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Jacob Edward Winschel Date

Effects of 5-HT_{2A} Receptor Antagonism on Cocaine Self-administration, Cocaine-induced Reinstatement, and Cocaine-induced Dopamine Overflow in Rhesus Monkeys

By

Jacob Edward Winschel

Master of Science

Graduate Division of Biological and Biomedical Science

Molecular and Systems Pharmacology

_____

Leonard Howell
Advisor

_____

Yoland Smith
Committee Member

_____

David Weinshenker
Committee Member

Accepted:

_____

Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____

Date

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Abstract

Effects of 5-HT_{2A} Receptor Antagonism on Cocaine Self-administration, Cocaine-induced Reinstatement, and Cocaine-induced Dopamine Overflow in Rhesus Monkeys

By Jacob Edward Winschel

Growing evidence indicates that serotonin (5-HT) systems modulate the effects of stimulants by altering dopamine system function. The serotonin 2A and 2C receptor (5-HT_{2A}R and 5-HT_{2C}R) subtypes have been found to exhibit particularly strong, and opposing, influence on dopamine systems. For example, 5-HT_{2A}R antagonists have been shown to inhibit stimulant-induced dopamine overflow in the nucleus accumbens (NAc) and to block both cue-induced and cocaine-primed reinstatement, although these effects have only been demonstrated in rats. These promising results suggest that 5-HT_{2A}R antagonists may be effective therapeutics against cocaine abuse and further investigation of their therapeutic potential is warranted. Therefore, using a within-subjects design in rhesus macaques (n=3), we evaluated the effects of the selective 5-HT_{2A}R antagonist M100,907 (M100) on intravenous cocaine self-administration and cocaine-primed reinstatement. In separate subjects, we evaluated the role of 5-HT_{2A}Rs in cocaine-induced dopamine overflow using *in vivo* microdialysis targeting the NAc (n=4) and the caudate nucleus (n=5). Consistent with previous studies, M100 (0.3 mg/kg, IM) did not significantly attenuate cocaine self-administration, even over a three day dosing regimen. In contrast, but also consistent with previous rat studies, M100 (0.3 mg/kg, IM) largely abolished cocaine-primed reinstatement across a range of cocaine priming doses. In accord with its lack of effect on cocaine self-administration, M100 (0.3 mg/kg, IM) did not significantly attenuate cocaine-induced dopamine overflow in the NAc. However, M100 (0.3 mg/kg, IM) significantly attenuated cocaine-induced dopamine overflow in the caudate nucleus. The concordance between the effects of M100 on cocaine-induced dopamine overflow in the caudate and cocaine-induced reinstatement supports a growing body of evidence highlighting the distinct role of the caudate in reinstatement and habit-related behaviors. Furthermore, since reinstatement procedures are the most widely accepted preclinical model of drug relapse, these findings suggest that 5-HT_{2A}R antagonism may be a viable approach to relapse prevention in cocaine-dependent subjects. Supported by DA00517, DA 10344, and RR00165.

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List of Abbreviations

5,7-DHT	5,7-dihydroxytryptamine
5-HT	5-hydroxytryptamine; serotonin
5-HT_2R	serotonin 2 receptor family
5-HT_{2A}R	serotonin 2A receptor subtype
5-HT_{2B}R	serotonin 2B receptor subtype
5-HT_{2C}R	serotonin 2C receptor subtype
5-HTP	5-hydroxytryptophan
aCSF	artificial cerebrospinal fluid
ANOVA	analysis of variance
DA	dopamine
DAT	dopamine transporter
DOI	1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane
F	Friday
FR20	fixed ratio 20
GABA	gamma-amino butyric acid
GPCR	G-protein coupled receptor
HPLC	high-performance liquid chromatography
IM	intramuscular
IV	intravenous
M100	M100,907
M	Monday
MDMA	3,4-methylenedioxymethamphetamine
mg/kg	milligrams per kilogram
mg/kg/inf	milligrams per kilogram per infusion
MI	myocardial infarction

mPFC	medial prefrontal cortex (in the rat)
NAc	nucleus accumbens
NE	norepinephrine
NET	norepinephrine transporter
NHP	nonhuman primate
PCA	parachloroamphetamine
PCPA	parachlorophenylalanine
PET	positron emission tomography
PFC	prefrontal cortex
SA	self-administration
SD	standard deviation
SEM	standard error of the mean
SERT	serotonin transporter
SN	substantia nigra
SNc	substantia nigra pars compacta
SNr	substantia nigra pars reticulata
SSRI	selective serotonin reuptake inhibitor
Th	Thursday
TRP	L-tryptophan
Tu	Tuesday
veh	vehicle
VTA	ventral tegmental area
W	Wednesday

Chapter 1: Introduction

Background and Rationale

Drug Abuse Prevalence and Statistics

Drug abuse is a widespread and devastating problem in the United States and around the world which contributes heavily to crime, health problems, hospitalizations, and loss of productivity, while financially and socially burdening users as well as society. The number of drug users in the US is staggering: the 2011 National Survey on Drug Use and Health (NSDUH) estimated that in 2010, 22.6 million or 8.9% of Americans age 12 and older were past month ("current") illicit drug users (Substance Abuse and Mental Health Services Administration, 2011). Compounding the problem is the fact that illicit drug use appears to have been increasing over the past decade. Comparing the NSDUH's 2010 results with those of the years 2002 to 2009 indicates that illicit drug use prevalence was higher in 2010 than at any other time since at least 2002. In addition, the 2012 Monitoring the Future Study (MFS) estimated that 40% of 12th graders used an illicit drug in 2011 and that 17.6% of 12th graders used an illicit drug other than marijuana (Johnston et al., 2012). This study corroborates the trend of increasing illicit drug use in the NSDUH, showing that prevalence has increased every year but one since 2007 (Johnston et al., 2012).

In addition to a relatively high prevalence, drug abuse is a significant and growing health and economic burden, constituting one of the most costly health problems in the US. The 2011 National Drug Intelligence Center report, The Economic Impact of Illicit Drug Use on American Society, estimated the total cost to society of illicit drug use in health care, productivity loss, crime, incarceration, and drug enforcement in 2007 as ~$193 billion (National Drug Intelligence Center, 2011). To put this into perspective: the National Institute on Digestive and Kidney Diseases estimated that diabetes costs the US more than $174 billion/year, Finkelstein, et al.

(2009) estimated obesity to cost more than $147 billion in 2008, and the CDC reported that between 1995 and 1999, cigarette smoking cost ~$157 billion/year and heart disease cost ~$316 billion in 2010. In addition, the 2012 Drug Abuse Warning Network (DAWN) report reported that, of the 4.9 million drug-related emergency department visits in 2010, ~1.17 million, or 23.8%, involved illicit drugs (Substance Abuse and Mental Health Services Administration, 2012). Cocaine is considered one of the most prevalent and harmful drugs to users and society.

Prevalence of Cocaine Abuse and Dependence

Cocaine is one of the most widely abused drugs in the US: in 2011, nearly 15% of Americans had tried cocaine in their lifetime and ~5.2% had tried it by their senior year of high school; the NSDUH reported that in 2010, ~1.5 million people (0.6%) age 12 and older were current users (Johnston et al., 2012). In that report, cocaine was the 4th most commonly illicitly used drug, with only marijuana (~17.4 million or 6.9%), pharmaceutical analgesics as a class (5.1 million or 2.0%), and tranquilizers as a class (2.2 million or 0.88%) currently used by more people (use of alcohol and tobacco greatly eclipses all of these illicit drugs). The survey also reported that ~1 million people (0.4%) met the criteria for cocaine abuse or dependence in 2010. Importantly, ~650,000 people tried cocaine for the first time in 2010; this amounts to ~1,700 new initiates per day. Particularly striking is the frequency of cocaine use in young adults (age 18-25), with 1.5% currently using cocaine. In addition, the MFS found that 1.4% of 8th graders, 1.9% of 10th graders, and 2.9% of 12th graders had used cocaine in the year before the study, which illustrates the scope of abuse of this dangerous drug in the most vulnerable populations.

Health-Related Effects of Cocaine

Cocaine is a dangerous drug that causes serious health consequences with both short- and long-term use. Adverse events caused by cocaine span the physiological and the psychological and can include myocardial infarction (MI, heart attack), stroke, seizures, sudden death, anxiety,

panic attacks, and psychosis. Although most stimulants have some risk of causing cardiovascular adverse effects, the risk of MI with cocaine use is much greater than that of other stimulants because of the several opposing pharmacological effects of cocaine in heart vascular and muscle tissue (Bauman et al., 1994). Adverse events related to cocaine use are so prominent that emergency department visits involving cocaine use occur at a greater frequency than those involving the use of any other illicit drug (Substance Abuse and Mental Health Services Administration, Treatment Episode Data Set, 2012). Furthermore, rates of cocaine use and drug injection are associated with increased risk of contracting HIV/AIDS (Patrick et al., 2012). In 2010, of the ~1.17 million visits to US emergency departments comprising patients whose condition involved use of illicit drugs, amazingly 41.7% involved cocaine use, more than visits involving any other single drug except alcohol. In addition, of the nearly 2 million admissions to substance abuse treatment centers in 2009, primary cocaine abuse accounted for 9% and a remarkable 24% of all treatment admissions in 2009 involved cocaine abuse of some severity.

The large scope of cocaine abuse in the United States and the crippling health, crime, and economic burdens associated with it have fueled intensive efforts to understand the behavioral pharmacological effects of cocaine and to develop pharmacotherapeutics for cocaine abuse and addiction. Despite significant headway in identifying the neuroanatomical and neurochemical mechanisms that mediate the effects of cocaine, no drugs have been approved to reduce cocaine intake, craving, or relapse to cocaine taking.

Behavioral Neuropharmacology of Cocaine

Monoamine Systems and the Abuse-Related Effects of Cocaine

The three primary monoamines—dopamine (DA), serotonin (5-HT), and norepinephrine (NE)—are important neurotransmitters involved in the output and regulation of behavior, especially via the key roles they play in cognition, stress response, mood/pleasure, motivation, and locomotion. Oftentimes, monoamines appear to act as neuromodulators, fine-tuning the

activity of inputs and outputs of the neural pathways and brain regions that act as the basic machinery for behavior. Additionally, monoamine systems are important players in the gamut of psychiatric and neurological disorders; accordingly, therapeutics that modulate these systems have revolutionized the treatment of a variety of disorders (Moore and Bloom, 1978). Monoamine modulators that affect the dopaminergic systems—including Levo-DOPA, the synthetic precursor to DA which treats Parkinson's disease, and amphetamine (Adderall) and methylphenidate (Ritalin), which treat attention deficit disorder and narcolepsy—and drugs that act on serotonergic systems such as selective serotonin reuptake inhibitors (SSRIs), which treat depression, anxiety, social phobias, and an increasingly long list of disorders—have contributed enormously to the understanding and treatment of psychiatric and neurological disorders.

Cocaine, which is naturally produced by the Coca Erythroxylaceae plant (Chychula and Okore, 1990), has very high abuse liability, as demonstrated by the epidemiological studies above. Cocaine can cause a range of negative and positive psychological effects, including euphoria, mental and physical stimulation, confidence or craving, anxiety, depression, egocentrism, aggression, etc., respectively (Chychula and Okore, 1990; Gawin, 1989). The effects of cocaine that are thought to be directly related to, or responsible for, its psychotropic effects and high abuse liability—its "abuse-related" effects—are mediated both by acute changes in monoamine systems in well-defined regions of the brain (Wise, 1998) and adaptive changes that are not as well elucidated, but which are a highly active area of current research (Kalivas, 2007; Kalivas, 2009; Kalivas and Volkow, 2005; Knackstedt and Kalivas, 2009).

Cocaine causes its many psychological and behavioral effects by enhancing monoamine signaling via inhibition of monoamine transporter proteins found on the presynaptic terminals of monoamine-expressing neurons. The three primary monoamine transporter targets of cocaine are dopamine transporter (DAT), norepinephrine transporter (NET), and serotonin transporter (SERT) (Kuhar et al., 1991; Volkow et al., 1997). These transporters normally bind to their respective monoamines (DAT binds to DA, NET to NE, SERT to 5-HT) and transport them from

the synapse into the presynaptic terminals so they can be packaged into synaptic vesicles and later released (Iversen, 1971). Cocaine inhibits the reuptake of monoamines via each of the three monoamine transporters with roughly equal potency (many studies across mammalian species and various techniques report that cocaine inhibits each transporter with a K_i of ~200-700 nM) (Han and Gu, 2006; Ritz et al., 1988; Rothman et al., 2001). This blockade of transporters over a period of time elevates the concentration and prolongs the duration of monoamines at synapses after administration (Fletcher et al., 1999b; Reith et al., 1986). The increased concentrations and delayed synaptic clearance of monoamines result in augmented and prolonged activation of monoamine receptors, enhancing the activity of downstream signaling pathways which mediate many of the effects of cocaine.

Monoamine regulation of parameters of behavior, such as motivation, locomotion, and reinforcement, has been found to be critical in the abuse-related effects of drugs. In particular, a vast body of work has established that DA plays a major role in processes including reinforcement, euphoria (and other positive affective states), motivation, attention and focus, cognition, and motor output (Kelley and Berridge, 2002; Wise, 1998), but that 5-HT and NE systems have some influence over these functions (Uhl et al., 2002). One of the primary focuses of the study of cocaine abuse is the process of reinforcement of drug-taking behavior, since cocaine's reinforcing properties are thought to be a central facet of its addictiveness. Considerable research has indicated that the inhibition of DAT by cocaine, but not NET or SERT, is directly responsible for the reinforcing effects of cocaine (Kuhar et al., 1991; Ritz et al., 1987). It follows that the increase in DA concentration in the brain consequent to DAT inhibition by cocaine is the cause of its reinforcing effects. Indeed, many seminal studies have demonstrated a tight link between increases in extracellular levels of DA in the brain and the reinforcing effects of not just cocaine, but most drugs of abuse. Further evidence of this principle is the correlation between the rate of entry into the brain and reinforcing effectiveness of drugs; the high speed with which

cocaine reaches the brain and increases DA levels is a crucial factor in the robustness of its reinforcing and addictive effects (Kimmel et al., 2008; Kimmel et al., 2007).

Dopamine Systems

An extraordinary body of research has revealed many of the functional neuroanatomical, neurochemical, regulatory, and behavioral characteristics of the related mesolimbic and mesocortical DA systems which are commonly considered the primary projections of the "reward pathway." This system is sometimes thought of as the final common pathway of all drugs of abuse and reinforcing stimuli, since nearly all drugs of abuse increase DA levels in a terminal region of this pathway, the nucleus accumbens (NAc) (Pierce and Kumaresan, 2006). Although there are several DA systems in the brain, the two systems thought to be most important in drug abuse and in the effects of psychostimulants are the mesolimbic/mesocortical and the nigrostriatal systems (Moore and Bloom, 1978; Wise, 1998). Currently, these two dopamine pathways are largely thought to be the neuroanatomical substrates of most of the subjective (Volkow et al., 1997), locomotor stimulant, reinstatement, and reinforcing effects of cocaine and other abused stimulants. In the recent past, the mesolimbic pathway was much more closely associated with these effects (Kuhar et al., 1991; Wise, 1998) whereas the nigrostriatal pathway was more closely associated with the stereotypy caused by stimulants. However, new roles for the nigrostriatal pathway have been recently elucidated which strongly implicate it in many behavioral effects of cocaine.

The mesolimbic DA system is thought to be the central mediator of effects such as reinforcement in animals and humans, and reward, pleasure, and aversion in humans. The most fundamental portion of the mesolimbic DA system consists of dopaminergic projections from the ventral tegmental area (VTA) DA nucleus to the ventral striatum, which is primarily the NAc. Additional important outputs from the VTA include most of the cortex in primates and prefrontal cortex (PFC), amygdala nuclei, hypothalamus, and hippocampus in primates and rats (Haber and

Knutson, 2010; Moore and Bloom, 1978); the substantia nigra (SN) pars compacta (SNc) DA nucleus also has minor projections to the NAc (Haber and Knutson, 2010). In rats, VTA projections to the PFC onto glutamatergic pyramidal neurons in rat medial PFC (mPFC) have well-validated reciprocal fibers which synapse onto VTA DA neurons (and another, unidentified neuron type), release glutamate into the VTA, and elicit feedback DA release from VTA projection neurons into the mPFC and into the NAc (Carr and Sesack, 2000; Sesack and Carr, 2002). However, there is no evidence of glutamatergic corticonigral projections that synapse on DA neurons in the VTA or SN in primate, and the corticonigral projection identified appears to be very small, terminating invariably on non-DA-labeled neurons (Frankle et al., 2006). It is well-established that non-contingent presentation of salient stimuli or contingent presentation of reinforcing stimuli such as cocaine, other drugs of abuse, or stimuli such as food increases levels of DA in the NAc; with drugs of abuse, this occurs at doses that are reinforcing in self-administration (SA) tests (Kuhar et al., 1991; Wise, 1998). Furthermore, this increase in NAc DA is implicated in the locomotor stimulant effects of drugs (Wise, 1998; Wise and Bozarth, 1987), and the discriminative stimulus and conditioned place preference effects of cocaine.

The nigrostriatal system projects primarily from the SNc DA nucleus to the dorsal striatum (primarily the caudate and putamen in primates; in rats, the dorsal striatum is the striatum and the NAc core) and it has been historically implicated primarily in (non-psychostimulant-induced) locomotor output and control; accordingly, abnormal signaling within the nigrostriatal pathway is linked to locomotor dysfunction, such as that found in Parkinson's Disease or in extra-pyramidal diskinetic side effects of anti-psychotic drugs. However, recent research has uncovered a vital role of the SNc and the dorsal striatum in behaviors that have been well-trained (Kalivas, 2007; Schmidt et al., 2005).

There is considerable evidence that the reinforcing effects of cocaine are primarily mediated by the increased extracellular DA levels that result from inhibition of DAT and not from the effects of cocaine on 5-HT or NE (De Wit and Wise, 1977; Kuhar et al., 1991; Ritz et

al., 1987). For example, SERT (Howell and Byrd, 1995) or NET (Woolverton, 1987) inhibitors alone do not function as reinforcers, whereas DAT-selective inhibitors do (Howell and Byrd, 1991). Furthermore, the potencies of many cocaine-like drugs to produce reinforcing effects in SA experiments strongly correlate to their affinities for DAT, but not SERT or NET (Ritz et al., 1987). In addition, the intensity of the subjective effects of cocaine in humans is well-correlated to occupancy of DAT (Volkow et al., 1997). More generally, it has been found that nearly all drugs self-administered by animals are abused by humans and vice versa (Deneau et al., 1969; Johanson and Balster, 1978). Since nearly all drugs self-administered by animals cause increased DA levels in the NAc, this finding demonstrates that importance of NAc DA levels in reinforcement. Importantly, this means that if a treatment can reduce the DA-increasing or reinforcing effects of a drug, it may reduce the abuse liability of that drug. (Mello and Negus, 1996)

Serotonin Systems Modulate the Effects of Cocaine

Most research has focused on DA as the primary mediator of the abuse-related effects of cocaine, even though cocaine also increases extracellular 5-HT and NE, largely because 5-HT- and NE-selective drugs failed to reduce the reinforcing effects of cocaine (De Wit and Wise, 1977) and amphetamine (Yokel and Wise, 1975). Although DA is primarily implicated in the reinforcing and abuse-related effects of drugs, a role for 5-HT in brain reward systems and in the modulation of DA systems has been amply demonstrated. To this end, recent work has demonstrated that drugs that enhance extracellular 5-HT (e.g. SSRIs) or 5-HT biosynthetic precursor availability (L-tryptophan and 5-HTP), and drugs that activate or inhibit specific 5-HT receptor subtypes can modulate DA systems, through which they can control DA increases elicited by psychostimulants such as cocaine and their behavioral effects (Anastasio et al., 2011; Breese et al., 1974; Bubar and Cunningham, 2006; Bubar and Cunningham, 2008; Bubar et al., 2003; Bubar et al., 2011; Filip et al., 2004; Filip et al., 2006; Walsh and Cunningham, 1997).

Serotonin System Neuroanatomy and Receptor Subtypes

The serotonin system contains at least 14 distinct receptor subtypes, spanning 7 different families (5-HT_1 – 5-HT_7), through which 5-HT acts (Barnes and Sharp, 1999; Hoyer et al., 2002). 5-HT fiber distribution is very diffuse in the brain, so 5-HT has access to nearly every brain region (Weerts et al., 2007). Positron emission tomography (PET) studies labeling SERT proteins throughout the brain with highly selective SERT ligands demonstrate the diffuseness of this system (Weerts et al., 2007). Since SERT is found largely on the axon terminals of 5-HT neurons, it follows that these terminals reach nearly every region of the brain and that 5-HT may act as a global modulator of the brain. This is in stark contrast to DA systems, which are less diffuse and target more strongly specific brain regions (Haber and Knutson, 2010).

The diffuse distribution of the 5-HT system might at first seem to not lend itself to careful control of the activation state of different brain regions and neurotransmitter systems by the brain. On the other hand, the number of distinct receptor subtypes, with different mechanisms, downstream effector targets, and transcriptional and translational supports this ability. Of the 14 identified 5-HT receptors, all but 1 is a G-protein coupled receptor, at least 1 receptor couples to each of $G_{\alpha q}$, $G_{\alpha i/o}$, and $G_{\alpha s}$, and one receptor, 5-HT_3, is a ligand-gated ion channel. Furthermore, as demonstrated by PET studies employing selective 5-HT receptor ligands, 5-HT receptors show highly variable brain region distribution (Cornea-Hebert et al., 1999; Lopez-Gimenez et al., 1997; Lopez-Gimenez et al., 2001; Pompeiano et al., 1994; Ward and Dorsa, 1996). Lastly, even within a single brain region such as the VTA, there are usually many different types of neurons (Doherty and Pickel, 2000; Nocjar et al., 2002), each likely expressing a different profile of 5-HT receptors and other receptors. Altogether, these facets of the 5-HT system likely don't support precise control over neurochemistry with globally acting drugs like SSRIs, but strongly support precise pharmacological targeting of specific brain regions and systems by allowing for the targeting of different receptor subtypes.

Serotonergic Modulation of Dopamine Systems

5-HT projections from raphe neurons target each region of the mesolimbic DA system enriched in specific 5HT receptors (Kuhar et al., 1991; Wise, 1998). Thus, the 5-HT system is anatomically and functionally well-suited to modulate DA release by VTA neurons and the excitability, firing, and neurotransmitter release of downstream neurons. Indeed, 5-HT attenuates DA neuron firing (Kapur and Remington, 1996). Correspondingly, a significant and growing body of literature demonstrates the important role of 5-HT systems in modulating the effects of cocaine. For example, acute pretreatment with the SSRI fluoxetine reduced break-points on a progressive-ratio schedule of IV cocaine SA in rats (in progressive-ratio schedules, the response requirement to obtain a single injection of drug increases throughout a session), and fluoxetine decreased SA response rates under other schedule conditions, suggesting that increasing extracellular 5-HT levels decreases the reinforcing effects of cocaine (Carroll et al., 1990a; Richardson and Roberts, 1991). In addition, our lab showed that SSRIs attenuate the behavioral stimulant effects of cocaine, suggesting that 5-HT also modulates motor pathways of cocaine (Howell and Byrd, 1995). Importantly, SSRIs also inhibit the striatal DA overflow elicited by cocaine at doses of SSRIs that attenuate reinforcing effects but have no effect on the behavioral stimulant effects of cocaine (Czoty et al., 2002).

Synthetic precursors to 5-HT, such as L-tryptophan (TRP) and 5-hydroxytryptophan (5-HTP), which can acutely increase 5-HT levels considerably, also modulate abuse-related effects (Breese et al., 1974; Cheney and Goldstein, 1971; Raleigh et al., 1980). For example, when TRP was administered before rats began a progressive-ratio schedule of cocaine SA, they reached significantly lower break-points than vehicle-injected controls (McGregor et al., 1993). In other studies, TRP attenuated fixed-ratio cocaine SA (Carroll et al., 1990b) and 5-HTP reduced amphetamine-induced locomotion (Breese et al., 1974; Mabry and Campbell, 1973). These studies are consistent with the hypothesis that 5-HT inhibits DA and effects of cocaine. Because

increased 5-HT levels inhibit reinforcing effects of different drugs in many different schedules of reinforcement in primates and nonprimates, these preclinical data served as a strong justification for the scale-up into human clinical trials. Unfortunately, across many clinical trials, SSRI treatment did not decrease cocaine relapse, as measured by percent cocaine-positive urine samples (Grabowski et al., 1995).

Despite evidence, in direct contrast to animal studies, that interventions that increase 5-HT levels may not affect human relapse or addiction, there is also evidence that decreasing 5-HT levels (5-HT depletion) can affect the abuse-related effects of stimulants, further reinforcing the key role 5-HT plays in stimulant abuse. The effects of depletion with parachlorophenylalanine (PCPA), parachloroamphetamine (PCA), and 5,7-dihydroxytryptamine (5,7-DHT) are fairly well-studied, but depletion with the biosynthetic inhibitor PCPA is the best studied in relation to the abuse-related effects of drugs. Studies investigating the effect of 5-HT depletion with PCPA on the locomotor stimulant effects of amphetamine (Breese et al., 1974) and cocaine (Herges and Taylor, 1999) clearly demonstrated that 5-HT depletion enhances the locomotor stimulant effects of these drugs. Several studies also investigated the effects of 5-HT depletion with 5,7-DHT on SA of cocaine (Loh and Roberts, 1990) or amphetamine (Lyness et al., 1980); in two of these studies, the authors concluded that 5-HT depletion increased the reinforcing effects of the drug tested. However, there were conflicting results across experimental groups and conditions as well as considerable methodological flaws that obscure interpretation of the results. More rigorous studies addressed some of these problems and each found no effect of 5,7-DHT treatment on SA (Fletcher et al., 1999a). For example, a follow-up study from one group that initially observed an enhancement of the reinforcing effects of cocaine (Loh and Roberts, 1990) demonstrated that 5,7-DHT depletions enhanced both food and cocaine SA break-points (Roberts et al., 1994). Some acute TRP-depletion studies also support the hypothesis that 5-HT plays a role in the dopaminergic effects of cocaine: in one human study, TRP-depletion enhanced cocaine-induced increases in striatal DA levels (Cox et al., 2011).

One important conclusion that might be made from the experiments outlined above is that 5-HT clearly modulates DA systems and that and even the behavior of animals in relation to drugs that alter DA system function, but that global alterations in 5-HT tissue or extracellular levels are not very effective. Taking also into account the large number of 5-HT receptor subtypes spanning most of the brain, one practical explanation for this paradox is that 5-HT systems are not well-suited to alter DA system function through *global* increases or decreases in 5-HT signaling, but rather through *selective* modulation of 5-HT signaling via one or a few receptor subtype or brain regions. A practical prediction of this hypothesis is that agonists or antagonists with greater receptor selectivity will better succeed in altering the effects of stimulants than compounds that alter global 5-HT signaling. Indeed, the substantial body of work investigating compounds with greater receptor selectivity appears to support that prediction. One of the most promising 5-HT receptor families implicated in serotonergic modulation of DA system function and the effects of stimulants is the 5-HT_2 receptor (5-HT_2R) family, within which the 5-HT_{2A} receptor (5-HT_{2A}R) is particularly exciting.

5-HT_2 Receptors Modulate Effects of Stimulants

Mounting evidence indicates that the 5-HT_2 family of 5-HT receptors has especially strong influence over DA systems and also the effects of stimulants that are associated with DA system activation. Particular focus has been given on the 5-HT_{2A}R and 5-HT_{2C}R subtypes, which appear to have nearly directly opposing regulatory influence over DA system function(Di Matteo et al., 2008; McMahon et al., 2001). Thus, the consensus of the literature is that systemically administered selective agonists for 5-HT_{2A}Rs enhance, and antagonists attenuate, DA system activity and the behavioral and neurochemical effects of stimulants (Di Matteo et al., 2008; McMahon et al., 2001). On the other hand, 5-HT_{2C}Rs appear to exert essentially opposite effects on DA systems from 5-HT_{2A}Rs, and 5-HT_{2C}R agonists thus attenuate and antagonists enhance DA systems and the effects of stimulants.

Both receptors are found in each primary region of the mesolimbic dopamine pathway—the VTA, the striatum (including the NAc), and the frontal cortex. The effects of manipulations of these receptors by ligands on DA system function, the effects of stimulants, and behavior have been measured by various methods, including *in vivo* microdialysis, electrophysiology, and behavioral tests such as SA, reinstatement, fixed interval schedules of reinforcement, and locomotor tests. The results of these experiments are largely in agreement with the molecular, cellular, and tissue level experiment results that these receptors have a unique position of influence on DA system function and its related behavioral outputs. This thesis will focus on 5-HT_{2A}Rs but a general understanding of 5-HT_{2C}Rs will aid in the understanding of the results of many experiments with ligands for these receptors since many of these compounds bind to both receptors.

Molecular Pharmacology of 5-HT_{2A} Receptors

The 5-HT_2 family of receptors is composed of 3 distinct receptor subtypes: 5-HT_{2A}R, 5-HT_{2B}R, and 5-HT_{2C}R (Hannon and Hoyer, 2008). These subtypes have about 50% sequence identity, complicating the development of selective ligands for these receptors (Hannon and Hoyer, 2008). An important facet of a drug targeting 5-HT_{2A}Rs or 5-HT_{2C}Rs is the omission of any agonist activity at 5-HT_{2B}Rs, since direct or indirect (5-HT releasers like fenfluramine) agonists for these receptors are thought to cause valvulopathies and pulmonary hypertension associated with anorectic drugs like Phen-Fen (Hannon and Hoyer, 2008).

5-HT_{2A}Rs and 5-HT_{2C}Rs are both members of the family of receptors called G-protein coupled receptors (GPCRs), which are the targets of nearly half of all therapeutic drugs. All proteins in this very large family of receptors are 7 transmembrane-domain receptors. GPCRs are usually found on the extracellular membranes of cells and transduce signals from outside to within the cell (Karnik et al., 2003; Kristiansen, 2004).

Distribution

The distribution of 5-HT_{2A}Rs in the brain has been assessed through mRNA hybridization ("in situ hybridization") and autoradiography, the results of which are in good agreement within species (Hannon and Hoyer, 2008; Hoyer et al., 2002; Pazos et al., 1985; Pazos et al., 1987). These studies demonstrate the 5-HT_{2A}Rs are distributed throughout most of the brain, but at high density within the cerebral cortex in both rats and primates and at intermediate density within the limbic areas and basal ganglia (including the caudate-putamen, and NAc). Interestingly, autoradiography studies found intermediate signal strength within the striatum, including the NAc, but found also that there was a large non-specific binding signal in that area that was not displaceable by any known 5-HT_2R family ligand. In addition, one study employing both in situ hybridization and binding of radiolabeled M100,907 (M100), a selective 5-HT_{2A}R antagonist, found that the basal ganglia of monkeys had very low specific binding of the ligand and no mRNA coding for the 5-HT_{2A}R (Lopez-Gimenez et al., 2001). In addition, there are also species differences of the receptor distribution. For example, although rat studies using radiolabeled M100 found a medium density of 5-HT_{2A}Rs within the caudate-putamen and NAc, monkey studies using the same ligand found a very low density. Also, monkey brain in situ hybridization studies did not detect any transcript within the caudate-putamen and substantia nigra whereas rat studies did; contrastingly, monkey studies detected higher levels than rat studies of labeling within the VTA, from which DA neurons project to the NAc and frontal cortex.

5-HT_{2A}Rs are thought to be found primarily in postsynaptic sites within the cortex on glutamatergic pyramidal neurons and on gamma-amino butyric acid (GABA)-expressing medium-spiny neurons (MSNs) in the striatum. Since 5-HT_{2A}Rs are thought to be found in VTA DA neurons (Doherty and Pickel, 2000; Nocjar et al., 2002), MSNs of the striatum, and pyramidal neurons in the cortex, and since each of these regions projects to each other within the

mesolimbic dopamine pathway, there is thus strong support for a role of these receptors in DA system function and the effects of stimulants.

Neurochemical and Behavioral Effects of 5-HT_{2A} Receptor Agonists

Indeed, as suggested by the distribution of 5-HT_{2A}Rs in the brain, 5-HT_{2A}R ligands modulate DA systems and various neurochemical and behavioral effects of stimulants, primarily demonstrated in rodents. Both agonists and antagonists of these receptors have been shown to alter these metrics, although with different patterns of effects. The agonist tested in these studies was the psychedelic phenethylamine hallucinogen 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) which is a 5-$HT_{2A/2C}$R partial agonist.

5-HT_{2A}R agonists have been shown to alter metrics of DA system function, including DA neuron firing rates and DA concentration in different brain regions. For example, systemic administration or local administration within the rat median prefrontal cortex (mPFC, thought to be analogous to primate dorsolateral prefrontal cortex) of DOI, increased the firing and burst firing rate of VTA DA neurons and DA release in the mPFC and the VTA. In another study, systemic administration of DOI increased DA levels in the prefrontal cortex. Importantly, the effects in both of these studies were completely abolished by systemic administration of M100, demonstrating the 5-HT_{2A}R specificity of the mechanism of these effects. Importantly, systemic administration of M100 does not alter DA system function in that it does not alter the release of DA in the prefrontal cortex or the NAc or alter firing rates of VTA DA neurons.

Microperfusion of the 5-HT_{2A}R agonist DOI into the NAc of rats was found to elevate extracellular DA levels to 200% baseline, an level similar to that caused reinforcing doses of dopaminergic drugs (ref). This increase was blocked by co-perfusion with LY53,857, a nonselective 5-HT_2 antagonist (Bowers, 2000). In another study, DOI infused directly into the rat NAc elevated DA by up to 473% of baseline, a massive increase by any measure. This increase was blocked by pre- and co-perfusion of LY53,857 or ketanserin, a nonselective 5-$HT_{2A/2C}$

inhibitor. These results serve to demonstrate the profound effect that 5-HT_{2A}Rs can elicit on DA systems. In another study, when DOI was perfused directly into the medial prefrontal cortex of conscious rats, it elevated extracellular levels of DA there (Pehek, 2001); this effect was blocked by coperfusion with M100, again supporting the direct role of 5-HT_{2A}Rs in these effects.

Neurochemical and Behavioral Effects of 5-HT_{2A} Receptor Antagonists

Although 5-HT_{2A}R antagonists do not seem to have effects on DA system function alone (Fletcher et al., 2002; Kehne et al., 1996a; McMahon and Cunningham, 2001), they clearly attenuate the DA-elevating effects of stimulants and behavioral effects mediated by enhanced DA system activity as well as block 5-HT_{2A}R agonist-induced DA elevations (Gobert and Millan, 1999; Pehek et al., 2001). For example, in an *in vivo* voltammetry study, ketanserin, a 5-$HT_{2A/2C}$ antagonist, attenuated the elevation of DA (and 5-HT) levels in the NAc elicited by an injection of cocaine (Broderick et al., 2004). Ketanserin also attenuated the locomotor stimulant effects of cocaine in that study, an effect replicated with more selective compounds. Additionally, in another study, local injection of the selective 5-HT_{2A}R antagonist SR46349B into the VTA inhibited the DA overflow and locomotion elicited by systemic administration of D-amphetamine (Auclair et al., 2004).

Hypotheses

With these previous results in mind, we hypothesized that M100 would block cocaine-primed reinstatement and cocaine-induced increases in DA in the NAc and caudate nucleus of monkeys, but that it would not reduce the reinforcing effects of cocaine. To address these hypotheses, using a within-subjects design in rhesus macaques, we evaluated the effects of selective 5-HT_{2A}R antagonism with M100 on IV cocaine SA and cocaine-primed reinstatement. In addition, with two separate groups of monkeys, we assessed the effect of 5-HT_{2A}R antagonism

on baseline DA levels and cocaine-induced increases in DA levels using *in vivo* microdialysis targeting either the NAc or the caudate.

Chapter 2: Materials and Methods

Subjects

These studies were conducted in NHPs rather than rodents due to the many advantages of NHP models of drug abuse, among other reasons. First, several of the experiments in this study were similar to studies that had been performed in rodents; it was sought to expand upon those results. Also, in a common drug screening process for the evaluation of a potential therapeutic for the treatment of cocaine abuse, NHP SA studies are typically the last step (Carroll et al., 1999), in accordance with the *replacement* principle supported by the *Guide for the Care and Use of Laboratory Animals*; previous findings in experiments similar to those in the present study strongly supported the potential of the test compound, M100. Furthermore, NHP SA studies have high predictive validity in the assessment of the abuse liability of drugs (Deneau et al., 1969; Johanson and Balster, 1978) and can also effectively predict alterations in abuse liability by pretreatment drugs, as evidenced by the concordance between NHP SA studies and clinical data of the several drugs approved for treatment of drug abuse (Mello and Negus, 1996). The only reports on the interaction between M100 and the behavioral and neurochemical effects of cocaine have been obtained in rodent models except for a single report in NHPs (Fantegrossi et al., 2002) which suggested that M100 was effective, but that the experiment lacked statistical power.

In the interest of producing evidence with the greatest translational value, it is often advantageous for preclinical researchers to employ the animal models that are believed to model the human condition best. Rhesus monkey models of drug abuse are thought to have high translational value in addition to other advantageous characteristics. For example, monkeys are believed to have a phylogenetically, anatomically, and neurochemically more similar brains and more similar proteomes to humans than those of rodents (Weerts et al., 2007). There are also important similarities in distribution of brain neurotransmitters and neurons including,

importantly, very similar organizations of DA projections to the prefrontal cortex between NHPs and humans that support the translational value of models using these species (Goldman-Rakic et al., 1992). Additionally, the pharmacokinetic properties—including metabolism, a characteristic known to vary greatly between species—of several drugs have been found to be much more similar between humans and NHPs than with rats (Ward and Smith, 2004; Weerts et al., 2007).

Three (RRg4, RLl4, RMv3) adult female rhesus monkeys (macaca mulatta) weighing 7.5-10 kg served as subjects for all behavioral experiments. Four (RJt7, RLt7, RNb7, and RHp8) adult female rhesus monkeys weighing 7-9.5 kg served as subjects of *in vivo* microdialysis experiments targeting the NAc. Four (RKa10, RGg9, RZq8, and RDn8) adult female rhesus monkeys weighing 7-9.5 kg and one (RKn8) adult male rhesus monkey weighing ~12 kg served as subjects of *in vivo* microdialysis experiments targeting the caudate nucleus. Importantly, none of the subjects of *in vivo* microdialysis experiments took part in behavioral experiments and none were currently taking part in other behavioral experiments at the time of this work. In addition, subjects of microdialysis studies targeting the NAc did not take part in experiments targeting the caudate, or vice versa.

All subjects were housed individually, fed LabDiet 5037 Monkey Diet Jumbo Chow (PMI Nutrition International, Brentwood, MO), fresh fruits, and vegetables once-daily, and fed cereal, peanuts, or popcorn twice daily as enrichment. Additional enrichment included forage boards and balls, chew toys, and sheets of paper to tear. Water was continuously available in each subject's home cage and subjects did not undergo food-restriction procedures. All subjects had served in previous pharmacological studies and were exposed to drugs with similar mechanisms of action and similar behavioral and neurochemical effects to those presented herein, including cocaine, amphetamine, and methylenedioxymethamphetamine (MDMA) (Lindsey et al., 2004; Sawyer and Howell, 2011; Wilcox et al., 2002).

All protocols and animal care and handling strictly followed the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*, the American Association for

Accreditation of Laboratory Animal Care (AAALAC), and were approved by the Institutional Animal Care and Use Committee of Emory University.

Surgeries

Self-Administration and Reinstatement

All surgeries were performed on monkeys using aseptic techniques with appropriate anesthesia, preoperative antibiotics, and postoperative analgesia. To permit intravenous (IV) injections of drug and saline solutions in animals that underwent SA and reinstatement procedures, each monkey was surgically implanted with a chronic indwelling venous catheter and subcutaneous vascular access port using aseptic techniques, as described previously (Wilcox et al., 2002). In order to prevent infection during surgery, subjects were administered preoperative antibiotics Rocephin (ceftriaxone, 25 mg/kg, IM). Surgical anesthesia was initiated by an injection of Telazol (tiletamine HCl + zolazepam HCl; 4 mg/kg, IM) and maintained with isoflurane under the supervision of a veterinary technician. After anesthesia was achieved, a subcutaneous vascular access port (Access Technologies, Skokie, IL) was first sutured to the muscles in the central region of the back of the animal. Next, one of the femoral or jugular veins was accessed using a cut-down procedure. One end of a silicone catheter (0.65 mm ID, 1.75 mm OD; Access Technologies, Skokie, IL) was inserted into the vein and routed through its interior until its proximal end terminated near the junction of the superior and inferior vena cavae at the entrance to the right atrium. This placement ensured both rapid distribution of injected drugs due to the high turbulence of this region and immediate entrance into systemic circulation via the heart. Once inserted, the portion of catheter outside of the vein near its entrance into the vein was sutured to muscles underneath the vein to fasten it while the vein was tied onto the catheter. The other end of the catheter was then routed subcutaneously via trocar to the middle of the back, where it was securely connected to the subcutaneous vascular access port sutured to the muscles in the back.

Port and catheter patency were then tested and insured by inserting a syringe needle into the port and flushing with heparinized saline (3 mL of 100 U/mL). After surgery, subjects were administered the analgesic buprenorphine (0.01 mg/kg, IM), returned to their home cages, and administered Banamine (flunixin meglumine, 1 mg/kg IM) every 6 h for 24 h to minimize pain and discomfort after the surgery. Subjects were allowed 10-14 days to recover from surgery before beginning experiments. Catheters were flushed at least once per week with 1.5 mL of 100 U/mL heparinized saline to maintain their patency. Periodically, ports and catheters would become occluded. When this occurred, the port and catheter were surgically removed using aseptic techniques under Telazol (5 mg/kg, IM) anesthesia with supplemental, as-needed ketamine (variable dose, IM). Preoperative antibiotic and postoperative analgesic regimens were administered as described above. Subjects were then given 2 weeks to recover from surgery, before entering catheter and port implantation surgery as described above.

Microdialysis

Microdialysis test subjects were prepared with surgically implanted chronic indwelling catheters in identical fashion to those of the subjects of the SA and reinstatement studies described above, and as described previously (Wilcox et al., 2002). Subjects of microdialysis studies targeting either the caudate nucleus or the NAc underwent identical surgical techniques, including targeting of cannulae. In addition, each subject was then implanted with bilateral CMA/11 guide cannulae (CMA Microdialysis, North Chelmsford, MA) that was targeted stereotaxically to the head of the caudate nucleus as previously described (Czoty et al., 2000; Wilcox et al., 2005). Cannulae targeting the caudate nucleus additionally targeted the NAc, which is positioned several millimeters deeper in the brain (ventral to the caudate). Magnetic resonance imaging (MRI) was employed to determine where to implant intracranial cannulae. T1- weighted images of the whole brain were acquired using a 1.5 Tesla Philips NT scanner (Philips Medical Systems, The Netherlands). Guide cannulae were then implanted bilaterally into the caudate

nucleus, following sterile guidelines. Preoperative antibiotics (Rocephin, 25 mg/kg) were given on the day of surgery in order to prevent infection. The subject was sedated with Telazol (4.0 mg/kg) and anesthesia was maintained with isoflurane during surgery. The subject was positioned in a stereotaxic frame, and coordinates were determined from the subject's own MRI. A small burr hole was drilled directly above the caudate nucleus, and the CMA/11 guide cannulae (CMA Microdialysis, North Chelmsford, MA) were inserted to the appropriate depth. Titanium and teflon screws attached to the skull were used to anchor cranioplastic cement in order to support the guide cannulae. Recording chambers with caps (Crist Instruments, Hagerstown, MD) were placed around the guide cannulae and anchored to the skull using the cranioplastic cement to prevent the animal from damaging the implant site. Next, the skin around the implant site was sutured closed. Two stainless steel stylets were inserted into the guide cannulae while they were not in use. Monkeys were allowed to recover from surgery for at least 2 weeks before beginning microdialysis experiments. All subjects received postoperative Banamine (1.0 mg/kg) every 6 h for 24 h, or longer if they exhibited signs of discomfort.

Apparatus

Self-administration and Reinstatement

On behavioral testing days, subjects were seated individually in commercially-available primate chairs (Primate Products, Miami, FL) from the time that they were removed from their home cages until they were returned. A response panel (operant panel) with a single lever was mounted on to the front of the chair before testing. The panels included green, red, and white stimulus lights above the lever. Once a panel was attached to a chair, the chair was placed inside a ventilated, sound-attenuating chamber (Med Associates Inc., St. Albans, VT) which included a white noise generator to reduce the effects of ambient noise on behavior. Next, a Huber needle (Access Technologies) was inserted into a subcutaneous port on the subject's back that empties into the venous circulation. The Huber needle was then connected via polyvinyl chloride tubing

to a 35 mL syringe containing either saline or cocaine solution which was attached to a motor-driven syringe pump (Model PHD2000, Harvard Apparatus, Holliston, MA) located outside the chamber. Each syringe was connected to the Huber needle tubing by a 0.2 μm pore-size disc filter. Syringe pumps and stimulus lights in all chambers were simultaneously controlled by a computer which was running Med-PC IV software (Med Associates Inc., St. Albans, VT) and which was connected to each chamber. Daily sessions lasted about 1 h and 20 min and monkeys were never seated in primate chairs for >4 consecutive hours.

Microdialysis

The microdialysis techniques employed in these studies were similar to those previously described (Wilcox et al., 2005). During microdialysis sessions, subjects were seated in commercially available primate chairs (Primate Products, Inc., Miami, FL). Animals were each tested no more often than once every two weeks. Testing sessions lasted about 5 h total, during which time subjects were housed within a sound-attenuating, ventilated chamber (Med Associates Inc., St. Albans, VT). Primate chairs were arranged to limit the movement of the animals to prevent them from pulling out perfusion tubes and to keep them positioned such that connections between dialysis probes and perfusion tubes were preserved.

Procedure

Initial Self-administration Experiments

Drug SA procedures model ongoing drug intake, also called drug maintenance. This is the central phase of the drug addiction cycle which begins with introduction/induction and then leads into the cycle of maintenance, withdrawal, and relapse. One of the main applications of the SA model is to evaluate compounds that might be able to reduce the reinforcing effects of a drug of abuse without otherwise disrupting the behavior of the subject in a nonspecific manner. Since the reinforcing effects of drugs are highly correlated to the abuse liability of drugs, this model is

thought to have high translational value to the human condition. The drug SA procedure measures the rate at which a subject responds in a manner that causes the administration of a drug; the magnitude of the maximum response rate attained under the schedule parameters is thought to be related to the effectiveness of a drug at reinforcing the responding of the subject. Therefore, if a pretreatment reduces the maximum response rate, it suggests that the pretreatment attenuates the effectiveness of that drug as a reinforcer and thus attenuates its abuse liability or positive effects.

In the present study, subjects were trained to self-administer IV cocaine according to a 3-component fixed ratio 20 (FR20) schedule of cocaine reinforcement. SA sessions lasted 80 min and were conducted 5 days per week (Monday – Friday) whenever possible. Timing and coordination of experimental events were controlled by a Med-PC IV program running on a computer interfaced with the syringe pump, the operant lever, and the lights. The white light was only and always illuminated during active time, during which time responses on the lever count towards the fixed ratio. In contrast, responses made while the white light was not illuminated had no programmed consequences. The red light was only illuminated during drug infusion. In all SA experiments, the concentrations of cocaine solutions in drug syringes were calculated based on individual subjects' body weights as measured every 2 weeks, such that a 0.5 mL infusion contained the unit dose of cocaine desired.

The schedule of reinforcement consisted of three 15 min components, each separated by a 15 min intercomponent interval during which all lights were extinguished and lever presses had no programmed consequences. When the program was initialized, a 5 min delay period elapsed before any experimental stimuli were presented; during this time, lever presses had no programmed consequences. Immediately after the delay period elapsed, the house light (white light) was turned on, signaling the availability of cocaine reinforcement and the beginning of the first 15 min component. If a fixed ratio (FR) of 20 responses was completed during active time, the white light was extinguished, the red light was illuminated for 3 s, and the syringe pump was turned on, intravenously infusing the subject with 0.5 mL of cocaine solution (0.01, 0.03, or 0.1

mg/kg/inf; 10 mL/min flow rate) or vehicle solution over 3 s. After these 3 s elapsed, the red light and syringe pump were turned off and a time-out period was instituted over the next 1 min 57 s, during which time all lights were extinguished and responses on the lever had no programmed consequences.

After a time-out period, the white light was illuminated and responses were again counted towards the fixed ratio. After 15 min had elapsed from the start of the component, the component ended and all lights were extinguished. Over the course of the next 15 minutes, the intercomponent interval, lights remained extinguished and responses on the lever had no programmed consequences. This interval was followed by a second 15 min component, then a second intercomponent interval, and finally a third 15 min component. A maximum of 8 infusions could be received per component, for a total possible 24 infusions per session. Subjects typically received between 18 and 21 infusions at unit doses of cocaine of 0.01 and 0.03 mg/kg, and between 11 and 21 at a unit dose of 0.1 mg/kg. The primary dependent measure of this procedure, response rate, was calculated as the total number of responses emitted by a subject during active time divided by the total active time throughout the entire session. When group mean response rates over consecutive Friday (F), Monday (M), and Tuesday (Tu) sessions varied by < 20% from the mean over those 3 sessions, subjects' responding was considered stable and the average rate on M and Tu was considered the stable baseline. Once group responding was stable, the group moved on to SA experiments with drug pretreatments.

5-HT_{2A}R Antagonism Experiments

In order to begin evaluating the therapeutic potential of a 5-HT_{2A}R antagonist in for the treatment of cocaine abuse, an appropriate test drug had to be decided upon. Important factors in this decision included availability, strong evidence for selectivity for 5-HT_{2A}Rs, especially vs. 5-HT_{2B}Rs, and support for effectiveness at inhibiting behavioral effects of psychostimulants, especially in NHPs. Considering all these factors, the drug M100 was a clear choice: it was

currently available; it robustly attenuated behavioral effects of MDMA in NHPs and behavioral and neurochemical effects of many stimulants in rats across many studies; it has high in vitro and *in vivo* selectivity for 5-HT_{2A}Rs over many other receptors that modulate the pharmacological effects of cocaine; and, finally, its safety profile has been verified in preclinical studies in rhesus monkeys (Fantegrossi et al., 2002) and in human clinical trials (clinicaltrials.gov identifiers: NCT00464243 and NCT00495885), in which 2-phase 3 trials have been completed.

One of the most important properties of a 5-HT_{2A}R antagonist test drug is that absence of 5-HT_{2C}R activity, since nearly all ligands for 5-HT_{2A}Rs have appreciable relative activity at 5-HT_{2C}Rs (Pehek et al., 2006). This poses a problem since the functional outputs of these receptors are opposite each other in most assessments (Di Matteo et al., 2008). Most studies that assessed the *in vitro* affinity of M100 for 5-HT_{2A}Rs and other relevant receptors reported that M100 had between 100- (Kehne et al., 1996b) and 300-fold (Kehne et al., 1996a) greater affinity for 5-HT_{2A}Rs than 5-HT_{2C}Rs, and about 500-fold greater affinity for 5-HT_{2A}Rs than any other target, such as 5-HT_{2B}Rs, α_1-adrenergic receptors, D1, and D2 receptors (although one study found only ~40-fold selectivity for 5-HT_{2A}Rs vs. 5-HT_{2C}Rs *in vitro*, ~30-fold selectivity vs. H1 histamine receptors, and ~60-fold selectivity vs. α_1-adrenergic receptors (Pehek et al., 2006)). In contrast, one *in vivo* assessment found >1250-fold potency of M100 for 5-HT_{2A}Rs vs. 5-HT_{2C}Rs (Kehne et al., 1996a). These demonstrations of the appreciable selectivity of M100 for 5-HT_{2A}Rs vs. 5-HT_{2C}R indicate the relatively low likelihood of encountering a false-negative result due to *in vivo* loss of selectivity and subsequent 5-HT_{2C}R antagonism counteracting the effects of 5-HT_{2A}R antagonism. In fact, this possibility is important to consider, since loss of selectivity appears to have occurred in several previous studies, in which authors observed an effect of M100 opposite the effect expected and previously demonstrated. For example, The present study tested for this possibility in which

Although M100 is thus selective for 5-HT_{2A}Rs, any increase in the dose may have decreased selective antagonism of the 5-HT_{2A}R vs. 5-HT_{2C}R.5-HT_{2A}R over 5-HT_{2C}R of all available 5-HT_{2A}R antagonists. fothat 5-HT_{2A}R antagonists

An appropriate reaction to these results might be to question why a higher dose of M100 was not tested in this study, since we found some evidence that M100 attenuates the reinforcing effects of cocaine. Firstly, the dose of M100 tested in the present study (0.3 mg/kg) has been shown to be effective in neurochemical studies conducted in rhesus monkeys (Murnane et al., 2012) and in behavioral studies conducted in squirrel monkeys (Fantegrossi et al., 2009). Importantly, the same dose of M100 was effective in blocking cocaine-induced reinstatement in the present study. Therefore, this rules out the possibility that this dose of M100 was ineffective in SA tests because it was pharmacologically inactive. Additionally, one of the primary aims of this study was to provide insight into the role of 5-HT_{2A}Rs in regulation of DA signaling and modulation of the behavioral effects of cocaine; selectivity for this receptor is essential to make conclusions about its specific role in these measures.

M100 pretreatments were administered before experimental sessions for each of the 3 techniques employed in the present study. The dose (0.3 mg/kg, IM) and pretreatment time (1 h) of M100 injections in these experimental sessions were identical between each of the techniques in order to facilitate qualitative comparisons between results. Although SA experiments were held for 3 consecutive days in order to extend the results of the experiment, the effects on the first day are comparable to results of other experiments. The parameters of M100 dosing were chosen based on several factors. Most importantly, preliminary data obtained from systematic testing of pretreatment time and dose in rhesus monkeys in our lab revealed these parameters to be maximally effective in attenuation of the behavioral effects of a stimulant. Also, previous results demonstrated that this dose effectively inhibits the rate suppressant effects of racemic MDMA on a fixed interval stimulus termination schedule in squirrel monkeys (Fantegrossi et al., 2009) in

addition to attenuating the prolactin secretion-eliciting effects of R(-)-MDMA in rhesus monkeys (Murnane et al., 2012). Furthermore, a dose (0.1 mg/kg) one half-log unit below that tested in the present study was found to completely block R(-)-MDMA SA in rhesus monkeys (Fantegrossi et al., 2002). In contrast, in the same study, a range of doses (0.1, 0.3, and 1.0 mg/kg) of M100 were administered before cocaine SA sessions and, although there was some evidence of effectiveness, the effect did not reach statistical significance. Therefore, these previous results demonstrate that this dose of M100 is robustly behaviorally active. Although this dose (0.3 mg/kg) is lower than a dose (1.0 mg/kg) found to be ineffective in a cocaine SA study, there was no evident difference in effectiveness between these doses; additionally in the present study, a 3-day pretreatment regimen was utilize in order to evaluate any change in effectiveness over multiple days of treatment, since preliminary data and previous studies indicated enhanced effectiveness of serotonergic agents over repeated treatment days (Davidson et al., 2002).

Self-Administration with M100 Pretreatment

To determine whether 5-HT_{2A}R antagonism attenuates the reinforcing effects of cocaine in rhesus monkeys, M100 (veh or 0.3 mg/kg, IM) was administered to subjects before cocaine SA sessions. To determine whether 5-HT_{2A}R antagonism attenuates the reinforcing effects of cocaine and whether 3-days of treatment would increase the effectiveness of the pretreatment, M100 (veh or 0.3 mg/kg, IM) was administered to subjects in their home cages 1 h before SA sessions on Wednesday (W), Thursday (Th), and F of a week in which monkeys were stable over the previous F, M, and Tu. Each pretreatment (veh or M100) was administered to each subject before SA of each unit dose of cocaine (0.01, 0.03, and 0.1 mg/kg). Within each 3-day treatment block, all animals received the same treatment for all 3 days and the treatment order within each dose was randomized. After the first 3-day treatment block at a given cocaine dose, subjects continued SA sessions without pretreatments until they stabilized again. In order to ensure that pre-pretreatment baseline rates would be similar and comparisons could be made between treatment blocks,

subjects only entered the second 3-day treatment block within a given cocaine dose if the current 3-day stability period rate varied < 20% from the previous 3-day stability period rate. The order of cocaine SA unit doses was 0.03, 0.01, then 0.1 mg/kg.

Reinstatement

The reinstatement model is based on drug SA procedures, but it is designed to model the relapse phase of the drug addiction cycle. Reinstatement is the best-accepted animal model of relapse; one of its primary applications is the screening of potential pharmacotherapeutics for treatment of cocaine relapse. This is especially important since there are currently no drugs approved by the FDA to prevent or minimize the consequences of cocaine relapse. Relapse follows the drug withdrawal phase, and treatments that target this phase to prevent relapse may have some advantages to those targeting the maintenance phase. For example, during maintenance of drug administration, subjects are probably less likely to be seeking or receiving treatment, they likely have limited financial resources to obtain pharmaceuticals for treatment, and they may less able or willing to consistently and appropriately take medication than during the withdrawal phase. Although there have been a handful of false positives in the reinstatement model (drugs that worked in the model, but not in humans), most of the drugs that are effective at preventing relapse in humans (such as buprenorphine or methadone for opioid addiction) are effective in blocking reinstatement and most stimuli that induce relapse in humans induce reinstatement in animals (Mello and Negus, 1996). These qualities of the model lend credit to its ability to generalize to humans.

The reinstatement procedure employed in this study comprised 3-steps, beginning with stabilization of group SA response rates, continuing with extinction of responding, and ending with the reinstatement test. Between each reinstatement test session, animals' responding was restabilized at the group peak dose of cocaine, and their responding was again extinguished. To begin, subjects were trained to self-administer cocaine according to the SA parameters described

above at the unit dose that produced the maximum group mean response rate (the "peak dose," a unit dose of 0.03 mg/kg). Stability criteria during reinstatement experiments were different from those during SA experiments: subjects were considered stable if animals responded with < 20% variance in daily group mean response rate over any 3 consecutive sessions, irrespective of day. After group responding stabilized, animals underwent extinction sessions.

In extinction sessions, saline was substituted for cocaine in the syringe, so that responses on the lever were not reinforced with cocaine infusions and instead generated saline infusions. In keeping with the standard protocol in our lab, the red stimulus light was not illuminated when infusions were delivered, in contrast to drug SA procedures, in order to preserve any conditioned reinforcing effects the light may have attained. This was done to maximize response rates during reinstatement sessions and facilitate the detection of an effect of drug interaction. All other parameters of the extinction sessions were identical to those in drug SA sessions. Extinction conditions were instituted daily until the group mean response rate met extinction criteria (group mean rate < 20% of the 3-day average rate when stabilized). Subjects were then repeatedly stabilized at the peak cocaine dose and their responding extinguished until group responding extinguished within 2 sessions of instituting extinction conditions. Reinstatement tests were begun the day after this last round of stabilization and extinction.

Reinstatement test sessions were identical to extinction sessions as described above except that the red stimulus light was illuminated during saline infusions and, immediately before the session was initialized, subjects were administered a noncontingent bolus infusion of cocaine ("cocaine prime;" 0.1, 0.3, or 0.56 mg/kg, IV) to reinstate lever-responding behavior. Thus, responses on the lever were not reinforced by cocaine, but instead generated saline infusions coincident with illumination of the red stimulus light, whose presentation was previously paired to cocaine reinforcement. Accordingly, responses on the lever were induced by the combination of the reinstating effects of the cocaine prime and the conditioned reinforcing effects of the red stimulus light. Therefore, these reinstatement tests are more accurately described as drug- + cue-

induced reinstatement tests. The red stimulus light was presented with saline infusions in reinstatement test sessions because our lab has found that the combination of a drug prime and conditioned reinforcer presentations produces more robust reinstatement effects than a drug prime alone. This is advantageous because a greater peak reinstatement effect facilitates the statistical identification of an attenuation of reinstatement by M100 pretreatment.

In order to determine whether 5-HT_{2A}R antagonism attenuates the reinstating effects of a cocaine prime, subjects were administered M100 (veh or 0.3 mg/kg, IM) 1 h before cocaine-primed reinstatement test sessions. On each reinstatement test day, all three subjects received the same pretreatment (veh or M100) 1 h before each receiving the same dose of cocaine prime (0.1, 0.3, or 0.56 mg/kg). To obtain a reinstatement dose-response function (rate vs. dose cocaine prime administered after veh pretreatment), reinstatement tests began with a dose of cocaine (0.3 mg/kg) known in our lab to often be the peak reinstating dose of cocaine using similar fixed ratio schedules. Since this dose robustly engendered responding, a dose one half-log unit lower (0.1 mg/kg) was tested; this cocaine prime induced a low response rate. Therefore, the next dose administered (0.56 mg/kg) was one quarter-log unit above the first dose tested. This dose also induced a low response rate. With these three cocaine doses, a clear inverted U-shaped pattern emerged, so no further doses were tested. At each priming dose of cocaine two reinstatement sessions were held, between which subjects' responding was stabilized and then extinguished. In each session, either M100 or vehicle pretreatment was administered; order of pretreatment session was randomized.

In vivo microdialysis

In vivo microdialysis is a powerful method of directly observing the neurochemical changes within the brain. Our lab performs *in vivo* microdialysis studies using probes that target the caudate nucleus or the NAc of adult rhesus monkeys. By sampling the cerebrospinal fluid of these regions of the monkeys' brains and then analyzing the samples for neurochemical content,

we can view temporal and quantitative changes in neurochemistry elicited by drugs of abuse, potential treatment drugs, and a combination of both. One reason this method is so powerful is because it helps to elucidate the specific neurochemical mechanisms underlying the effect of drugs and drug interactions on behavior. The illumination of the neurochemical mechanisms of interactions between drugs of abuse and potential treatments is key in understanding the pharmacological determinants of drugs that mediate their abuse and the relationship between alterations in measures of abuse liability and neurochemistry. Furthermore, data on the neurochemistry of drug interactions within multiple brain regions, as obtained in the present experiments, has enhanced mechanistic and predictive value. This information is highly valuable for the development of effective pharmacotherapeutics for drug abuse.

In addition to identifying the mechanisms underlying the alteration of the effects of drugs of abuse by potential therapeutics, this technique can aid in the identification of neurochemical explanations for unexpected results or failure of a promising treatment in evaluations of abuse liability. For example, if M100 pretreatment is found to alter behavioral effects of cocaine that are strongly correlated to DA systems, such as reinforcing effects, a sound conclusion is that M100 alters DA system function. In addition, since the reinforcing effects of cocaine and other drugs are strongly correlated to DA levels in the striatum, a sensible prediction would be that M100 specifically alters the cocaine-induced increase in extracellular DA levels in the striatum. This prediction can be tested directly with *in vivo* microdialysis. A result demonstrating an attenuation of cocaine-induced DA overflow would strongly support the mechanistic predictions made from behavioral experiments and facilitate more incisive and conclusive future experiments towards developing treatments for cocaine abuse. On the other hand, if M100 failed to attenuate the reinforcing effects of cocaine, neurochemical data demonstrating unaffected DA levels would help explain the negative result. Importantly, this technique also allows experimenters to verify the absence of neurochemical effects linked to undesirable clinical side effects, such as increases

or decreases in baseline DA levels by a potential drug alone, since these might hamper further development.

In the present study, microdialysis experiments targeting the caudate were begun by inserting 24-mm stainless-steel microdialysis probes with 4-mm membranes (CMA Microdialysis, North Chelmsford, MA) into each subject's surgically implanted guide cannulae. For experiments targeting the NAc, 28-mm stainless-steel microdialysis probes with 4-mm membranes (CMA Microdialysis, North Chelmsford, MA), after which subjects were positioned in sound-attenuating test chambers. After probe insertion, a 1 h equilibrium period was allowed to elapse, and then samples were collected every 10 min for 3.5 h. M100 pretreatment (0.3 mg/kg, IM) was administered 30 min after sampling was initiated to provide a pre-pretreatment and pretreatment period for assessment of any effects of M100 pretreatment alone on DA levels. Cocaine (1.0 mg/kg, IV) was then administered 1 h after M100 pretreatment, allowing 1 h for any effects of M100 pretreatment to emerge. Cocaine was infused through the previously implanted indwelling venous catheter, via the subcutaneous vascular access port. Samples were collected over the next 2 h. Probe functionality was confirmed for each microdialysis session, before and after the session, by determining the change in DA concentration after DA had traversed the probe from a reference solution containing a known concentration of DA (i.e. probe recovery). In addition, the viability of the sampling site was confirmed via retrodialysis of a solution with a high-potassium concentration (100 mM) which was ionically matched to artificial cerebrospinal fluid. DA concentrations of microdialysis samples were quantified using high-performance liquid chromatography (HPLC) with electrochemical detection and samples were analyzed by comparing sample chromatograms to those of solutions of known DA concentrations with EZChrom Elite software (version 3.1; Scientific Software, Pleasanton, CA).

High-Performance Liquid Chromatography

HPLC with electrochemical detection was used to quantify DA levels in dialysate samples as described previously (Banks et al., 2009; Kimmel et al., 2007). The HPLC machine was composed of a small bore column (3.2 mm × 150 mm × 3 μm), an ESA 582 model solvent delivery pump with the flow rate set to 0.6 ml/min, and an ESA model 542 autosampler (ESA, Inc., Chelmsford, MA). Electrochemical detection was performed using a guard cell (ESA model 5020; potential 350 mV), a dual-channel analytical cell (ESA model 5040), and an ESA model Coulochem II detector. The analytical cell's oxidation channel was programmed to a voltage of −150 mV, and its reduction channel was programmed to 275 mV. The commercially available mobile phase used for dopamine quantification (MD-TM; ESA, Inc.) was composed of octanesulfonic acid (1.7 mM), triethylamine (100 μl/l), sodium dihydrogen phosphate (75 mM), acetonitrile (10%), and EDTA (25 μM). After mixing, the solution was brought to a pH of 3 by adding phosphoric acid.

Data Analysis

For all SA and reinstatement data (Figures 3.1, 3.2, 3.3, and 3.4), the dependent measure analyzed was the group mean (±SEM) response rate (responses/sec). For baseline dose-effect curve analysis (Figure 3.1), a one-way repeated measures ANOVA was employed, with unit dose of cocaine as the factor. Post hoc comparisons were made with Dunnett's test to compare data at each cocaine dose to the saline data. Data in Figures 3.2, 3.3, 3.4 were analyzed using two-way repeated measures ANOVAs with factors of pretreatment (Figures 3.2, 3.3, and 3.4), and day (Figure 3.2), cocaine unit dose (Figure 3.3), or cocaine prime dose (Figure 3.4). Post hoc comparisons were made using Dunnett's test (Panel (A) of Figure 3.2; Figure 3.4) and paired t-tests (Panel (A) of Figure 3.2). For *in vivo* microdialysis data, datapoints at -20, -10, and 0 min, which corresponded to the group mean (±SEM) DA concentration, were averaged to create a baseline. All datapoints were then normalized as a percentage of this baseline. Data in both panels

of Figure 3.5 were analyzed using two-way ANOVAs with factors of time (baseline, 10, 20, and 30) and pretreatment. Post hoc comparisons were made using Dunnett's tests (Panels (A) and (B)) and paired t-tests (Panel (B)).

All graphical data presentations were created by using Prism 4 (GraphPad Software Inc., San Diego, CA), all statistical tests were performed by using SigmaStat 3 (Systat Software, San Jose, CA), and significance was arbitrated at $p < 0.05$ for all statistical tests.

Drugs

Cocaine HCl (National Institute on Drug Abuse, Bethesda, MD) was dissolved in 0.9% saline. M100,907, generously provided by Kenner C. Rice, Chief of the Chemical Biology Research Branch and Chief of the Drug Design and Synthesis Section of the Intramural Research Program of the National Institute on Drug Abuse, was dissolved to a concentration of 1.06 mg/mL in 0.9% saline. Doses of drugs were determined from the weights of the salts.

Chapter 3: Results

Cocaine Self-administration

Each subject self-administered cocaine at multiple unit doses, and responding was allowed to stabilize at each dose before drug interactions were evaluated. Subjects self-administered cocaine at each dose for about 3 months. The effects of different unit doses of cocaine on lever-pressing behavior are shown in Figure 3.1, which displays the response rate of each of the 3 subjects as well as the group average. Individual subject data represent the response rate (mean±SD) of two 2-day averages at each dose of cocaine; group data represent an average of the response rate (mean±SEM) of the 3 subjects. Group mean response rates varied across cocaine unit dose, producing the typical inverted U-shaped dose-response function often observed in SA experiments with drugs of abuse, including stimulants (Mello and Negus, 1996). The dose of cocaine that maintained the highest mean rate of responding—the "peak dose," which was 0.03 mg/kg—engendered very high rates of behavior: 2.16 ± 0.62 responses/sec. Response rates diminished to a very low level, 0.12 ± 0.07 responses/sec, within two days after saline was substituted for cocaine under extinction conditions. Subjects usually did not respond on the lever until the house light was illuminated and responding would usually stop immediately when the house light was extinguished, demonstrating strong schedule control in these animals (data not shown).

Although SA response rates varied over a large range across cocaine doses, the number of cocaine infusions received by monkeys across these doses varied by much less. A ~3.8-fold difference in mean rate between cocaine doses of 0.03 and 0.01 mg/kg corresponded to a ~1.3-fold difference in number of infusions at those doses. Furthermore, despite a ~13.5-fold difference between the maximum and minimum response rates among subjects, there was only a Although SA response rates varied over a large range across cocaine doses, the number of

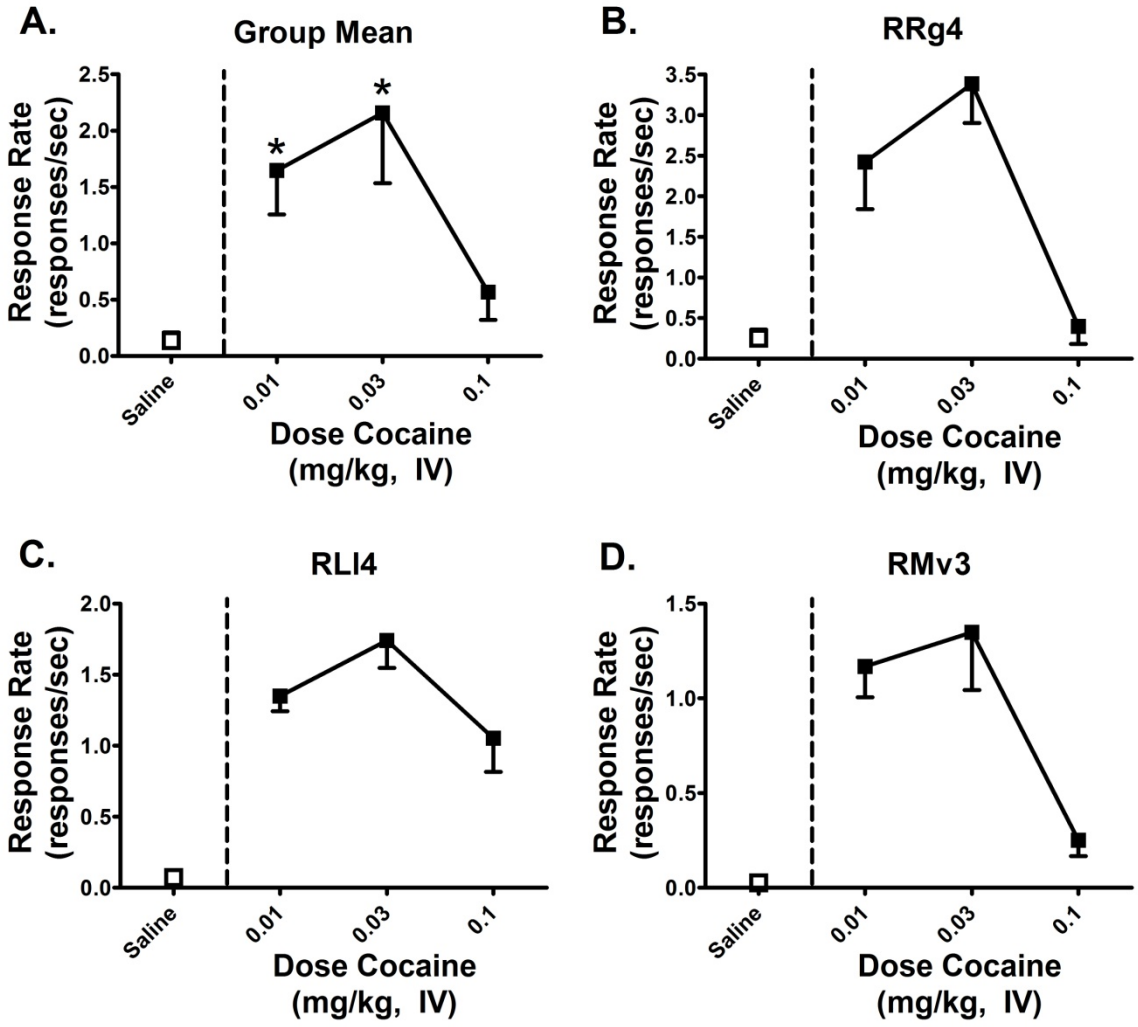

Figure 3.1. Dose-response curves for cocaine (0.01, 0.03, and 0.1 mg/kg/infusion) and saline vehicle SA in rhesus monkeys (n=3) trained to lever-press on a fixed ratio 20 schedule. (*A*) Group mean rate (mean±SEM). (*B*)-(*D*) Individual subject mean rate (mean±SD) for subjects RRg4, RLl4, and RMv3, respectively. Unfilled squares represent average response rate in sessions that satisfied extinction criteria (saline, 2 sessions). Filled squares represent the average of the baseline response rates when responding was stable before M100 or vehicle pretreatments (see Figure 3.2). *Abscissae*: dose of cocaine. *Ordinates*: response rate.

cocaine infusions received by monkeys across these doses varied by much less. A ~3.8-fold difference in mean rate between cocaine doses of 0.03 and 0.01 mg/kg corresponded to a ~1.3-fold difference in number of infusions at those doses. Furthermore, despite a ~13.5-fold difference between the maximum and minimum response rates among subjects, there was only a ~1.6-fold difference in the number of infusions. Notably, the dose-effect functions for individual subjects followed a very similar pattern, and each subject's peak dose was the same, as shown in Figure 3.1. Therefore, sensitivity to the reinforcing effects of cocaine was very similar across subjects under the present SA conditions.

To determine whether cocaine acted as a reinforcer under these experimental conditions, the group data were analyzed with one-way repeated measures ANOVA, which identified a main effect of dose ($F_{3,6} = 8.19$, $p = 0.015$). Post hoc Dunnett's tests indicated that cocaine doses of 0.01 and 0.03 mg/kg maintained rates of responding significantly greater than saline ($p < 0.05$) and were thus reinforcing. Response rates decreased at the highest unit dose due to higher drug intake.

M100 pretreatment

Next, we investigated whether M100 would attenuate the reinforcing effects of cocaine when administered over 3 consecutive pretreatment days. The three panels in Figure 3.2 depict cocaine SA response rates at multiple unit doses of cocaine over the 5-day weeks in which M100 or vehicle was administered as a pretreatment. Note that in each panel of Figure 3.2, the two baseline (BSL) datapoints each represent an average over the M & Tu of each pretreatment week (subjects were not administered pretreatments on these days), whereas days 1, 2, and 3 represent W, Th, and F (when subjects were administered pretreatments) of the same week. At the lowest dose of cocaine (Panel (A), 0.01 mg/kg), although baseline response rates were very similar, after 3 days of M100 pretreatment, group mean response rate was reduced to ~47% of baseline. Two-way repeated measures ANOVAs revealed main effects of pretreatment ($F_{1,2} = 26.96$, $p = 0.035$)

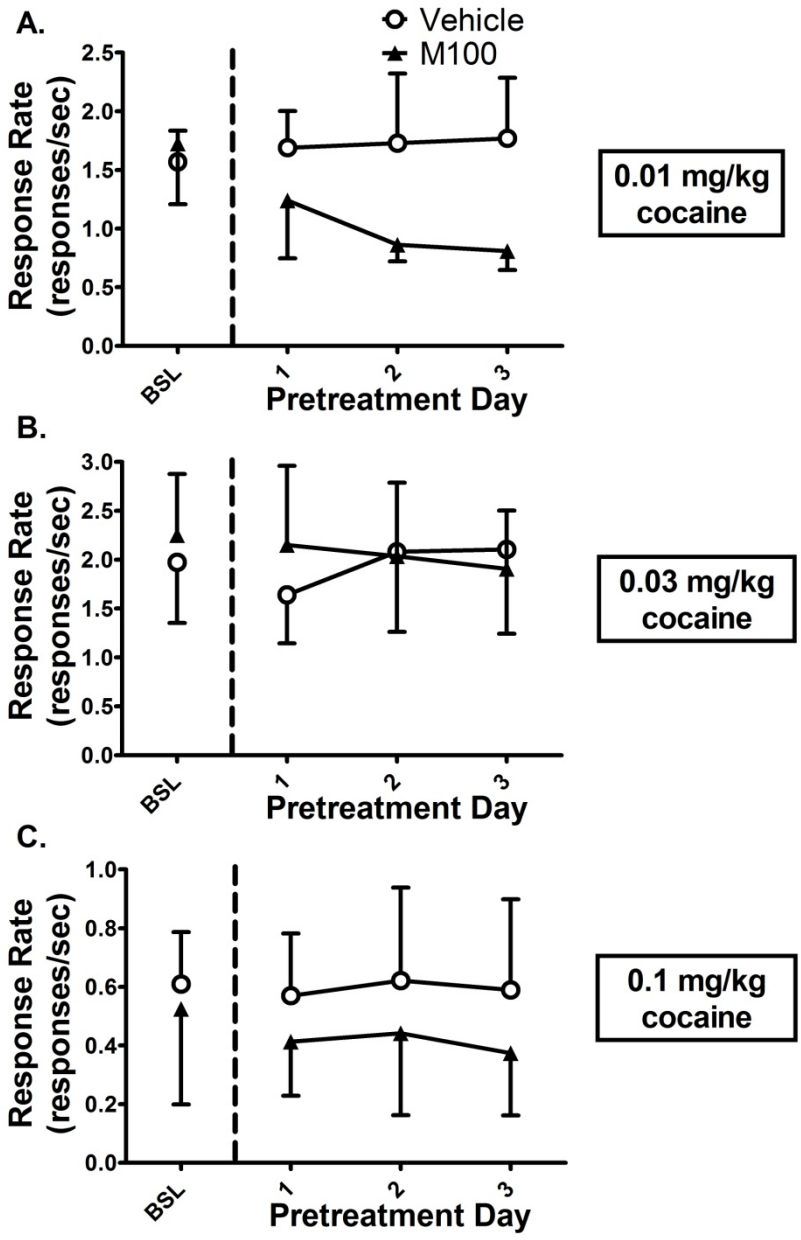

Figure 3.2. The effects of 3 consecutive days of M100 pretreatment (0.3 mg/kg) on SA of cocaine at (A) 0.01, (B) 0.03, and (C) 0.1 mg/kg. Empty circles represent vehicle pretreatment weeks and filled triangles represent M100 pretreatment weeks. Data are expressed as group (n=3) response rates (mean±SEM) . BSL represents the average of M & Tu baseline days, and days 1, 2, and 3 represent W, Th, and F of each 5-day treatment week at the specified dose of cocaine. *Abscissae*: pretreatment day. *Ordinates*: response rate.

and day ($F_{3,6} = 6.38$, $p = 0.027$) only at the lowest dose tested. No significant differences between any datapoints were identified in post hoc comparisons.

Although a two-way repeated-measures ANOVA of the data in Figure 3.2 revealed a main effect of day and pretreatment at the lowest dose of cocaine, the lack of a significant difference between any two datapoints indicates that the effectiveness of M100 pretreatment does not substantially change over multiple days of treatment. Therefore, the data for each dose of cocaine were grouped into 3-day averages. Figure 3.3 displays the group mean SA response rate as a function of cocaine unit dose (0.01, 0.03, and 0.1 mg/kg) after receiving M100 or vehicle pretreatments, where each data point represents the average of a 3-day treatment block. Two-way repeated measures ANOVA indicated that there was no main effect of pretreatment or dose. Therefore, these data as a whole demonstrate a failure of M100 to attenuate the reinforcing effects of cocaine across a range of doses of cocaine with varying reinforcing effectiveness.

Reinstatement

The results of M100 pretreatments on cocaine- + cue-induced reinstatement tests are summarized in Figure 3.4, in which the group mean rates of responding elicited by a range of non-contingent primes of cocaine are plotted against the dose of cocaine prime administered. To the left of the dashed line are the response rates of the extinction condition sessions in which extinction criteria were reached and which directly preceded the reinstatement sessions to the right. Cocaine bolus priming doses (0.1, 0.3, and 0.56 mg/kg), given immediately before sessions began, induced increased rates of lever-pressing, and an inverted U-shaped dose-effect curve was observed. The group response rate (mean±SEM) elicited by the peak reinstating dose of cocaine (0.3 mg/kg) after subjects received vehicle pretreatments was 0.81 ± 0.27 responses/sec. These response rates are lower than those observed in cocaine SA sessions (maximum reinstatement rate/maximum SA rate = 0.38).

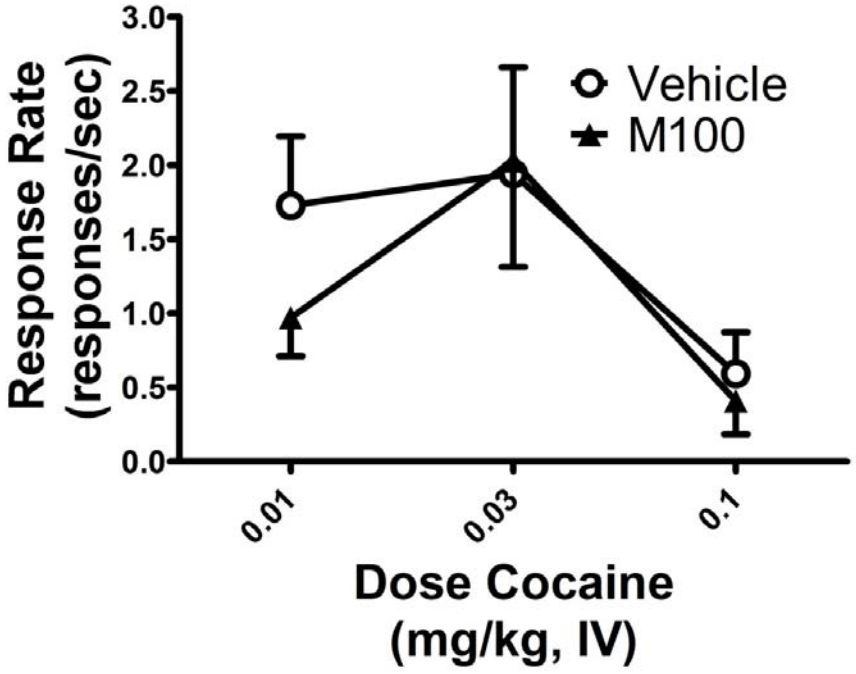

Figure 3.3. Cocaine SA (0.01, 0.03, and 0.1 mg/kg) dose-response function expressed as group (n=3) response rates (mean±SEM) averaged over 3-day M100 (0.3 mg/kg; unfilled circles) or vehicle (filled triangles) treatment blocks. *Abscissa*: dose of cocaine. *Ordinate*: response rate.

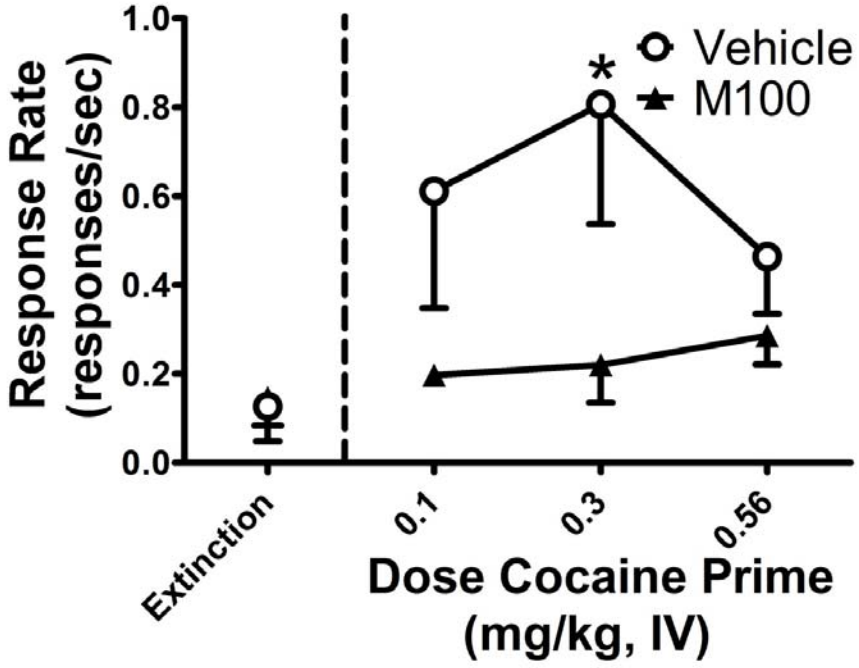

Figure 3.4. The effects of M100 pretreatment on cocaine- + cue-induced reinstatement of behavior previously maintained by cocaine. Data are expressed as group (n=3) response rate (mean+SEM) during SA sessions under extinction conditions (to the left of the dashed line) or reinstatement (to the right of the dashed line) conditions. Extinction datapoints represent behavior that satisfied extinction criteria in sessions that immediately preceded reinstatement sessions. Reinstatement datapoints represent rates in reinstatement sessions in which subjects received vehicle (unfilled circles) or M100 (0.3 mg/kg; filled triangle) pretreatments 1 hr before a bolus cocaine (0.l, 0.3, or 0.56 mg/kg) prime. Asterisks (*) indicate a significant difference ($p < 0.05$) from the saline extinction point within the same treatment condition as the given datapoint. *Abscissa*: dose of cocaine prime. *Ordinate*: response rate.

Figure 3.4 also includes the rates observed during extinction sessions that immediately preceded reinstatement sessions, although importantly, subjects were not pretreated during these extinction sessions. All extinction sessions that preceded all reinstatement trials within a given treatment condition were averaged, producing the two extinction datapoints in the figure. Accordingly, a total of 6 extinction sessions preceded vehicle pretreatment reinstatement sessions, and 7 preceded M100 pretreatment reinstatement sessions. Extinction session averages were,0.13 ± 0.078 and 0.14 ± 0.059 responses/sec, respectively

Two-way repeated measures ANOVA of the reinstatement results indicated a main effect of priming dose ($F_{3,6} = 8.684$, $p = 0.013$), but not pretreatment. A subsequent post hoc Dunnett's test indentified a significant difference between the response rate after a dose of 0.3 mg/kg cocaine and the rate on pre-reinstatement extinction days ($p < 0.05$). Therefore, a cocaine priming dose of 0.3 mg/kg significantly reinstated lever-pressing behavior. However, the same dose of cocaine did not reinstate lever pressing following M100 pretreatment; thus, M100 blocked the reinstatement effect elicited by cocaine.

In Vivo Microdialysis

In order to shed light on the neurochemical and neurobiological mechanisms underlying drug interactions on behavior, subjects underwent *in vivo* microdialysis experiments with dialysis probes targeting either the caudate (n=5) or the NAc (n=4). Figure 3.5 summarizes the results of these experiments, where data points represent the group mean percentage of baseline DA concentration in either the (A) NAc or (B) caudate after an IV injection of cocaine (1.0 mg/kg). Cocaine elicited similar increases in DA in the two brain regions after M100 vehicle pretreatment, with levels reaching 1010 ± 342% of baseline in the NAc and 840 ± 58% of baseline in the NAc. These values are within the range that would be expected under these conditions (Kirkland Henry et al., 2009). After pretreatment with M100, DA levels reached 757 ± 176% of baseline in the NAc, but only 266 ± 54% of baseline in the caudate.

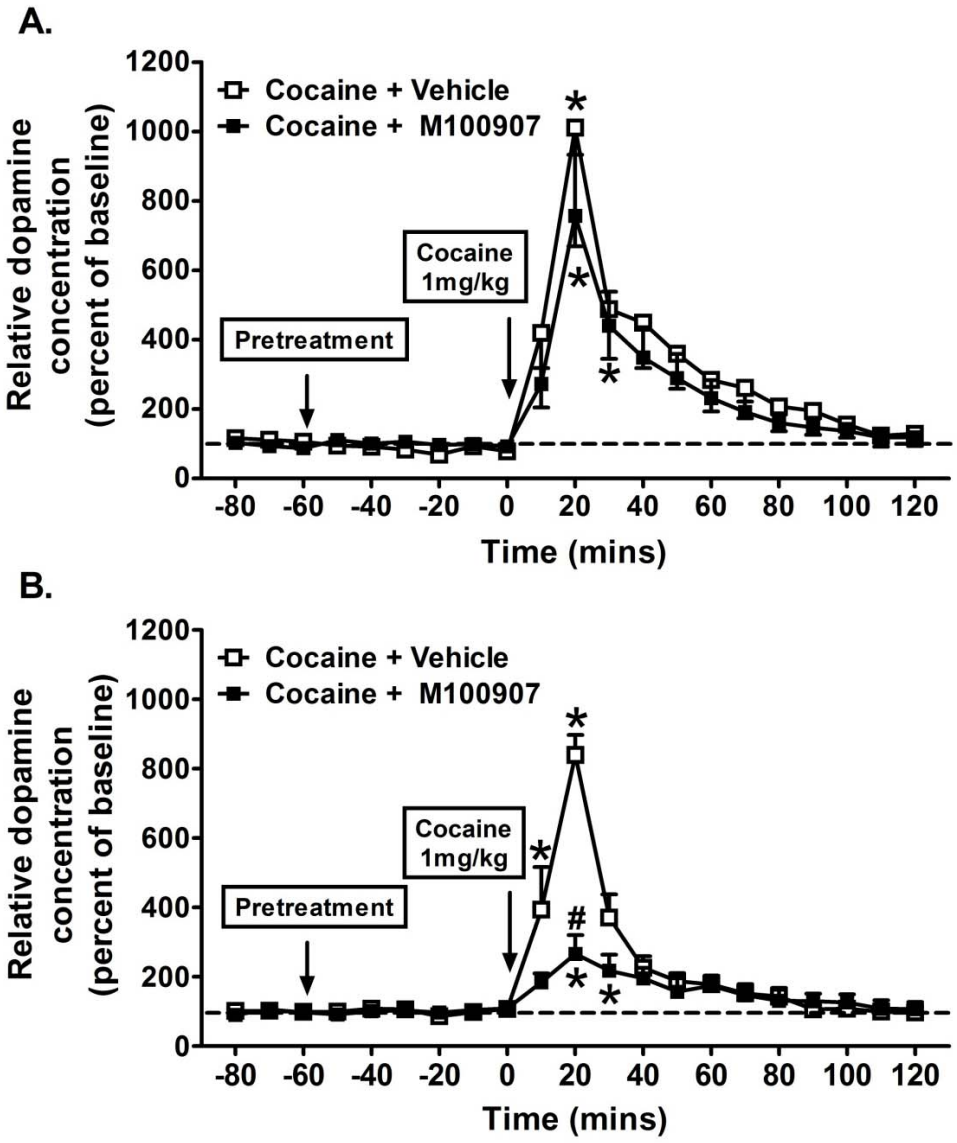

Figure 3.5. Modulation of cocaine-induced increases in DA in (A) NAc (n=4) and (B) caudate (n=5) by M100 (0.3 mg/kg). Data are expressed as group relative percent (mean±SEM) of baseline DA concentration under vehicle (unfilled squares) or M100 (filled squares) pretreatment conditions. Baseline represents the average of DA concentrations measured at time points -20, -10, and 0 min. Pound signs (#) indicate a significant difference between vehicle and M100 pretreatment conditions ($p < 0.05$). Asterisks (*) located above (vehicle) or beneath (M100) datapoints indicate a significant difference from baseline ($p < 0.05$). *Abscissae*: time in relation to cocaine injection. *Ordinates*: dopamine concentration as percent of baseline.

Two-way repeated measures ANOVAs identified a main effect of time ($F_{3,9} = 14.150$, $p < 0.001$) in the NAc and main effects of time ($F_{3,12} = 25.457$, $p < 0.001$) and pretreatment ($F_{1,4} = 10.741$, $p = 0.031$) as well as a significant interaction in the caudate. Post hoc Dunnett's tests of NAc data identified a significant difference ($p < 0.05$) from baseline after vehicle pretreatment at 20 mins and after M100 pretreatment at 20 and 30 mins. Furthermore, Dunnett's tests of caudate data identified a significant difference from baseline after vehicle pretreatment at 10 and 20 mins and after M100 pretreatment at 20 and 30 mins. Importantly, paired t-tests revealed that, although there was no difference in baseline DA levels in the caudate between M100 and vehicle pretreated subjects, there was a significant difference at 20 mins. Therefore, M100 pretreatment attenuated the cocaine-induced increases in DA concentration in the caudate, but not the NAc. Although M100 substantially attenuated the cocaine-induced DA increases in the caudate, DA levels still rose significantly above baseline levels.

Chapter 4: Discussion

Summary of Rationale

The primary objectives of the current project were 1) to evaluate the effectiveness of the selective 5-HT_{2A}R antagonist M100,907 (M100) in attenuating distinct but related measures of the abuse liability of cocaine in rhesus monkeys, and 2) to provide evidence of the neurochemical mechanism underlying the effect of M100 on behavior maintained by cocaine. Of particular importance was the extension of preclinical data supporting the therapeutic potential of M100 in the treatment of stimulant abuse with this multifaceted, mechanistic study in rhesus monkeys, since the previous work in this field was largely limited to rat studies and *in vitro* studies, each of which have considerable limitations. These experiments are a logical continuation of the work evaluating the role of 5-HT_2Rs in modulation of DA signaling and the effects of stimulants; more broadly, the results of this work are in accord with the extensive literature indicating significant serotonergic modulation of the mesolimbic and mesocortical DA systems. Indeed, the integration of the results of the present *in vivo* neurochemistry and behavioral pharmacology procedures powerfully addresses the complex mechanism of 5-HT_{2A}R antagonist-mediated modulation of the effects of stimulants. Finally, since these experimental techniques in rhesus monkeys have excellent predictive (Moser et al., 2011) and translational value (Weerts et al., 2007), our results may act as a critical validation of the potential clinical value of M100 in reducing stimulant abuse.

Results Summary

The results of this study corroborated previous studies in nonhuman primates (NHPs) (Fantegrossi et al., 2002) and rats (Fletcher et al., 2002; Nic Dhonnchadha et al., 2009) that M100 is largely ineffective at attenuating the reinforcing effects of cocaine across a range of SA doses.

In contrast, we report for the first time in rhesus monkeys that M100 completely abolishes cue- + cocaine-primed reinstatement, extending the results found previously in rats reinstated by administration of a cocaine prime in the absence of cocaine-paired cues (Fletcher et al., 2002) or cocaine-paired cues alone (Nic Dhonnchadha et al., 2009). Additionally, we report that, in the first investigation of the effects of selective 5-HT_{2A}R antagonism on cocaine-induced DA increases in monkeys, M100 attenuated cocaine-induced DA increases in the caudate, whereas it had no effect on cocaine-induced increases in DA in the NAc or on baseline DA in either brain region.

Overview

Over several decades of research into the addictive properties of drugs, a clear relationship has emerged between abuse liability of drugs and increases in DA levels within the NAc. The most well-validated model for evaluation of the abuse liability of a drug is the SA model, which measures the reinforcing effects of a drug through its ability to engender behavior that leads to contingent administration or availability of the test drug (Moser et al., 2011). Assessments of the reinforcing effects of a gamut of drugs with this model demonstrated a remarkably close concordance between the list of drugs that maintained SA and those with abuse liability of that drug in humans, as measured by preponderance of evidence of human abuse of that drug (Johanson and Balster, 1978). Additional studies consistently revealed that drugs with reinforcing effects usually cause increases in extracellular DA levels in the NAc at doses that maintain SA. Furthermore, a strong correlation was found between the potencies of various dopamine transporter inhibitors to produce reinforcing effects and their potency to bind to the DAT, strongly supporting the hypothesis that the reinforcing effects of cocaine were a direct result of increases in DA caused by DAT inhibition (Ritz et al., 1987). Additionally, studies on SA into specific regions of the brain found that animals self-administered cocaine directly into the NAc shell(McKinzie et al., 1999), part of the ventral striatum, but not the dorsal striatum or NAc

core, which displays similarity to dorsal striatum. In addition, SA studies in rats revealed that the reinforcing effects of cocaine were principally related to DA increases in the NAc (Pettit and Justice, 1989; Pettit and Justice, 1991). Although many drugs, like cocaine and amphetamine, induce increases in DA in the caudate/putamen region of the dorsal striatum (Madras and Kaufman, 1994), there is not a clear relationship between dorsal striatal DA increases and reinforcing effects or other behavioral effects of drugs of abuse.

Although it has become clear that DA plays a central role in reinforcing and abuse-related effects of drugs, a considerable body of evidence indicates that serotonergic systems modulate DA systems, thereby altering the neurochemical and behavioral effects of drugs. This serotonergic modulation of DA associated effects of drugs of abuse is mirrored by the neuroanatomical organization of the 5-HT projection in relation to the DA systems (Carr and Sesack, 2000; Sesack and Carr, 2002). 5-HT neurons, projecting from the raphe nuclei, provide afferent input to each region of the mesolimbic/mesocortical DA systems (Azmitia and Segal, 1978; Fibiger and Miller, 1977; Fuxe, 1965). Preclinical studies evaluating the effectiveness of drugs with broad effects on 5-HT systems, such as SSRIs (Carroll et al., 1990a; Richardson and Roberts, 1991) and L-tryptophan (Carroll et al., 1990b), at attenuating the abuse-related effects of cocaine yielded promising results: in several cases, these drugs attenuated the reinforcing (Czoty et al., 2002) and DA-increasing effects (Czoty et al., 2002) of cocaine in NHPs and rats, although there were also several negative results (Walsh and Cunningham, 1997). Unfortunately, this success was not matched by clinical trials seeking to reduce cocaine abuse with SSRIs. The reasons for the failures of these drugs in clinical trials may include the coexpression of functionally opposed 5-HT receptor subtypes on VTA DA neurons or within the NAc or PFC, or DA neurons may be innervated by a pair of functionally opposed afferent neurons driven by increased brain 5-HT concentrations. Therefore, to avoid issues like these, researchers have since primarily engaged in identifying specific 5-HT receptor targets and receptor-selective ligands that may have more specific effects on DA systems in order to successfully treat cocaine abuse.

5-HT$_{2A}$Rs: Early Work to Mechanistic Findings

Investigations of individual 5-HT receptor subtypes as potential targets for the treatment of cocaine abuse have identified 5-HT$_{2A}$Rs as particularly promising. Although 5-HT$_{2A}$R agonists are unsuitable for clinical use since they mostly comprise the "classical hallucinogens" (Jakab and Goldman-Rakic, 1998), early work with selective 5-HT$_{2A}$R antagonists revealed several desirable characteristics of this drug class for the treatment of drug abuse. One of the most prominent qualities of these drugs is the nearly complete lack of measurable behavioral or neurochemical effects after administration to normal subjects in the absence of a drug challenge or other treatment (Kehne et al., 1996a; Moser et al., 1996). Thus, the effects of these drugs are often described as being state-dependent or as antagonistic of phasic DA release, since they appear to only attenuate the effects of phasically-activate DA systems (e.g., burst-firing of VTA neurons projecting to the PFC) (Gobert and Millan, 1999; Pehek et al., 2001; Pehek et al., 2006). In fact, the effects of selective 5-HT$_{2A}$R antagonists are generally don't fit in the categories of dopaminergic or anti-dopaminergic drugs, even though they antagonize the neurochemical (Auclair et al., 2004; Porras et al., 2002; Schmidt et al., 1992) and behavioral (McMahon and Cunningham, 2001; Moser et al., 1996) effects of stimulants including cocaine, amphetamine, and MDMA; since they only appear to affect activated systems, they exhibit few of the characteristic effects of dopaminergic drugs. For example, they lack the primary and adverse effects commonly exhibited by DA indirect agonists (i.e. methylphenidate, which has stimulant effects and abuse liability) or DA receptor antagonists (i.e. haloperidol, which has antipsychotic effects, but which has sedative and sometimes dyskinetic effects) (Carroll et al., 1999). This is a critical factor since severe side effects like these accompany many of the drugs that were most successful in preclinical evaluations of therapeutics for cocaine abuse and they were primary determinants that prevented their eventual clinical use (Carroll et al., 1999). The absence of these serious side effects of selective 5-HT$_{2A}$Rs, coupled with their robust inhibitory effects on the

behavioral and neurochemical effects of stimulants greatly supports the potential therapeutic value of these drugs.

One of the clinical approaches to the treatment of cocaine abuse is to attempt to reduce the reinforcing effects of cocaine, which are thought to be a primary determinant in the drug-taking behavior of abusers. Therefore, several previous studies have evaluated the effectiveness of selective 5-HT_{2A}R antagonism to attenuate the reinforcing effects of cocaine in rodents (Fletcher et al., 2002; Nic Dhonnchadha et al., 2009) and NHPs (Fantegrossi et al., 2002) across a range of behaviorally active doses, but none has found an attenuation of SA responding. In the only previous study on the effect of 5-HT_{2A}R antagonism on cocaine SA in monkeys, it was reported that M100 produced a large and dose-dependent downward-shift in the cocaine SA dose-response curve at the highest M100 dose tested (1.0 mg/kg), but the effect did not reach statistical significance (Fantegrossi et al., 2002). In the present results, with a dose one half-log unit below the highest dose in the previous study (0.3 mg/kg), we observed that M100 was largely ineffective in attenuating the reinforcing effects of cocaine, although there was some limited evidence of a modest suppression of response rates at the lowest dose of cocaine that may have increased effectiveness slightly over the 3 days of pretreatment. At the other cocaine doses, there was no evidence of an effect of pretreatment, and when all 3 doses were analyzed together, no significant effect was obtained. Therefore, in accord with the previous NHP study, it appears that M100 weakly suppresses the reinforcing effects of cocaine at marginally reinforcing doses, but that doses of cocaine that engender stronger behavior override this effect. We interpret this effect as merely an example of the basic principle of operant behavior—that behavior maintained by robust reinforcers is more sensitive to disrupting stimuli than behavior maintained by weak reinforcers.

An appropriate reaction to these results might be to question why a higher dose of M100 was not tested in this study, since we found some modest evidence that M100 attenuates the reinforcing effects of cocaine. Firstly, the dose of M100 tested in the present study (0.3 mg/kg)

has been shown to be effective in neurochemical studies conducted in rhesus monkeys (Murnane et al., 2012) and in behavioral studies conducted in squirrel monkeys (Fantegrossi et al., 2009). Importantly, the same dose of M100 was effective in blocking cocaine-induced reinstatement in the present study. Therefore, this rules out the possibility that this dose of M100 was ineffective in SA tests because it was pharmacologically inactive. It is important to note that, although M100 is often considered highly selective for the 5-HT_{2A}R, most *in vitro* binding studies have found about a 100-fold selectivity for the 5-HT_{2A}R over the 5-HT_{2C}R (Kehne et al., 1996a; Kehne et al., 1996b). Therefore, it is conceivable that, under the conditions of the present work, M100 exhibited appreciable antagonism for the 5-HT_{2C}R since this dose appears to be near the top of the animal equivalent range (Fantegrossi et al., 2002; Kehne et al., 1996a). This is especially important since a previous study in rats found, at the highest M100 dose tested (3.0 mg/kg), a dose which is equivalent in rats to the dose used in the present study in monkeys, an effect of M100 identical to a less selective 5-$HT_{2A/2C}$R (Bonaccorso et al., 2002). Additionally, recent work by our lab (Manvich et al., 2012) and others' (Bubar and Cunningham, 2006) demonstrates that 5-HT_{2C}R antagonism enhances cocaine-induced DA release in the NAc, likely by inhibiting GABAergic interneurons in the VTA(Bubar et al., 2011), causing consequent disinhibition of VTA DA neurons projecting to the NAc (Manvich et al., 2012). A powerful means to address this possibility was included in our microdialysis methods; we allowed 60 minutes after administration of M100 for any effects of M100 alone to evolve. Based on the hypothesis about the effect of 5-HT_{2C}R antagonists of NAc DA levels, one would predict an increase in baseline DA levels after administration of a dose of M100 that acted as a functional antagonist at 5-HT_{2C}Rs, but we observed no changed in DA levels in either NAc or caudate during the pretreatment-only period. Notably, despite significantly attenuating the cocaine-induced elevation of DA in the caudate, M100 did not fully block DA increases elicited by cocaine.

The results of our SA experiments are the most powerful evidence yet that 5-HT_{2A}R antagonists do not reduce maintenance of cocaine SA procedures, but M100 has found much greater success in a preclinical model of relapse—reinstatement. Our results are in agreement with the rodent literature; we found that M100 completely abolished the reinstatement of responding induced by a cocaine-cue and noncontingent prime of cocaine administered before an extinction session. As reinstatement procedures are the most widely accepted preclinical model of drug relapse, these findings suggest that 5-HT_{2A}R antagonism may be a viable approach to relapse prevention in cocaine-dependent subjects. These results are particularly poignant, considering the present lack of effective pharmacotherapies for cocaine addiction. Treatment of relapse in drug addicts is a particularly promising treatment approach due to its many advantages over treatment of maintenance, during which time drug-dependent individuals are probably less likely to take medication consistently and appropriately, in addition to other factors.

In order to provide insight into the direct neurochemical and neurobiological mechanisms underlying the modulation of the reinforcing and reinstating effects of cocaine by M100, we performed *in vivo* microdialysis experiments in separate groups of monkeys with dialysis implants targeting the caudate and NAc. These microdialysis experiments are the first to assess the *in vivo* neurochemical interaction between M100 and cocaine in NHPs. Systemic administration of M100 has no effect on baseline DA levels in the NAc or caudate, in perfect accordance with the preponderance of evidence indicating that M100 exhibits no effects on baseline neurochemistry or behavior. For example, systemic administration of 5-HT_{2A}R antagonists alone lacks effects on locomotion (Fletcher et al., 2002; Kehne et al., 1996a; McMahon and Cunningham, 2001), DA cell firing (Palfreyman et al., 1993; Sorensen et al., 1993) and dorsal striatal (Ichikawa and Meltzer, 1995; Schmidt et al., 1992), NAc (Bonaccorso et al., 2002; De Deurwaerdere and Spampinato, 1999; Ichikawa and Meltzer, 1995; Kuroki et al., 2003), and PFC DA levels (Bonaccorso et al., 2002; Kuroki et al., 2003; Pehek et al., 2006). Next, although we observed a typical elevation of DA levels in both regions in response to a

cocaine challenge, interestingly, M100 pretreatment attenuated caudate, but not NAc DA increases. This finding also corroborates the results of most preclinical studies, in that the effects of M100 were only evident following a drug challenge (Kehne et al., 1996a; McMahon et al., 2001; Porras et al., 2002).

In the present work, we observed that M100 did not attenuate the reinforcing effects of cocaine or cocaine-induced increases in DA in the NAc, whereas M100 blocked cocaine-induced reinstatement and cocaine-induced DA increases in the caudate. The overlap in these functional effects of M100 on behavior and neurochemistry is supported by previous work relating the neurochemical effects of drugs to behavior (Howell and Murnane, 2008; Howell and Wilcox, 2002). Furthermore, this coordination between the neurochemical and behavioral effects of M100 provides evidence that the neurochemical effects are direct indicators of the mechanisms through which 5-HT_{2A}Rs act to modulate DA systems in these studies. Since such a strong link exists over decades of behavioral neuroscience research, there is ample support for the hypothesis that M100 fails to alter the reinforcing effects of cocaine because it does not modulate cocaine-induced DA increases in the NAc.

The selectivity we observed in 5-HT_{2A}R-mediated modulation of caudate vs. NAc DA levels is a significant divergence from the majority of previous reports, as the majority of the previous work demonstrated inhibition of NAc DA release by 5-HT_{2A}R antagonists (Auclair et al., 2004; Porras et al., 2002) or strongly implicated the VTA in the effects of 5-HT_{2A}R antagonists (Auclair et al., 2004; McMahon et al., 2001). Although the reasons for this discrepancy remain speculative, we propose that it is the result of a combination of factors including significant species differences in DA systems, their regulation by 5-HT_{2A}R, and the test drug used (amphetamines vs. cocaine). In the present study, we observed that M100 attenuated cocaine-induced increases in DA selectively in the dorsal striatum, which deviates starkly from the majority of previous preclinical research; the primary difference between the present study and previous work is the species in which the work was done. Whereas the present experiments

were performed with NHPs, nearly all previous studies with direct insight into the role of 5-HT_{2A}Rs in the regulation or modulation of DA systems have employed rodent models (McMahon et al., 2001; Moser et al., 1996), mostly in rats. Although little mention is made of possible functional differences between rodents and primates in the previous preclinical studies, nor of their relationship to human neuroanatomy and neuropharmacology, there is substantial evidence that the DA and 5-HT systems of rats differ considerably from those of primates. For example, primates have a much more developed prefrontal cortex, with several more specialized compartments implicated in the effects of stimulants (Frankle et al., 2006; Haber and Knutson, 2010). Indeed, there is strong evidence for differential neuroanatomical (Carr and Sesack, 2000) and neurochemical (Frankle et al., 2006) correlates of prefrontal control (Haber and Knutson, 2010) of drug-taking and relapse-like behaviors between primates and rodents.

After early studies demonstrated that M100 blocks that hyperlocomotive and DA-releasing properties of amphetamine and MDMA, several follow-up studies in rats that attempted to unveil the specific mechanism of action of these effects bore the conclusion that M100 likely merely inhibits the capacity of these drugs to stimulate the increased synthesis of DA within VTA and SNc DA neurons (Lucas and Spampinato, 2000; Schmidt et al., 1992). The value of these previous results for the interpretation of the present results is especially evident given that there are no published studies on the effects of selective 5-HT_{2A}R antagonism on cocaine-induced DA release. However, cocaine acts through a very different mechanism than amphetamines, primarily blocking reuptake rather than primarily inducing release of DA (and other monoamines), so the results of these neurochemical and mechanistic studies may not be applicable to the present results (Carboni et al., 1989). Additionally, since cocaine has been found to *decrease* DA synthesis after administration (Galloway, 1990), rather than increase it, as MDMA does (Schmidt et al., 1992) and amphetamine is thought to, the extrapolation of the results of these previous mechanistic investigations of the interaction of M100 with amphetamine-based drugs to the effects of M100 on cocaine seems foolish. Although there are no published studies on the effects

of M100 on cocaine-induced DA increases in the brain, work by the Cunningham group investigating the effect of M100 on cocaine-induced hyperlocomotion when administered systemically or by local infusion into the VTA or NAc may shed light on the *in vivo* mechanism of 5-HT_{2A}R-mediated DA modulation (McMahon and Cunningham, 2001; McMahon et al., 2001).

In this set of publications, it was demonstrated that M100 blocks cocaine-induced hyperlocomotion when administered systemically (McMahon and Cunningham, 2001) or intra-VTA (McMahon et al., 2001) to rats but not when administered intra-NAc. Many previous studies have demonstrated a tight correlation between stimulant-induced hyperlocomotion and DA increases in the NAc (Tzschentke and Schmidt, 2000), so changes in baseline locomotion or response to cocaine in cocaine-induced hyperlocomotion assays can be viewed as a proxy measure for changes in DA overflow in the NAc. As such, these experiments suggest that M100 blocks cocaine-induced DA overflow in the NAc when administered systemically or intra-VTA, but not when administered intra-NAc. This suggests that 5-HT_{2A}Rs on VTA DA neurons normally facilitate the firing of either VTA DA neurons projecting to the NAc or those projecting to the mPFC and that DA there enhanced DA overflow in the NAc likely via corticostriatal afferents (Haber and Knutson, 2010).

A recent, excellent report of the whole-brain distribution of 5-HT_{2A}Rs and 5-HT_{2A}R-encoding mRNA in NHPs indicates that the likely primary binding site of M100 within the NHP brain, via which it modulates the behavioral and neurochemical effects of stimulants, is the prefrontal cortex. This study, which included a rhesus monkey brain, revealed, with autoradiography using [^{3}H]M100907 and *in-situ* hybridization, a remarkably dense clustering of 5-HT_{2A}Rs throughout the PFC of the NHP brain, especially within layers III and IV and especially in the temporal and cingulate cortices. Importantly, the primate brain has very low expression of 5-HT_{2A}Rs throughout the basal ganglia and the SN, although there is low expression in the VTA. This is in stark contrast to a report by the same group in which the rat

brain is labeled with [^{3}H]M100907; the rat brain exhibited considerably greater 5-HT_{2A}R expression within the SN, caudate, and NAc. The selectivity observed in the present study, in which M100 attenuated cocaine-induced increases in DA in the caudate but not the NAc, was a surprising result since previous rat data appeared to support a VTA- (and likely SNc-) based 5-HT_{2A}R-mediated mechanism. However, based on these primate data on 5-HT_{2A}R distribution and expression, the likely hypothesis would be an attenuation of NAc cocaine-induced DA overflow and less to no effect in the caudate.

Among the many differences between rodents and primates in substrates that mediate the effects of cocaine and related stimulants is the activation of mesocortical VTA DA (and GABA) afferents to the PFC, a pathway which has been elucidated in depth through rodent electrophysiological and labeling studies (Carr and Sesack, 2000; Ichikawa et al., 2001; Ichikawa and Meltzer, 1995; Kuroki et al., 2003). This may be a particularly important factor in elucidation of the differences between rodent and primate studies of the effects of 5-HT_{2A}R ligands on DA systems relation to the modulation of stimulants by 5-HT_{2A}Rs, since the mesocortical DA projection from the VTA to the PFC has been widely hypothesized to play a critical role in the mediation of the primary effects of stimulants (Carr and Sesack, 2000). For example, it was discovered, in rats, that activation of the mPFC activates VTA neurons that release DA into the mPFC, and also, by an unknown mechanism, increases NAc DA release. This effect is very likely mediated by 5-HT_{2A}Rs since they are found expressed in high concentration in the mPFC (Cornea-Hebert et al., 1999; Lopez-Gimenez et al., 1997; Lucas and Spampinato, 2000) and since 5-HT_{2A}R agonists cause the activation of VTA neurons and the reciprocal release of DA into the PFC (Ichikawa and Meltzer, 1995; Lucas and Spampinato, 2000; Pehek et al., 2006). Overall, a significant number of studies have investigated the role of these pathways in schizophrenia, drug abuse, parkinson's disease, and other disorders of the CNS (Bortolozzi et al., 2005; Carr and Sesack, 2000; Kuroki et al., 2003; Pehek et al., 2006). Despite all this work, the relevance to man was never investigated, such as through primate research, until recently. In this study, in primate

brain, it was found that there were very few prefrontocortical afferents that synapse in midbrain DA nuclei (Frankle et al., 2006). This work may have revealed a key difference between results of rat studies of 5-HT_{2A}R modulation of stimulant effects and those in the present study.

Another set of hypotheses on the role of 5-HT_{2A}Rs in abuse-related effects of stimulants focuses on 5-HT_{2A}Rs located within the primary DAergic loci, the VTA, the NAc, SN, and the dorsal striatum. These hypotheses have garnered considerable support from studies of 5-HT_{2A}R localization and distribution, especially from immunocytochemical and immunohistochemical studies of these regions, which consistently reported very high expression of this receptor in the regions investigated, and particular attention was paid to VTA (Bubser et al., 2001; Lopez-Gimenez et al., 1997; Pompeiano et al., 1994). Surprisingly, the localization of 5-HT_{2A}Rs and mRNA in these areas studies generally supports the likelihood of 5-HT_{2A}R modulation of SN neurons and dorsal striatum, due to the common finding that 5-HT_{2A}Rs were likely expressed in these regions at medium expression levels. This finding wasn't totally consistent though; some studies reported very low expression of receptor or mRNA across most DAergic regions (Cornea-Hebert et al., 1999). Although distribution and expression studies can be highly informative for interpretation of results or for predicting the functional regulation of a system, it is important to note that few of these studies employed techniques with enough resolution to detect specific neural types.

Contrastingly, most studies are in agreement that systemic administration of a 5-HT_{2A}R agonist, such as the nonselective 5-$HT_{2A/2C}$R agonist DOI, enhances the activity of mesolimbic/mesocortical DA systems. Receptor selectivity in the production of this effect is commonly demonstrated through blockade of agonist-mediated effects by pretreatment with a selective antagonist. For example, systemic administration of DOI was shown to enhance mPFC DA release, and this effect was blocked by systemic (and Gobert and Millan) or intra-mPFC administration of M100 (Pehek et al., 2001). DOI also enhances glutamate levels in the VTA (Pehek et al., 2006), an effect which might enhance DA release to downstream targets through

excitation of VTA DAergic cells. Interestingly, intracortical infusion of M100 blocked this effect, which can be explained by the high density of 5-HT_{2A}Rs in cortical pyramidal neurons (Bubser et al., 2001; Lopez-Gimenez et al., 1997; Pompeiano et al., 1994; Santana et al., 2004), some of which synapse onto VTA mesocortical and mesolimbic neurons(Carr and Sesack, 2000).

Although a link between the reinforcing effects of cocaine and DA elevations specifically in the NAc has been well-established, the relevance of the caudate to the behavioral effects of cocaine has been less clear. In contrast, emerging evidence of the neurochemical and neurobiological substrates of cocaine-induced and cue-induced reinstatement has revealed an important role of the caudate. In addition, recent work has clarified the roles and interactions of various brain regions and associated neurotransmitters in drug-maintained behaviors in addition to cue-induced and drug-induced reinstatement (Kalivas, 2007; Kalivas, 2009; Kalivas and Volkow, 2005). Many of these results stem from the recent in-depth characterization of the complex functional neuroanatomic relationship between midbrain DA nuclei, striatal regions, and prefrontal cortical regions. For example, diverse research has indicated an important role of the dorsal striatum, including the caudate and putamen, in the integration of limbic, cognitive, and motor inputs that is required for the execution of complex behaviors which are influenced by a host of motivational and predictive factors (Haber et al., 2006; Haber and Knutson, 2010). It is well known that the striatum of mammals is logically ordered along a ventromedial to dorsolateral axis from regions related more to limbic systems in the ventral striatum (NAc) to cognitive and associative regions in the medial striatum (caudate/putamen) finally terminating at motor regions of the dorsal striatum (Haber et al., 2000). Each of these regions receives efferent input from prefrontocortical regions that mirror their ventral to dorsal gradient. Integration is thought to occur through a complex striatonigralstriatal (SNS) system that connects ventral and dorsal striatum, as well as midbrain DA nuclei through an “ascending spiral” formation (Haber et al., 2000; Haber and Knutson, 2010). At the end of this pathway is the dorsal striatum, which is thought to be a central associative junction, since it receives limbic information passed from the

ventral striatum and associative information from the medial striatum; the dorsolateral caudate outputs to the entire cortex (Haber et al., 2000).

Recent advances in studies of neuroadaptive changes in the striatum have coalesced into a compelling and wide-ranging theory that describes the changes in neuroanatomical and neurochemical control of behavior as an animal repeatedly encounters a salient environmental event, such as administration of a psychostimulant (Kalivas, 2007). A key neurotransmitter involved in these recent studies is glutamate, the primary neurotransmitter released from terminals of projections from the PFC. These findings bear great value in the interpretation of the current results, as they tie together 5-HT_{2A}Rs, the likely downstream effects of antagonism of 5-HT_{2A}Rs, behavior, and neurochemistry. As is clear from binding and mRNA hybridization and immunocytochemical studies, 5-HT_{2A}Rs are most densely and widely expressed across the PFC, primarily on glutamatergic pyramidal neurons (Jakab and Goldman-Rakic, 1998; Lopez-Gimenez et al., 2001). Many studies pointed towards dense apical dendrite localization and high somatodendritic localization. Importantly, the predicted effect of M100 on these cells, reduction of glutamate release from pyramidal glutamatergic projections, was verified in a recent study (Ansah et al., 2011).

This theory holds that the behavioral response to initial administrations of a drug rely largely on DA release from the VTA into the NAc, amygdala, and PFC, but that repeated exposures build increasingly powerful associations made between drug administration and environmental cues, such that contextual cues take on DA-releasing effects. These associative processes are thought to be centrally driven by drug-induced DA release via mesocortical pathways into the PFC (Kalivas, 2007; Kalivas, 2009). Therefore, once the responding of an animal has been extinguished, exposure to drug-associated cues in the SA environment induces reinstatement. Mechanistic experiments revealed that cue-induced reinstatement is mediated by cue-induced DA release into the PFC and amygdala, which causes the activation of glutamatergic projections between PFC and amygdala and to the VTA and NAc. Further investigations

convincingly linked cocaine-primed reinstatement to glutamate release into the NAc core by PFC glutamatergic pyramidal neurons (McFarland et al., 2003). An important caveat to the decisiveness of this result is that these experiments were carried out with rats, which were trained under the simplest possible operant schedule for the shortest time to achieve results; other experiments continued investigation of the role of neuroplastic changes throughout the DA systems across a broad range of conditions.

Since increased duration of behaviors tied to limbic responses, such as SA, continually increase associations between cues and brain responses, it follows that regions that are implicated in associations between limbic and motor regions would increase in sensitivity to cues and become more sensitive to disruption. This is the basis for the hypothesis, which has accumulated substantial support, that dorsal striatum plays a central role in reinstatement responding. This theory integrates the recent findings related to functional connections and neural circuits such as the striatonigrostriatal loop to studies of habitual behaviors. It is well described that as the duration of SA by an animal increases, the circuits that mediate the control over behavior and neurochemical response continue to undergo neuroplastic changes (Porrino et al., 2007). These changes are accompanied by a gradual shift in the striatal response to cocaine administration from more ventromedial (NAc) regions of the striatum towards more dorsolateral regions (caudate) (Kalivas, 2007; Porrino et al., 2007). In line with this finding, animals trained under schedules that require more intensive training and under which the behavioral output of animals is considerably greater than other schedules (both in absolute number of responses per unit time and in number of responses emitted per reinforcer delivered), such as second-order schedules (Belin and Everitt, 2008; Ito et al., 2002) and progressive ratio schedules (Suto et al., 2011), undergo neuroplastic changes to the striatum that render them sensitive to inhibition of the dorsolateral caudate. Additionally, after training under these schedules, inactivation of the dorsolateral caudate of animals prevents cue- + cocaine-induced reinstatement (Kantak et al., 2002), without

affecting cue-induced-reinstatement. Together, these recent findings provide strong support for the direct role of dorsal striatum in cocaine-induced reinstatement responding.

The findings related to progressively increased dorsal striatum control over extended SA are directly related to the hypothesis that glutamate dysfunction occurs over repeated psychostimulant intake. MSNs in the dorsal striatum receive DAergic input from midbrain DA neurons in addition to glutamatergic input from cortical neurons, some of which are projections from the anterior cingulate. Anterior cingulate binds the greatest amount of M100 of all brain regions in primates, with among the highest levels of mRNA hybridization; these receptors are thought to be found on pyramidal neurons (Lopez-Gimenez et al., 2001). Previous findings demonstrating that M100 decreases glutamate levels in the dorsal striatum (Ansah et al., 2011) coalesce with the glutamate hypothesis of reinstatement and recent elucidation of the importance of the dorsal striatum to suggests that M100 inhibits cocaine-induced reinstatement by reducing prefrontal glutamatergic drive from associative cortices to dorsal striatum. The present work supports, in NHPs, recent studies that have synthesized the advances in research of the neuroanatomical and neurochemical substrates of repetitive/habitual behaviors, but furthermore, the present work has revealed the considerable potential of M100 for treating cocaine abuse.

Therapeutic potential

The results of reinstatement tests clearly demonstrate the effectiveness of selective 5-HT_{2A}R antagonism with M100 in the suppression of cocaine-induced reinstatement. These exciting results point to the potential clinical effectiveness of this compound in the prevention of relapse to cocaine-taking in cocaine addicts, an exciting possibility for several reasons. First, the present lack of effective pharmacotherapeutics to treat cocaine or stimulant abuse enhances the value of any drug that demonstrates real potential. Second, the use of NHP models of cocaine abuse, which are the current highest level of mammalian research that can be performed before human research and which provide a substantially enhanced translational value over experiments

performed with rodent or lower mammal models, further augment the power and value of these results. Third, M100 has completed 2-Phase 3 clinical trials, demonstrating its excellent safety profile in humans, one of the most selective filters in the drug development pipeline; this would additionally reduce the financial burden on the developer of a medication ready for clinical tests since its safety has been amply demonstrated heretofore. Fourth, the development of a successful drug to combat psychostimulant abuse would be a boon to the preclinical drug abuse research world, who would have access to a positive control for relapse models, with which the validity of various models could be evaluated, the potential of experimental treatments could be compared, and the parameters that predispose an individual to relapse to drug taking or remain in remission could be revealed. Finally, since M100 is highly amenable to isotope labeling for PET studies, it would allow for the development of 5-HT_{2A}R availability as a brain marker for predisposition to relapse.

In addition to all the aforementioned benefits, the elucidation of M100 as an effective antipsychotic drug could reveal additional positive effects of treatment in addicts, possibly enhancing clinical treatment success. M100 has a robust history as a promising antipsychotic drug in large-scale preclinical trials, in accordance with its pharmacological relationship to atypical antipsychotics, which prominently antagonize 5-HT_{2A}Rs in addition to D2 receptors (with sometimes considerably greater potency for 5-HT_{2A}Rs) (Markowitz et al., 1999). For example, M100, like other atypical antipsychotics, appears to have equivalent antipsychotic effectiveness, but with a reduced incidence of debilitating side effects, like the extrapyramidal motor deficits that develop over chronic treatment. Although demonstration of the safety and antipsychotic effectiveness of M100 in clinical use would be a great boon to medicine in itself, it would likely enhance the therapeutic potential of M100 as a treatment for stimulant abuse for two reasons. It is well-known in the clinical literature, and especially among emergency room clinicians, that stimulant abuse can manifest as psychosis—such as amphetamine psychosis—in otherwise psychologically healthy individuals. Stimulant abuse can also exacerbate induce relapse to

psychosis in individuals with preexisting psychosis, even after a single dose (for a comprehensive review of the effects of stimulants on individuals with schizophrenia, see (Curran et al., 2004)). Furthermore, there is high comorbidity between psychotic disorders and substance abuse, including abuse of stimulants (Mueser et al., 1992). The potential clinical antipsychotic effectiveness of M100 might additionally prevent any drug relapse-inducing effects of relapse to psychosis in an individual in drug remission who has preexisting psychosis.

Summary

The most important result of this study comes from the reinstatement experiments. The results of reinstatement experiments, which are the best-accepted model of relapse and which have the added translational value inherent in rhesus monkey research, suggest that M100 or another selective 5-HT_{2A}R antagonist may have clinical utility as a treatment to prevent relapse to cocaine taking in cocaine dependent individuals. Furthermore, the results of *in vivo* microdialysis experiments, with which we assessed the direct *in vivo* pharmacological interaction between M100 and cocaine, provide powerful support for a burgeoning hypothesis which implicates the dorsal striatum as critically important in the generation of habitual or relapse-like responding. Furthermore, in light of previous assessments of 5-HT_{2A}R distribution and expression in addition to recent revelations about the central role played by glutamate in behaviors related to addiction, we hypothesize that the divergence between 5-HT_{2A}R-mediated modulation of cocaine-induced DA levels in the caudate and the NAc are a result of the differences in 5-HT_{2A}R-mediated glutamate release into those regions. This difference in 5-HT_{2A}R-mediated glutamate release is a function of the difference in expression of 5-HT_{2A}Rs on glutamatergic pyramidal neurons projecting from Layer V between cortical regions with greater limbic function and those with more associative, somatosensory, or motor function.

When considered as a whole, the results reported in this study are highly valuable, both in the demonstration of the therapeutic potential of M100 in the treatment of cocaine addiction and

in the elucidation of the complex mechanism underlying the interaction between 5-HT_{2A}Rs and mesocortical/mesolimbic DA systems. Furthermore, the well-accepted exceptional translational value of NHP research indicates the enhanced likelihood that the results of the present study will provide the foundation for a future positive clinical or preclinical outcome.

Chapter 5: References

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