats' lever pressing was extinguished for a minimum of 7 days and rats only began reinstatement testing if they had 28 or fewer active lever presses for at least 2 consecutive days immediately prior to the reinstatement session. The final 2 days of extinction training prior to each reinstatement session served as the extinction baseline.

Food Self-administration and Extinction

Following surgery and recovery, rats were given 20 g rat chow per day following each self-administration, extinction, and reinstatement session. The food-seeking experiments followed previous protocols established by McFarland and Kalivas (2001) in order to ensure robust

reinstatement responding later during testing. Initially, active lever presses produced a single food pellet (45 mg; BioServ) on an FR1 schedule along with the same light/tone cues used in the cocaine self-administration studies. As training progressed, the reinforcement schedule, including both the food pellet and the cues, increased to FR3 and then FR5. This schedule of reinforcement was used to help ensure robust reinstatement, as established by McFarland and Kalivas (2001). Rats were required to receive 100 pellets per day for at least 3 days before moving onto the next schedule. Extinction procedures began when these criteria were achieved on the FR5 schedule. Rats' active lever pressing was considered extinguished once active lever presses reached <10% of the active lever presses achieved on the final day of self-administration for 2 continuous days, with a minimum of 7 days of extinction training.

Microinjections

Intra-AId or intra-PIc microinjections were given prior to each reinstatement test. Microinjectors (with 2 and 3 mm projections for the AId and PIc, respectively) were connected to PE20 tubing, which was attached to 10-µl Hamilton syringes controlled by an infusion pump. The microinjections were 0.2 µl/side, given at a rate of 0.3 µl/min. Following each microinjection, injectors were left in position for 1 min to allow for diffusion. Immediately following the microinjection, rats were placed into the operant chamber for their appropriate reinstatement test. Microinjected drugs consisted of the $GABA_{B/A}$ receptor agonists baclofen and muscimol (BM, given as a cocktail at 1 and 0.1 mM, respectively), dissolved in artificial cerebrospinal fluid (aCSF) as the vehicle, or the corticotropin-releasing factor receptor-1 (CRF1) antagonist antalarmin (6.0 mM), dissolved in a 70% DMSO/30%

aCSF solution as the vehicle. Doses of drugs were chosen based on previous studies (Blacktop *et al*, 2011; LaLumiere *et al*, 2012).

Reinstatement Testing

Each reinstatement test lasted 2 h and, during the reinstatement session, active lever presses never produced a cocaine infusion. Between reinstatement tests, lever pressing was re-extinguished to baseline for a minimum of 3 days using the same criteria described above. For all reinstatement tests, microinjections occurred immediately before testing. For cued reinstatement for either cocaine seeking or food seeking, active lever presses produced the light and tone cues that were previously paired with the drug infusion or delivery of food pellet during self-administration. The cued reinstatement of food seeking was performed on the same FR1 schedule that the rats engaged in cued reinstatement of cocaine seeking used. The cocaine-prime reinstatement used in Experiment 1 consisted of an injection of cocaine (10 mg/kg, i.p.) immediately before the reinstatement session. For food-prime reinstatement, 2 pellets were placed in the food hopper before the start of the session and for the first 30 min of the session a single pellet was non-contingently dispensed into the hopper every 2 min, following previously published procedures (McFarland *et al*, 2001). The remaining 90 min of the food-prime reinstatement session were a standard extinction session. During the duration of the food-prime reinstatement session, active lever presses had no consequence. The cue + food-prime reinstatement combined both sets of procedures described above.

Experiment 1. In the first experiment, the AId was inactivated prior to the cued reinstatement testing described above via BM microinjections. To determine whether AId inactivation had any effect alone, a subset of rats that underwent the cued reinstatement also underwent an inactivation-alone test, in which the AId was inactivated prior to a standard extinction session. A separate group of rats received BM microinjections into the AId prior to a cocaine-prime reinstatement.

Experiment 2. As previous work has identified the PIc as a critical subregion of the IC for mediating nicotine craving and relapse (Forget *et al*, 2010; Pushparaj *et al*, 2013), the second experiment examined whether the cued reinstatement findings from Experiment 1 extended to the PIc. Prior to the reinstatement testing, rats received BM microinjections into the PIc to inactivate the region.

Experiment 3. As prior work has suggested that CRF within the IC may be involved in addiction processes (Naqvi *et al*, 2009), Experiment 3 examined whether CRF1 receptor blockade in the AId alters the reinstatement of cocaine-seeking behavior. Therefore, rats received intra-AId microinjections of either the CRF1 receptor antagonist antalarmin or vehicle immediately prior to undergoing cued reinstatement.

Experiment 4. In order to determine whether the AId plays a similar role in the reinstatement of food-seeking behavior, the AId was inactivated via BM microinjections immediately prior

to cued, food-prime, or cue + food-prime reinstatement. In this case, all rats underwent all three reinstatement tests in the described order.

Histological Analysis

Rats were overdosed with sodium pentobarbital (100 mg/kg, i.p.) and intracardially perfused using phosphate-buffered saline. All brains were placed in 3.7% formaldehyde for a minimum of 24 h. Coronal slices (75 μm thick) were taken and mounted onto gelatin-coated slides. Sections were stained with Cresyl violet and each animal was analyzed for accurate placement of microinjector termination points. Data from any rat whose injection tracts terminated outside the borders of the AId or PIc were excluded from analysis.

Data Analysis

Reinstatement lever pressing data was analyzed using two-way analyses of variance (ANOVA) with both comparisons as repeated measures (extinction vs. reinstatement; aCSF vs. drug). Post-hoc analysis was completed using Holm-Sidak's multiple comparison tests. *p*-values of less than 0.05 were considered significant. All measures were expressed as mean $\pm$ SEM. Each group's n is indicated in the figure.

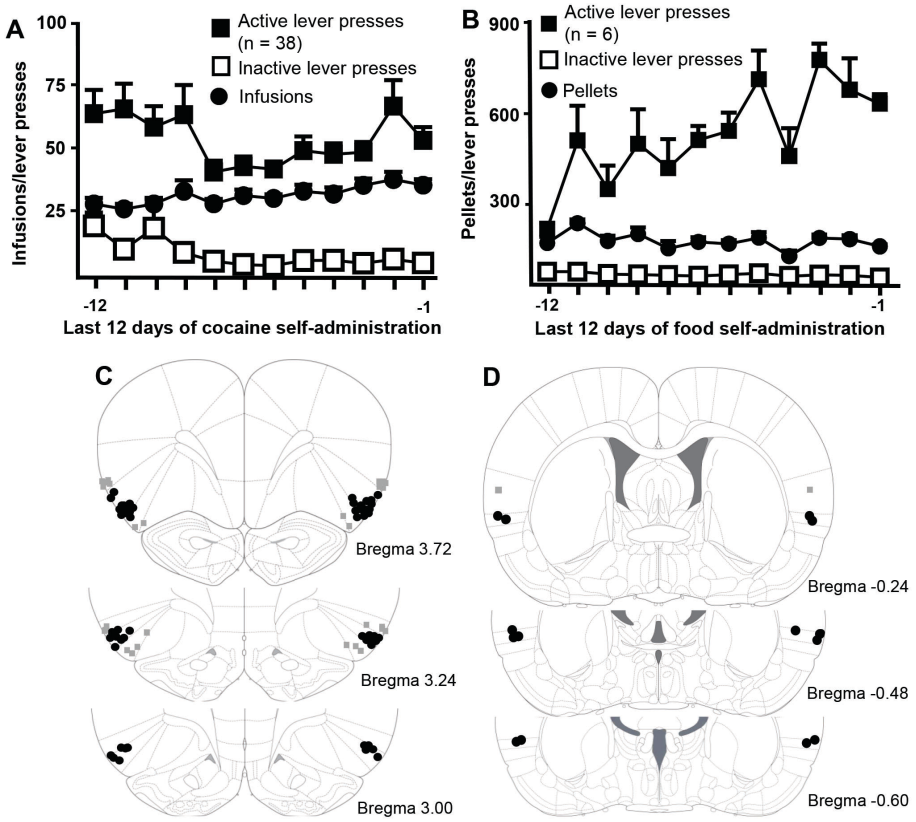

Figure 8. Self-administration data and histological representations. **A,** Number of active and inactive lever presses and cocaine infusions for the last 12 days of cocaine self-administration for all rats included in the final analysis. **B,** Number of active and inactive lever presses and food pellets for the last 12 days of food self-administration for all rats included in the final analysis. **C and D**, Diagrams showing the termination of needle-tracks for microinjections aimed at the AId and PIc, respectively. Black circles indicate correct placements. Gray squares indicate incorrect placements. Figures adapted from Paxinos and Watson (2007), and A/P coordinates (in mm) are given relative to Bregma.

Results

Out of a total of 82 rats used in the present experiments, 44 rats were included in the final data. Rats were excluded due to misplaced (15 rats) or unverifiable microinjection termination locations (11 rats), failure to acquire self-administration (1 rat), clogged cannula (3 rats), illness or death of rat prior to completion of reinstatement testing (8 rats). When determining the termination of microinjector tips, conservative criteria were used and any rats in which one or both injector tracts were not clearly visible were excluded, which resulted in the relatively high number of rats excluded due to misplaced or unverifiable

microinjection locations. Figure 8A shows the number of active and inactive lever presses and cocaine infusions over the final 12 days of cocaine self-administration. Figure 8B shows the number of active and inactive lever presses and food pellets received over the final 12 days of food self-administration. Figures 8C and D show the location of the microinjector tips, both correctly and incorrectly placed, in the AId and PIc, respectively.

Experiment 1. AId Inactivation Reduces Cued Reinstatement of Cocaine-seeking Behavior

Figure 9 shows the active and inactive lever presses (panels A-C and D-F, respectively) across the different reinstatements examined in Experiment 1, in which the AId was inactivated prior to the reinstatement tests. AId inactivation significantly reduced active lever presses for cued reinstatement (Figure 9A). Because two rats had cued reinstatement data that were more than two standard deviations beyond the mean (one for aCSF and one for BM), they were excluded from the cued reinstatement analysis. A two-way repeated-measures ANOVA of active lever presses indicated a significant effect of reinstatement ($F_{(1, 9)} = 15.91$, $p < 0.01$), a significant effect of microinjection ($F_{(1, 9)} = 10.40$, $p < 0.05$), and a significant interaction ($F_{(1, 9)} = 12.57$, $p < 0.01$). Post-hoc tests revealed that, although both treatment groups showed increased active lever pressing during reinstatement compared to the extinction baselines ($p < 0.05$), the BM-treated group had significantly fewer active lever presses compared to the aCSF-treated group ($p < 0.05$). AId inactivation alone (Figure 9B), when given before an extinction session, had no effect on active lever pressing. A two-way repeated-measures ANOVA of active lever presses indicated no effect of reinstatement ($F_{(1, 5)} < 1$, $p > 0.05$), no effect of microinjection ($F_{(1, 5)} = 2.304$, $p > 0.05$), and no interaction ($F_{(1, 5)} < 1$, $p > 0.05$). Figure 9C shows the active and inactive lever presses from the rats that

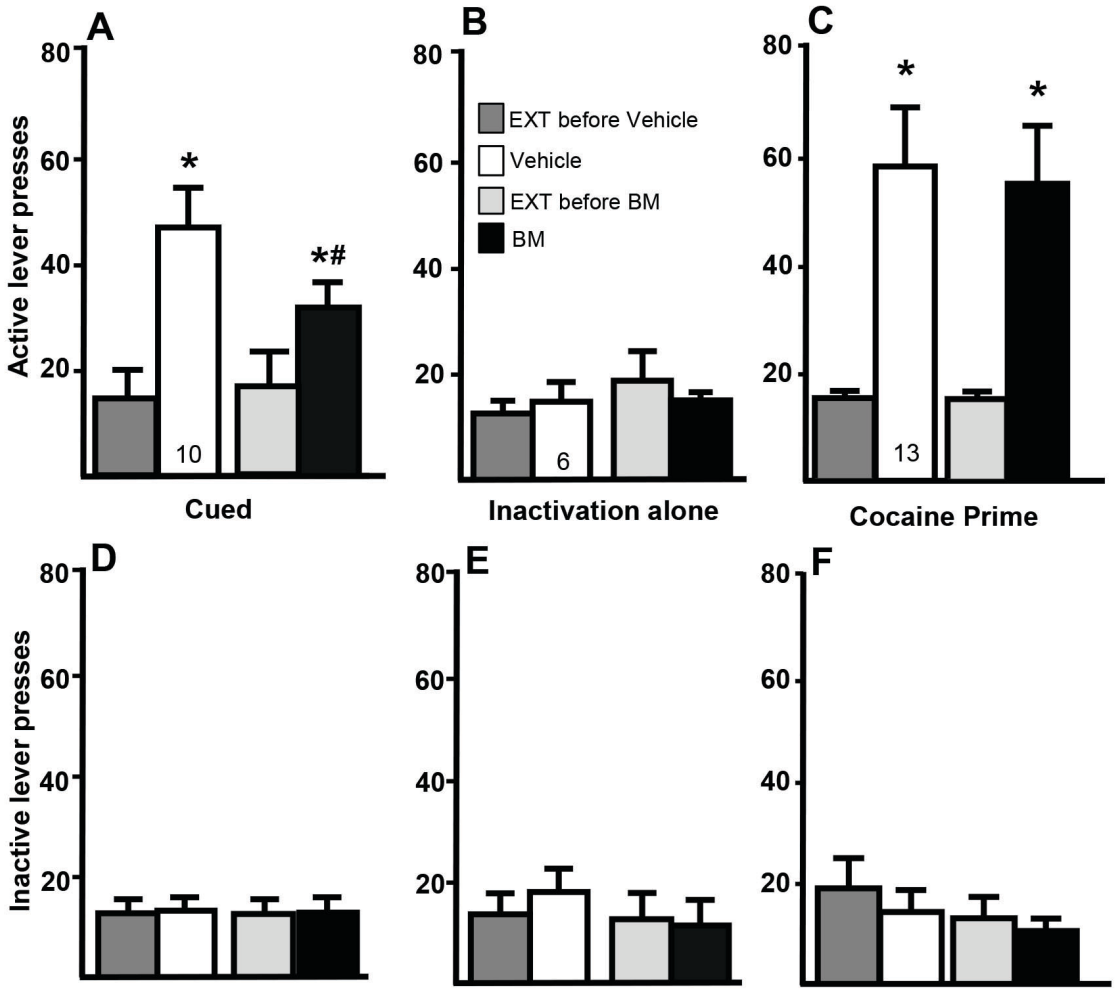

Figure 9. AId inactivation reduces cued reinstatement of cocaine-seeking behavior. Panels A-C show the active lever presses and panels D-F show the inactive lever presses across the different tests for Experiment 1. **A,** Intra-AId microinjections of BM significantly reduced active lever pressing during cued reinstatement compared to vehicle-controls. **B,** Intra-AId microinjections of BM had no effect on active lever presses when given alone prior to a standard extinction session in a subset of rats used in panel A. **C,** Intra-AId microinjections of BM had no effect on active lever presses when given prior to a cocaine-prime reinstatement test. **D-F,** There were no effects of reinstatement or microinjections on inactive lever presses across any of the tests. *, $p < 0.05$ compared to extinction baseline. #, $p < 0.05$ compared to vehicle-control group. EXT, Extinction baseline.

underwent the cocaine-prime reinstatement. A two-way repeated-measures ANOVA of active lever presses indicated a significant effect of reinstatement ($F_{(1, 12)} = 18.65$, $p < 0.01$), no effect of microinjection ($F_{(1, 12)} < 1$, $p > 0.05$), and no interaction ($F_{(1, 12)} < 1$, $p > 0.05$). Both BM- and vehicle-treated rats had significant reinstatement compared to their extinction baseline ($p < 0.01$). There were no significant differences in inactive lever pressing across any of the reinstatements.

Experiment 2. PIc Inactivation has no Effect on Cued Reinstatement of Cocaine-seeking Behavior

Figure 10 shows the active and inactive lever presses (panels A and B, respectively) for rats with PIc inactivation during cue-induced reinstatement. Rats treated with vehicle or BM showed equivalent levels of active lever presses (Figure 10A). A two-way repeated-measures ANOVA of active lever presses indicated a significant effect of reinstatement ($F_{(1,5)} = 8.209$, $p < 0.05$), no effect of microinjection ($F_{(1,5)} < 1$, $p > 0.05$), and no interaction ($F_{(1,5)} = 1.192$, $p > 0.05$). Post-hoc tests found significant differences between the extinction baseline and both the vehicle- and BM-treated rats ($p < 0.05$). Active lever pressing for the BM group was not significantly different from the vehicle group. There were no significant differences in inactive lever presses for any of the reinstatement tests.

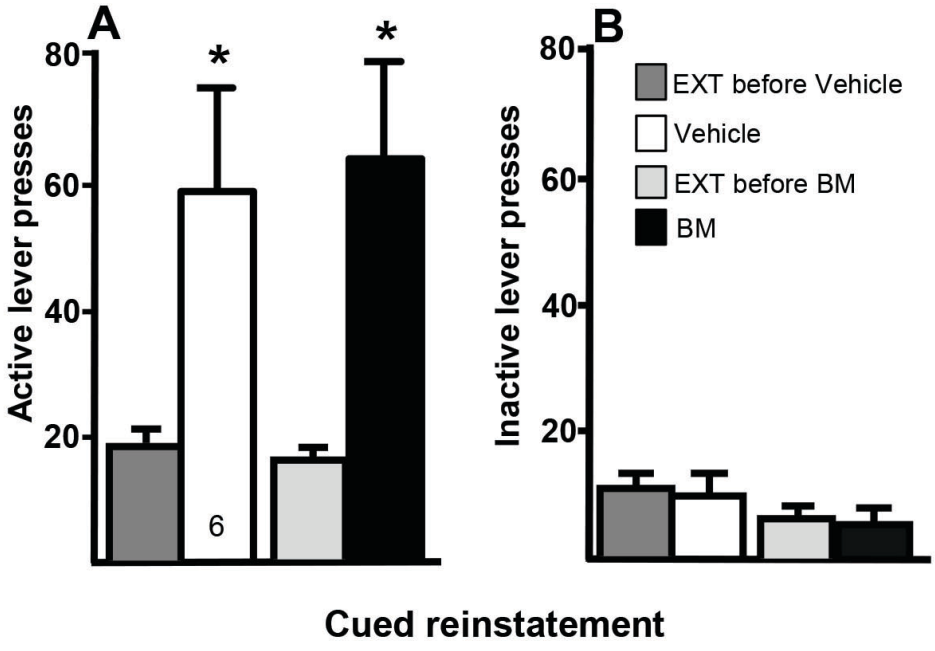

Figure 10. PIc inactivation has no effect on cued reinstatement of cocaine-seeking behavior. **A and B,** Active and inactive lever presses, respectively, for Experiment 2. Intra-PIc microinjections of BM had no effect on active lever presses during cued reinstatement compared to vehicle-control injections. There were no effects of reinstatement or microinjections on inactive lever presses. *, $p < 0.05$ compared to extinction baseline. EXT, Extinction baseline.

Experiment 3. Blockade of Intra-AId CRF1 Receptors Reduces Cued Reinstatement of Cocaine-seeking Behavior

Because the results of Experiments 1 and 2 indicated that the AId, but not the PIc, regulated reinstatement of cocaine seeking, Experiment 3 examined the role of the CRF1 receptors in the AId only. Figure 11 shows the active and inactive lever presses (panels A and B, respectively) during cue-induced reinstatement, in which intra-AId microinjections of the CRF1 receptor antagonist antalarmin were given prior to the reinstatement test. The results were similar to those of Experiment 1 with AId inactivation. Blockade of CRF1 receptors in the AId significantly reduced active lever presses for cued reinstatement (Figure 11A). A two-way repeated-measures ANOVA of active lever presses indicated a significant effect of reinstatement ($F_{(1, 6)} = 18.40$, $p < 0.01$), a significant effect of microinjection ($F_{(1, 6)} = 8.384$, $p < 0.05$), and a significant interaction ($F_{(1, 6)} = 8.138$, $p < 0.05$). Post-hoc tests revealed that aCSF-treated rats showed a significant increase in active lever pressing compared to their

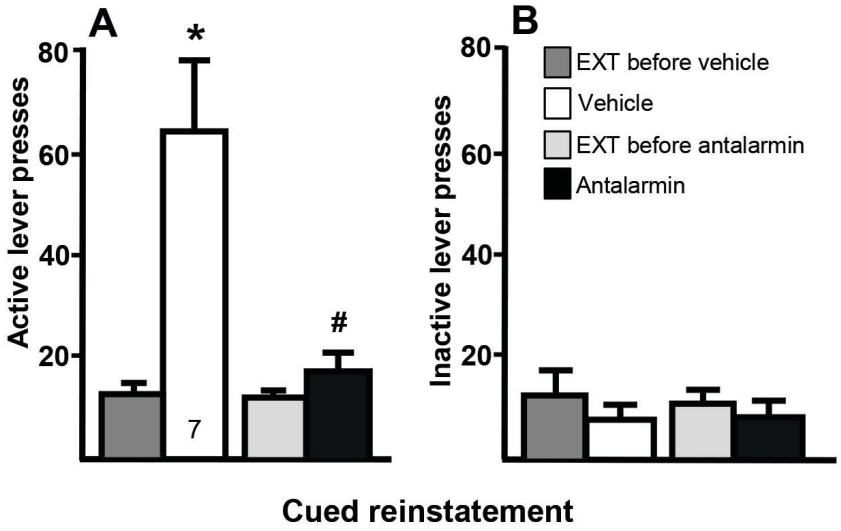

Figure 11. Blockade of intra-AId CRF1 receptors reduces cued reinstatement of cocaine-seeking behavior. **A and B,** Active and inactive lever presses, respectively, for Experiment 3. Intra-AId microinjections of the CRF1 receptor antagonist antalarmin (6.0 mM) reduced active lever presses during cued reinstatement compared to vehicle-control injections. There were no effects of reinstatement or microinjections on inactive lever presses. *, $p < 0.05$ compared to extinction baseline. #, $p < 0.05$ compared to vehicle-control group. EXT, Extinction baseline.

extinction baseline and the antalarmin-treated group ($p < 0.05$), whereas the antalarmin-treated group did not significantly differ in active lever presses compared to its extinction baseline ($p > 0.05$). There were no significant differences in inactive lever presses for any of the reinstatement tests ($p > 0.05$).

Experiment 4. AId Inactivation has no Effect on the Reinstatement of Food-seeking Behavior

Figure 12 shows the active and inactive lever presses (panels A-C and D-F, respectively) for the reinstatement of food-seeking behavior from Experiment 4. Overall, the results indicated no effect of AId inactivation on food-seeking behavior. A two-way repeated-measures ANOVA of active lever presses during cued reinstatement indicated a significant effect of reinstatement ($F_{(1, 5)} = 49.04$, $p < 0.001$), no effect of microinjection ($F_{(1, 5)} < 1$, $p > 0.05$), and no interaction ($F_{(1, 5)} = < 1$, $p > 0.05$). Both BM and vehicle-treated rats had trends toward a significant cued reinstatement compared to their extinction baselines ($p < 0.09$). A two-way repeated-measures ANOVA of active lever presses during food-prime reinstatement indicated a significant effect of reinstatement ($F_{(1, 5)} = 20.84$, $p < 0.01$), no effect of microinjection ($F_{(1, 5)} < 1$, $p > 0.05$), and no interaction ($F_{(1, 5)} < 1$, $p > 0.05$). Both BM and vehicle-treated rats had significant food-prime reinstatement compared to their extinction baselines ($p < 0.05$). A two-way repeated-measures ANOVA of active lever presses during cue + food-prime reinstatement indicated a significant effect of reinstatement ($F_{(1, 5)} = 17.00$, $p < 0.01$), no effect of microinjection ($F_{(1, 5)} < 1$, $p > 0.05$), and no interaction ($F_{(1, 5)} < 1$, $p > 0.05$). Both BM and vehicle-treated rats had trends toward a significant cue + food-prime reinstatement compared to their extinction baselines ($p < 0.07$). Visual inspection of the data in all cases did not suggest that AId inactivation had any effect on food-seeking

reinstatement. There were no significant differences in inactive lever pressing in all cases ($p > 0.05$).

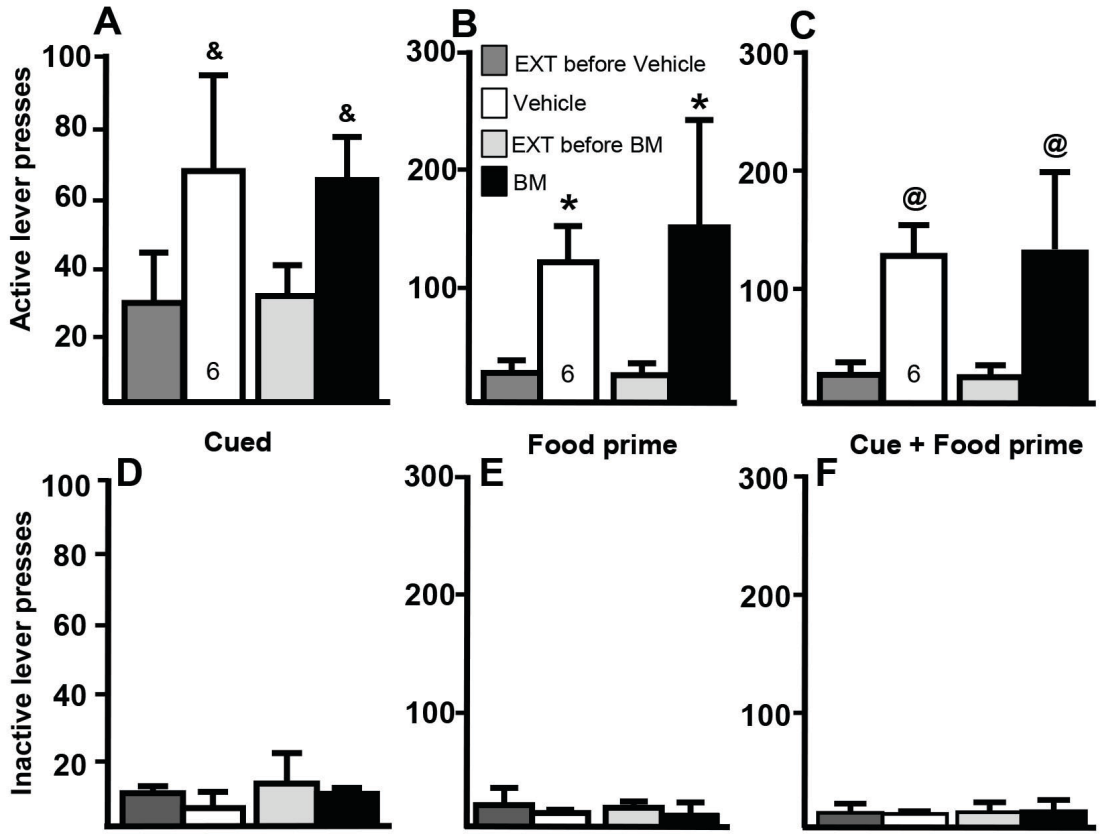

Figure 12. AId inactivation has no effect on the reinstatement of food-seeking behavior. Panels **A-C** show the active lever presses and panels **D-F** show the inactive lever presses across the different reinstatement tests. **A, B, and C,** Intra-AId microinjections of BM had no effect on active lever pressing during cued, food-prime, and cue + food-prime reinstatement. **D, E, and F,** There were no effects of reinstatement or microinjections on inactive lever presses across any of the reinstatement tests. *, $p < 0.05$ compared to extinction baseline. @, $p < 0.07$ compared to extinction baseline. &, $p < 0.09$ compared to extinction baseline. EXT, Extinction baseline.

Discussion

The present findings indicate that AId activity and the CRF1 receptors within the AId regulate cocaine-seeking behavior. AId inactivation decreased cocaine seeking for cued reinstatement but had no effect on cocaine-prime reinstatement, suggesting a differential role for the AId depending on the type of reinstatement. In contrast, PIc inactivation had no effect on cued reinstatement. Similar to the findings with AId inactivation, blockade of the CRF1 receptors in the AId reduced cued reinstatement. Additional experiments found that AId inactivation had no effect on reinstatement of food-seeking behavior, suggesting a selective role for the AId in drug seeking.

The IC has been increasingly implicated as a critical component for relapse, as imaging studies have demonstrated that drug-associated cues elicit IC activity across multiple drugs of abuse (Brody *et al*, 2002; Kilts *et al*, 2004; Myrick *et al*, 2004). Moreover, IC damage in humans produces a profound loss of nicotine craving and relapse (Naqvi *et al*, 2007). Similarly, rodent studies have found that PIc inactivation and electrical stimulation reduce nicotine self-administration and reinstatement induced by nicotine-associated cues or a nicotine prime (Forget *et al*, 2010; Pushparaj *et al*, 2013). The present results, however, indicate that PIc inactivation had no effect on cued reinstatement to cocaine seeking. In contrast, AId inactivation as well as intra-AId administration of a CRF1 receptor antagonist altered cocaine-seeking behavior produced by cued reinstatement. A role for the AId in regulating such behavior is consistent with previous work suggesting that the AId is involved in amphetamine- and cocaine-related behavior, including relapse (Contreras *et al*, 2012; Di Pietro *et al*, 2006; Pelloux *et al*, 2013).

Although studies investigating the role of the anterior insular cortex in addiction are limited, previous work using coordinates for AId microinjections akin to the ones used in the present study found that AId inactivation via lidocaine reduced odor context-dependent cue-induced reinstatement of cocaine seeking but had no effect on sound context-dependent cue-induced reinstatement (Di Pietro *et al*, 2006). Although the present experiments found that AId inactivation attenuated lever pressing when the AId was inactivated prior to the cued reinstatement, the current methods were different from those of the Di Pietro *et al*, as the prior study used a contextual cue (sound) and a discrete contingent cue (light) in contrast to the contingent tone + light cues used in the current study. A potentiation of cocaine seeking following IC manipulation has previously been observed by Pelloux *et al* (2013) in which lesions of the anterior IC (including the AId) were found to increase cocaine-seeking behavior when animals were reintroduced to the drug-taking context following an abstinence period (Pelloux *et al*, 2013). The procedures used in the present experiments, however, were significantly different from those used by Pelloux *et al*. Nonetheless, taken together, these results suggest that the role of the AId in cocaine-seeking behavior may be rather complex.

The IC is a critical region for the mediation of interoceptive cues (Goldstein *et al*, 2009; Paulus and Stewart, 2014), and these interoceptive cues appear to be critical to addiction and relapse. In the presence of external cues, it has been argued that the IC receives information regarding these cues and that this drives the recall of drug-specific interoceptive cues, which produce subjective craving and relapse behaviors (Naqvi *et al*, 2009; Naqvi *et al*, 2014). In the present study, however, the cues are delivered in a response-contingent manner and are

thus conditioned reinforcers, though they may also act as antecedents to future lever pressing during the session. Therefore, whether the cues used in the present study induce the recall of interoceptive cues in rodents is difficult to ascertain, though the present results are consistent with the hypothesis that the AId is involved in behavior related to drug-associated cues. As each drug of abuse produces its own unique set of interoceptive cues (Naqvi *et al*, 2014), it is possible that the discrepancy between the nicotine findings and the present cocaine-seeking findings with regard to the PIc and AId are due to such differences. That the present results indicate that AId inactivation did not alter the reinstatement of food seeking suggest that the AId is not generally involved in the reinstatement of reward-related behavior. Given that the AId maintains a population of CRF1 receptors (Potter *et al*, 1994; Sanchez *et al*, 1999) and that it has been suggested that CRF in the AId may be involved in the mediation of these interoceptive cues (Naqvi *et al*, 2009), the present work also examined the role of CRF1 receptors in the AId in regulating reinstatement and found that CRF1 receptor blockade reduced cued reinstatement. To our knowledge, these are the first findings demonstrating a role for CRF in the AId in drug-seeking behavior.

The differences in results between the present work and the nicotine studies may also involve differences in anatomical connections and/or differential activation of structures during reinstatement. The present study targeted the granular cortex in the PIc, which projects to the agranular regions but otherwise appears to maintain relatively few connections with other forebrain regions likely involved in drug addiction (Shi and Cassell, 1998). In contrast, the AId region targeted in the current experiments projects to the nucleus accumbens core and most regions of the amygdala, maintains reciprocal connections with the prelimbic and

infralimbic cortices, and also receives input from the medial orbital cortex (Hoover and Vertes, 2007, 2011; Shi *et al*, 1998; Vertes, 2004; Voorn *et al*, 2004). The anatomical connections suggest that the PIc may act upstream of the AId anatomically and at least functionally for nicotine seeking, but the PIc appears to play no role in cue-induced cocaine seeking, suggesting a distinct circuit for such reinstatement. Indeed, Naqvi and Bechara have hypothesized that drug-associated cues activate the IC via the ventromedial PFC and the amygdala (2009) which connect with the AId directly, providing a circuit by which cued reinstatement can bypass the PIc, though it is not clear why this is not the case for nicotine seeking. As the AId projects to the nucleus accumbens core (Reynolds and Zahm, 2005) and inactivation of the core or blockade of glutamate receptors in the core prevents cued and cocaine-prime reinstatement (Backstrom and Hyytia, 2007; Cornish and Kalivas, 2000; Fuchs *et al*, 2004a; McFarland *et al*, 2001), activity in this pathway may be responsible for the present cued reinstatement results. As AId inactivation alone had no effect on lever pressing, it appears that the AId does not act similarly to the infralimbic cortex in suppressing cocaine-seeking behavior (e.g. Peters *et al*, 2008).

Studies have examined other regions of the lateral PFC, including the nearby lateral orbitofrontal cortex (lOFC), in cocaine-seeking behavior. Intriguingly, lOFC lesions made prior to self-administration potentiate contextual and cocaine-prime reinstatement and have no effect on cued reinstatement, whereas lesions made after self-administration have no effect on contextual reinstatement (Fuchs *et al*, 2004b; Lasseter *et al*, 2009). However, lOFC inactivation, via BM microinjections given before the test trial akin to the present study's design, was found to impair cued and contextual reinstatement and have no effect on

cocaine-prime reinstatement, similar to the present results. Based on examination of the histology results from these previous studies, the lOFC microinjections appear to be approximately 0.5 mm anterior to the present microinjections and 1-2 mm medial to the present microinjections, making it unlikely that the 0.2 ul microinjections used in the current study spread to the lOFC. Nonetheless, the present findings, together with previous studies, suggest that lPFC subregions are differentially involved in the reinstatement of drug seeking and deserve increased attention, particularly considering the apparent heterogeneity of function among these subregions.

Given that there must be CRF release for antalarmin to have any effects and that general inactivation is not activity dependent, it is surprising that the inactivation appeared to be less robust at impairing cued reinstatement than the CRF1 receptor blockade. However, comparing two different drugs is difficult, as it is possible that the relative doses produced smaller or larger areas of physiological effects. The antalarmin dose that was used, therefore, may have resulted in greater spread of physiologically effective concentrations of antalarmin compared to the spread of the physiologically effective concentrations of the GABA receptor agonists. If this is the case, higher doses of BM may be required to produce the same results that were seen with antalarmin. Given that different doses of lidocaine produced different results in the Di Pietro *et al* (2006) study and given the relatively large size of the AId, especially in the rostral-caudal dimension, it is possible that increased doses of BM would produce more robust attenuation of cued reinstatement.

Conclusions

The present results indicate a critical role for the AId and specifically CRF1 receptors in the AId in the cued reinstatement of cocaine-seeking behavior. Of interest, PIc inactivation had no effect on the reinstatement of cocaine seeking, suggesting subregional specificity for these effects. Moreover, AId inactivation had no effect on the reinstatement of natural reward (food) seeking, suggesting that the present results are not due to general effects on the reinstatement of operant behavior. The present findings also indicate that AId activity and the effects of CRF1 receptor activation in the AId differentially influence such behavior depending on the type of reinstatement. These findings provide significant evidence of the critical nature of the AId in the circuitry underlying cued reinstatement of cocaine-seeking behavior.

CHAPTER 4. D1 RECEPTOR ANTAGONISM IN THE DORSAL AGRANULAR INSULAR CORTEX DISRUPTS COCAINE SEEKING AND INACTIVATION OF INSULAR CORTEX SUBREGIONS DIFFERENTIALLY ALTERS HEROIN SEEKING

The neural circuitry underlying reinstatement to both cocaine and heroin seeking involves the medial prefrontal cortex (mPFC) (Jasinska et al., 2015; Moorman et al., 2015; Koob and Volkow, 2016). However, recent work has made a case for the importance of the lateral prefrontal cortex in drug seeking, with a particular emphasis on the insular cortex (IC), a region of the lateral prefrontal cortex known for its involvement in interoceptive processing (Goldstein et al., 2009; Naqvi and Bechara, 2010). Pharmacologically inactivating the caudal portion of the IC reduces nicotine self-administration as well as subsequent nicotine seeking during reinstatement testing (Forget et al., 2010). In contrast, blocking activity in this region has no effect on cocaine-seeking behaviors. Rather, inhibition of the more rostral portion of the IC, the dorsal agranular insular cortex (AId), reduces cocaine seeking (Cosme et al., 2015), suggesting that distinct subregions of the IC have specific and divergent influences on drug seeking.

Although the precise mechanisms underlying the role of the AId in cocaine seeking are unknown, dopaminergic activity may be one key contributor, given that the prefrontal cortex broadly receives dopaminergic inputs from the ventral tegmental area. Additionally, studies investigating the role of mPFC dopamine on reinstatement have found normal dopamine signaling in this region to be essential for cocaine seeking, as blocking dopamne receptor activity in both the dorsal and ventral mPFC, the prelimbic (PL) and infralimbic (IL) corticies respectively, reduces reinstatement to cocaine seeking (McFarland and Kalivas,

2001; Cosme et al., 2016). However, increasing activity at PL D1 receptors also results in a reduction of cocaine seeking, suggesting that there is an optimal level of PFC D1 receptor activity for successful cocaine-seeking behaviors (Devoto et al., 2016).

Although anatomically categorized as part of the lateral prefrontal cortex, the AId, like the prelimbic subregion of the mPFC, sends glutamatergic projections to the nucleus accumbens core, a structure that is involved in mediating cocaine-seeking behaviors and similarly drives cocaine seeking (Chikama et al., 1997; Van De Werd and Uylings, 2008; Kutlu et al., 2013). Thus, given that these two structures share several similarities, and dopaminergic signaling in the PL is important for drug seeking, it is possible that dopamine within the AId also regulates cocaine seeking. Previous studies found that blocking D1 receptors in the IC reduces both cocaine and nicotine self-administration (Di Pietro et al., 2008a; Kutlu et al., 2013). Whereas, blocking activity at D2 receptors within the prefrontal cortex has produced conflicting results, with several studies demonstrating a lack of effect on drug seeking (Kutlu et al., 2013; Cosme et al., 2016) and others indicating a disruption of cocaine seeking following systemic and intra-mPFC D2 receptor activity disruption (Sun and Rebec, 2005; Liu et al., 2010). Therefore, despite the known role of insular D1 receptors in mediating drug-taking behaviors, further studies are required to determine the precise role of dopaminergic signaling in the IC during reinstatement to cocaine seeking.

The present study also examined the role of the IC in mediating heroin seeking. As previously described, the distinct subregions of the IC play discrete roles in mediating drug seeking, as the caudal portion of the IC drives nicotine seeking and the rostral portion, the

AId, mediates cocaine seeking. However, no studies have examined the role of the IC during heroin seeking. Prior research suggests that the circuits underlying cocaine and heroin seeking are distinct, though overlap exists (Badiani et al., 2011; Moorman et al., 2015) for instance, although blocking activity in the substantia nigra, central and basal amygdala, and nucleus accumbens shell reduces heroin-prime reinstatement, inactivation of these same regions has no effect on cocaine-prime reinstatement (McFarland and Kalivas, 2001; Rogers et al., 2008). Such circuitry differences also extend to the prefrontal cortex where the infralimbic cortex, a medial PFC subregion, inhibits cocaine seeking but alternatively promotes heroin seeking (Peters et al., 2013). Given these differences, it is unclear how the IC will regulate heroin seeking despite its known role in the regulation of cue-induced cocaine seeking.

Materials and Methods

Subjects

Male Sprague–Dawley rats (250–275 g at time of arrival; Charles River Laboratories; n =75) were single-housed on a 12-h reverse light cycle and kept at a constant temperature, with food and water ad libitum in an AAALAC-approved vivarium. All animals acclimated to the vivarium for at least 5 days before undergoing surgery. All procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by University of Iowa Institutional Animal Care and Use Committee.

Surgery

Rats were anesthetized with ketamine (100 mg/kg, i.m.) and xylazine (6 mg/kg, i.m.). Meloxicam (2 mg/kg) was given immediately following anesthetic injections as well as the day immediately following surgery. To complete the catheter implantation a 13-cm piece of Silastic tubing was threaded under the skin from the back of the rat to the ventral side and inserted into the right jugular vein. A silicone ball affixed 4 cm from the inserted end of the catheter served as a stopping point for implantation. Catheters were secured with 3 surgical knots on either side of the silicone ball using silk sutures. The opposite end of the catheter was externalized through a small hole in the skin between the animal's shoulder blades. The externalized end was connected to a 22-gauge guide cannula that was secured in the middle of a harness that was looped around the rat's forelimbs. Rats were then placed in a small animal stereotaxic instrument (Kopf Instruments, Tujunga, CA). 5 jewelers' screws were affixed to the skull surface and bilateral cannulae (Plastics One, Roanoke, VA) were implanted and secured with dental cement, aimed at either the AId or the PIc with the following coordinates: AId: 3.1 mm anterior to and 4.2 mm lateral from Bregma and 3.6 mm ventral from the skull surface (cannula aimed 2 mm above the AId); PIc: 0.5 mm posterior to and 6.0 mm lateral from Bregma and 3.9 mm ventral from skull surface (cannula aimed 3 mm above the PIc). The coordinates were developed based on prior work (Cosme et al., 2015). Following surgery, all animals received 3 ml sterile saline subcutaneously and a topical application of the local anesthetic bupivacaine to the animal's head, chest, and back. Obdurators were placed in all cannulae and maintained throughout reinstatement testing. Rats were then returned to their home cages to recover for 5–7 days. During this time, catheters

were flushed daily with 0.1 ml of heparinized saline (100 USP) to ensure catheter patency and 0.07 ml of the antibiotic enrofloxacin (22.7 mg/ml) to reduce the chance of an infection.

Self-administration and Extinction

All self-administration experiments occurred in standard operant chambers (Med Associates, Fairfield, VT) that contained two retractable levers, a house light, a cue light, and a tone generator (4500 Hz). Rats were food deprived 24 h before a 15-h overnight food-training session, during which each active lever press resulted in a single food pellet (45 mg) on a fixed ratio 1 (FR1) schedule. After food training, rats received 20 g of food daily, which continued throughout all training and testing. Before the start of self-administration, all animals had their catheters checked for patency using 0.1 ml of sodium brevital (10 mg/ml). If rats did not show a loss of muscle tone following brevital administration, a second catheter was inserted into the non-compromised vein. One day following successful completion of food training, self-administration began.

Cocaine Self-administration

Rats underwent 2-h self-administration sessions where presses on the active lever produced a single infusion of cocaine (50 µl infusion of 200 µg cocaine dissolved in sterile saline, given over 2.18 s; cocaine kindly provided by NIDA) and a 5-s light and tone cue on an FR1 schedule. A 20-s timeout period followed each infusion. Inactive lever presses had no consequence. Rats underwent daily self-administration 6 days per week for a minimum of 12 days. In order to move into extinction, rats were required to take at least 15 infusions of

cocaine per day for at least 10 days, including the last 3 days of self-administration, and demonstrate discrimination between the active and inactive lever.

Heroin Self-Administration

Rats underwent 3-h self-administration sessions where presses on the active lever produced a single infusion of heroin, given over 2.18 s (heroin kindly provided by NIDA). During the first stage of self-administration training, active lever presses produced a 50 µl infusion of .05 mg heroin dissolved in sterile saline. Once rats completed 2 stage one sessions in which they earned at least 5 infusions on each day of training, they moved on to the second stage of self-administration. During stage 2, each lever press produced a 50 µl infusion of .0225 mg heroin. All infusions in both stage one and two of self-administration were paired with the presentation of a 5-s light and tone cue on an FR1 schedule. A 20-s timeout period followed each infusion. Inactive lever presses had no consequence. Rats underwent daily self-administration 6 days per week for a minimum of 12 days. In order to move into extinction, rats were required to complete at least 10 days of stage-two self-administration and take at least 15 infusions of heroin per day for at least 7 days, as well as demonstrate discrimination between the active and inactive lever.

Extinction Training

During extinction, active lever presses did not produce drug infusions or the light and tone cues. Rats' lever pressing was extinguished for a minimum of 7 days and rats only began reinstatement testing if they fell below the active lever pressing criteria for at least two consecutive days immediately prior to the reinstatement session (fewer than 25 active lever

presses for cocaine self-administering rats and fewer than 30 active lever presses for heroin self-administering rats). The final 2 days of extinction training immediately preceding each individual reinstatement session served as the extinction baseline for that respective test.

Microinjections

Intra-AId or intra-PIc microinjections were given before each reinstatement test. Microinjectors (with 2 and 3 mm projections for the AId and PIc, respectively) were connected to PE20 tubing, which was attached to 10-µl Hamilton syringes controlled by an infusion pump. The microinjections were 0.2 µl/side, given at a rate of 0.3 µl/min. Following each microinjection, injectors were left in position for 1 min to allow for diffusion. Immediately following the microinjection, rats were placed into the operant chamber for their assigned reinstatement test. Microinjected drugs consisted of the D1 receptor antagonist SCH 23390 (0.1 µg/side), the D2 receptor antagonist sulpiride (30 ng/side), and the $GABA_{B/A}$ receptor agonists baclofen and muscimol (BM, given as a cocktail at 1 and 0.1 mM, respectively) each dissolved in artificial cerebral spinal fluid (aCSF) as the vehicle. Doses were chosen based on prior research (Lalumiere et al., 2004).

Reinstatement Testing

At least one day prior to the start of reinstatement testing, microinjectors were lowered into each cannula to habituate animals to the injection process and assure cannula patency. Each reinstatement test lasted 2 h for cocaine- and 3 h for heroin-treated rats. During the reinstatement session, active lever presses never produced a drug infusion. Between reinstatement tests, lever pressing was reextinguished to baseline levels for a minimum of 3

days using the same criteria described above. For all reinstatement tests, microinjections occurred immediately before testing. For cued reinstatement, active lever presses produced the light and tone cues that were previously paired with the drug infusion during self-administration training. For drug-prime reinstatement, rats received a single systemic injection of cocaine (15 mg/kg, i.p.) or heroin (0.5 mg/kg, subcutaneously) immediately before the start of the reinstatement session.

Experiment 1A. Rats underwent cocaine self-administration training followed by extinction and subsequent reinstatement testing. Prior to reinstatement testing rats received intra-AId injections of the D1 receptor antagonist SCH 23390 or its vehicle control. Rats either underwent cued (n = 8) or cocaine-prime reinstatement testing (n = 6) and performed their assigned reinstatement test twice (acsf v SCH 23390 in a counterbalanced design).

Experiment 1B. Rats underwent cocaine self-administration training followed by extinction and subsequent reinstatement testing. Prior to reinstatement testing rats received intra-AId injections of the D2 receptor antagonist sulpiride or its vehicle control. Rats either underwent cued (n = 13) or cocaine-prime reinstatement testing (n = 8) and performed their assigned reinstatement test twice (acsf v sulpiride in a counterbalanced design).

Experiment 2A. Rats underwent heroin self-administration training followed by extinction and subsequent reinstatement testing. Prior to reinstatement testing rats received intra-AId injections of the $GABA_{B/A}$ receptor agonists B/M or their vehicle control. Rats either

underwent cued (n = 9) or heroin-prime reinstatement testing (n = 10) and performed their assigned reinstatement test twice (acsf v BM in a counterbalanced design).

Experiment 2B. Rats underwent heroin self-administration training followed by extinction and subsequent reinstatement testing. Prior to reinstatement testing rats received intra-PIc injections of the $GABA_{B/A}$ receptor agonists B/M or its vehicle control. Rats either underwent cued (n = 11) or heroin-prime reinstatement testing (n = 10) and performed their assigned reinstatement test twice (acsf v BM in a counterbalanced design).

Histological Analysis

In order to determine correct cannula placement, rats were overdosed with sodium pentobarbital (100 mg/kg, i.p.) and intracardially perfused using phosphate-buffered saline. All brains were placed in 3.7% formaldehyde for a minimum of 48 h. Coronal slices (75 μm thick) were taken and mounted onto gelatin-coated slides. Sections were stained with Cresyl violet and each animal was analyzed for accurate placement of microinjector termination points. Data from rats whose injection tracts terminated outside the borders of the AId or the PIc were excluded from analysis.

Data Analysis

Active lever press data were analyzed using two-way analyses of variance (ANOVA) with both comparisons as repeated measures (extinction vs reinstatement; aCSF vs drug). Post hoc analysis was completed using Holm–Sidak's multiple comparison tests. P-values of < 0.05

were considered significant. All measures are expressed as mean ± SEM. Each group's n is indicated in the figure.

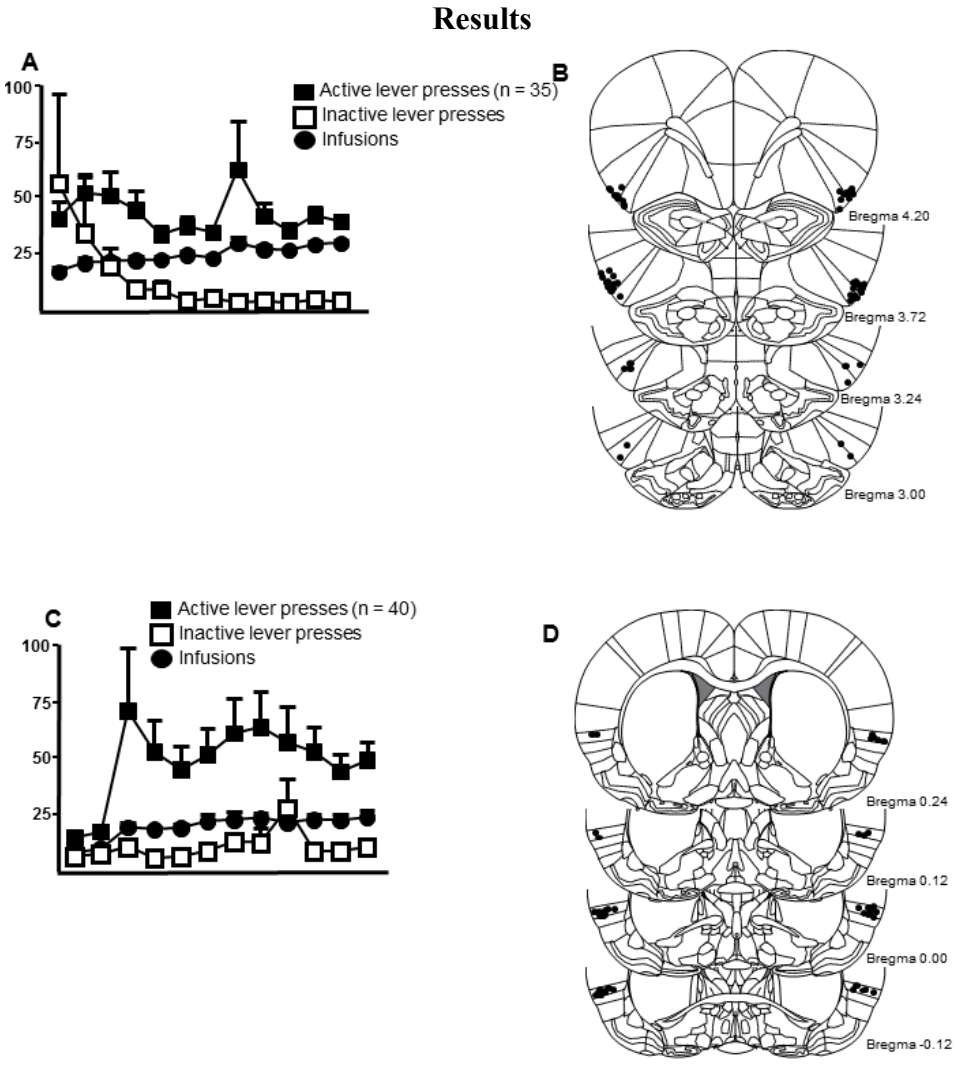

Figure 13. Self-administration data and histological representations. **A,** Number of active and inactive lever presses and cocaine infusions for the last 12 days of cocaine self-administration. **B**, Diagrams showing the termination of needle tracks for microinjections aimed at the AId. **C,** Number of active and inactive lever presses and heroin infusions for the last 2 days of stage one heroin self-administration and the last 10 days of stage two heroin self-administration. **D**, Diagrams showing the termination of needle tracks for microinjections aimed at the PIc. Figures are adapted from Paxinos and Watson (2007), and A/P coordinates (in mm) are given relative to Bregma.

Figure 13A shows the number of active and inactive lever presses and cocaine infusions over the final 12 days of cocaine self-administration. Figures 13B shows the termination site of

the microinjector tips in the AId. Figure 13C shows the number of active and inactive lever presses for the final two days of stage one heroin administration and the final ten days of stage two heroin administration. Figure 13D shows the termination site of the microinjector tips in the PIc.

Experiment 1A. D1 receptor Blockade in the AId Reduced Cued and Cocaine-prime Reinstatement

D1 receptor blockade within the AId significantly reduced active lever presses during cued reinstatement (Figure 14A). A two-way repeated-measures ANOVA of active lever presses

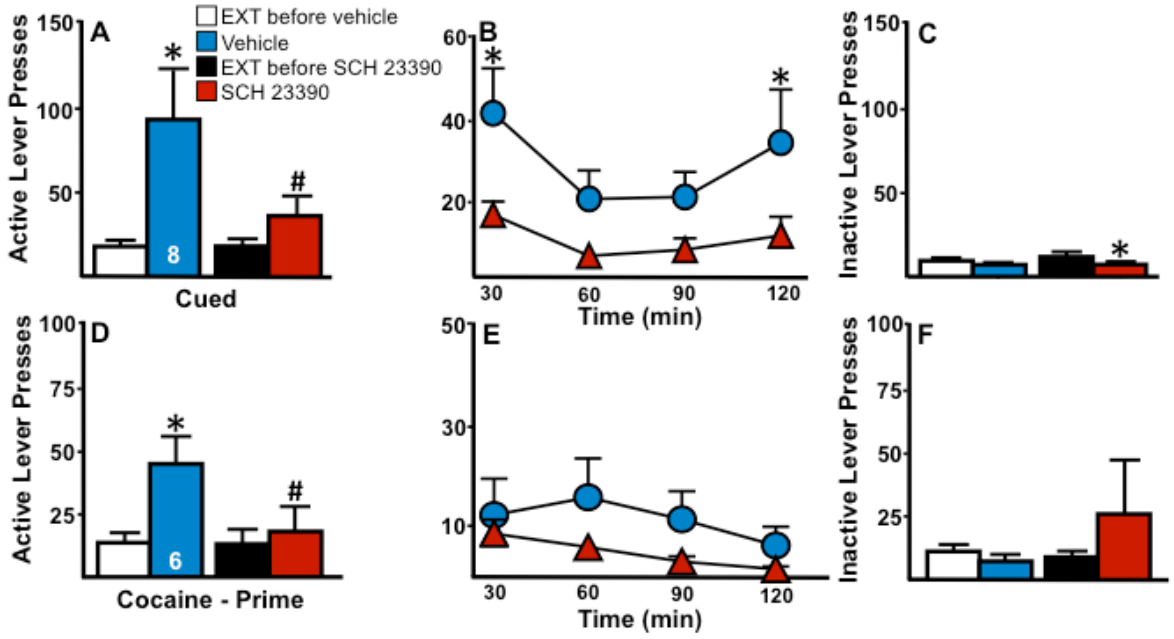

Figure 14. Intra-AId D1 receptor blockade via SCH 23390 (SCH) microinjections reduces cued and cocaine-prime reinstatement. Note that the y-axis is set to 150 for cued reinstatement and 100 for cocaine-prime reinstatement to account for the consistent difference in reinstatement levels. **A,** Intra-AId microinjections of the D1 receptor antagonist SCH significantly reduced active lever presses during cued reinstatement compared to vehicle controls (n = 8). **B,** Intra-AId microinjections of SCH significantly reduced active lever pressing during the first and last 30 mins of a 2 h cued reinstatement test compared to vehicle controls. **C,** Intra-AId microinjections of SCH significantly reduced inactive lever presses during cued reinstatement compared to extinction baseline **D,** Intra-AId microinjections of SCH significantly reduced active lever presses during cocaine-prime reinstatement compared to vehicle controls (n = 6). **E,** Intra-AId microinjections of SCH significantly reduced active lever pressing during a 2 h cocaine-prime reinstatement test compared to vehicle controls. **F,** Intra-AId microinjections of SCH had no effect on inactive lever presses during cocaine-prime reinstatement. *$p < 0.05$ compared with extinction baseline. #$p < 0.05$ compared to vehicle-control group. EXT, extinction baseline.

indicated a significant effect of reinstatement ($F_{(1, 7)} = 11.03$, $p < 0.05$), a significant effect of microinjection ($F_{(1,7)} = 6.98$, $p < 0.05$) and a significant interaction ($F_{(1,7)} = 7.49$, $p < 0.05$). Post hoc tests revealed that active lever presses during cued reinstatement following vehicle treatment were significantly higher than extinction baseline ($p < 0.05$). However, active lever presses during cued reinstatement following SCH treatment were no different from extinction baseline and were significantly lower than the vehicle control group ($p < 0.05$). To determine whether the D1 receptor blockade altered reinstatement in a time-dependent manner, active lever presses during the reinstatement tests were divided into 30 min bins. A two-way repeated measures ANOVA of these data revealed a significant effect of microinjection ($F_{(1,7)} = 7.24$, $p < 0.05$), no effect of time ($F_{(3,21)} = 2.52$, $p > 0.05$), and no significant interaction ($F_{(3,21)} = 0.99$, $p > 0.05$). Post hoc tests revealed active lever presses for the SCH-treated animals were significantly lower during the 0-30 min and 91-120 min bins ($p < 0.05$) (Figure 14B). A two-way repeated measures ANOVA of inactive lever presses indicated a significant effect of reinstatement ($F_{(1,7)} = 8.20$, $p < 0.05$), no significant effect of microinjection ($F_{(1,7)} = .94$, $p > 0.05$), and no interaction ($F_{(1,7)} = 1.03$, $p > 0.05$) (Figure 14C). Post hoc tests revealed that during cued reinstatement inactive lever presses following SCH treatment were significantly lower compared to extinction baseline.

D1 receptor blockade within the AId also significantly reduced active lever presses during cocaine-prime reinstatement (Figure 14D). A two-way repeated-measures ANOVA of active lever presses indicated a significant effect of reinstatement ($F_{(1, 5)} = 6.77$, $p < 0.05$), a significant effect of microinjection ($F_{(1,5)} = 10.69$, $p < 0.05$) and a significant interaction ($F_{(1,5)} = 8.92$, $p < 0.05$). Post hoc tests revealed that active lever presses during reinstatement

with vehicle treatment were significantly higher than the extinction baseline ($p < 0.05$), however active lever presses during cocaine-prime reinstatement following SCH treatment were not different from extinction baseline ($p > 0.05$) and were significantly lower than the vehicle control group ($p < 0.05$). A two-way repeated measures ANOVA of active lever presses during cocaine-prime reinstatement divided into 30 min bins revealed a significant effect of microinjection ($F_{(1,5)} = 9.83$, $p < 0.05$), no effect of time ($F_{(3,15)} = 1.03$, $p > 0.05$), and no significant interaction ($F(3,15) = 0.17$, $p > 0.05$). Post hoc tests revealed no significant differences in active lever presses between the control group and SCH-treated animals (Figure 14E). A two-way repeated measures ANOVA of inactive lever presses during cocaine-prime reinstatement indicated no effect of reinstatement ($F_{(1,5)} = 0.37$, $p > 0.05$), no significant effect of microinjection ($F_{(1,5)} = 0.68$, $p > 0.05$), and no significant interaction ($F_{(1,5)} = 1.07$, $p > 0.05$) (Figure 14F).

Experiment 1B. D2 receptor Blockade in the AId Had No Effect on Cued or Cocaine-prime Reinstatement

D2 receptor blockade within the AId had no effect on active lever presses during cued reinstatement (Figure 15A). A two-way repeated measures ANOVA of active lever presses indicated a significant effect of reinstatement ($F_{(1, 11)} = 19.88$, $p < 0.05$), no significant effect of microinjections ($F_{(1, 11)} = 1.65$, $p > 0.05$), and no significant effect interaction ($F_{(1, 11)} = 1.67$, $p > 0.05$). Post hoc tests revealed that active lever presses during cued reinstatement were significantly higher than the extinction baseline for the sulpiride-treatment group ($p < 0.05$) and showed a trend toward being different from those of the control group ($p = .07$). To determine whether the D2 receptor blockade altered cued reinstatement in a time-dependent

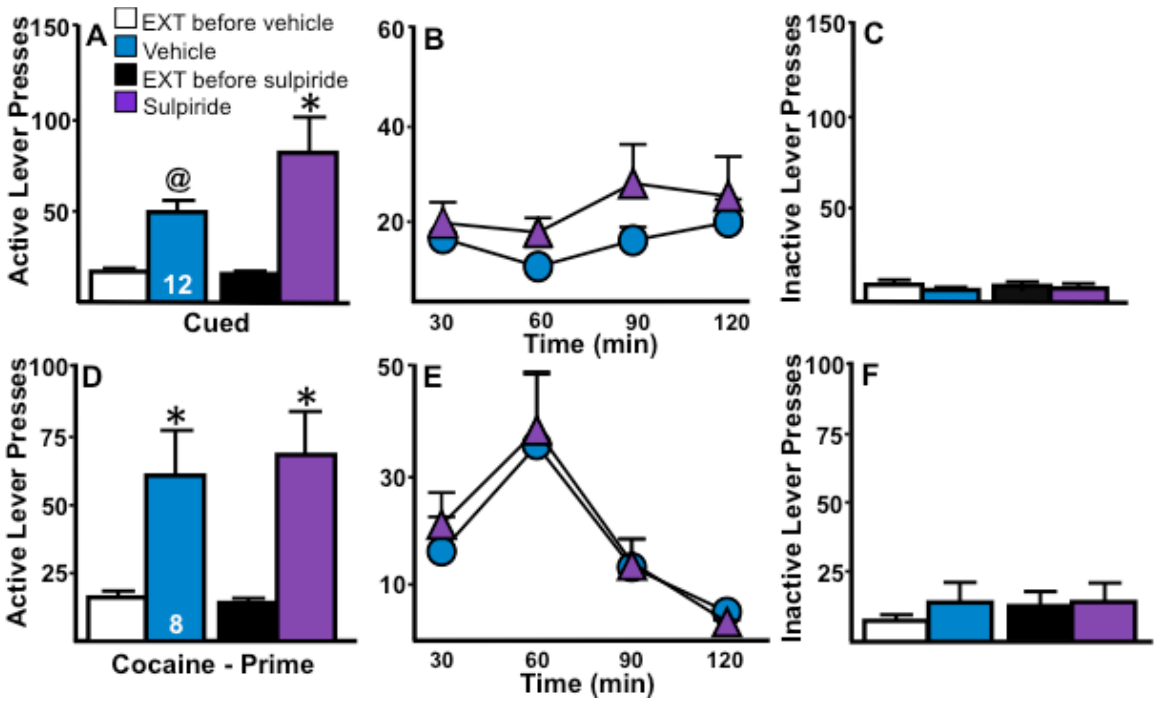

Figure 15. Intra-AId D2 receptor blockade via sulpiride microinjections has no effect on cued or cocaine-prime reinstatement. **A,** Intra-AId microinjections of the D2 receptor antagonist sulpiride had no effect on active lever presses during cued reinstatement (n = 12). **B,** Intra-AId microinjections of sulpiride had no effect on active lever pressing during cued reinstatement testing compared to vehicle controls when analyzed as 30 min bins. **D,** Intra-AId microinjections of sulpiride had no effect on active lever presses during cocaine-prime reinstatement (n = 8). **E,** Intra-AId microinjections of sulpiride had no effect on active lever pressing during cocaine-prime reinstatement testing compared to vehicle controls when analyzed as 30 min bins. **C+F,** Intra-AId microinjections of sulpiride had no effect on inactive lever presses during cued or cocaine-prime reinstatement. *$p < 0.05$ compared with extinction baseline. #$p < 0.05$ compared to vehicle-control group. EXT, extinction baseline.

manner, active lever presses during the reinstatement tests were divided into 30 min bins. A two-way repeated measures ANOVA of these data revealed no significant effect of microinjection ($F_{(1,11)} = 1.58$, $p > 0.05$), no significant effect of time ($F_{(3,33)} = 2.53$, $p > 0.05$), and no interaction ($F_{(3,33)} = 0.40$, $p > 0.05$) (Figure 15B). A two-way repeated measures ANOVA of inactive lever presses during cued reinstatement revealed no significant effect of reinstatement ($F_{(1,11)} = 0.96$, $p > 0.05$), no significant effect of microinjection ($F_{(1,11)} = 0.06$, $p > 0.05$), and no significant interaction ($F_{(1,11)} = 1.70$, $p > 0.05$) (Figure 15C).

D2 receptor blockade within the AId also had no effect on active lever presses during cocaine-prime reinstatement (Figure 15D). A two-way repeated measures ANOVA of active

lever presses indicated a significant effect of reinstatement ($F_{(1, 7)} = 9.43$, $p < 0.05$), no significant effect of microinjections ($F_{(1, 7)} = 0.18$, $p > 0.05$), and no significant interaction ($F_{(1, 7)} = 0.41$, $p > 0.05$). Post hoc tests revealed that active lever presses during cocaine-prime reinstatement were significantly higher that the extinction baseline for both the control and sulpiride-treatment groups ($p < 0.05$). To determine whether the D2 receptor blockade altered cocaine-prime reinstatement in a time-dependent manner, active lever presses during the reinstatement tests were divided into 30 min bins. A two-way repeated measures ANOVA of these data revealed no significant effect of microinjection ($F_{(1,6)} = 0.17$, $p > 0.05$), a significant effect of time ($F_{(3,18)} = 6.52$, $p > 0.05$), and no interaction ($F_{(3,18)} = 0.14$, $p > 0.05$) (Figure 15E). A two-way repeated measures ANOVA of inactive lever presses during cocaine-prime reinstatement revealed no significant effect of reinstatement ($F_{(1,7)} = 1.04$, $p > 0.05$), no significant effect of microinjection ($F_{(1,7)} = 0.93$, $p > 0.05$), and no significant interaction ($F_{(1,7)} = 1.68$, $p > 0.05$) (Figure 15F).

Experiment 2A. AId Inactivation Potentiated Heroin Seeking During Cued Reinstatement

AId inactivation significantly potentiated heroin seeking during cued reinstatement (Figure 16A). A two-way repeated measures ANOVA of active lever presses indicated a significant reinstatement effect ($F_{(1,8)} = 16.61$, $p <0.05$), a significant microinjection effect ($F_{(1,8)} = 8.72$, $p < 0.05$), and a significant interaction ($F_{(1,8)} = 8.39$, $p < 0.05$). Post hoc tests revealed that active lever presses during cued reinstatement were significantly higher than extinction baseline for both the vehicle- and BM- treated groups ($p < 0.05$). Additionally, active lever pressing for BM-treated animals was significantly higher compared to the vehicle control group ($p < 0.05$). To determine whether pharmacological inactivation altered cued

reinstatement in a time-dependent manner, active lever presses during the reinstatement tests were divided into 30 min bins. A two-way repeated measures ANOVA of these revealed a significant effect of microinjection ($F_{(1,8)} = 9.62$, $p < 0.05$), a significant effect of time ($F_{(5,40)} = 3.61$, $p < 0.05$), and a significant interaction ($F_{(5,40)} = 4.31$, $p < 0.05$). Post hoc tests revealed that active lever presses for the BM-treated group were significantly higher during the 60-90 minute bin compared to the control group (Figure 16B). A two-way repeated measures ANOVA of inactive lever presses during cued reinstatement revealed no significant effect of reinstatement ($F_{(1,8)} = 0.67$, $p > 0.05$), no significant effect of microinjection ($F_{(1,8)} =$

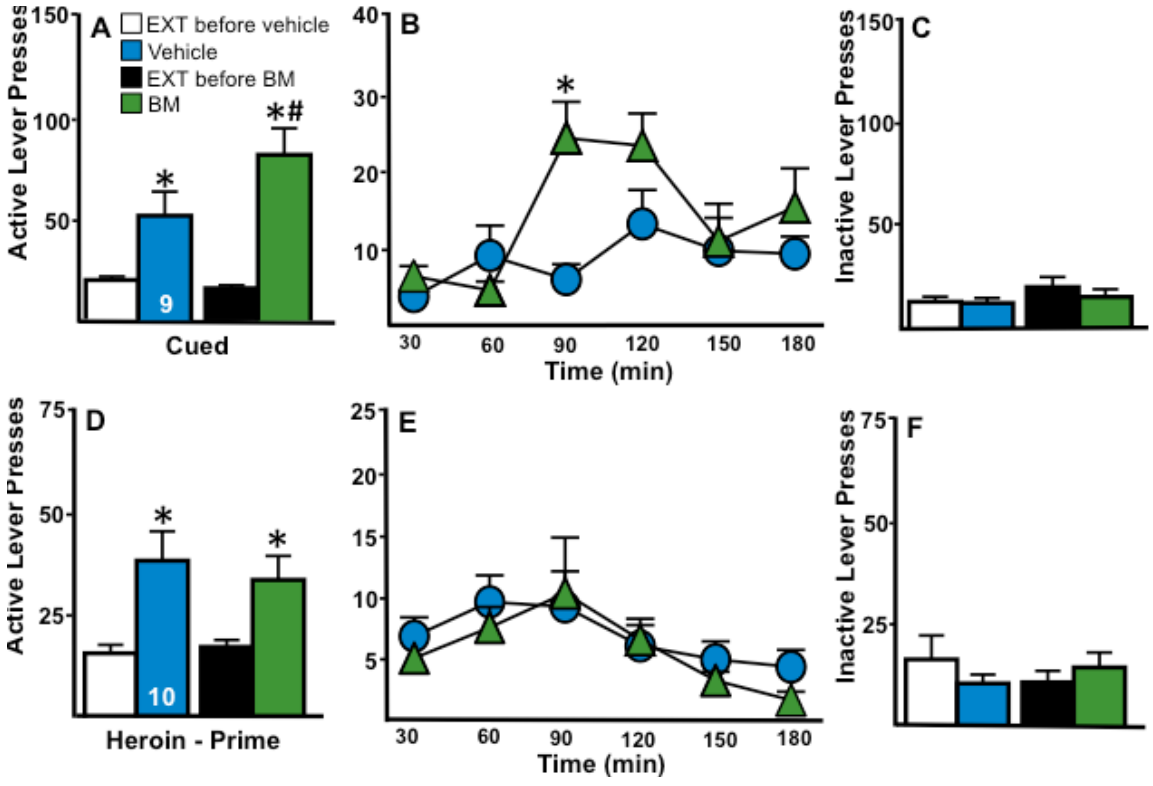

Figure 16. Intra-AId inactivation via BM microinjections potentiated cued reinstatement to heroin seeking. **A,** Intra-AId microinjections of the GABA agonist cocktail BM potentiated active lever presses during cued reinstatement (n = 9). **B,** Intra-AId microinjections of BM significantly increased active lever pressing during the 60-90 minute bin compared to vehicle controls when analyzed as 30 min bins. **D,** Intra-AId microinjections of BM had no effect on active lever presses during heroin prime reinstatement (n = 10). **E,** Intra-AId microinjections of BM had no effect on active lever pressing during heroin prime reinstatement testing compared to vehicle controls when analyzed as 30 min bins. **C+F,** Intra-AId microinjections of BM had no effect on inactive lever presses during cued or heroin prime reinstatement. *$p < 0.05$ compared with extinction baseline. #$p < 0.05$ compared to vehicle-control group. EXT, extinction baseline.

1.21, $p > 0.05$), and no significant interaction ($F_{(1,8)} = 0.86$, $p > 0.05$) (Figure 16C).

AId inactivation had no effect on active lever pressing during heroin-prime reinstatement (Figure 16D). A two-way repeated measures ANOVA revealed a significant effect of reinstatement ($F_{(1,9)} = 16.87$, $p < 0.05$), no effect of microinjection ($F_{(1,9)} = 0.12$, $p > 0.05$), and no significant interaction ($F_{(1,9)} = 0.46$, $p > 0.05$). Post hoc tests revealed that active lever presses during heroin-prime reinstatement were significantly higher than the extinction baseline for the vehicle- and BM-treated groups ($p < 0.05$). To determine whether pharmacological inactivation altered heroin-prime reinstatement in a time-dependent manner, active lever presses during the reinstatement tests were divided into 30 min bins. A two-way repeated measures ANOVA of these data revealed no effect of microinjection ($F_{(1,9)} = 0.54$, $p > 0.05$), a significant effect of time ($F_{(5,45)} = 3.98$, $p < 0.05$), and no significant interaction ($F_{(5,45)} = 0.30$, $p > 0.05$) (Figure 16E). A two-way repeated measures ANOVA of inactive lever presses during cocaine-prime reinstatement revealed no significant effect of reinstatement ($F_{(1,9)} = 0.11$, $p > 0.05$), no significant effect of microinjection ($F_{(1,9)} = 0.06$, $p > 0.05$), and no significant interaction ($F_{(1,9)} = 2.24$, $p > 0.05$) (Figure 16F).

Experiment 2B. PIc Inactivation Blocked Heroin Seeking During Cued Reinstatement

PIc inactivation significantly reduced heroin seeking during a cued reinstatement test (Figure 17A). A two-way repeated measures ANOVA of active lever presses indicated a significant reinstatement effect ($F_{(1,10)} = 20.38$, $p < 0.05$), a significant microinjection effect ($F_{(1,10)} = 5.83$, $p < 0.05$), and a significant interaction ($F_{(1,10)} = 6.69$, $p < 0.05$). Post hoc tests revealed that active lever presses during cued reinstatement were significantly higher than extinction

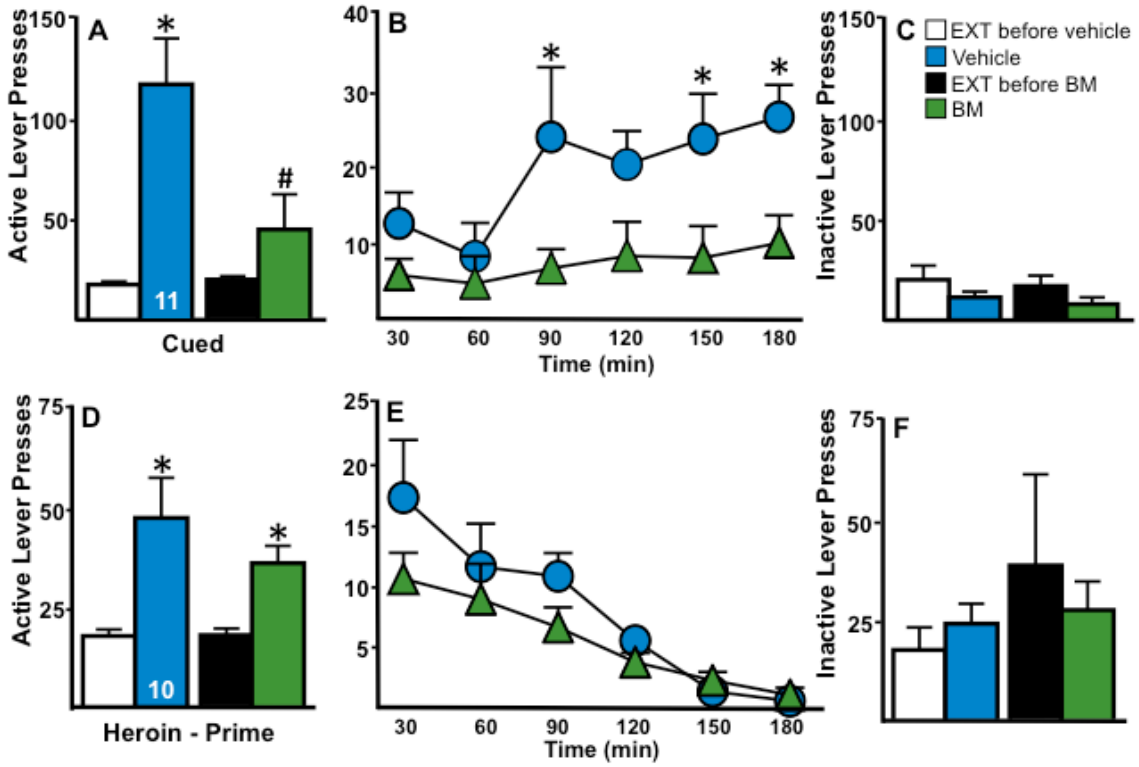

Figure 17. Intra-PIc inactivation via BM microinjections blocked cued reinstatement to heroin seeking. **A,** Intra-PIc microinjections of the GABA agonist cocktail BM reduced active lever presses during cued reinstatement (n = 11). **B,** Intra-PIc microinjections of BM significantly increased active lever pressing overall, though post-hoc tests found no significant differences when analyzed as 30 min bins. **D,** Intra-PIc microinjections of BM had no effect on active lever presses during heroin prime reinstatement (n = 10). **E,** Intra-PIc microinjections of BM had no effect on active lever pressing during heroin prime reinstatement testing compared to vehicle controls when analyzed as 30 min bins. **C+F,** Intra-PIc microinjections of BM had no effect on inactive lever presses during cued or heroin prime reinstatement. *$p < 0.05$ compared with extinction baseline. #$p < 0.05$ compared to vehicle-control group. EXT, extinction baseline.

baseline for the vehicle-treated group ($p < 0.05$), whereas active lever pressing for BM-treated animals was not significantly differently compared to extinction baseline and was significantly lower compared to that of the control group ($p < 0.05$). To determine whether the pharmacological inactivation altered cued reinstatement in a time-dependent manner, active lever presses during the reinstatement tests were divided into 30 min bins. A two-way repeated measures ANOVA of these data revealed a significant effect of microinjection ($F_{(1,10)} = 6.25$, $p < 0.05$), a significant effect of time ($F_{(5,50)} = 3.17$, $p < 0.05$), and no significant interaction ($F_{(5,50)} = 1.20$, $p > 0.05$). Post hoc tests revealed that active lever

presses for the BM-treated group were significantly lower during the 60-90 minute bin, the 120-150 minute bin, and the 150-180 minute bin compared to the control group (Figure 17B). A two-way repeated measures ANOVA of inactive lever presses during cued reinstatement revealed a significant effect of reinstatement ($F_{(1,10)} = 7.57$, $p < 0.05$), no significant effect of microinjection ($F_{(1,10)} = 0.59$, $p > 0.05$), and no significant interaction ($F_{(1,10)} = 2.24$, $p > 0.05$). Post hoc tests revealed no significant differences in active lever pressing between vehicle- and BM-treated groups (Figure 17C).

PIc inactivation had no effect on heroin seeking during a heroin prime reinstatement test (Figure 17D). A two-way repeated measures ANOVA of active lever presses indicated a significant reinstatement effect ($F_{(1,9)} = 15.06$, $p < 0.05$), no significant microinjection effect ($F_{(1,9)} = 1.18$, $p > 0.05$), and no significant interaction ($F_{(1,9)} = 1.37$, $p > 0.05$). Post hoc tests revealed that active lever presses during heroin prime reinstatement were significantly higher than extinction baseline for both the vehicle- and BM-treated groups ($p < 0.05$). To determine whether pharmacological inactivation altered heroin-prime reinstatement in a time-dependent manner, active lever presses during the reinstatement tests were divided into 30 min bins. A two-way repeated measures ANOVA of these data revealed no significant effect of microinjection ($F_{(1,9)} = 2.02$, $p > 0.05$), a significant effect of time ($F_{(5,45)} = 13.73$, $p < 0.05$), and no significant interaction ($F_{(5,45)} = 1.11$, $p > 0.05$) (Figure 17E). A two-way repeated measures ANOVA of inactive lever presses during cocaine-prime reinstatement revealed no significant effect of reinstatement ($F_{(1,9)} = 0.07$, $p > 0.05$), no significant effect of microinjection ($F_{(1,9)} = 1.40$, $p > 0.05$), and no significant interaction ($F_{(1,9)} = 0.45$, $p > 0.05$) (Figure 17F).

Discussion

The present data suggest that dopamine activity within the AId is critical for cocaine seeking and indicate that distinct subregions of the IC differentially regulate heroin seeking. Blocking D1 receptors in the AId reduced both cued and cocaine-prime reinstatement, whereas blocking D2 receptors had no effect on cocaine seeking, similar to previous studies showing D2 receptor blockade in the AId has no effect on nicotine seeking (Kutlu et al., 2013). Additionally, pharmacological AId inactivation potentiated cued reinstatement to heroin seeking and had no effect on heroin-prime reinstatement. In contrast, PIc inactivation reduced cued reinstatement to heroin seeking and had no effect on heroin-prime reinstatement. These data are the first to establish a role for the IC in heroin seeking and suggest that each IC subregion independently regulates drug seeking.

The Role of Insular Dopamine in Drug Seeking

Previous work investigating insular dopamine during cocaine use focused on cocaine self-administration (Di Pietro et al., 2008b); in contrast, our study examined how dopamine within the AId influences reinstatement to cocaine seeking. Although drug-taking and drug-seeking are distinct stages of drug use, AId D1 receptor blockade consistently reduces both behaviors as blocking these receptors reduced overall cocaine intake during self-administration (Di Pietro et al., 2008b) as well as cued and cocaine-prime reinstatement in the present experiments. Although D1 receptor blockade in the AId disrupted cocaine seeking, both blocking and overexpressing D1 receptors in the PL subregion of the mPFC reduces cocaine seeking, indicating that an optimal level of dopamine receptor activity within

the PL is required for normal reinstatement (Brenhouse et al., 2015; Devoto et al., 2016). Likewise, both inactivation and electrical stimulation of the AId disrupts nicotine seeking, suggesting that similar to the PL an optimal level of IC activity is required for drug seeking (Pushparaj et al., 2013; Pushparaj et al., 2015a). However, it is unknown whether this effect is dopamine dependent as it is in the mPFC.

Although our previous work found that AId inactivation via GABA agonist microinjections has no effect on cocaine-prime reinstatement (Cosme et al., 2015), the present results indicate that D1 receptor blockade in the AId inhibited this form of cocaine seeking. These results are not the first to reflect this discrepancy, indeed GABA agonist-induced inactivation of either the IL or NAshell has no effect on reinstatement, whereas D1 receptor blockade in these same regions reduces cocaine seeking (McFarland and Kalivas, 2001; Anderson et al., 2006; Cosme et al., 2016). Considering that pharmacological inactivation via GABA agonist administration is often used as a "first pass" to determine the involvement of a distinct structure in behavior, these data suggest that, under some circumstances, this approach may produce "false negatives" in the identification of circuits and structures regulating a behavior. However, as GABA agonists should produce a larger, and less targeted, disruption of activity, it remains surprising that the more limited manipulation altered behavior.

Given the behavioral differences observed following GABA agonist-based inactivation and D1 receptor blockade in the AId, it is possible that, by limiting our manipulation to dopamine receptors, we preferentially targeted cocaine prime-responsive neural ensembles within the AId, resulting in more precise behavioral control. Indeed, work investigating the effects of

chronic cocaine use on dopamine signaling suggests that acute exposure to cocaine (such as a cocaine-prime) following chronic cocaine exposure leads to a predominance of D1 over D2 receptor signaling within the dorsal striatum (Park et al., 2013). A predominance of D1 signaling in the AId following cocaine self-administration may facilitate cocaine-prime reinstatement, as D1 receptor activity is known to signal the rewarding effects of cocaine, and blocking activity at these receptors may inhibit reinstatement as was observed in the current study. Moreover, an imbalance of D1 versus D2 receptor signaling would explain why no effects were observed with D2 receptor blockade.

Insular Differences with Regards to Cocaine and Heroin Seeking

Although previous studies have implicated the IC in drug seeking for several drugs of abuse (nicotine, alcohol, cocaine, and amphetamine), prior research had not investigated the IC during heroin seeking (Di Pietro et al., 2008b; Forget et al., 2010; Contreras et al., 2012; Pushparaj et al., 2015a; Pushparaj and Le Foll, 2015). Our findings indicate that AId inactivation potentiated cued heroin seeking, whereas PIc inactivation reduced this form of reinstatement. In contrast to our findings, previous work has found that IC subregions either act similarly to regulate drug seeking (e.g., nicotine seeking), or one region may completely lack any influence over drug seeking (e.g., the PIc during cocaine seeking) (Forget et al., 2010; Cosme et al., 2015; Pushparaj et al., 2015a). In contrast, our results suggest that the AId and PIc act in opposition to one another to regulate cue-driven heroin seeking, a finding similar to what has been observed in the PL and IL during cocaine seeking (McFarland and Kalivas, 2001; Peters et al., 2008; Moorman et al., 2015), though surprisingly the PL and IL do not have opposing roles for heroin seeking (Rogers et al., 2008). Considering that the

ventral agranular insular cortex (AIv; immediately ventral to AId) projects to the IL, and we did not exclude cannula tracks in the dorsal AIv, it is possible that the inhibitory influence of the AId in heroin seeking reflects a connection with the inhibitory mechanisms of the IL responsible for suppressing cocaine seeking (Reep, 1984). The PIc in contrast may influence heroin seeking via an indirect connection to the NAcore through the AId, as the NAcore appears to be part of a final common pathway for the reinstatement of drug seeking (Moussawi and Kalivas, 2010; Naqvi and Bechara, 2010).

Additionally, distinct neural ensembles within the AId may mediate cocaine and heroin seeking, as is true in the IL, which similarly influences cocaine and heroin seeking in opposite directions (albeit in the opposite direction from what is observed in the AId). Indeed, in the IL, only a select population of neurons have been found to suppress alcohol seeking (Pfarr et al., 2015), and single unit recordings in the rodent mPFC found that only ~20% of neurons showed similar responses to both cocaine and heroin administration (Chang et al., 1998). These findings suggest that distinct populations throughout the PFC mediate motivated behavior towards cocaine and heroin, and this finding may extend to the IC as well. Another explanation for the present results may be the interoceptive properties associated with cocaine versus heroin reinstatement. The AId is known to mediate the interoceptive effects of drugs. However, these signals can carry both positive and negative hedonic values and distinct stages of our paradigm may have elicited different interoceptive cues. For example, cocaine produces aversive properties during self-administration, as evidenced by a runway task in which animals show an approach/avoidance response towards obtaining a cocaine infusion; these same rats, however, successfully reinstate cocaine seeking

following the presentation of cocaine-associated cues (Su et al., 2011). In contrast, heroin-administering rats will run without hesitation for a heroin infusion but do not show increased heroin seeking following the presentation of heroin-associated cues in the same task (Su et al., 2011). Although the precise mechanism behind this result is unknown, one possibility may be a change in drug-associated interoceptive cues following withdrawal, in which case AId inactivation during heroin seeking may remove negative interoceptive cues, thus potentiating reinstatement, whereas AId inactivation during cocaine seeking may block positive cocaine-associated interoceptive cues to reduce reinstatement.

Conclusions

The present findings indicate that, like AId inactivation, blocking D1 receptors reduced cued reinstatement of cocaine seeking. However, although AId inactivation had no effect on cocaine-prime reinstatement, our results demonstrate that D1 receptor blockade within the AId reduced this type of cocaine seeking. D2 receptor blockade had no effect on any form of reinstatement. The current results also suggest that the AId and PIc regulate cue-driven heroin seeking in opposite directions but do not influence heroin-prime reinstatement. Thus, the present findings add to the complexity of AId functioning with regard to drug-seeking behaviors and provide further evidence that, like other regions of the prefrontal cortex, the IC can both inhibit and promote drug seeking depending on the type of drug and the type of reinstatement being examined.

CHAPTER 5: GENERAL DISCUSSION

Mapping out the neural circuitry underlying reinstatement of drug seeking has been a critical question in neurobiological research for decades, with the prefrontal cortex emerging as a region promiscuously involved in numerous types of reinstatement. However, studies investigating the role of prefrontal subregions in drug seeking have yet to identify the mechanisms underlying the inhibitory influence of the infralimbic cortex (IL) on cocaine seeking. Additionally, although the medial prefrontal cortex (mPFC) has been extensively investigated for its various roles in drug seeking, a clear role for the subregions of the lateral PFC in cocaine seeking has not been established despite evidence indicating that disrupting insular cortex (IC) activity, a subregion of the lateral PFC, influences several forms of drug seeking in both humans and animals.

In chapter 2, we found that IL and medial orbitofrontal activity (mOFC) dopamine activity is necessary for specific types of reinstatement. Although previous studies have shown an inhibitory influence for the IL in cocaine seeking, our results suggest that the IL can also promote cocaine seeking under certain circumstances, as blocking D1 receptors in this region reduced cued reinstatement but had no effect on cocaine-prime reinstatement. In contrast, blocking D1 receptors in the mOFC reduced all forms of reinstatement that were examined, establishing distinct roles for IL and mOFC D1 receptor activity during cocaine seeking. Blocking D2 receptors in both the IL and mOFC had no effect on any form of reinstatement, indicating the influence of dopamine in these regions is specific to D1 receptor activation. These results further suggest that the IL acts to drive drug seeking under certain conditions and are the first to demonstrate a role for the mOFC in cocaine seeking.

Chapter 3 investigated the role of the IC in both cocaine and food seeking, along with the role of AId CRF activity in reinstatement behaviors. Inactivating the AId reduced cued reinstatement and had no effect on cocaine-prime reinstatement, whereas this same manipulation had no effect on food seeking, signifying the role of the AId in cocaine seeking does not generalize to natural rewards. Additionally, inactivating the PIc had no effect on any type of cocaine seeking. Thus, although the PIc has previously been implicated in nicotine and amphetamine seeking, it does not mediate cocaine seeking, suggesting that each IC subregion has an independent influence over drug seeking. Finally, blocking CRF1 receptors in the AId inhibited cued reinstatement, indicating a possible mechanism by which the AId mediates reinstatement.

Chapter 4 continued our exploration into the role of the AId in cocaine seeking by investigating both the contribution of AId dopamine in mediating cocaine seeking, as well as the role of the IC in heroin seeking. Initially, blocking AId D1 receptors reduced cued reinstatement as well as cocaine-prime reinstatement, whereas blocking D2 receptors had no effect on any type of reinstatement. These data are particularly interesting considering that pharmacological inactivation of the AId had no effect on cocaine-prime reinstatement. Further, inactivating the AId *potentiated* heroin seeking during cued reinstatement, and had no effect on heroin prime reinstatement. In contrast, blocking the PIc *reduced* heroin seeking during cued reinstatement and similarly had no effect on heroin prime reinstatement. These data are the first to demonstrate a role for the IC in heroin seeking, and lay out a mechanism whereby the AId may manipulate cocaine seeking.

The Role of Interoception in Drug Seeking

Although the IC plays a role in numerous cognitive processes, its involvement in interoception provides the strongest foundation for how the IC participates in generating motivated behaviors towards drugs of abuse. Initially, the IC is highly interconnected with both limbic and thalamic nuclei (Craig, 2009), making it well suited for integrating visceral information with cognitive representations of the world to form a conscious experience. This complex integration within the IC hinges on the processing of interoceptive and exteroceptive cues to form an internal representation of these stimuli referred to as interoception. These cues are any perturbations to the body that may effect homeostasis, such as changes in temperature, cardiovascular functions, taste, pain, and touch (Craig, 2002, 2003), such cues are critical for survival as they alert us to potentially dangerous changes in homeostatic function. However, interoception is not a neutral representation of our internal state but rather carries either a positive or negative valence, creating a system whereby changes to the body become associated with specific emotional outcomes and motivational states (Naqvi and Bechara, 2009). Damasio (2000) proposes an "as if" representation of bodily states perpetuated by the IC wherein both interoceptive and exteroceptive cues along with external stimuli predictive of said cues are integrated with past experiences to form a stable interoceptive representation that can be recalled "as if" the original stimuli were present, even in the absence of the interoception generating stimuli. Considering that the IC is at the center of translating changes in the body into conscious awareness via emotional and motivational properties, its involvement with drug seeking is a reasonable outcome.

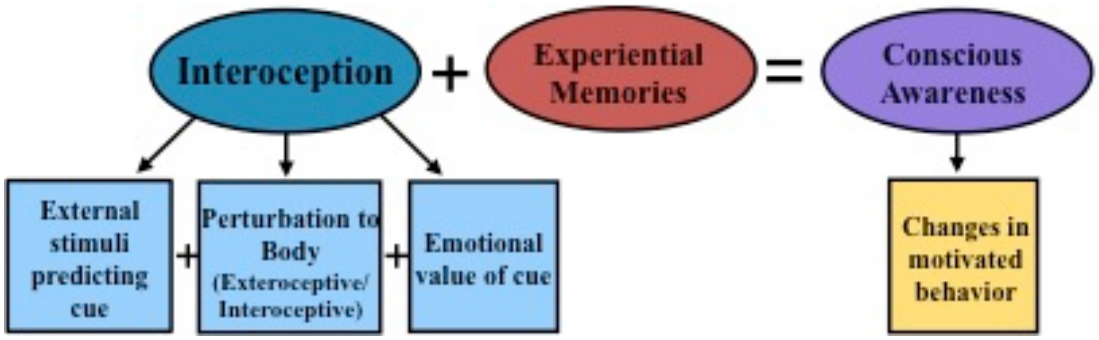

Figure 18. A schematic diagram of the interoceptive information processed in the IC. An interoceptive representation is formed via integration of the external stimuli predictive of either an interoceptive or exteroceptive cue, the physical components of the cue that are acting on the body, and the emotional value associated with a given cue. This interoceptive representation is then combined with the knowledge of prior experiences to form a conscious awareness that can influence motivated behavior.

All drugs of abuse induce a unique set of both exteroceptive and interoceptive cues, such as the feeling of smoke in one's airway or the prick of a needle during heroin administration. Given the drastic differences between drug-specific cues, the internal representations forned from these cues may account for the discrete urges drug users experience for their specific drug of choice, despite the fact that many abusive drugs have the same end result – an increase in nucleus accumbens dopamine (Naqvi and Bechara, 2009, 2010). With influence from the reviews regarding interoception and drug abuse described above (Damasio, 2000; Goldstein et al., 2009; Naqvi and Bechara, 2009; Picard and Craig, 2009; Naqvi and Bechara, 2010), I have synthesized several hypotheses to form a theoretical framework that explains the manner in which the IC influences drug seeking (Figure 18). Initially interoception, or the encoding of changes in the body, is generated via the integration of three distinct components: the external stimuli predicting the exteroceptive or interoceptive cue (the bottle of alcohol), the physical attributes of the interoceptive or exteroceptive cue (taste of alcohol), and the emotional values associated with the cue (content, happy, craving, etc.). The IC has been linked to each of these components via imaging studies and experiments using animal

models of behavior (for more details see: (Craig, 2002; Phan et al., 2002; Kilts et al., 2004; Myrick et al., 2004)). Therefore, interoception is the internal representation of the attributes comprising a change in the body's normal state; the IC then combines this interoceptive representation with the knowledge of prior experiences to form a conscious awareness, which influences motivated behavior. Under this framework, it is possible that drug-associated stimuli, such as a light and tone cue or a systemic drug prime, may evoke the interoceptive representation of drug use in the "as if" manner described earlier, which would drive drug seeking even in the absence of drug delivery as is observed during reinstatement.

This framework further suggests several explanations for the data we have presented. Naqvi and Bechara (2009) suggest that the interoceptive processing in the IC reflects two different types of learning that may induce dopamine-dependent neural plasticity in the IC. First, drug users learn to assign positive hedonic value to seemingly unpleasant experiences such as the foreign feeling of smoke in one's airway and the prick of a needle. Second, the IC aids in the association of external stimuli with both exteroceptive and interoceptive cues, setting up an association whereby drug-associated stimuli can drive drug seeking. Therefore, during our experiments it is possible that disrupting activity in the IC led to an "unlearning" of these associations, resulting in decreased cued reinstatement. Additionally, withdrawal from drug use produces a unique set of interoceptive cues that differ from those present during drug administration and these withdrawal related cues may modulate the interoceptive representations formed during self-administration. Indeed, although cocaine withdrawal induces mild discomfort, heroin withdrawal is widely considered an aversive physical experience. Therefore the aversive qualities of heroin withdrawal may modulate the

interoceptive representation formed during heroin self-administration in such a way that the IC subsequently inhibits heroin seeking during reinstatement testing, an outcome that would explain why the AId promotes cocaine seeking and inhibits heroin seeking in our paradigm.

Finally, it is possible that the different subregions of the IC differentially encode interoceptive information, with the AId encoding aversive information and the PIc encoding positive information for some classes of drugs of abuse. Prior work has shown a specific role for the AId in aversive processing, as inactivation of this region prevents conditioned place aversion for naloxone-precipitated morphine withdrawal but had no effect on the development of morphine induced conditioned place preference (Li et al., 2013). This would explain why the AId and PIc play inconsistent roles in the mediation of heroin and cocaine seeking, as exposure to distinct drug associated cues may recall interoceptive representations with different valences, with heroin-associated cues reflecting an aversive interoceptive representation. Thus, inactivating the AId may remove the negative interoception produced in the presence of heroin-associated stimuli, leaving only positive hedonic properties resulting in a potentiation of heroin seeking.

To test the role of the AId in aversive interoceptive processing we utilized a cocaine conditioned place aversion model, in which animals are systemically infused with cocaine via an intra jugular catheter and then placed into one side of a two-sided apparatus following a 15-minute delay. Although rats placed into the conditioning chamber immediately following a systemic cocaine injection develop a preference for that side, rats placed in the same chamber 15 minutes after a cocaine injection develop an aversion to the cocaine-paired

side. Through this testing, I was successfully able to show aversion to the side paired with a delayed cocaine injection, however AId inactivation during the training phase had no effect on the acquisition of this aversion. Thus, we were unable to confirm the role of the AId in negative aversive processing. However, given the discrepancies between our cocaine-prime reinstatement data after inactivation or post D1 antagonism, it is possible that blocking D1 receptors could reveal a role for the AId in conditioned place aversion towards cocaine that wasn't observed with general inactivation.

Importance of Distinct Insular Pathways

In order to better explain the complex role of the prefrontal cortex in mediating drug seeking, future studies will need to manipulate downstream prefrontal pathways to determine if distinct efferent connections mediate specific behaviors. One key IC efferent pathway is a reciprocal connection with the mPFC, wherein the AId connects to the PL and the PIc connects to the IL (Figure 19) (Li et al., 1998). These pathways may shed light on the discrepancies observed in my data in which the AId inhibits and the PIc promotes cued heroin seeking, as the mPFC shows similar differences in mediating drug seeking. Indeed, the PL is known to promote cocaine seeking whereas the IL inhibits cocaine seeking, demonstrating that subregions within the same structure do not necessarily mediate drug seeking in the same direction (McLaughlin and See, 2003; Peters et al., 2008). When looking at the subregions of the mPFC and lateral PFC in pairs based on their reciprocal connections (AId and PL; PIc

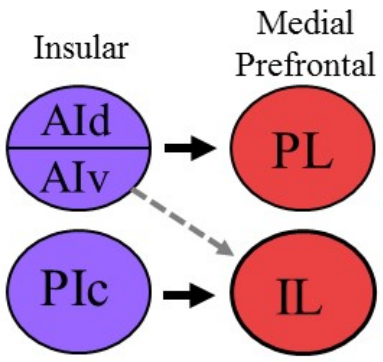

Figure 19. Schematic diagram of the connections between insular and medial prefrontal cortex subregions.

and IL; see Figure 19) we find that much like the PL, the AId drives cued cocaine seeking establishing a consistent role for these two connected regions in the promotion of cocaine seeking (Figure 20). In contrast, during cocaine seeking the PIc and IL play distinct roles from their PFC counterparts (AId and PL, respectively), as the PIc shows no influence over cocaine seeking and the IL inhibits cocaine seeking (evidenced by a decrease in cocaine seeking following IL activation (Peters et al., 2008)).

	PL	IL	AId	PIc
Cued (Cocaine)	↑	↓	↑	≠
Cocaine-Prime	↑	↓	≠	≠
Cued (Heroin)	↑	↑	↓	↑
Heroin-Prime	↑	↑	≠	≠

Figure 20. Cocaine seeking outcomes during cued and drug-prime reinstatement tests with various prefrontal structures inactivated Upwards arrow indicates structure drives that form of reinstatement, downward arrow indicates that structure inhibits the form of reinstatement.(PL = prelimbic; IL = infralimbic; AId = dorsal agranular insular cortex; PIc = posterior insular cortex.)

Looking to heroin reinstatement, the PIc and IL both drive cued reinstatement to heroin seeking, whereas the AId and PL no longer act similarly to each other but rather the AId inhibits and the PL promotes cued reinstatement to heroin seeking. Therefore, for cocaine seeking it is possible that the AId→PL pathway is driving cued reinstatement, with the IL providing inhibition via its projection to the nucleus accumbens shell, whereas the PIc→IL pathway appears to drive heroin seeking, with the AId providing inhibitory influence (LaLumiere et al., 2012a). In both of these cases, the structure providing inhibitory influence does not influence drug-seeking in the same way that its prefrontal counterpart does, suggesting inhibitory circuits diverge to include regions beyond what is required for the promotion of drug seeking. Although more recent tracing studies indicate that the AId and

PIc maintain dense projections to distinct mPFC regions, Reep (1984), notes that the ventral agranular insular cortex (AIv; located directly beneath the AId) also projects to the IL (Figure 19). Throughout our studies, we did not exclude animals' whose cannula tracks terminated in the AIv, thus in the case of cued heroin seeking where we observed the AId inhibiting reinstatement, it is possible that the AIv engaged the inhibitory mechanisms in the IL responsible for cocaine seeking inhibition in order to inhibit heroin seeking. However, given that AId inactivation alone had no effect on lever pressing, it appears that the AId does not act similarly to the infralimbic cortex in all circumstances (e.g. Peters et al. (2008)). Still, it is possible that the AId interacts with the infralimbic cortex and, under certain conditions, suppresses drug seeking.

As described earlier, prior research has established a final common pathway in the reinstatement of cocaine seeking, which includes the glutamatergic projection from the mPFC to the nucleus accumbens core (NAcore) and the GABAergic projection from the NAcore to the ventral pallidum (Moran et al., 2005). The IC integrates with the final common pathway via direct glutamatergic projections to both the mPFC and the NAcore, however it is unclear how these two distinct projections influence cocaine seeking. Initially, sensory input from the thalamus passes through the granular and dysgranular insular cortices on the way to the agranular insular cortex where the AId integrates sensory information with cognitive representations to form interoceptive representations (Arguello et al., 2017). Therefore, it is possible that sensory information, such as the light and tone cues present during cued reinstatement, is first processed in the thalamus and subsequently generates motivated behavior via an indirect projection to the mPFC via the IC. However, the AId also sends direct projections to the NAcore. Given that previous studies found optogenetically

inhibiting the PL→NAcore pathway blocks both cued and cocaine-prime reinstatement, and the AId and PL similarly regulate cocaine seeking, it is reasonable to believe that the AId may similarly mediate cocaine seeking via its glutamatergic projection to the NAcore (Stefanik et al., 2015). To tease out what projections are essential in mediating the AId's influence over cocaine-seeking future experiments should specifically silence the AId→PL and AId→NAcore pathways via optogenetics or chemogenetics in order to determine the differential involvement of these pathways in cocaine seeking.

Discrepancies in Heroin versus Cocaine Seeking

Our data demonstrate that the AId mediates cocaine and heroin seeking in opposite directions (Figure 20), as this IC subregion drives cocaine seeking and inhibits heroin seeking. One methodological characteristic that may account for this discrepancy is the manner in which rats were housed during experiments (Badiani et al., 2011). Several experiments have suggested that housing rats in the same operant chambers where they are permitted to take drugs (resident rats) produces different responses towards drugs of abuse than housing rats in a separate chamber that is distinct from the testing chamber (non-resident rats), as was done in all of the experiments I have presented here (Caprioli et al., 2007a). Non-resident rats self-administer greater amounts of cocaine and have higher breakpoints for cocaine under a progressive-ratio program than their resident counterparts and administer reduced amounts of heroin compared to resident rats (Caprioli et al., 2007b; Caprioli et al., 2008). Additionally, in contrast to resident rats, which prefer heroin when given a choice between cocaine and heroin administration, non-resident rats are cocaine preferring, demonstrating a circuit whereby heroin administration is inhibited in non-resident rats (Caprioli et al., 2009). Badiani et al. (2011) suggests inhibition of heroin use in a non-resident environment may be

advantageous, as the sedative effects of heroin can be dangerous in a non-home environment, whereas the arousing effects of cocaine may be valuable in a new and unfamiliar environment. Caprioli et al (2007) further details these behavioral differences may be due to changes in the interoceptive cues produced by abusive drugs under distinct housing conditions. Given that the AId processes interoceptive cues, and is also involved in mediating context-based reinstatement, it is possible that this region is involved in mediating the different proclivities towards abusive drugs described above. Under this theoretical framework it is possible that via processing in the AId our non-residents rats have increased motivation towards cocaine, and a diminished motivation to administer heroin. Thus, inactivating the AId, as was done in the experiments presented in chapters 3 and 4, may reduce the heightened rewarding properties of cocaine leading to a decrease in cocaine seeking compared to controls, and the same manipulation may block the AId's inhibitory influence over heroin seeking leading to a potentiation in reinstatement as our data show.

Another explanation for the differences between our cocaine versus heroin seeking data may lie in the generation of reward prediction error (RPE) signals. EEG recordings during a gambling task revealed that cocaine users show impaired negative reward prediction error signaling compared to healthy controls, which may contribute to their continued relapse even in the face of adverse consequences (Parvaz et al., 2015). Interestingly, the AId has been implicated in the generation of RPE signals (Jo and Jung, 2016). During a reward prediction task in which rats were trained to respond for a water reward following one of 5 different audio cues that each indicated a different probability of reward delivery, neural recordings revealed that certain populations of anterior insular neurons increased their firing following a negative outcome as a function of the previously expected value in a pattern consistent with

RPE signaling. Given this involvement, it is possible that the deficits observed in cocaine addicts are related to structural changes within the AId that inhibit RPE signals from successfully encoding information about the omission of a reward. If cocaine induced changes within the AId lead to a sensitized response toward an error, inactivating the AId in our experiment may restore the impaired RPE signaling, therefore allowing rats to better encode the omission of reward, resulting in decreased cued reinstatement. As no studies have indicated a deficit in RPE signals in heroin users, it is possible that AId inactivation following heroin use disrupts normal encoding of reward omission, resulting in a potentiation of cued reinstatement. Our data further support this hypothesis, as D1 antagonism in the AId revealed an effect on cocaine-prime reinstatement that was not observed with general inactivation. Considering that RPE signals are known to be dopaminergic in nature, it is possible that the amount of dopamine receptor activation needed to successfully encode reward omission within the AId falls along an inverted U with either too little or too much dopamine receptor activation resulting in impaired RPE signaling (Hart et al., 2014; Keiflin and Janak, 2015). Under this premise, cocaine abuse may lead to an overexpression of D1 receptors in the AId that impairs reward omission encoding. Thus, blocking activity at these receptors may allow for optimal RPE signaling during reinstatement, resulting in decreased cocaine seeking.

Methodological Considerations

Several of the methodological details of our experiments may provide insight into the observed results. Initially, although we did not see any influence for the AId in food seeking, our experiments used standard rat chow, as did Forget et al. (2010), which similarly showed no effect for the PIc in food seeking behavior. However, Baldo et al. (2016) found AId

inactivation decreased total intake of a highly palatable chocolate drinks as well as feeding duration, with no effect on water or food intake in food-deprived rats. These results suggest that the AId may process the value of reward based on taste perception and establishes the IC as a multifunctional structure that is involved in generating motivated behavior towards both drugs of abuse and highly palatable rewards. Moving forward it would be interesting to determine if the IC efferents producing reward-seeking behaviors are identical for natural palatable awards and abusive drugs.

Additionally, the location of our manipulations, as well as the technique by which we silenced activity, may have influenced our data. The IC is a heterogeneous structure that spans a significant portion of the brain, and although we developed strict criteria for structural boundaries in the dorsal/ventral plane, we did not impose any inclusion criteria for the anterior/posterior plane, which resulted in manipulations along the entire length of the IC. Previous work has found that identical manipulations along the length of a structure can have different behavioral outcomes, as GABAergic activation in the anterior NAshell results in positive feeding behavior and GABA agonism in the posterior shell produces defensive behavior (Reynolds and Berridge, 2001). Thus, it is possible that as we move along the anterior/posterior axis of the IC, the role of the IC in drug seeking may shift, with some areas driving drug seeking and others inhibiting drug seeking. Another possibility is that different neuronal populations within the IC are involved in mediating reinstatement for specific drugs of abuse, meaning there could be a unique population mediating cued versus drug-primed reinstatement and cocaine versus heroin seeking. An ideal method by which to study this proposed involvement would be using a transgenic approach in combination with

optogenetics wherein we are able to limit transduction of cells to the neurons specifically active during reinstatement as has been done previously (Tayler et al., 2013). To accomplish this we could utilize *fos-tTA* reporter mice, as the removal of doxycycline (DOX) from these animals' diets permits cFos activity to induce tTA, H2B-GFP, and Cre expression. To allow for the inhibition of these cFos expressing IC neurons, a floxed version of ArchT would be injected into the region prior to a reinstatement test. AId neurons involved in reinstatement would then be tagged by taking animals off DOX during the reinstatement test to allow for ArchT and H2B-GFP expression in cFos positive cells. This method would allow us to determine if the populations mediating cued and cocaine-prime reinstatement are identical, which could explain why we saw different results with our cocaine-prime data following general inactivation and D1 antagonism, as blocking D1 receptors may have targeted a specific subset of neurons.

Conclusions

These data are the first to establish a role for the IC in both cocaine and heroin seeking, and further contribute to the complex role of the PFC in cocaine seeking. Although the AId drives cued reinstatement to cocaine seeking, it inhibits cued reinstatement to heroin seeking. Additionally, the PIc plays no role in cocaine seeking but opposes the AId in heroin seeking by driving cued reinstatement. These types of inconsistencies regarding the role of the IC in cocaine seeking are described in detail elsewhere, however few theories have been proposed as to how this region can both drive and inhibit drug seeking under different circumstances. Therefore, I propose that future studies investigating the role of the IC use pathway specific manipulations to determine how IC efferents differentially effect drug seeking.

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