Effects of Distinct Populations of Adenosine Receptors in the Ventral Striatum on Cocaine Seeking

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Effects of Distinct Populations of Adenosine Receptors in the Ventral Striatum on Cocaine Seeking

Thesis directed by Assistant Professor Ryan K. Bachtell, Ph.D.

Abstract

Drug-associated cues or pharmacological stimuli induce cocaine seeking by enhancing dopamine and glutamate neurotransmission in the nucleus accumbens (NAc). Adenosine is an inhibitory neuromodulator of dopamine and glutamate signaling and represents a viable target for decreasing relapse vulnerability. Postsynaptic adenosine A₁ receptors and adenosine A₂A receptors co-localize with dopamine receptors on distinct populations of medium spiny neurons in the NAc. The co-localization of dopamine and adenosine receptors is meaningful in that adenosine receptor stimulation antagonizes dopamine receptor signaling and alters the activity of NAc output pathways. Presynaptic adenosine receptors are expressed on glutamate terminals in the NAc where A₁ receptors inhibit and A₂A receptors enhance glutamate release in the NAc. The overarching goal of these studies was to determine the how distinct populations of adenosine receptors modulate striatal signaling to influence cocaine seeking. Our results indicate that adenosine receptors oppositely modulate cocaine seeking depending on the receptor subtype and their synaptic locale. Postsynaptic adenosine A₂A receptor stimulation in the NAc decreases cocaine seeking by disrupting dopamine D₂ receptor signaling in the NAc. Postsynaptic blockade of adenosine A₂A receptors enhances cocaine seeking by facilitating dopamine D₂ receptor signaling. Blockade of
presynaptic adenosine $A_{2A}$ receptors, on the other hand, reduces cocaine seeking, potentially by tempering augmented glutamate release that drives reinstatement. Lastly, adenosine $A_1$ receptor stimulation or presynaptic adenosine $A_{2A}$ receptor blockade during extinction produces long-term changes in relapse susceptibility. The findings suggest that modulating specific populations of NAc adenosine receptors are influential in cocaine seeking and may represent viable pharmacotherapeutic strategies.
Dedication

This work is dedicated to my grandparents: Tom Myers, Judy Myers, Joann O’Neill, and Pete O’Neill.
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“No more time to tell how
This is the season of what
Now is the time of returning
With thought jewels polished and gleaming

Now is the time past believing
The child has relinquished the reign
Now is the test of the boomerang
Tossed in the night of redeeming

Eight sided whispering hallelujah hatrack
Seven faced marble eye transitory dream doll
six proud walkers on jinglebell rainbow
Five men writing in fingers of gold
Four men tracking down the great white sperm whale
Three girls wait in a foreign dominion

Ride in the whale belly
Fade away in moonlight
Sink beneath the waters
to the coral sand below”

-Robert Hunter
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Chapter 1: Introduction and Overview

History and Significance

Cocaine is a naturally occurring alkaloid found in the leaves of the *Erythroxylon coca* plant. Cocaine makes up only 1% of the coca leaf when grown in high altitudes, and people living in the Andes mountains have chewed these leaves for at least 1200 years (Altman *et al.*, 1985). Ancient civilizations in South America, Mexico, Indonesia and the West Indies used coca leaves for medicinal, religious and ceremonial reasons. The arrival of the Spanish in South America eventually brought coca to Europe where the cocaine alkaloid was first isolated in the mid-1800s by a German PhD student. It became a popular additive to wines, tonics, and patent medicines, and was even marketed as a cure for morphine addiction in the late 1800s (Goldstein *et al.*, 2009). However, by the turn of the century cocaine’s addictive properties and potential for serious medical complications became apparent and in 1914 it became illegal in the United States. By the 1920s cocaine use had significantly declined partially due to the introduction of amphetamine (Dackis and O’Brien, 2001). It wasn’t until the 1960s that cocaine resurfaced as a popular drug of abuse with a glamorous reputation (Goldstein *et al.*, 2009). By the late 1970s epidemic levels of cocaine addiction developed in the United States, this was especially spurred by the advent of “crack,” an affordable free-base form of cocaine (Dackis *et al.*, 2001).

Cocaine abuse in the United States persists as a significant public health problem. The National Survey on Drug Use and Health estimates that more than 34
million Americans over the age of 12 have used cocaine at least once in their lifetime, and 2.1 million are current users of cocaine (SAMHSA, 2008). Cocaine is the most common drug-related cause of emergency department visits in the United States (NIDA, 2009). More than 1.3 million people in the United States needed treatment for cocaine addiction, but less than 300,000 were actually admitted in to treatment facilities in 2008 (SAMHSA, 2008). Unfortunately, rates of relapse following treatment for addiction are high, between 40-60% (NIDA, 2009). Not only does addiction impact the addicted individual’s life negatively, frequently leading to incarceration, job loss and homelessness, its negative consequences reach beyond the individual resulting in higher crime rates and approximately 181 billion dollars in economic cost to the United States every year (NIDA, 2009).

The Nature of Cocaine Addiction

Addiction can be described as cyclic pattern of use, abstinence, and relapse. Initial drug use is voluntary and activates the reward pathways of the brain resulting in euphoria in the user. These positive reinforcing effects of the drug are what primarily drive continued use in the beginning stages of addiction. However, chronic cocaine use produces neuroadaptations and users frequently experience withdrawal symptoms (Cornish and Kalivas, 2001; Dackis et al, 2001; Leshner and Koob, 1999; Thomas et al, 2008). These adaptations mediate the transition from use motivated by the positive reinforcing effects of cocaine to use motivated by the negative reinforcing effects of cocaine, like decreasing unpleasant withdrawal symptoms. Much research has
suggested that repeated cocaine use decreases behavioral control, and addicts in this stage of addiction find themselves going to extraordinary lengths to acquire cocaine (Cornish et al., 2001; Dackis et al., 2001; Leshner et al., 1999; Thomas et al., 2008; Volkow and Fowler, 2000; Volkow et al., 1992). At this stage, many addicts will attempt to quit using. Unfortunately, because cocaine addiction involves the primary reward centers of the brain it takes on the characteristics of a primary survival drive, thus maintaining abstinence difficult and relapse common (Dackis et al., 2001; Leshner et al., 1999).

Criteria put forth by the American Psychiatric Association (2013) for diagnosing substance use disorder are divided into 4 clusters: impaired control (e.g. inability to decrease drug use), social impairment (e.g. failure to fulfill major obligations due to drug use), risky use (e.g. recurrent use in hazardous situations), and pharmacologic dependence (e.g. withdrawal symptoms when not using or using less). Cocaine addiction is characterized by compulsive drug use despite negative consequences, and high rates of relapse even after long periods of abstinence. Continued cocaine seeking is driven by the desire to feel the euphoria or “high” associated with administration coupled with the need to suppress the negative symptoms (e.g. anhedonia and anxiety) that accompany withdrawal from chronic use (Kiyatkin, 1994; Leshner et al., 1999). Cocaine craving and relapse to drug taking in abstinent human addicts is typically precipitated by one of three major stimuli: a stressful life event, a stimulus previously associated with drug use, or reexposure to cocaine itself (Leshner et al., 1999; Schmidt and Pierce, 2010; Shaham et al., 2003).
Intravenous Drug Self-Administration and Reinstatement

The scientific community has developed several models of cocaine addiction. Intravenous (IV) self-administration of cocaine, in which animals perform an operant task (e.g. lever-pressing) to self-administer a drug via an IV catheter, is the most common animal model of relapse (Kalivas and McFarland, 2003; Schmidt et al, 2010). To model relapse, rodents typically undergo a period of cocaine self-administration followed by extinction of the drug-reinforced behavior (e.g. previously drug-paired lever pressing) in the absence of cocaine. Once animals have adequately extinguished, the ability for stress, drug-associated stimuli, or cocaine to reinstate non-reinforced lever pressing is assessed. This model of relapse has proved to have excellent face validity in that stimuli that trigger cocaine craving in human addicts also produce reinstatement in animals (Epstein et al, 2006). The predictive validity of this model, however, is unclear since we have yet to uncover a successful pharmacotherapy to prevent relapse to cocaine addiction (Epstein et al, 2006). Despite this drawback, the reinstatement model of addiction has good construct validity that has proven useful in elucidating the cellular and molecular mechanisms as well as the neural circuitry underlying cocaine seeking behavior (Kalivas et al, 2003; Nestler, 2005; Volkow et al, 2000).

Mechanisms of Cocaine Action in the Dopamine System

Cocaine blocks the reuptake of all the monoamines dopamine, norepinephrine, and serotonin although neuropharmacological studies have established a critical role for
central nervous system dopamine in the acute rewarding effects of cocaine. Mice lacking expression of the dopamine transporter (DAT) gene failed to show increased locomotor activity to cocaine (Giros et al., 1996), and showed dramatically decreased self-administration of cocaine compared to serotonin transporter (SERT) knockout mice (Thomsen et al., 2009). Additionally, mice engineered to express a cocaine-insensitive DAT show decreased cocaine reward in cocaine conditioned place preference and self-administration paradigms (Chen et al., 2006; Thomsen et al., 2009). Low doses of dopamine receptor antagonists reliably block cocaine conditioned place preference (Pruitt et al., 1995), development and expression of cocaine sensitization (Fontana et al., 1993), and acquisition of cocaine self-administration (De Wit and Wise, 1977; Leshner et al., 1999; Woolverton, 1986; Woolverton and Johnson, 1992).

There are two primary dopamine systems within the brain. The nigrostriatal system projects from the substantia nigra to the caudate putamen, and the mesocorticolimbic dopamine system that projects from the ventral tegmental area (VTA) to the nucleus accumbens (NAc), frontal cortex and amygdala (Thomas et al., 2008). The nigrostriatal system is typically associated with movement generation for well-established behavior patterns. The mesocorticolimbic system, on the other hand, has been primarily implicated in goal-directed learning and motivated behavioral outcomes. Thus, this system has been associated with the reinforcing actions of cocaine based on a number of studies. First, this pathway way is necessary for the reinforcing effects of cocaine since selective destruction of dopamine neurons in the mesocorticolimbic pathway with 6-hydroxydopamine abolished cocaine self-administration (Roberts et al,
Second, increased extracellular dopamine in the NAc is a common characteristic of acute responses to addictive substances, including cocaine (Di Chiara, 1995; Di Chiara and Imperato, 1988; Pontieri et al, 1995).

Neurons originating in the VTA release dopamine into terminal areas such as the NAc upon presentation of salient stimuli and during episodes of reward-based learning. The NAc consists (~90%) of medium spiny GABA projection neurons (MSNs) and a variety of inhibitory (GABA-expressing) and excitatory (acetylcholine-expressing) interneurons. Of these, the MSNs have received the greatest attention since they are the primary projection neurons and form two distinct output pathways (Aubert et al, 2000; Steiner and Gerfen, 1998). The direct pathway MSNs express the opioid peptide dynorphin and project back to the VTA, while the indirect pathway MSNs express the opioid peptide enkephalin and innervate the ventral pallidum, a key output structure of the ventral striatum (Cornish et al, 2001; Lu et al, 1998). In addition to the direct and indirect pathways being distinguished by opioid peptide expression they also display distinct expression of the two main subtypes of dopamine receptors, dopamine D₁ and dopamine D₂ receptors (Lu et al, 1998). The direct pathway expresses dopamine D₁ receptors and the indirect pathway expresses dopamine D₂ receptors (see figure 1.1). There is a subset of MSNs that express both dopamine D₁ and D₂ receptors, however these neurons make up less than 10% of the neurons within the NAc and the functional significance and neuroanatomical projection sites are less clear (Girault, 2012).

Dopamine D₁ and D₂ receptors are distinguished based on their G-protein coupling and the downstream effects on cAMP production and PKA-mediated signaling
Figure 1.1 Direct and Indirect Pathways of the Ventral Striatum. Dopamine from the ventral tegmental area stimulates dopamine receptors on medium spiny GABA neurons in the nucleus accumbens. Dopamine D₁ receptors are expressed on the direct pathway which projects to the ventral tegmental area, and dopamine D₂ receptors are expressed on the indirect pathway which projects to the ventral pallidum.
Dopamine D₁ receptors signal through Gα₄/olf to stimulate adenylyl cyclase, leading to the production of cAMP, and the activation of PKA (Sibley et al., 1998). In contrast, dopamine D₂ receptors signal through Gαᵢ/o inhibiting adenylyl cyclase, decreasing production of cAMP, and limiting PKA activation (Lachowicz et al., 1997). DARPP-32, a phosphoprotein regulated by cAMP and dopamine, is a major target of PKA and is highly expressed in dopamine responsive striatal and cortical neurons (Borgkvist and Fisone, 2007; Svenningsson et al., 2005). DARPP-32 integrates signals from multiple neurotransmitters to bidirectionally modulate PKA activity, and plays a critical role in the regulation of downstream signal transduction pathways (Greengard et al., 1999). Dopamine D₁ receptor stimulation increases PKA phosphorylation of DARPP-32 which then inhibits PP1, an inhibitor of PKA, to further enhance PKA activation (Greengard et al., 1999). Conversely, dopamine D₂ receptor stimulation results in dephosphorylation of PP2B, and this converts DARPP-32 into a potent inhibitor of PKA (Borgkvist et al., 2007; Svenningsson et al., 2005). Interestingly, the loss DARPP-32 in dopamine D₁ neurons decreases cocaine-induced locomotion, while loss of DARPP-32 in dopamine D₂ neurons increases cocaine-induced locomotion (Bateup et al., 2010).

Postsynaptic dopamine receptor stimulation also modulates the direct and indirect pathway neurons by influencing glutamate input to the ventral striatum. Studies have indicated that activation of the PKA cascade through dopamine D₁ receptor stimulation has direct effects on the trafficking and function of ionotropic glutamate receptors. Dopamine D₁ receptor activation of PKA increases surface expression of
GluA1 containing α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors and N-methyl-D-aspartate (NMDA) receptors (Hallett et al., 2006; Snyder et al., 2000), and also enhances NMDA receptor activated currents in these neurons (Cepeda et al., 1993; Liu et al., 2004). Dopamine D₁ receptor stimulation has also been shown to alter Ca²⁺ and K⁺ channels to enhance glutamate signaling (Gerfen and Surmeier, 2011; Lobo and Nestler, 2011; Surmeier et al., 2007). Conversely, dopamine D₂ receptor stimulation decreases the activity of PKA to inhibit the excitability of the indirect pathway. In fact, dopamine D₂ receptor stimulation promotes the dephosphorylation of serine 845 residue on the GluA1 subunit to decrease surface expression of AMPA receptors (Hakansson et al., 2006), and decreases AMPA receptor currents in indirect pathway neurons (Cepeda et al., 1993). Dopamine D₂ receptor stimulation also alters Ca²⁺, Na⁺, and K⁺ channels to decrease glutamate signaling (Gerfen et al., 2011; Lobo et al., 2011; Surmeier et al., 2007). Thus, dopamine input to the NAc modulates excitatory glutamate input onto medium spiny neurons to ultimately determine behavioral output.

Both types of dopamine receptors play an important role in mediating the addictive properties of psychostimulants like cocaine. It appears that dopamine D₁ receptors are more important for the initial rewarding/stimulating effects of cocaine, but that dopamine D₂ receptors may ultimately become more important in mediating the effects of chronic cocaine use. Studies in non-cocaine addicted humans have also shown that the subjective effects of methylphenidate, a drug that mimics the mechanism of cocaine, are correlated with dopamine D₂ receptor occupancy (Volkow et al., 1999b). In this study, lower levels of dopamine D₂ receptor occupancy were correlated with
increased “liking” (Volkow et al, 1999b), but in chronic cocaine users cue-induced craving was positively correlated with dopamine D2 receptor occupancy (Wong et al, 2006). This idea is further supported by the fact that antagonism of dopamine D1 receptors block the acquisition and expression of cocaine conditioned place preference, but dopamine D2 receptor antagonism has no effect (Cervo and Samanin, 1995). In locomotor sensitization models of addiction, dopamine D1 and D2 receptors are necessary for the development of cocaine sensitization, but only dopamine D2 receptors are necessary for its expression (Fontana et al, 1993).

Additionally, PET studies of human cocaine addicts show decreases in dopamine D2 receptor expression, but no change in dopamine D1 receptors (Martinez et al, 2004; Volkow et al, 1993; Volkow et al, 1990). Interestingly, chronic cocaine self-administration in rats has been shown to increase the expression of dopamine D2 receptors existing in high affinity state, which may explain why a decrease is observed in human addicts (Briand et al, 2008). However, in self-administration models both dopamine D1 and D2 antagonists administered in the nucleus accumbens decrease cocaine self-administration (Barrett et al, 2004), but another study found that after 2 week access to cocaine dopamine D1 receptor antagonism had less pronounced effects on progressive ratio responding of animals exposed to long access cocaine self-administration sessions (Ramoa et al, 2014). In reinstatement paradigms systemic administration of dopamine D2 receptor, but not dopamine D1 receptor agonists, induces cocaine seeking (Self et al, 1996). In fact, systemic administration of dopamine D1 receptor agonists or antagonists block cocaine-primed reinstatement (Khroyan et al,
2000). It is important to note, however, that stimulation of both dopamine D$_1$ and D$_2$ receptors in the NAc is sufficient to produce reinstatement responding (Bachtell et al, 2008; Schmidt and Pierce, 2006b).

**Neuroadaptations in Glutamate Transmission**

Glutamate is the main excitatory neurotransmitter in the central nervous system, and it is estimated that more than 80% of the synapses in the brain use glutamate (Siegel et al, 2006). For many years research focused on the contribution of dopamine to addictive processes, but in the past 15 years glutamate has emerged as an increasingly important neurotransmitter in relapse and reinstatement of cocaine seeking behavior. Glutamate input to the NAc (see figure 1.1) comes from several sources including the medial prefrontal cortex (mPFC), amygdala, and hippocampus (Britt et al, 2012; Carlezon and Thomas, 2009). While each of these brain regions can regulate certain aspects of reinstatement responding, numerous studies have revealed that glutamate signaling from mPFC to the NAc is necessary to induce cocaine seeking (Cornish et al, 1999; Cornish and Kalivas, 2000; Kalivas, 2004; McFarland and Kalivas, 2001; McFarland et al, 2003). Extracellular glutamate levels in the nucleus accumbens are largely unaffected by acute cocaine administration, but after chronic cocaine taking and withdrawal animals show disrupted glutamate homeostasis (Kalivas, 2009). This disrupted glutamate homeostasis is defined by decreased basal levels of extracellular glutamate, but exacerbated increases in extracellular glutamate in the NAc in response to a cocaine challenge (Kalivas, 2009). Studies have established that the decreases in
basal glutamate levels observed after withdrawal from chronic cocaine self-administration are a result of reduced activity of the cystine glutamate-antiporter (Baker et al, 2003a; Baker et al, 2003b).

Glutamate signaling is mediated by several subfamilies of ionotropic receptors including: NMDA receptors and AMPA receptors. These tetrameric receptors are made up of different subunit compositions that provide functional diversity among the subfamilies. The contribution of NMDA receptors to cocaine addiction is unclear, conflicting reports have been published indicating both decreases and increases of NR1 and NR2A/B receptor subunits following withdrawal from cocaine-self administration (Gass and Olive, 2008; Lu et al, 2003; Self et al, 2004), and intra-accumbens administration of both NMDA receptor agonists and antagonists produce reinstatement in rats (Cornish et al, 1999; Cornish and Kalivas, 2000; Famous et al, 2007). AMPA receptors, on the other hand, show increases in GluA1 subunit surface expression following chronic cocaine self-administration (Conrad et al, 2008). Interestingly, re-exposure to cocaine has been shown reduce surface expression of GluA1 subunits 24 hrs later (Boudreau et al, 2007). However, increases in phosphorylation of GluA1 subunits immediately following dopamine D1 receptor stimulation appear to mediate cocaine seeking (Anderson et al, 2008; Hobson et al, 2013).

Glutamate signaling is also mediated by metabotropic glutamate receptors (mGluRs) that modulate intracellular signaling pathways through their associated G proteins. There are three main families of mGluRs: group I (mGluR 1 and 5), group II (mGluR 2 and 3), and group III (mGluR 4 and 6-8). Systemic or intra-NAc blockade of
mGluR5 decreases cocaine-primed reinstatement (Kumaresan et al, 2009; Lee et al, 2005). Stimulation of mGluR2/3 has been shown to decrease cocaine-primed reinstatement, however these effects were also seen on food seeking indicating possible non-specific effects (Peters and Kalivas, 2006). Although it is not fully clear how the receptors that mediate glutamate transmission are regulated following chronic cocaine self-administration, the necessity of glutamate release from the mPFC to the accumbens in cocaine-induced drug seeking has been established.

Following cocaine self-administration and extinction a systemic injection of cocaine produces a robust increase in glutamate in the NAc, which can be attenuated by inactivation of the mPFC (McFarland et al, 2003) or by AMPA receptor blockade in the NAc (Cornish et al, 2000). Similarly, cocaine administered directly into the mPFC induces reinstatement that can be blocked by antagonism of AMPA receptors in the NAc (Park et al, 2002). Cocaine-primed reinstatement is also associated with decreased extracellular GABA in the ventral pallidum, presumably arising from indirect pathway neurons in the accumbens. Thus, this decreased GABA input to the ventral pallidum is dependent on glutamate release from the mPFC to the NAc (Tang et al, 2005; Torregrossa et al, 2008). Taken together these finding strongly support the idea that activation of glutamatergic projections from the mPFC to the NAc are responsible for cocaine seeking, and this is further substantiated by human studies demonstrating that cocaine craving in addicts is accompanied by increased mPFC activation (Volkow et al, 1999a; Volkow et al, 2005).
Past and Present Pharmacotherapies for Cocaine Addiction

There are currently no approved pharmacotherapies for cocaine addiction. In the United States, treatment for cocaine addiction ranges from residential in-patient treatment facilities to intensive outpatient programs, cognitive behavioral therapy, individual psychotherapy, family therapy, and self-help groups. Typically treatment focuses on detoxification, behavioral modifications, and strategies to cope with craving. While non-pharmacological therapies are likely affecting brain function, the ultimate solution to decreasing relapse susceptibility may be the discovery of pharmacotherapies that can normalize the neuroadaptations produced by chronic cocaine use.

Since the 1980s numerous treatments for cocaine addiction have been assessed, and relatively few have shown any significant effects. Because cocaine reward heavily relies on increased dopamine signaling antipsychotics, therapeutics that mainly block dopamine receptors, were some of the earliest treatments for cocaine dependence. A meta-analysis of 10 studies found that antipsychotics did not differ from placebo in decreasing the number of cocaine use days, severity of addiction, or cocaine craving (Kishi et al, 2013). Unfortunately, in addition to having no effect on cocaine dependence, antipsychotics also produced severe side effects (e.g. depression, dizziness, akathisia) leading to greater rates of intolerability-related discontinuation (Kishi et al, 2013). Dopamine agonists have also been assessed as a potential substitution-based pharmacotherapy for cocaine addiction. Although the side effects were less severe, no improvement in abstinence from cocaine was seen (Amato et al, 2011). Due to cocaine’s effects on serotonin reuptake, antidepressants have been...
examined for effectiveness in producing abstinence from cocaine. Similarly, a meta-
alysis of these studies found no significant effects on abstinence from cocaine
compared to placebo (Pani et al, 2011).

More recently, interest in glutamate mechanisms of cocaine addiction led to the
development of d-cycloserine as a therapeutic for treatment of cocaine dependence. D-
cycloserine is a partial agonist at NMDA receptors, and has been shown to facilitate
extinction of cocaine self-administration in rodents (Thanos et al, 2011a; Thanos et al,
2011b). However, in a randomized, placebo-controlled study of human addicts, d-
cycloserine did not facilitate extinction of cocaine craving, but may have enhanced it
(Price et al, 2013). No differences were observed between groups in post-extinction
cocaine use (Price et al, 2013).

The most recently developed pharmacotherapy for cocaine addiction is N-
acetylcysteine, a prodrug that drives the cystine-glutamate antiporter. N-acetylcysteine
shows promise as an antirelapse medication. As previously mentioned, disruption of the
cystine-glutamate antiporter is responsible for the decreased levels of basal glutamate
in the nucleus accumbens following chronic cocaine self-administration (Baker et al,
2003a; Baker et al, 2002). Administration of N-acetylcysteine restores basal glutamate
levels and normalizes corticostriatal function (Moussawi et al, 2011). N-acetylcysteine
administered prior to extinction training or during abstinence prevents reinstatement to
cocaine seeking for two weeks after administration by reversal of the neuroplasticity
required for reinstatement (Madayag et al, 2007; Moussawi et al, 2011; Reichel et al,
2011). Preliminary studies in human cocaine addicts show decreased cocaine craving to
an experimenter delivered IV injection of cocaine following 4 days of N-acetylcysteine administration (Amen et al, 2011), decreased responsiveness to cocaine cues while taking N-acetylcysteine, and facilitates termination/reduction of cocaine use in treatment seeking individuals (Mardikian et al, 2007). In a larger double-blind placebo-controlled trial N-acetylcysteine failed to decrease cocaine use in cocaine-dependent volunteers, but significantly decreased craving and time to relapse in subjects who had already achieved abstinence before entering the trial (LaRowe et al, 2007).

Adenosine: A Neuromodulator of Dopamine and Glutamate Signaling

Adenosine is a neuromodulator of both dopamine and glutamate signaling and for this reason, among others, it has become a viable target for decreasing relapse vulnerability (Fuxe et al, 2007a). Adenosine is tonically released into the synapse through bidirectional nucleoside transporters. Phasic release occurs through the vesicular release and subsequent metabolism of adenosine triphosphate (ATP) in the synapse (Fredholm and Dunwiddie, 1988; Fredholm et al, 1984; Svenningsson et al, 1999a). Adenosine signals through two main receptor subtypes expressed in the central nervous system, adenosine A1 and A2A receptors (Ferre, 1997; Ferre et al, 1992). Adenosine A1 receptors are widely expressed in the brain, existing presynaptically on most glutamate and dopamine terminals and postsynaptically on cortical and hippocampal pyramidal neurons (Fuxe et al, 2007a; Linden, 1991). Importantly, adenosine A1 receptors are also expressed postsynaptically on MSNs in the striatum and are highly colocalized with dopamine D1 receptors in the direct pathway (see figure
1.1) (Aubert et al, 2000; Fuxe et al, 2007a). Adenosine A$_{2A}$ receptors, on the other hand, have dense expression in the striatum, but little expression in other brain regions (Svenningsson et al, 1999b). Adenosine A$_{2A}$ receptors are typically postsynaptic on MSNs in the striatum and are highly colocalized with dopamine D$_2$ receptors in the indirect pathway (see figure 1.1) (Ferre et al, 1993a; Svenningsson et al, 1997).

Presynaptic adenosine A$_{2A}$ receptors exist in smaller proportions and are localized to a subset of glutamate terminals that specifically synapse onto direct pathway MSNs (Quiroz et al, 2009; Rosin et al, 2003).

Both types of adenosine receptors are G protein coupled with adenosine A$_1$ receptors being inhibitory (G$_{\alpha_i/o}$) and adenosine A$_{2A}$ receptors being stimulatory (G$_{\alpha_{s/olf}}$) (Ferre et al, 1997; Linden, 1991; Svenningsson et al, 1999b). Importantly, the G protein coupling of adenosine A$_1$ and A$_{2A}$ receptors opposes the intracellular signaling cascades of dopamine D$_1$ and D$_2$ receptors, respectively, on MSNs where these receptors are colocalized (Ferre et al, 1997). In addition to their opposing intracellular signaling effects, adenosine and dopamine receptors form heteromeric receptor complexes (A$_1$-D$_1$ and A$_{2A}$-D$_2$) where adenosine and dopamine receptors have direct antagonistic interaction with one another (Ferre et al, 2004; Fuxe et al, 2008).

In fact, stimulation of adenosine A$_1$ receptors results in decreased binding affinity at dopamine D$_1$ receptors and facilitates the formation of the A$_1$-D$_1$ heteromeric receptor complex (Ferre et al, 1994b; Ferre et al, 1998; Fuxe et al, 2007a), while dopamine D$_1$ receptor stimulation decreases the expression of the A$_1$-D$_1$ receptor heteromers (Franco et al, 2003). Although most of this work has been done in vitro, co-immunoprecipitation
experiments have verified the existence of this heteromer in the rat striatum and have shown that repeated cocaine injections disrupt the expression of the A₁-D₁ heteromer (Toda et al., 2003). Interestingly, receptors maintain their heteromeric receptor complexes with co-administration of both an adenosine A₁ receptor agonist and a dopamine D₁ receptor agonist, but show decreased dopamine D₁ signaling to adenylyl cyclase indicating a possible uncoupling of the Gaαs/olf from the dopamine D₁ receptor (Gines et al., 2000). In the absence of the A₁-D₁ heteromeric complex the antagonistic interactions of the receptors play out through their intracellular signaling cascades. Thus, the presence of adenosine A₁ receptor agonists leads to a decrease in dopamine D₁-induced cAMP production, and the presence of adenosine A₁ receptor antagonists potentiate the production of cAMP in response to dopamine D₁ receptor stimulation (Ferre et al., 1998).

In the indirect pathway MSNs, adenosine A₂A receptor stimulation decreases the binding affinity of dopamine D₂ receptors likely due to a conformational change in the binding pocket (Salim et al., 2000). Additionally, adenosine A₂A receptor activation leads to a reduction of Gaαi/o coupling to the dopamine D₂ receptor (Ferre et al., 1993b). Adenosine A₂A receptor stimulation has also been shown to counteract dopamine D₂ receptor-induced intracellular calcium responses (Salim et al., 2000). Stimulation of dopamine D₂ receptors decreases firing rates of the indirect pathway MSNs, and this effect can be enhanced by antagonism of adenosine A₂A receptors and attenuated by adenosine A₂A agonists (Stromberg et al., 2000). Thus, in experimental conditions adenosine A₂A receptor agonists function similarly to dopamine D₂ receptor antagonists,
and removal of the adenosine A\textsubscript{2A} receptors' tonic inhibition on dopamine D\textsubscript{2} receptors results in decreased activation of the indirect pathway. As with adenosine and dopamine heteromeric receptor complexes in the direct pathway, cocaine decreases the expression of A\textsubscript{2A}-D\textsubscript{2} heteromers in the indirect pathway (Marcellino \textit{et al}, 2010). With regard to the opposing intracellular cascades of adenosine A\textsubscript{2A} and dopamine D\textsubscript{2} receptors, dopamine D\textsubscript{2} receptors can inhibit adenosine A\textsubscript{2A} receptor-induced increases in cAMP (Ferre \textit{et al}, 1993a; Fuxe \textit{et al}, 2005; Kull \textit{et al}, 1999; Svenningsson \textit{et al}, 1999a). Additionally, activation of adenosine A\textsubscript{2A} receptors facilitates increased excitability in the indirect pathway MSNs through amplified PKA activity that results in greater phosphorylation of AMPA and NMDA receptors and results in increased phosphorylation of DARPP-32, a target potently inhibited by dopamine D\textsubscript{2} receptor agonists (Fuxe \textit{et al}, 2007a; Fuxe \textit{et al}, 2007b; Hakansson \textit{et al}, 2006; Hakansson \textit{et al}, 2004). In microdialysis experiments, intra-NAc administration of an adenosine A\textsubscript{2A} receptor agonist increases extracellular levels of GABA in the ventral pallidum (Ferre \textit{et al}, 1994a), where decreases in GABA are necessary for reinstatement of cocaine seeking (Tang \textit{et al}, 2005; Torregrossa \textit{et al}, 2008).

Presynaptic expression of adenosine A\textsubscript{1} and A\textsubscript{2A} on glutamate terminals in the nucleus accumbens provide an important means to regulate phasic glutamate release (Orru \textit{et al}, 2011b). Here stimulation of adenosine A\textsubscript{1} receptors inhibits the release of glutamate, while stimulation of adenosine A\textsubscript{2A} receptors facilitates glutamate release (Ciruela \textit{et al}, 2006; Orru \textit{et al}, 2011b). On these glutamate terminals adenosine A\textsubscript{1} and A\textsubscript{2A} receptors have also been shown to form heteromeric receptor complexes (Ciruela \textit{et al}, 2006; ...
These heteromers appear to be a functional “molecular switch” that controls glutamate release in response to extracellular levels of adenosine (Ciruela et al., 2006). Consequently, adenosine A₁ and A₂A receptors have opposing effects on intracellular signaling, and low concentrations of adenosine promote signaling through adenosine A₁ receptors inhibiting glutamate release (Ciruela et al., 2006; Ferre et al., 2008; Quiroz et al., 2009). Under conditions of high extracellular adenosine, glutamate release is stimulated by signaling through the adenosine A₂A receptor, which inhibits adenosine A₁ receptor signaling through intramembrane receptor interaction (Ciruela et al., 2006; Ferre et al., 2007; Ferre et al., 2008; Quiroz et al., 2009). It is unclear how chronic cocaine administration effects extracellular adenosine levels, but repeated cocaine has been shown to increase adenosine tone in the VTA (Bonci and Williams, 1996).

Due to the ability of adenosine receptors to modulate activity of dopamine and glutamate in the striatum, adenosine receptors are practical targets for tempering the neuroadaptations seen after chronic cocaine use. In fact, several studies have indicated a role for adenosine receptors in cocaine-mediated behaviors. Increased in locomotor activity to acute administration of cocaine is reduced by the adenosine A₂A receptor agonist, CGS 21680, and enhanced by the adenosine A₂A receptor antagonist, MSX-3, (Poleszak and Malec, 2002b; Rimondini et al., 1997). Systemic A₂A receptor stimulation also impairs the initiation of cocaine self-administration (Knapp et al., 2001) and reduces cocaine sensitization (Filip et al., 2006). Non-selective antagonism of adenosine A₁ and A₂A receptors induces reinstatement (Green and Schenk, 2002; Weerts and Griffiths,
2003), and stimulation of either adenosine A₁ or A₂A receptors blocks the expression of cocaine sensitization (Hobson et al, 2012) and attenuates cocaine seeking (Bachtell and Self, 2009; Hobson et al, 2013; Weerts et al, 2003). Additionally, blockade of adenosine A₂A receptors reverses reward impairments produced by cocaine withdrawal (Baldo et al, 1999). Based on these findings, adenosine receptors appear to have the ability to modulate multiple cocaine-related behaviors through their effects on dopamine and glutamate transmission. The work presented here will focus on elucidating the specific role of several populations of adenosine receptors in cocaine seeking, and present a novel target for preventing relapse.
Chapter 2: Adenosine $A_{2A}$ Receptors in the Nucleus Accumbens Bi-directionally Alter Cocaine Seeking in Rats

Abstract

Repeated cocaine administration enhances dopamine $D_2$ receptor sensitivity in the mesolimbic dopamine system, which contributes to drug relapse. Adenosine $A_{2A}$ receptors are colocalized with $D_2$ receptors on NAc medium spiny neurons where they antagonize $D_2$ receptor activity. Thus, $A_{2A}$ receptors represent a target for reducing enhanced $D_2$ receptor sensitivity that contributes to cocaine relapse. The aim of these studies was to determine the effects of adenosine $A_{2A}$ receptor modulation in the NAc on cocaine seeking in rats that were trained to lever press for cocaine. Following at least 15 daily self-administration sessions and 1 week of abstinence, lever pressing was extinguished in daily extinction sessions. We subsequently assessed the effects of intra-NAc core microinjections of the $A_{2A}$ receptor agonist, CGS 21680 (4-[2-[[6-amino-9-(N-ethyl-b-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzenepropanoic acid hydrochloride), and the A2A receptor antagonist, MSX-3 (3,7-dihydro-8-[(1E)-2-(3-methoxyphenyl)ethyl]-7-methyl-3-[3-(phosphonooxy)propyl-1-(2-propynyl)-1H-purine-2,6-dione disodium salt hydrate), in modulating cocaine- and quinpirole-induced reinstatement to cocaine seeking. Intra-NAc pretreatment of CGS 21680 reduced both cocaine- and quinpirole-induced reinstatement. These effects were specific to cocaine reinstatement as intra-NAc CGS 21680 had no effect on sucrose seeking in rats trained to self-administer sucrose pellets. Intra-NAc treatment with MSX-3 modestly reinstated
cocaine seeking when given alone, and exacerbated both cocaine- and quinpirole-
induced reinstatement. Interestingly, the exacerbation of cocaine seeking produced by 
MSX-3 was only observed at sub-threshold doses of cocaine and quinpirole, suggesting 
that removing tonic $A_{2A}$ receptor activity enables behaviors mediated by dopamine 
receptors. Taken together, these findings suggest that $A_{2A}$ receptor stimulation reduces, 
while $A_{2A}$ blockade amplifies, D$_2$ receptor signaling in the NAc that mediates cocaine 
relapse.
Introduction

The mesolimbic dopamine (DA) system is involved in many aspects of addiction, including drug reward, craving, and relapse behaviors (Shaham et al, 2003; Shalev et al, 2002). Activation of this pathway through stress exposure, drug associated cues, and pharmacological stimuli is known to mediate relapse to cocaine seeking (Shaham et al, 2003). The mesolimbic DA system consists of DA cells in the VTA that project to the NAc among other forebrain targets.

Drugs of abuse stimulate DA release in the NAc that is mediated by two major classes of DA receptors that are distinguished by their intracellular signaling cascades among other aspects. DA binding at dopamine D₁ receptors increases adenylyl cyclase activity, while DA binding at dopamine D₂ receptors decreases the activity of this enzyme (Lachowicz et al, 1997). In addition, dopamine D₁ and D₂ receptors are primarily expressed on two distinct populations of NAc neurons, with dopamine D₁ receptors occurring mainly on dynorphin/substance P-expressing neurons and dopamine D₂ receptors on enkephalin-expressing neurons (Lu et al, 1998). These subpopulations of neurons comprise the direct and indirect striatal pathways, respectively, that differ in their projection targets as well as their influence on behavioral output (Aubert et al, 2000; Steiner et al, 1998).

Repeated cocaine administration produces alterations in DA receptor-mediated responses. Thus, repeated cocaine administration produces cross-sensitization with dopamine D₂ receptor agonists (Ujike et al, 1990), and while dopamine D₁ and D₂ receptors are necessary for the acquisition of behavioral sensitization, only dopamine
D$_2$ receptors are necessary for its expression (Fontana et al., 1993). In self-administration models, systemic and intra-accumbens stimulation of dopamine D$_2$ receptors produces robust reinstatement to cocaine seeking (Bachtell et al., 2005; De Vries et al., 1999; Dias et al., 2004; Khroyan et al., 2000; Schmidt et al., 2006b; Self et al., 1996), and dopamine D$_2$ receptors appear to mediate cue-induced relapse to cocaine seeking (Cervo et al., 2003; Gal and Gyertyan, 2006). Therefore, tempering dopamine D$_2$ receptor-mediated behaviors following chronic cocaine administration could prove useful in preventing relapse.

A known modulator of DA neurotransmission is adenosine. Adenosine activity is mediated by subtypes of adenosine receptors including adenosine A$_{2A}$ receptors that are heavily expressed in the striatum, where they are highly co-localized with dopamine D$_2$ receptors on enkephalin-containing neurons of the indirect pathway (Fink et al., 1992; Svenningsson et al., 1999b). Adenosine A$_{2A}$ receptors exert tonic inhibitory control over dopamine D$_2$ receptor signaling within the striatum (Farrar et al., 2010; Hakansson et al., 2006; Harper et al., 2006; Nagel et al., 2003; Weber et al., 2010). Thus, adenosine A$_{2A}$ receptor stimulation decreases DA binding at dopamine D$_2$ receptors (Ferre et al., 1991b). A recent study has suggested that this may be mediated by heteromeric receptor complexes comprised of adenosine A$_{2A}$ and dopamine D$_2$ receptors (Marcellino et al., 2010). Interestingly, cocaine was shown to reduce the expression of the A$_{2A}$-D$_2$ receptor heteromer, which may partially explain the enhanced dopamine D$_2$ receptor-mediated behaviors following repeated cocaine administration (Marcellino et al., 2010).
Recent studies have shown an involvement of adenosine $A_{2A}$ receptors in the behavioral effects of cocaine. For example, systemic adenosine $A_{2A}$ receptor stimulation impairs the initiation of cocaine self-administration (Knapp et al., 2001), reduces cocaine sensitization (Filip et al., 2006), and blocks reinstatement of cocaine seeking (Bachtell et al., 2009). The non-specific adenosine antagonist, caffeine, produces modest reinstatement (Green et al., 2002; Worley et al., 1994), while specific antagonism of adenosine $A_{2A}$ receptors enhances cocaine sensitization (Filip et al., 2006). It remains unclear whether these adenosine $A_{2A}$ receptor effects on cocaine behaviors are mediated by adenosine $A_{2A}$ receptors in the NAc. Therefore, the present study examines whether adenosine receptor effects on cocaine seeking are mediated by adenosine $A_{2A}$ receptors localized to the NAc. These experiments test the effects of intra-NAc adenosine $A_{2A}$ receptor stimulation or blockade on cocaine seeking in animals extinguished from cocaine self-administration. Local infusions of CGS 21680, a selective adenosine $A_{2A}$ receptor agonist, and MSX-3, a phosphatase prodrug of the adenosine $A_{2A}$ receptor antagonist MSX-2 (Muller et al., 1998; Sauer et al., 2000), were made into the medial division of the NAc core, a site where dopamine $D_2$ receptor stimulation is sufficient for reinstatement (Bachtell et al., 2005; McFarland and Kalivas, 2001).

**Materials and Methods**

*Animals*
Male Sprague-Dawley rats (Charles River, Wilmington, MA) initially weighing 275-325 grams were individually housed with food and water available ad libitum. All experiments were conducted during the light period of a 12-hr light/dark cycle in accordance with the guidelines established by the Institutional Animal Care and Use Committee at the University of Colorado at Boulder.

Surgery

Surgical implantation of jugular catheters and intracranial cannulae occurred in concert. Catheters were implanted into the jugular vein under halothane anesthesia (1-2.5%). Each rat was then placed into a stereotaxic instrument, the scalp was incised and retracted, and the head was positioned with Bregma and Lambda at the same depth coordinate. Screws were secured into the skull and holes were drilled in order to bilaterally insert guide cannulae into the NAc core (A/P: +1.7, M/L +/-1.5, D/V -5.7 from bregma; (Paxinos and Watson, 1998). Once inserted, the guide cannulae were fixed in place with dental cement. Dummy stylets extending 1 mm beyond the tip of the cannulae were placed into the guide cannulae to maintain patency. Animals showing signs of post-surgical distress were administered (S)-(+-)Ketoprofen (5mg/kg), a nonsteroidal anti-inflammatory analgesic (Carabaza et al, 1996). Catheters were flushed daily with 0.1 mL heparinized saline and rats were allowed 4-7 days recovery in their home cage before experimental procedures began.

Drugs
Adenosine $A_{2A}$ receptor agonist, CGS 21680 [4-[[6-Amino-9-(N-ethyl-b-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino] ethyl]benzenepropanoic acid hydrochloride] was purchased from Tocris Bioscience (Ellisville, MO). Adenosine $A_{2A}$ receptor antagonist, MSX-3 [3,7-dihydro-8-[(1E)-2-(3-methoxyphenyl)ethenyl]-7-methyl-3-[3-(phosphonooxy)propyl]-1-(2-propynyl)-1H-purine-2,6-dione disodium salt hydrate], dopamine $D_2$-selective agonist, Quinpirole [(-)-Quinpirole hydrochloride], and Cocaine hydrochloride were obtained from Sigma-Aldrich (St. Louis, MO). All drugs were dissolved in sterile-filtered physiological (0.9%) saline.

**Cocaine self-administration, extinction and reinstatement procedures**

Self-administration procedures were performed in operant conditioning chambers (Med-Associates, St. Albans, VT) equipped with two response levers and an infusion pump system. Animals were initially trained to lever press for sucrose pellets to facilitate acquisition of cocaine self-administration. After 24-48 hr of food-restriction, rats were trained to lever-press for sucrose pellets on a fixed ratio 1 (FR1) reinforcement schedule until acquisition criteria was achieved (100 sucrose pellets in one session). After lever-press training, animals were fed ad libitum for at least 1 day prior to surgery (see above).

After recovery from surgery, animals were allowed to self-administer intravenous cocaine (0.5 mg/kg/100 μL injection) on a fixed ratio 1 (FR1) reinforcement schedule in daily 4-hr sessions for 5–6 d/wk. Cocaine injections were delivered over 5 s concurrent with the illumination of a cue light above the active lever and was followed by a 15 s
time out period (TO 20s) when the house light remained off and responding produced no consequence. Inactive lever responses produced no consequence throughout testing.

After a minimum of 15 cocaine self-administration sessions, animals remained in their home cages for 7 days of forced abstinence. On days 8-13 following self-administration, animals returned to the operant conditioning chambers for extinction training. Extinction sessions occurred in the absence of cocaine reinforcement in 4-hr test sessions. Responses on the lever previously paired with cocaine injections during self-administration (drug-paired lever) and on the inactive lever were recorded but had no programmed drug or cue delivery.

Each reinstatement session was initiated with 2-hr of extinction conditions followed by a 2-hr reinstatement test period. In most experiments, an intra-NAc pretreatment was administered prior to a pharmacological prime (see below), which was immediately followed by the 2-hr reinstatement test period. Responses at both the previously drug-paired and inactive levers were recorded but resulted in no cue or drug delivery during testing.

\[A_{2A} \text{ antagonist (MSX-3)-primed reinstatement}\]

Two groups of animals were used to assess the effects of systemic and intra-NAc treatments of MSX-3 on reinstatement. MSX-3 is a prodrug of the selective adenosine \(A_{2A}\) receptor antagonist MSX-2 that is rapidly converted to its active form by phosphatases in vivo (Muller et al, 1998; Sauer et al, 2000), and has been shown to be
suitable for intracranial microinfusion (Hauber et al., 1998). Animals in one group were given systemic injections of MSX-3 (vehicle, 3, and 6 mg/kg, i.p.) following the extinction session. Animals in a separate group were given intra-NAc injections of MSX-3 (vehicle, 5, 10, and 20 ug/side). Immediately following the systemic treatments and 5 min after the intra-NAc microinjections the animals underwent 2-hr of reinstatement testing. Animals in both groups were tested under all conditions in a randomized order and received a maximum of 4 treatments. Responses at both levers were recorded, but resulted in no cue or cocaine delivery.

**Effects of A\(_{2A}\) receptor stimulation and blockade on cocaine-primed reinstatement**

The effects of intra-NAc adenosine A\(_{2A}\) receptor stimulation on cocaine-primed reinstatement were tested by a pretreatment of the adenosine A\(_{2A}\) agonist, CGS 21680 (vehicle, 0.5, 1.0, 2.5, 5.0 and 10 ng/side), 5 min prior to the priming injection of cocaine (vehicle or 15 mg/kg i.p.). In a separate group of animals the effects of systemic and intra-NAc adenosine A\(_{2A}\) receptor blockade on cocaine-primed reinstatement was tested by a pretreatment of the adenosine A\(_{2A}\) antagonist, MSX-3 (5 and 10 mg/side), 5 min prior to a priming injection of cocaine (vehicle, 5, or 10 mg/kg i.p.).

**Effects of A\(_{2A}\) receptor stimulation or blockade on D\(_2\) agonist-primed reinstatement**

The effect of intra-NAc adenosine A\(_{2A}\) receptor stimulation on dopamine D\(_2\) receptor-primed relapse behavior was assessed by a pretreatment of the adenosine A\(_{2A}\) agonist, CGS 21680 (vehicle or 2.5 ng/side), administered 5 min prior to quinpirole
treatment (0.3 mg/kg). The effect of intra-NAc adenosine A<sub>2A</sub> receptor antagonism on dopamine D<sub>2</sub> receptor-primed relapse behavior was assessed by administration of a pretreatment of the adenosine A<sub>2A</sub> antagonist, MSX-3 (vehicle and 10 ug/side, intra-NAc), 5 min prior to quinpirole treatment (vehicle, 0.1, 0.3, and 1.0 mg/kg, i.p.).

**Sucrose Reinstatement**

Animals were trained to self-administer sucrose pellets on an FR1:TO 20 sec schedule as described above. After 15 daily sessions (50 pellets/session), animals remained in their home cages for 7 days of “abstinence”, and were then subjected to extinction training in five daily 4-hr sessions. Following extinction training, animals were tested for reinstatement of sucrose seeking. A pretreatment of CGS 21680 (2.5 ng/side, intra-NAc microinfusion) was administered 5 min prior to sucrose reinstatement testing. Reinstatement testing was initiated by non-contingent sucrose pellet delivery in a single 2-hr test immediately following 2-hr of extinction conditions. During the reinstatement phase, animals were presented with the non-contingent delivery of a sucrose pellet every 2 min for the first 10 min of the session (total of 5 pellets). Responding at both levers was recorded, but resulted in no cues or sucrose pellet delivery.

**Locomotor Testing**

Locomotor activity was recorded in plexiglass chambers (San Diego Instruments) measuring 16x16x15 inches with 16 pairs of photobeams spaced 1 inch apart on both the x and y axes. All locomotor tests were performed in darkened chambers during the
light phase of the light:dark cycle. One week following the completion of the self-administration and reinstatement procedures, animals were habituated to the locomotor testing chambers for 2-hr (1 day prior to cocaine-induced locomotor activity testing). On test day animals were habituated for 1.5-hr, and given a pretreatment of CGS 21680 (vehicle, 2.5 or 5 ng/side, intra-NAc microinfusion). 5 min following the pretreatment, all animals received cocaine (15 mg/kg). Total locomotor activity as measured by number of beam breaks during the 2-hr testing period.

Histology and Microinjections

Microinjections were administered as pretreatments 5 min prior to challenge injections. All microinjections occurred in the NAc at a volume of 0.5-1.0 μL. Infusions occurred over a 1 min period, and the microinjectors were removed 1 min after the full volume of the infusion was given to ensure absorption into the tissues. In these experiments reinstatement was assessed over repeated sessions and animals received a maximum of 5 treatments in a randomized/counter-balanced order. All animals did not receive all treatments due to concerns of residual testing and weakening of reinstatement responding over repeated trials.

After all experimental procedures were complete, rats were euthanized with carbon dioxide gas and 1.0 μL/side of 0.1% cresyl violet was infused intracranially to verify cannulae tip placements. Placements were determined from coronally-sliced sections and recorded on histological maps. Data from rats with incorrect placements were excluded from these studies.
Statistical analyses

The numbers of animals in each group ranged from 4-17 and are reported for each experiment in the figure captions. All reinstatement data (dependent variables: active lever and inactive lever responses) were analyzed by a 2-way ANOVA with lever (within) and treatments with A2A agonists/antagonist-cocaine/quinpirole (between) as the factors unless otherwise noted. Significant interactions were followed up with simple main effects analyses (1-way ANOVA) and post hoc tests (Bonferroni’s comparisons). Sucrose reinstatement data were analyzed by two separate 2-way ANOVAs with session (within) and the CGS-21680/cocaine treatment (between) as the factors. Significant effects were followed up with appropriate post hoc tests. The effect of CGS-21860 pretreatment on cocaine-induced locomotor activity was analyzed by 1-way between subjects ANOVA. Statistical significance was set at p<0.05 for all tests.

Results

Intra-NAc adenosine A2A receptor stimulation dose-dependently blocks cocaine-induced reinstatement

Animals were trained to self-administer cocaine for 3 wks (avg intake: X = 74.0 ± 3.5) and lever responding was extinguished in daily sessions (Figure 2.1a and b). Figure 1c illustrates that an intra-NAc pretreatment of the adenosine A2A agonist CGS 21680 dose-dependently reduces cocaine-induced drug seeking. A significant lever X treatment interaction (F6,72=8.65; p<0.0001) and significant main effects of lever
Figure 2.1 Intra-NAc administration of the adenosine A$_{2A}$ agonist CGS 21680 dose-dependently blocked cocaine-induced reinstatement. (a) Average number of cocaine infusions in each 4 h session over the 3 week cocaine self-administration phase. (b) Extinction training was performed in 6 daily 4 h sessions. (c) The adenosine A$_{2A}$ receptor agonist, CGS 21680, dose-dependently reduced cocaine-induced active lever responding. (d) Injection sites of animals included in the data set. Number of animals per treatment group: 0.0 CGS/saline=17, 0.0 CGS/15 mg/kg cocaine=16, 0.5 ng CGS/15 mg/kg cocaine=6, 1.0 ng CGS/15 mg/kg cocaine=7, 2.5 ng CGS/15 mg/kg cocaine=13, 5.0 ng CGS/15 mg/kg cocaine=10, and 10.0 ng CGS/15 mg/kg cocaine=10. *Significant from 0.0 CGS/saline (p<0.0001 Bonferroni’s post-test); #significant from 0.0 CGS/15 mg/kg cocaine (p<0.0001 Bonferroni’s post-test).
(F_{1,72}=27.82; p<0.0001) and treatment (F_{6,72}=8.77; p<0.0001) were observed.

Subsequent analysis of the interaction found that the cocaine-prime in the absence of CGS 21680 significantly induced active lever pressing, that was dose-dependently decreased by an intra-NAc pretreatment with CGS 21680 (F_{6,72}=8.726; p<0.0001).

Significant effects of CGS 21680 were also observed on the inactive lever (F_{6,72}=2.929; p<0.05).

Intra-NAc adenosine A_{2A} receptor stimulation blocks D_{2} agonist-induced reinstatement

Animals in this experiment averaged 76.2 ± 5.07 cocaine infusions over the last 5 days of self-administration. Figure 2.2a demonstrates that a pretreatment of CGS 21680 (2.5 ng/side) blocks quinpirole-induced reinstatement. A significant treatment x lever interaction (F_{3,36}=23.67; p<0.0001) and significant main effects of treatment (F_{3,36}=24.16; p<0.0001) and lever (F_{1,36}=31.33; p<0.0001) were observed. Simple main effects analysis of the interaction found that quinpirole significantly increased active lever pressing, and that an intra-NAc pretreatment with CGS 21860 prevented this increase (F_{3,36}=24; p<0.0001). A simple main effects analysis of inactive lever and treatment found that quinpirole alone significantly increased inactive lever responding when compared with CGS 21680 alone (F_{3,36}=3.52; p<0.05). While systemic stimulation of adenosine A_{2A} receptors is not sufficient to completely block dopamine D_{2} agonist-induced reinstatement, our findings suggest that intra-NAc stimulation of adenosine A_{2A} receptors effectively inhibits quinpirole-induced reinstatement suggesting a localized interaction of adenosine A_{2A} and dopamine D_{2} receptors within the NAc.
Figure 2.2 Intra-NAc administration of the adenosine A$_{2A}$ agonist CGS 21680 blocks quinpirole-induced reinstatement. (a) An intra-NAc pretreatment of the A$_{2A}$ receptor agonist, CGS 21680 (2.5 ng per side), was sufficient to block D$_2$ agonist-induced reinstatement. (b) Inactive lever presses from the reinstatement session reveal that quinpirole alone significantly increases inactive lever presses, which was prevented by 2.5 ng CGS 21680. (c) Injection sites for animals included in the data set. Number of animals per treatment group: 0.0 CGS/saline=10, 2.5 ng CGS/saline=10, 0.0 CGS/0.3 mg/kg quinpirole=10, and 2.5 ng CGS/0.3 mg/kg quinpirole=10. *Significant from saline/saline (p<0.05 Bonferroni’s post-test). #Significant from saline/0.3 mg/kg quinpirole (p<0.05 Bonferroni’s post-test).
Effects of intra-NAc adenosine A$_{2A}$ receptor stimulation on cocaine-induced locomotor activity

At high doses, systemic adenosine A$_{2A}$ receptor stimulation via CGS 21680 can reduce locomotor activity (Barraco et al., 1993, 1994). To ensure that reduced lever responding was not a result of locomotor suppression we assessed the effects two effective doses of intra-NAc CGS 21680 (2.5 ng/side and 5 ng/side) on cocaine-induced locomotor activity. These tests were performed in a subset of animals that self-administered cocaine. Figure 2.3 illustrates that intra-NAc pretreatment of CGS 21680 at either dose does not produce statistically significant reductions in cumulative cocaine-induced locomotor activity over the two hour session ($F_{2, 10}=1.086$, $p=0.37$). However, qualitative differences in the time-course of cocaine-induced activity were observed at the higher dose (5 ng/side) of CGS 21680 (Figure 2.3b). Analysis of the locomotor time course revealed significant main effects of time ($F_{15, 160}=8.901$, $p<0.0001$) and group ($F_{2, 160}=5.908$, $p<0.01$), but no significant time x treatment interaction ($F_{30, 160}=0.3424$, $p=0.995$). Simple main effects analysis of treatment revealed a significant reduction in locomotor activity of the group receiving 5.0 ng/side CGS 21680 compared with the vehicle group ($p<0.05$).

Effects of intra-NAc adenosine A$_{2A}$ receptor stimulation on sucrose-reinstatement

As an additional control for potential motivational effects of adenosine A$_{2A}$ receptor stimulation we examined the effects of the minimally effective dose of CGS 21680 (2.5 ng/side) on reinstatement to sucrose-seeking using non-contingent delivery
Figure 2.3. Adenosine A$_{2A}$ agonist CGS 21680 does not alter cocaine-induced locomotor behavior. (a) No significant differences were observed in total cocaine-induced locomotor activity over the 2-h test period of animals between receiving a pretreatment with 0.0, 2.5 and 5.0 ng per side CGS 21680 before cocaine (15 mg/kg, intraperitoneally). (b) Time course of locomotor activity illustrating the last 30 min of the habituation period (~30 to 0 min), followed by the effects of 15 mg/kg cocaine (intraperitoneally) with and without a pretreatment of intra-NAc CGS 21680. Note the significant differences in the time course of the cocaine-induced locomotor activity at 5 ng per side CGS 21680 (p<0.01 Bonferroni's post-test). Number of animals per treatment group: 0.0 CGS/15 mg/kg cocaine=4, 2.5 ng CGS/15 mg/kg cocaine=5, and 5.0 ng CGS/15 mg/kg cocaine=4.
of sucrose pellets in animals previously trained to self-administer sucrose pellets (Figure 2.4a). Figure 2.4c shows significant sucrose seeking on the active lever in both groups (\(F_{1, 12}=48.71, p<0.0001\)) that was unaltered by the minimally effective dose of CGS 21860 (\(F_{1, 12}=1.618, p=0.23\)). A significant increase in inactive lever responding was observed during the reinstatement session compared with the extinction session, however in both the extinction session (\(p<0.05\)) and reinstatement session (\(p<0.0001\)) active lever pressing was significantly higher than inactive (data not shown). While an intra-NAc infusion of the adenosine A\(_{2A}\) receptor agonist, CGS 21680 (2.5 ng/side) is sufficient to block both cocaine- and quinpirole-induced reinstatement, it does not affect reinstatement to natural rewards. This suggests that the effects of the agonist on cocaine- and quinpirole-induced reinstatement in cocaine-exposed animals can be disassociated from its effects on sucrose seeking in cocaine-naïve animals.

**Systemic and intra-NAc blockade of adenosine A\(_{2A}\) receptors moderately reinstate cocaine seeking**

Animals in these experiments had an average of 71.44 ± 9.17 cocaine infusions over the last 5 days of self-administration. Figure 2.5a illustrates that a systemic blockade of adenosine A\(_{2A}\) receptors with MSX-3 significantly increases active lever pressing in a dose dependent manner. A significant treatment X lever interaction (\(F_{2, 31}=6.545; p<0.01\)) and significant main effects of treatment (\(F_{2, 31}=5.512; p<0.01\)) and lever (\(F_{1, 31}=12.8; p<0.01\)) were observed. Subsequent analysis of the interaction found
Figure 2.4 Sucrose reinstatement was unaffected by adenosine A$_{2A}$ receptor agonist CGS 21680. (a) Sucrose self-administration was conducted over 3 weeks, and animals’ latency to acquire 50 pellets was recorded. (b) Extinction training was performed in 5 daily sessions until active lever responding was reduced to levels comparable with inactive lever responding. (c) Significant sucrose reinstatement was observed compared with extinguished responding; however, an intra-NAc pretreatment of 2.5 ng per side CGS 21860 failed to alter sucrose seeking compared with vehicle control. Active lever responding is shown during the last hour of extinction (white bars, extinction) and the reinstatement phase (black bars, reinstatement). (d) Injection sites of animals included in the data set. Number of animals per treatment group: saline=7 and 2.5 ng CGS=7. *Significant from extinction (p<0.0001).
Figure 2.5 Systemic and intra-NAc blockade of adenosine A$_{2A}$ receptors via MSX-3 produces cocaine seeking. (a) Systemic administration of MSX-3 (3 and 6 mg/kg) increased active lever responding in a dose-dependent manner. Number of animals per treatment group: saline=13, 3 mg/kg MSX-3=7, and 6 mg/kg MSX-3=14. (b) Intra-NAc administration of MSX-3 (5, 10, and 20 μg per side) increased active lever responding in a dose-dependent manner. The number of animals per treatment group: saline=14, 5 μg MSX-3=6, 10 μg MSX-3=11, and 20 μg MSX-3=6. (c) Injection sites of animals included in the intra-NAc MSX-3 data set. *Significant from saline (p<0.01 Bonferroni's post-test).
that a systemic MSX-3 pretreatment (6 mg/kg) significantly induced active lever pressing ($F_{2, 31}=6.16; p<0.01$). There was no significant effect of systemic administration of MSX-3 on the inactive lever ($F_{2, 31}=1.666, p=0.21$).

Although a systemic blockade of adenosine $A_{2A}$ receptors resulted in a significant increase in active lever responding, the overall reinstatement produced appeared moderate compared to cocaine- and quinpirole-induced cocaine seeking. To determine if a blockade of adenosine $A_{2A}$ receptors localized to the NAc would produce a more robust reinstatement, we assessed the effects of intra-NAc MSX-3 on reinstatement. Animals in these experiments had an average of 69.45 ± 4.5 cocaine infusions over the last 5 days of self-administration. MSX-3 significantly increased active lever pressing in a dose dependent manner, but overall resulted in only a modest reinstatement (Figure 2.5b). A significant treatment X lever interaction ($F_{3, 33}=4.488; p<0.01$) and significant main effects of treatment ($F_{3, 33}=5.636; p<0.01$) and lever ($F_{1, 33}=16.8; p<0.001$) were observed. Subsequent analysis of the interaction found that local microinjections of MSX-3 (10 μg/side) significantly increased active lever pressing ($F_{3, 33}=5.499; p<0.01$). There was no significant effect of the intra-NAc MSX-3 treatment on the inactive lever ($F_{3, 33}=2.462, p=0.08$).

Intra-NAc blockade of adenosine $A_{2A}$ receptors potentiates cocaine- and $D_2$ agonist-induced reinstatement

Because a blockade of adenosine $A_{2A}$ receptors via MSX-3 alone resulted in only modest reinstatement to cocaine seeking, we hypothesized that an intra-NAc
pretreatment of MSX-3 may potentiate reinstatement to sub-threshold doses of cocaine and quinpirole by enabling more potent stimulation of NAc DA receptor stimulation. Animals in these experiments had an average of 70.02 ± 6.82 cocaine infusions over the last 5 days of self-administration. Figure 2.6b demonstrates that an intra-NAc pretreatment of MSX-3 significantly increased active lever responding to a sub-threshold dose of cocaine (5 mg/kg), which alone does not produce reinstatement. A significant treatment X lever interaction (F$_{4, 58}$=13.07; p<0.0001) and main effects of treatment (F$_{4, 58}$=9.279; p<0.0001) and lever (F$_{1, 58}$=83.06; p<0.0001) were observed. A simple main effects analysis of the interaction found that the pretreatment of MSX-3 significantly increased active lever responding to a sub-threshold dose of cocaine (F$_{4, 58}$=10.98; p<0.0001). While significant effects of treatment on the inactive lever were observed (F$_{4, 58}$=2.735, p<0.05), post hoc testing revealed no significant differences between treatment groups.

Additionally, we examined the effect of adenosine A$_{2A}$ receptor blockade on reinstatement induced by the dopamine D$_2$ agonist, quinpirole, to determine if removing the tonic inhibition of the adenosine A$_{2A}$ receptor over the dopamine D$_2$ receptor could enhance responding to dopamine D$_2$ receptor stimulation. Animals in these experiments had an average of 69.92 ± 4.09 cocaine infusions over the last 5 days of self-administration. Figure 2.6c illustrates that an intra-NAc pretreatment of MSX-3 potentiates active lever responding to a sub-threshold dose of quinpirole (0.1 mg/kg), which alone does not significantly increase active lever responding when compared with the vehicle-saline control. A significant treatment X lever interaction (F$_{7, 97}$=5.86;
Figure 2.6 Intra-NAc blockade of adenosine A$_{2A}$ receptors via MSX-3 potentiates reinstatement response to sub-threshold doses of cocaine and quinpirole. (a) An intra-NAc pretreatment with 10 µg per side MSX-3 potentiated active lever responding at a sub-threshold dose of cocaine (5 mg/kg) compared with vehicle pretreatment.

#Significant from saline/5 mg/kg cocaine (p<0.0001 Bonferroni’s post-test). The number of animals per treatment group: vehicle/saline=13, vehicle/5 mg/kg cocaine=13, vehicle/15 mg/kg cocaine=13, 10 µg MSX-3/5 mg/kg cocaine=12, and 10 µg MSX-3/15 mg/kg cocaine=12. (b) Injection sites of animals shown in MSX-3 effects on cocaine-induced reinstatement. (c) An intra-NAc pretreatment of MSX-3 (10 µg per side) significantly increases active lever responding at a sub-threshold dose of quinpirole (0.1 mg/kg) compared with vehicle pretreatment.

#Significant from saline/0.1 mg/kg quinpirole (p<0.05 Bonferroni’s post-test). The number of animals per treatment group: vehicle/saline=29, vehicle/0.1 mg/kg quinpirole=12, vehicle/0.3 mg/kg quinpirole=5, vehicle/1.0 mg/kg quinpirole=13, 10 µg MSX-3/saline=11, 10 µg MSX-3/0.1 mg/kg quinpirole=11, 10 µg MSX-3/0.3 mg/kg quinpirole=12, and 10 µg MSX-3/1.0 mg/kg quinpirole=12. (d) Injection sites of animal included in MSX-3 effects on quinpirole-induced reinstatement.
p<0.0001) and main effects of treatment (F\textsubscript{7, 97}=5.863; p<0.0001) and lever (F\textsubscript{1, 97}=88.87 p<0.0001) were observed. A subsequent analysis of the interaction revealed that an intra-NAc pretreatment of MSX-3 significantly increased active lever responding to a sub-threshold dose of quinpirole (F\textsubscript{7, 97}=5.908; p<0.0001). Again, significant effects of treatment on the inactive lever were observed (F\textsubscript{7, 97}=2.138, p<0.05), however subsequent post hoc testing revealed no significant differences between treatment groups.

**Discussion**

We have previously shown that systemic adenosine A\textsubscript{2A} receptor stimulation attenuates cocaine seeking induced by pharmacological stimuli and drug related cues (Bachtell *et al*, 2009). Here we elucidate the NAc as a primary site of action for these effects. Our findings reveal that pharmacological manipulation of adenosine A\textsubscript{2A} receptors within the NAc bi-directionally alters cocaine seeking in extinguished rats. We show that intra-NAc stimulation of adenosine A\textsubscript{2A} receptors attenuates cocaine seeking induced by pharmacological stimuli such as cocaine and quinpirole suggesting that adenosine A\textsubscript{2A} receptors represent a potential target for therapies aiming to curb relapse vulnerability. Because systemic and higher doses of intra-NAc adenosine A\textsubscript{2A} agonists reduce lever pressing for sucrose (Font *et al*, 2008) and reduce locomotor activity (Barraco *et al*, 1993, 1994), we examined the effects of the minimally effective CGS 21680 dose on sucrose seeking. We show that it is specific to cocaine, since adenosine A\textsubscript{2A} stimulation did not significantly reduce sucrose seeking. Further support
for this comes from a recent study showing that an accumbens specific knockout of adenosine $A_{2A}$ receptors does not alter wakefulness at baseline conditions (Lazarus et al, 2011).

We also demonstrate that intra-NAc blockade of adenosine $A_{2A}$ receptors produces modest cocaine seeking alone. However, combining intra-NAc blockade of adenosine $A_{2A}$ receptors with sub-threshold doses of cocaine and quinpirole results in robust cocaine seeking, suggesting that removing the inhibitory control that the adenosine $A_{2A}$ receptor exerts over the dopamine $D_2$ receptor allows a normally ineffectual dose of cocaine or quinpirole to induce reinstatement. Other models support this tonic inhibitory role of adenosine $A_{2A}$ receptors in behavioral regulation. For example, a recent study demonstrated that blocking adenosine $A_{2A}$ receptors and hence, removing the adenosine “brake”, produces wakefulness (Lazarus et al, 2011). Antagonism of adenosine $A_{2A}$ receptors also restores deficits in effort-related behaviors induced by dopamine $D_2$ receptor blockade (Nunes et al, 2010; Worden et al, 2009), suggesting that adenosine $A_{2A}$ receptors are a tonic modulator of dopamine $D_2$ receptor expressing neurons within the striatum (Harper et al, 2006; Nagel et al, 2003). Our data provide further support that adenosine $A_{2A}$ receptors exert tonic regulation of dopamine $D_2$ receptors and suggests that adenosine $A_{2A}$ receptors are an important modulator of DA-mediated behavior (Farrar et al, 2010; Hakansson et al, 2006; Harper et al, 2006; Nagel et al, 2003; Weber et al, 2010).

These findings agree with previous work showing that stimulation of adenosine $A_{2A}$ receptors counteracts cocaine-mediated behaviors, while antagonism augments
cocaine-mediated behaviors. Administration of an adenosine A2A agonist attenuates both the development and expression of behavioral sensitization to cocaine (Filip et al, 2006), impairs the acquisition of cocaine self-administration (Knapp et al, 2001), and reduces the expression of cocaine conditioned place preference (Poleszak and Malec, 2002a). Blockade of adenosine A2A receptors, on the other hand, enhances both acute and sensitized cocaine-induced locomotor activity (Filip et al, 2006), and enhances discriminative stimulus effects of both cocaine and methamphetamine (Justinova et al, 2003). Antagonism of adenosine A2A receptors during withdrawal also has reward related effects. Blocking adenosine A2A receptors during a brain stimulation reward task reversed the elevated reward threshold produced by cocaine withdrawal, suggesting that removing the tonic activity of adenosine A2A receptors enables DA signaling to restore reward deficits observed during drug withdrawal (Baldo et al, 1999). This explanation is supported by our findings that adenosine A2A receptor blockade produces cocaine seeking by enabling DA receptor stimulation at sub-threshold doses of both cocaine and a dopamine D2 agonist. Together, these findings indicate that pharmacological stimulation of adenosine A2A receptors opposes the behavioral effects of cocaine while pharmacological blockade of adenosine A2A receptors enhances cocaine’s effects.

Studies utilizing genetic deletion of adenosine A2A receptors generally show effects opposite to those reported with pharmacological blockade of adenosine A2A receptors. In fact, adenosine A2A receptor knockout mice display reduced locomotor activity to acute injections of amphetamine and cocaine and impaired development of
amphetamine sensitization (Chen et al, 2003). In addition, adenosine A$_{2A}$ receptor knockout mice show reduced responding for cocaine on an FR1, FR3 and progressive ratio schedule of reinforcement (Chen et al, 2000; Chen et al, 2003; Soria et al, 2006). It is possible that compensatory changes during development or the lack of neuroanatomical specificity of the adenosine A$_{2A}$ receptor knockout contribute to these conflicting results between the two experimental methods. Indeed, a recent study showed striatal-specific knockdown of adenosine A$_{2A}$ receptors enhances locomotor activity in response to cocaine, while a forebrain-specific knockdown of the adenosine A$_{2A}$ receptors reduces cocaine-induced locomotor activity (Shen et al, 2008). Our experiments corroborate these findings by demonstrating that adenosine A$_{2A}$ receptor blockade specifically in the NAc enhances cocaine seeking. Taken together, these findings suggest that adenosine A$_{2A}$ receptors localized in the striatum and NAc provide inhibitory control over cocaine-mediated behaviors such as cocaine seeking as suggested by the pharmacological manipulations of adenosine A$_{2A}$ receptors.

It should be emphasized that the present experiments targeted the medial division of the NAc core, an area is known to be involved in the reinstatement of cocaine seeking (Bachtell et al, 2005; Ito et al, 2004; McFarland et al, 2003). Recently, the NAc has been discussed in terms of a medial-lateral continuum based on “spiraling” dopaminergic innervation and functional consequences (Haber et al, 2000; Heimer et al, 1997; Ikemoto et al, 2005). Modulation of the dopamine input along this medial-lateral continuum supports these functional differences in cocaine seeking. Thus, manipulations of dopamine receptors in the medial divisions of the NAc (shell and
medial core) induce cocaine seeking, while similar manipulations in the lateral NAc core do not regulate cocaine seeking (Bachtell et al, 2005; Schmidt et al, 2006a; Schmidt et al, 2006b). Here, we show that increasing and decreasing adenosine receptor activity in the medial NAc core, is sufficient to inhibit and promote cocaine seeking, respectively.

The NAc is comprised primarily of medium spiny GABAergic neurons that include two distinct subpopulations of neurons that are differentiated by their cellular peptide expression, receptor subtype expression and unique projection targets (Aubert et al, 2000; Steiner et al, 1998). Dopamine D$_1$ receptors are found mainly on dynorphin/substance P-expressing neurons that comprise the direct pathway, while dopamine D$_2$ receptors occur mainly on enkephalin-expressing neurons that comprise the indirect pathway (Lu et al, 1998). DA stimulation of both populations in the NAc elicits cocaine seeking (Bachtell et al, 2005; Schmidt et al, 2006a; Schmidt et al, 2006b). Thus, tempering DA signaling in the NAc is an ideal way to prevent relapse. Adenosine A$_{2A}$ receptors are co-localized with dopamine D$_2$ receptors where they provide reciprocal regulation of dopamine D$_2$ receptors making them a suitable target to temper DA signaling (Canals et al, 2003; Ferre, 1997; Fuxe et al, 2003; Hillion et al, 2002; Svenningsson et al, 1999a; Svenningsson et al, 1998; Svenningsson et al, 1999b).

Adenosine A$_{2A}$ and dopamine D$_2$ receptors interact to alter signaling of medium spiny GABAergic neurons within the striatum through several mechanisms. For example, these receptors form heteromeric receptor complexes through electrostatic interactions (Canals et al, 2003; Fuxe et al, 2003; Hillion et al, 2002). Heteromeric formation of A$_{2A}$-D$_2$ receptors allows adenosine A$_{2A}$ receptor stimulation to inhibit ligand
binding to dopamine D<sub>2</sub> receptors and decrease G-protein coupling at the dopamine D<sub>2</sub> receptor (Ferre et al., 1991a; Fuxe et al., 1998; Hillion et al., 2002; Torvinen et al., 2005). As previously mentioned cocaine reduces the expression of the A<sub>2A</sub>-D<sub>2</sub> heteromer (Marcellino et al., 2010), which may underlie some of the changes in behavioral responses following chronic cocaine intake. It will be critical for future studies to determine the impact of chronic cocaine intake on heteromeric A<sub>2A</sub>-D<sub>2</sub> receptor expression and how selective pharmacological targeting of these heteromers may be relevant behaviorally.

In addition to the contribution of the A<sub>2A</sub>-D<sub>2</sub> receptor heteromers, adenosine A<sub>2A</sub> and dopamine D<sub>2</sub> receptors are coupled to excitatory and inhibitory G proteins, respectively. For example, stimulation of adenosine A<sub>2A</sub> receptors counteracts the effects of dopamine D<sub>2</sub> receptor stimulation on immediate early gene expression (Morelli et al., 1994; Svenningsson et al., 1999a) and opposes dopamine D<sub>2</sub> receptor-mediated signal transduction in the striatum (Yang et al., 1995). Their complementary intracellular signaling also has profound effects on cAMP production and neuronal excitability (Schiffmann et al., 2007; Svenningsson et al., 1999a; Tozzi et al., 2007) suggesting that reciprocal regulation of downstream targets of cAMP (e.g. PKA-mediated phosphorylation targets) may play a role in the modulation of cocaine seeking. While adenosine A<sub>2A</sub> receptors obviously play a significant role in modulating DA neurotransmission within the striatum, the cellular mechanisms of our effects on cocaine seeking remain obscure. While it is likely that both A<sub>2A</sub>-D<sub>2</sub> heteromeric receptors and adenosine A<sub>2A</sub> receptor intracellular signaling contribute to the modulation of these
behaviors, future studies should focus on the independent contributions in determining their role in modulating cocaine-mediated behaviors.

Adenosine $A_{2A}$ receptors are expressed on other cell types in the NAc providing other possible explanations for our results. For example, expression of adenosine $A_{2A}$ receptors on presynaptic glutamatergic terminals is involved in modulating striatal glutamate release and synaptic plasticity (Hettinger et al, 2001; Rodrigues et al, 2005; Troisi et al, 2005). Thus, stimulation of presynaptic adenosine $A_{2A}$ receptors increases striatal glutamate release and blockade of adenosine $A_{2A}$ receptors produces the opposite effect (Corsi et al, 2000; Corsi et al, 1999). It seems unlikely that our findings would result from adenosine $A_{2A}$-induced increases in glutamate release since stimulation of AMPA receptors in the NAc induces cocaine seeking, and blockade of AMPA receptors prevents both cocaine- and cue-induced drug seeking (Cornish et al, 1999). There is also evidence that adenosine $A_{2A}$ receptors are expressed on cholinergic interneurons (Tozzi et al, 2011), although this report conflicts with a previous study where adenosine $A_{2A}$ receptor mRNA was absent in cholinergic interneurons (Svenningsson et al, 1997). Cholinergic interneurons make up a small percentage (<5%) of the cell types in the NAc, but have significant effects on modulating both direct and indirect output pathways from the NAc (Kawaguichi et al, 1995; Tepper and Bolam, 2004). It was recently shown that simultaneous blockade of adenosine $A_{2A}$ receptors and stimulation of dopamine $D_2$ receptors decreases firing of cholinergic interneurons, which consequently reduces the muscarinic $M_1$ receptor activity on medium spiny GABAergic neurons of the striatum (Tozzi et al, 2011). Thus, our findings that an
adenosine $A_{2A}$ antagonist enhances cocaine seeking may result from reduced muscarinic activity. Recent work does not support this notion since blockade of muscarinic receptors in the NAc attenuates cocaine seeking (Mark et al, 2006; Yee et al, 2011). Thus, it is unclear whether our manipulations on adenosine $A_{2A}$ receptors within the NAc are having a large effect on cholinergic interneurons. Future studies will help to elucidate the interactions between adenosine $A_{2A}$ and dopamine $D_2$ receptors on cholinergic interneurons.

Overall, the results of these experiments suggest an important role of adenosine $A_{2A}$ receptors in the modulation of cocaine seeking in an animal model of relapse. We demonstrate that intra-NAc stimulation of adenosine $A_{2A}$ receptors blocks both cocaine- and quinpirole-induced drug seeking while intra-NAc adenosine $A_{2A}$ receptor blockade enhances cocaine seeking. While, the antagonistic interaction between adenosine $A_{2A}$ and dopamine $D_2$ receptors on striatal neuronal transmission is supported by these experiments, the relative contribution of heteromeric and non-heteromeric complexes is unknown. Together, our results suggest that interactions between adenosine $A_{2A}$ and dopamine $D_2$ receptors influence striatal signaling that mediates cocaine seeking, but future studies should examine the specific cellular mechanisms by which adenosine $A_{2A}$ stimulation reduces dopamine $D_2$ receptor mediated behaviors. Finally, the results of this study illuminate the potential for $A_{2A}$ receptor stimulation as an effective strategy for reducing the relapse susceptibility.
Chapter 3: Opposing effects of pre- and postsynaptic adenosine $A_{2A}$ receptor blockade on cocaine seeking

Abstract

Drug-associated cues or pharmacological stimuli are known to mediate cocaine seeking through enhanced dopamine and glutamate neurotransmission in the NAc. Previous work has shown that adenosine receptors modulate reinstatement to cocaine seeking as well as other cocaine-mediated behaviors. Adenosine $A_{2A}$ receptors are expressed on both pre- and postsynaptic terminals in the NAc. Postsynaptic $A_{2A}$ receptors in the NAc are colocalized on dopamine D$_2$ expressing medium spiny GABAergic neurons where they reduce dopamine neurotransmission. Presynaptic $A_{2A}$ receptors are expressed on glutamate terminals in the NAc where they enhance glutamate transmission onto MSNs. The goal of these studies was to examine the differential effects of pre- and postsynaptic $A_{2A}$ receptor blockade on cocaine seeking. Rats were trained to lever press for cocaine in daily self-administration sessions on a fixed-ratio 1 schedule for 10 consecutive days. After one day of abstinence, lever pressing was extinguished in 8-10 daily extinction sessions. We subsequently identified whether a systemic administration of a presynaptic $A_{2A}$ receptor antagonist, SCH 442416, and a postsynaptic $A_{2A}$ receptor antagonist, KW 6002, would reinstate cocaine seeking. Higher doses of KW 6002 (0.3, 1.0, 3.0 mg/kg, i.p.) induced cocaine seeking, while SCH 442416 (1.0, 3.0 mg/kg, i.p.) had no effect. We next examined the effect of pre- and postsynaptic $A_{2A}$ receptor blockade on cocaine-induced reinstatement. Systemic
administration of cocaine (15 mg/kg, i.p.) produced robust reinstatement that was blunted by pretreatment with the presynaptic A<sub>2A</sub> antagonist, SCH 442416 (1.0 mg/kg, i.p.). On the other hand, blockade of postsynaptic A<sub>2A</sub> receptors by KW 6002 (0.3 mg/kg, i.p.) amplified cocaine-induced reinstatement (5 and 15 mg/kg, i.p.). We hypothesize that SCH 442416 reduces reinstatement by dampening excessive cocaine-induced glutamate release in the NAc from the prefrontal cortex (PFC) that is necessary for cocaine seeking. In order to assess this hypothesis, cocaine seeking was induced by either an intra-PFC injection of AMPA (0.8 nM/side) or cocaine (200 mg/side). A systemic pretreatment with the presynaptic A<sub>2A</sub> antagonist, SCH 442416 (1.0 mg/kg, i.p.) significantly reduced both intra-PFC AMPA and cocaine induced reinstatement. These findings suggest that presynaptic A<sub>2A</sub> receptors may be a viable target in tempering the augmented glutamate release that plays a key role in driving reinstatement.
**Introduction**

A major obstacle in the successful treatment of cocaine addiction is the persistence of cocaine craving and prevalence of relapse. In rodents, chronic cocaine self-administration has been shown to alter glutamate and dopamine signaling, and these neurotransmitter systems play a significant role in cocaine seeking (Cornish *et al.*, 2001; Kalivas, 2004; Schmidt *et al.*, 2005; Schmidt *et al.*, 2010). Increased glutamate transmission in the NAc is both necessary and sufficient for reinstatement of cocaine seeking (Cornish *et al.*, 2001; McFarland *et al.*, 2003). Similarly, dopamine D₁ and D₂ receptors in the NAc also play a significant role in reinstatement of cocaine seeking (Anderson *et al.*, 2003; Anderson *et al.*, 2006; Bachtell *et al.*, 2005; Schmidt *et al.*, 2006a). Developing novel pharmacotherapies that modulate the signaling of these systems may be key to decreasing relapse susceptibility in addicts.

There is clear evidence that adenosine acts as a modulator of both glutamate and dopamine through actions at two receptor subtypes expressed in the brain, adenosine A₂A and A₁ receptors (Goodman and Synder, 1982; Svenningsson *et al.*, 1997). Adenosine A₂A receptors are most densely expressed in the striatum (Dixon *et al.*, 1996). Within the accumbens, postsynaptic adenosine A₂A receptors are co-localized on dopamine D₂ expressing indirect pathway medium spiny GABA neurons (MSNs) (Fink *et al.*, 1992; Svenningsson *et al.*, 1999b). Postsynaptic adenosine A₂A receptor stimulation opposes dopamine D₂ receptor signaling (Ferre *et al.*, 1991b), and several studies have shown that adenosine A₂A receptors exert tonic inhibition over dopamine D₂ receptors (Farrar *et al.*, 2010; Hakansson *et al.*, 2006; Harper *et al.*, 2006).
Previous work suggests that adenosine A\textsubscript{2A} receptors can regulate cocaine behaviors. For example, stimulation of adenosine A\textsubscript{2A} receptors reduces sensitization to cocaine (Filip et al, 2006; Hobson et al, 2012), acquisition of cocaine self-administration (Knapp et al, 2001), and reinstatement to cocaine seeking (Bachtell et al, 2009; O'Neill et al, 2012). In contrast, blockade of adenosine A\textsubscript{2A} receptors enhances cocaine sensitization (Filip et al, 2006) and augments reinstatement responding induced by cocaine or a dopamine D\textsubscript{2} receptor agonist (O'Neill et al, 2012). These effects are thought to arise from the antagonistic interactions between postsynaptic adenosine A\textsubscript{2A} receptors and dopamine D\textsubscript{2} receptors.

Phasic increases in glutamate are mediated by presynaptic adenosine A\textsubscript{1} and A\textsubscript{2A} receptors in the nucleus accumbens (Orru et al, 2011b). Stimulation of adenosine A\textsubscript{1} receptors decreases glutamate release, while stimulation of adenosine A\textsubscript{2A} receptors enhances the release of glutamate (Ciruela et al, 2006; Orru et al, 2011b; Quiroz et al, 2009). Interestingly, presynaptic adenosine A\textsubscript{2A} receptors are preferentially located on presynaptic glutamate terminals that synapse onto dopamine D\textsubscript{1} receptor expressing MSNs (Quiroz et al, 2009) and there has been recent interest in how presynaptic adenosine A\textsubscript{2A} receptors contribute to striatal signaling (Ciruela et al, 2006; Orru et al, 2011a). In the dorsal striatum, administration of an antagonist that targets adenosine A\textsubscript{2A} receptors expressed presynaptically decreased glutamate release evoked by electrical stimulation of the cortex (Orru et al, 2011a). Additionally, binding assays for several other adenosine A\textsubscript{2A} receptor antagonists were performed, and SCH 442416 exhibited a much lower affinity for binding in adenosine A\textsubscript{2A} receptor and dopamine D\textsubscript{2} receptors.
receptor expressing cells compared to cells expressing only adenosine $A_{2A}$ receptors (Orru et al., 2011a). Interestingly, a recent paper has found that a presynaptic adenosine $A_{2A}$ receptor antagonism shifts THC self-administration dose response curves to the left, consistent with a decrease in its reinforcing effects, while postsynaptic adenosine $A_{2A}$ receptor antagonism resulted in a shift to the left (Justinova et al., 2014). Previous work from our lab has also shown that blockade of presynaptic adenosine $A_{2A}$ receptors during extinction training results in lasting decreases in susceptibility to drug-primed reinstatement (O'Neill et al., 2014).

The studies presented here distinguish the effects of antagonizing presynaptic vs. postsynaptic adenosine $A_{2A}$ receptors on cocaine seeking. Given the ability of presynaptic adenosine $A_{2A}$ receptor blockade to reduce glutamate release (Orru et al., 2011a), and postsynaptic adenosine $A_{2A}$ receptor blockade to facilitate dopamine $D_2$ receptor signaling (Farrar et al., 2010; Ferre et al., 1994a; Fuxe et al., 2007a), we predict that these antagonists will produce bidirectional effects on cocaine seeking. Thus, we hypothesize that presynaptic adenosine $A_{2A}$ receptor antagonism will reduce reinstatement of cocaine seeking and postsynaptic $A_{2A}$ receptor blockade will enhance cocaine seeking.

**Methods**

**Animals**

Male Sprague-Dawley rats (Charles River, Wilmington, MA) weighing between 300-350 grams were individually housed with food and water available *ad libitum*. 
Experiments were conducted during the light period of a 12-h light/dark cycle in accordance with the guidelines established by the Institutional Animal Care and Use Committee at the University of Colorado Boulder.

**Surgery**

Surgical implantation of jugular catheters and intracranial guide cannulae were implanted under halothane anesthesia (1-2.5%). Jugular catheter implantation was performed according to previously published methodology (O'Neill et al, 2012). Following jugular catheter implantation, some rats (see experimental procedures below) were fitted into a stereotaxic instrument where the scalp was incised and retracted. The head was positioned to place bregma and lambda at the same depth coordinate. Titanium screws were secured into the skull and holes were drilled allowing bilateral insertion of guide cannula into either the NAc shell (A/P: +1.7; M/L: ±0.8; D/V: -5.7 from bregma) or the mPFC (A/P: +2.7; M/L: ±0.6; D/V: -3.2 from bregma) (Paxinos et al, 1998). The guide cannula was then fixed in place by dental cement and dummy stylets extending 1 mm beyond the tip of the guide cannulae were inserted to maintain patency. Following surgery animals were administered (S)-(+-)ketoprofen (5 mg/kg), a non-steroidal anti-inflammatory analgesic (Carabaza et al, 1996) and Baytril (enrofloxacin) (5 mg/kg), a fluoroquinolone antibiotic (Vancutsem et al, 1990). Catheters were flushed daily with 0.1 mL of heparinized saline and rats recovered for a minimum of 4 days before experimental procedures began.
Drugs

The postsynaptic adenosine A\textsubscript{2A} receptor antagonist, KW 6002 ((E)-8-(3,4-Dimethoxystyryl)-1,3-diethyl-7-methylxanthine, 8-[(1E)-2-(3,4-Dimethoxyphenyl)ethenyl]-1,3-diethyl-3,7-dihydro-7-methyl-1H-purine-2,6-dione), presynaptic adenosine A\textsubscript{2A} receptor antagonist, SCH 442416 (2-(2-Furanyl)-7-[3-(4-methoxyphenyl)propyl]-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine; 5-amino-7-(3-(4-methoxyphenyl)propyl)-2-(2 furyl)pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine; 5-Amino-7-[3-(4-methoxy)phenylpropyl]-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine), AMPA receptor agonist, AMPA (\(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), and cocaine hydrochloride were obtained from Sigma-Aldrich (St Louis, MO). All drugs were dissolved in sterile-filtered physiological (0.9\%) saline, except SCH 442416 and KW 6002. SCH 442416 was dissolved in 33\% DMSO and 66\% ddH\textsubscript{2}O. KW 6002 was dissolved in 50\% DMSO and 50\% ddH\textsubscript{2}O.

Cocaine Self-Administration, Extinction and Reinstatement Procedures

Self-administration procedures were performed in operant conditioning chambers (Med-Associates, St Albans, VT) equipped with two response levers and an infusion pump system. Animals were initially trained to lever press for sucrose pellets to facilitate acquisition of cocaine self-administration (O’Neill et al, 2012). After lever-press training, animals were fed ad libitum for at least 24 hr before the catheter and intra-cranial cannula implantation surgery.
Following recovery from surgery, animals were allowed to self-administer intravenous cocaine (0.5 mg/kg/100 μl injection) on a fixed ratio 1 (FR1) reinforcement schedule in daily 2 hr sessions for 10 consecutive days. Cocaine injections were delivered over 5 sec concurrent with the illumination of a cue light above the active lever followed by an additional 15 sec time out period (TO 20 sec) when the house light remained off and responding was without consequence. Inactive lever responses produced no consequence throughout testing.

Twenty-four hours after the last self-administration session, animals underwent 8 daily extinction sessions. Extinction sessions occurred in the absence of cocaine reinforcement in 2 hr test sessions. Thus, responses on the lever previously paired with cocaine injections during self-administration (active lever) and on the inactive lever were recorded, but had no programmed drug or cue delivery. In all experiments, each reinstatement session was initiated with 2 hr of extinction conditions, followed by a 2 hr reinstatement test period. Reinstatement was initiated by a variety of manipulations (see below). During both the extinction phase and reinstatement phase, responses at both the previously active and inactive levers were recorded, but resulted in no cue or drug delivery during testing.

**Adenosine A<sub>2A</sub> Antagonist-Primed Reinstatement**

Following a 2 hr extinction session animals were given systemic injections of either the presynaptic adenosine A<sub>2A</sub> antagonist, SCH 442416 (vehicle, 1.0 mg/kg, and 3.0 mg/kg, i.p.), or the postsynaptic adenosine A<sub>2A</sub> antagonist, KW 6002 (vehicle, 0.3
mg/kg, 1.0 mg/kg, or 3.0 mg/kg, i.p.). After 5 min, animals were returned to the self-administration chambers for a 2 hr test period. Animals were tested under most doses in a randomized order and received a maximum of 4 treatments. Responses at both levers were recorded, but resulted in no cue or cocaine delivery.

*Effects of Pre- and Postsynaptic A<sub>2A</sub> Receptor Blockade on Cocaine-Primed Reinstatement*

The effects of presynaptic A<sub>2A</sub> receptor blockade on cocaine-primed reinstatement were examined by giving a systemic pretreatment of SCH 442416 (vehicle or 1.0 mg/kg, i.p.) followed by cocaine (vehicle or 15 mg/kg, i.p.) 5 minutes later. After the administration of cocaine, animals were immediately placed in the self-administration chambers for the 2 hr reinstatement test period. The effects of postsynaptic A<sub>2A</sub> receptor blockade on cocaine-primed reinstatement were tested by giving a systemic pretreatment with KW 6002 (vehicle or 0.3 mg/kg, i.p.) followed, 5 min later, by a systemic injection of cocaine (vehicle, 5 mg/kg, or 15 mg/kg, i.p.). We chose the 5 mg/kg cocaine dose and 0.3 mg/kg KW 6002 dose since neither produce reliably reinstatement and could reveal a synergistic effect on reinstatement responding. Animals were then immediately returned to the operant chambers for a 2 hr reinstatement session.

*Effects of Pre- and Postsynaptic A<sub>2A</sub> Receptor Blockade on Reinstatement Induced by Cortical Stimulation*
We next examined the effects of presynaptic and postsynaptic $A_{2A}$ receptor blockade on cortical stimulation-induced reinstatement. Animals were pretreated with systemic injections of either the presynaptic adenosine $A_{2A}$ antagonist, SCH 442416 (vehicle or 1.0 mg/kg, i.p.), or the postsynaptic adenosine $A_{2A}$ antagonist, KW 6002 (vehicle or 0.3 mg/kg, i.p.) prior to intra-PFC infusions of either AMPA (0.8 nM/side) or cocaine (200 μg/side). Following microinjection, animals were placed into the self-administration chambers for the 2 hr reinstatement test.

*Effects of Pre- and Postsynaptic $A_{2A}$ Receptor Blockade on Reinstatement Induced by Nucleus Accumbens Stimulation*

The effects of presynaptic and postsynaptic $A_{2A}$ receptor blockade on reinstatement induced by stimulation of the nucleus accumbens was also examined. Animals were pretreated with systemic injections of either the presynaptic adenosine $A_{2A}$ antagonist, SCH 442416 (vehicle or 1.0 mg/kg, i.p.), or the postsynaptic adenosine $A_{2A}$ antagonist, KW 6002 (vehicle or 0.3 mg/kg, i.p.) prior to intra-NAc infusion of AMPA (0.8 nM/side) or cocaine (200 μg/side).

*Microinjections and Histology*

All microinjections were delivered to the PFC or NAcc at a volume of 0.5 μl over a 1-min period. The microinjectors were remained in the cannula for 1 min after the full volume of the infusion was given to ensure absorption of drug into the tissues. After completion of each experiment, rats were euthanized with a Fatal-Plus solution and 1.0
µl of 0.1% cresyl violet was infused bilaterally to verify cannulae tip placements.

Placements were determined from coronally sliced sections and recorded on histological maps. Data from rats with incorrect placements were excluded from these studies.

**Statistical Analyses**

Reinstatement data (dependent variables: active lever and inactive lever responses) were analyzed by a two-way ANOVA with lever (within) and treatments with A$_{2A}$ pre/post antagonists–AMPA/cocaine (between) as the factors, unless otherwise noted. Significant interactions were followed up with post hoc tests (Bonferroni's comparisons). Statistical significance was set at p<0.05 for all tests.

**Results**

*Systemic Blockade of Postsynaptic Adenosine A$_{2A}$ Receptors Produces and Enhances Cocaine-Induced Reinstatement*

Animals were trained to self-administer cocaine for 10 days (average intake: 42.0 ± 3.9) and lever responding was extinguished in daily sessions (Figure 3.1a and b). Figure 3.1c shows that administration of KW 6002, a postsynaptic adenosine A$_{2A}$ receptor antagonist, dose dependently increased cocaine seeking. A significant treatment X lever interactions (F$_{3,81}$=9.37, p<0.0001), and significant main effects of treatment (F$_{3,81}$=9.63, p<0.0001) and lever (F$_{1,81}$=49.01, p<0.0001) were observed. Post hoc testing revealed that KW 6002 (1.0 and 3.0 mg/kg) significantly increased active
lever pressing compared to vehicle (1.0 mg/kg: $t_{81}=4.57$, $p<0.001$; 3.0 mg/kg: $t_{81}=4.37$, $p<0.001$) and the lowest dose (0.3 mg/kg) of KW 6002 (1.0 mg/kg: $t_{81}=3.45$, $p<0.01$; 3.0 mg/kg: $t_{81}=3.66$, $p<0.001$). There was no significant effect of systemic KW 6002 administration on the inactive lever.

We also examined the effect of postsynaptic adenosine $A_{2A}$ receptor blockade on cocaine-induced drug seeking. Animals in this experiment had an averaged 39.6 ± 4.3 cocaine infusions over the last 5 days of cocaine self-administration (data not shown). Pretreatment with KW 6002, at a dose with no effect on cocaine seeking, enhanced reinstatement responding to a cocaine prime (Figure 3.1d). Analysis revealed a significant treatment X lever interaction ($F_{3,31}=3.94$, $p<0.05$) and significant effects of treatment ($F_{3,31}=3.91$, $p<0.05$) and lever ($F_{1,31}=23.39$, $p<0.0001$). Post hoc testing found that 15mg/kg of cocaine increased active lever pressing compared to only 5 mg/kg cocaine ($t_{31}=2.42$, $p<0.05$), and that treatment with KW 6002 prior to administration of 15 mg/kg cocaine increased active lever presses compared to 15 mg/kg of cocaine alone ($t_{31}=3.08$, $p<0.01$). Active lever pressing following treatment with KW 6002 and 15 mg/kg cocaine was also found to be significantly increased compared to KW 6002 and 5 mg/kg cocaine ($t_{31}=3.13$, $p<0.01$) and 5 mg/kg cocaine alone ($t_{31}=4.76$, $p<0.001$). There
Figure 3.1 Postsynaptic $A_{2A}$ receptor antagonism produces reinstatement and enhances cocaine-induced reinstatement. A) Animals self-administered cocaine in over the course of 10 consecutive days in 2-hr sessions. B) Following 24hrs of forced abstinence in the home cage animals underwent 8 consecutive days of extinction training in 2-hr sessions. C) Systemic administration of KW6002 produces cocaine seeking in a dose dependent manner. Right) Pretreatment with KW6002 increases reinstatement responding induced by 15 mg/kg of cocaine. *** p< 0.001 Indicates significant from vehicle; ~ p<0.05 Indicates significant from vehicle--5 mg/kg cocaine; ## p< 0.01 Indicates significant from vehicle--15 mg/kg cocaine; ^ p<0.01 Indicates significant from KW 6002--5 mg/kg cocaine
were no significant effects of KW 6002 or cocaine administration on inactive lever responding.

*Postsynaptic Adenosine A$_{2A}$ Antagonism Increases Cocaine Seeking Induced by Cortical Stimulation*

Because we found that postsynaptic adenosine A$_{2A}$ receptor blockade produced reinstatement and exacerbated cocaine-primed reinstatement, we decided to examine its effects on reinstatement induced by mPFC stimulation. Animals in these experiments had an average intake of 43.8 ± 3.7 daily cocaine infusions for the last 5 days of self-administration (data not shown). Intra-mPFC infusion of AMPA produced reinstatement that was enhanced by pretreatment with KW 6002 (Figure 3.2a). A significant treatment X lever interaction was observed ($F_{3,42}=17.07; p<0.0001$), and significant main effects of lever ($F_{1,42}=78.35; p<0.0001$) and treatment ($F_{3,42}=21.39; p<0.0001$). Bonferroni’s posttests revealed that intra-mPFC AMPA infusion significantly increased active lever responding compared to vehicle ($t_{42}=7.15; p<0.001$) and KW 6002 alone ($t_{42}=4.15$, $p<0.001$), and pretreatment with KW 6002 exacerbated this effect (veh/Intra-PFC AMPA: $t_{42}=4.51$, $p<0.001$; veh/KW 6002: $t_{42}=7.08$, $p<0.001$; veh: $t_{42}=9.58$, $p<0.001$). There were no significant differences in inactive lever presses.

Similarly, local microinjections of cocaine in the PFC produced robust increases in active lever presses, which were exacerbated by postsynaptic adenosine A$_{2A}$ receptor antagonism (Figure 3.2b). A significant treatment X lever interaction ($F_{3,42}=9.93; p<0.0001$), and significant main effects of lever ($F_{1,42}=44.63; p<0.0001$) and treatment
Figure 3.2 Effects of postsynaptic A<sub>2A</sub> receptor blockade on reinstatement induced by prefrontal cortex stimulation. A) Intra-mPFC AMPA induces reinstatement which is enhanced by a pretreatment of KW 6002. B) Intra-mPFC cocaine also produces reinstatement which is amplified prior KW 6002 administration. C) Microinjection sites of animals included in the data set. *** p< 0.001 Indicates significant from vehicle-vehicle; ^ p< 0.001 Indicates significant from KW 6002-vehicle; ### p<0.001 Indicates significant from vehicle--Intra-PFC AMPA; # p<0.05 Indicates significant from vehicle--Intra-PFC AMPA
(F<sub>3,42</sub>=14.40; p<0.0001) were found. Post hoc testing found that intra-mPFC administration of cocaine increased active lever presses compared to vehicle (t<sub>42</sub>=6.35, p<0.001) and KW 6002 (t<sub>42</sub>=3.86, p<0.001) treated animals, and treatment with KW 6002 before intra-mPFC cocaine administration resulted in amplified reinstatement responding compared to cocaine alone (t<sub>42</sub>=2.54; p<0.05), KW 6002 alone (t<sub>42</sub>=5.23, p<0.001), and vehicle (t<sub>42</sub>=7.06, p<0.001). There were no significant effects of treatment on the inactive lever.

*Postsynaptic Adenosine A<sub>2A</sub> Antagonism Increases Cocaine Seeking Induced by Nucleus Accumbens Stimulation*

Stimulation of dopamine receptors or AMPA receptors in the NAc shell can produce reinstatement (Bachtell *et al.*, 2005; Cornish *et al.*, 2000). Blockade of postsynaptic adenosine A<sub>2A</sub> receptors in the nucleus accumbens shell has been shown to enhance cocaine seeking stimulated by systemic administration of cocaine (O'Neill *et al.*, 2012). In this experiment we induced reinstatement with local injections of AMPA into the NAc shell to examine whether postsynaptic adenosine A<sub>2A</sub> receptor antagonism would intensify intra-NAc AMPA-primed cocaine seeking. Animals in these experiments self-administered cocaine for 10 consecutive days and had an average intake of 39.4 ± 4.1 daily cocaine infusions for the last 5 days of self-administration (data not shown). Intra-NAc administration of AMPA following 2 hours of extinction increased active lever pressing, and this effect was enhanced by a pretreatment of KW 6002 (figure 3.3a). Analysis revealed a significant treatment X lever interaction (F<sub>2,31</sub>=47.23, p<0.0001)
Figure 3.3 Effects of postsynaptic A<sub>2A</sub> receptor blockade on reinstatement induced by nucleus accumbens stimulation. A) Intra-NAc AMPA produces reinstatement that is augmented by KW 6002. B) Intra-NAc cocaine also produces reinstatement. C) Microinjection sites of animals included in the data set. *** p< 0.001 Indicates significant from vehicle-vehicle; ### p< 0.001 Indicates significant from vehicle--Intra-NAc AMPA
and significant main effects of treatment ($F_{2,31}=52.03$, $p<0.0001$) and lever ($F_{1,31}=171.1$, $p<0.0001$). Bonferroni’s posttests indicated that administration of AMPA into the NAc shell induced reinstatement compared to vehicle ($t_{31}=8.23$, $p<0.001$), and that blockade of postsynaptic $A_{2A}$ receptors heightened cocaine seeking compared to vehicle ($t_{31}=13.80$, $p<0.001$) and intra-NAc AMPA alone ($t_{31}=5.75$, $p<0.001$). Responses on inactive levers were not affected by treatment.

Additionally, we found that both KW 6002 and vehicle treatments followed by local microinjections of cocaine into the nucleus accumbens shell initiated cocaine seeking in extinguished animals (figure 3.3b). A significant treatment $\times$ lever interaction ($F_{2,28}=12.70$, $p<0.0001$), and significant main effects of treatment ($F_{2,28}=12.80$, $p<0.0001$) and lever ($F_{1,28}=53.53$, $p<0.0001$) were found. Subsequent analysis uncovered a significant increased in reinstatement responding in animals treated with intra-NAc cocaine compared to vehicle treated animals ($t_{28}=5.72$, $p<0.001$). Pretreatment with the postsynaptic adenosine $A_{2A}$ antagonist prior to intra-NAc cocaine infusion also produced cocaine seeking in comparison with vehicle treated animals ($t_{28}=5.87$, $p<0.001$). There were no significant effects of treatment on inactive lever presses.

**Presynaptic Adenosine $A_{2A}$ Receptor Antagonism Decreases Cocaine Seeking**

Blockade of presynaptic adenosine $A_{2A}$ receptors during extinction training has been shown to decrease subsequent cocaine seeking induced by cocaine (O’Neill et al., 2014). This suggests that antagonism of presynaptic adenosine $A_{2A}$ receptors may have
opposing effects to postsynaptic adenosine $A_{2A}$ receptor antagonism, which has been shown to increases cocaine related behaviors (Filip et al., 2006; O'Neill et al., 2012). Because systemic administration of KW 6002 produces dose-dependent increases in cocaine seeking, we first examined the effect of systemic injection of SCH 442416 on animals extinguished from cocaine self-administration. Animals in this experiment had an average intake of $51.9 \pm 5.9$ infusions of cocaine for the last 5 days of cocaine self-administration (data not shown). Administration of SCH 442416 at both 1.0 mg/kg and 3.0 mg/kg had no effect on active lever pressing (Figure 3.4a), however we did observe a significant main effect of lever ($F_{1,24}=13.05$, $p<0.01$) indicating that animals in all groups pressed the active lever significantly more than the inactive lever.

Next we examined the effects of SCH 442416 on cocaine-primed reinstatement. Animals in this experiment averaged $42.3 \pm 4.9$ cocaine infusions over the last 5 cocaine self-administration sessions (data not shown). Systemic cocaine injection (15 mg/kg) produced cocaine seeking which was blunted by a pretreatment with SCH 442416 (1.0 mg/kg) (Figure 3.4b). Statistical analysis revealed a significant treatment X lever interaction ($F_{2,21}=24.25$, $p<0.0001$) and significant main effects of treatment ($F_{2,21}=24.02$, $p<0.0001$) and lever ($F_{1,21}=71.42$, $p<0.0001$). Post hoc tests indicated that 15 mg/kg cocaine increased active lever presses compared to vehicle ($t_{21}=9.78$, $p<0.001$). Pretreatment with SCH 442416 prior to 15 mg/kg of cocaine significantly decreased active lever presses compared with cocaine alone ($t_{21}=5.61$, $p<0.001$), but active lever responding of this group was still significantly increased compared to vehicle ($t_{21}=4.17$, $p<0.001$). No differences in inactive lever pressing were observed.
Figure 3.4 Presynaptic A_{2A} receptor antagonism has no effect on cocaine seeking and blunts cocaine-induced reinstatement. A) Systemic administration of SCH 442416 has no effect on cocaine seeking. B) Pretreatment with SCH 442416 blunts cocaine-primed reinstatement. *** p< 0.001 Indicates significant from vehicle-vehicle; ### p< 0.001 Indicates significant from vehicle-15 mg/kg cocaine.
Presynaptic Adenosine A\textsubscript{2A} Receptor Antagonism Blocks Cocaine Seeking Induced by Cortical Stimulation

Systemic administration of the presynaptic adenosine A\textsubscript{2A} receptor antagonist, SCH 442416, has been shown to decrease glutamate release in the striatum induced by electrical stimulation of the PFC (Orru et al., 2011a). Because increased in glutamate release in the nucleus accumbens has been linked to reinstatement (McFarland et al., 2003) we examined the effect of SCH 442416 on reinstatement induced by prefrontal cortical stimulation. In these experiments, animals self-administered cocaine for 10 consecutive days (average intake for last 5 sessions: 50.6 ± 5.9). Intra-mPFC AMPA, again, produced reinstatement that was blocked by pretreatment with SCH 442416 (figure 3.5a). A significant treatment X lever interaction (F\textsubscript{2,41}=23.83, p<0.0001) and significant main effects of treatment (F\textsubscript{2,41}=19.48, p<0.0001) and lever (F\textsubscript{1,41}=47.72, p<0.0001) were found. Bonferroni’s posttests indicated that local microinjections of AMPA significantly increased responding on the active lever compared to vehicle (t\textsubscript{41}=8.46, p<0.001), pretreatment with SCH 442416 abolished this effect (t\textsubscript{41}=7.61, p<0.001). Inactive lever responding was not affected by any of the treatments.

Similarly, intra-mPFC cocaine induced reinstatement that was eliminated by blockade of presynaptic adenosine A\textsubscript{2A} receptors (figure 3.5b). Analysis showed a significant treatment X lever interaction (F\textsubscript{2,41}=9.18, p<0.001) and significant main effects of treatment (F\textsubscript{2,41}=10.91, p<0.001) and lever (F\textsubscript{1,41}=25.60, p<0.0001). Subsequent post hoc tests revealed increased cocaine seeking following intra-mPFC cocaine compared to vehicle (t\textsubscript{41}=6.06, p<0.001), and administration of SCH 442416
Figure 3.5 Effects of presynaptic A$_{2A}$ receptor blockade on reinstatement induced by prefrontal cortex stimulation. A) Intra-mPFC AMPA induces cocaine seeking that is blocked by SCH 442416. B) Intra-mPFC cocaine also induces cocaine seeking. Again, pretreatment with SCH 442416 attenuates cocaine seeking. C) Microinjection sites of animals included in the data set. *** p < 0.001 Indicates significant from vehicle-vehicle
prior to intra-mPFC cocaine significantly decreased cocaine seeking compared to intra-mPFC cocaine alone \( (t_{41}=4.66, p<0.001) \). Lever presses on the inactive lever were not significantly different.

**Presynaptic Adenosine A\(_{2A}\) Receptor Antagonism has no Effect on Cocaine Seeking Induced by Nucleus Accumbens Stimulation**

Administration of AMPA or cocaine into the nucleus accumbens shell induces cocaine seeking, presumably by activating postsynaptic glutamate and dopamine receptors, respectively. We hypothesized that blockade of presynaptic adenosine A\(_{2A}\) receptors would not affect reinstatement mediated by direct stimulation of postsynaptic glutamate or dopamine receptors. In these experiments animals averaged 39.4 ± 4.1 cocaine infusions over the last 5 days of self-administration. As before, intra-NAc AMPA increased active lever presses, but pretreatment with the presynaptic adenosine A\(_{2A}\) receptors antagonist did not decrease reinstatement (figure 3.6a). A significant treatment X lever interaction \( (F_{2,42}=20.15, p<0.0001) \) and significant main effects of treatment \( (F_{2,42}=17.27, p<0.0001) \) and lever \( (F_{1,42}=88.36, p<0.0001) \) were observed. Post hoc testing revealed that both intra-NAc AMPA alone \( (t_{42}=8.25, p<0.001) \) and pretreatment with SCH 442416 prior to intra-NAc AMPA infusion \( (t_{42}=6.17, p<0.001) \) significantly increased cocaine seeking compared to vehicle. No effects of treatment were found on inactive lever responding.

Similarly, intra-NAc cocaine infusion increased responding on the active lever, but pretreatment with SCH 442416 failed to reduce reinstatement (figure 3.6b). Analysis
Figure 3.6 Effects of presynaptic $A_{2A}$ receptor blockade on reinstatement induced by intra-NAc stimulation. A) Intra-NAc AMPA produces cocaine seeking, and pretreatment with SCH 442416 has no effect. B) Intra-NAc cocaine also induces reinstatement that is not altered by pretreatment with SCH 442416. C) Microinjection sites of animals included in the data set. *** $p<0.001$ Indicates significant from vehicle-vehicle.
revealed a significant treatment X lever interaction ($F_{2,39}=11.80, p<0.0001$) and significant main effects of treatment ($F_{2,39}=17.57, p<0.0001$) and lever ($F_{1,39}=52.65, p<0.0001$). Bonferroni’s posttests showed that both SCH 442416 followed by intra-NAc cocaine infusion ($t_{39}=5.71, p<0.001$) and intra-NAc cocaine infusion alone ($t_{39}=7.11, p<0.001$) significantly increased reinstatement responding compared with vehicle. No effect of treatment on inactive lever responding was observed.

**Discussion**

The results of the studies presented here show that blockade of postsynaptic or presynaptic adenosine A$_{2A}$ receptors produces opposing effects on cocaine seeking. We observed a dose dependent increase in cocaine seeking to KW 6002 alone, which has previously been described as an exclusively postsynaptic adenosine A$_{2A}$ receptor antagonist (Orru et al., 2011a). We also found that pretreatment with KW 6002 enhanced cocaine-primed reinstatement. Similarly, cocaine seeking induced by AMPA or cocaine infusion into the mPFC or by AMPA infusion into the NAc was augmented by pretreatment with KW 6002.

These results are supported by previous literature showing that adenosine A$_{2A}$ receptors exert tonic inhibition over dopamine D$_2$ receptors (Farrar et al., 2010; Hakansson et al., 2006; Harper et al., 2006), and confirms previous work indicating that blockade of adenosine A$_{2A}$ receptors in the NAcc can facilitate cocaine seeking (O’Neill et al., 2012). As previously mentioned, adenosine A$_{2A}$ receptors have their densest expression in the striatum and are highly co-localized with dopamine D$_2$ receptors on
striatopallidal MSNs (Fink et al, 1992; Svenningsson et al, 1999b) where they have opposing intracellular cascades. Stimulation of dopamine D2 receptors in the NAc is necessary for cocaine-primed reinstatement (Anderson et al, 2006; Bachtell et al, 2005; Schmidt et al, 2006a), and decreasing inhibition from adenosine A2A receptors amplifies signaling through these receptors (Filip et al, 2006; Hakansson et al, 2006; Harper et al, 2006). For example, blockade of adenosine A2A receptors with KW 6002 prevents the effort-related behavioral deficits induced by a dopamine D2 receptor antagonist (Nunes et al, 2010) and mimics the cellular signaling effects of a quinpirole, a dopamine D2 receptor agonist (Hakansson et al, 2006).

Reinstatement responding was robustly enhanced by postsynaptic adenosine A2A receptor blockade in nearly all studies measuring cocaine seeking with the notable exception of cocaine seeking induced by cocaine delivery into the NAc. This suggests that inhibition of postsynaptic adenosine A2A receptors, presumably located on the indirect pathway MSNs, increases cocaine seeking regardless of whether dopamine or glutamate initiates reinstatement. Although the enhancement of reinstatement with pretreatment of KW 6002 was not seen with cocaine infusion into the NAc, we suspect there may have been a ceiling effect given that cocaine in the NAc with or without KW 6002 pretreatment produced robust cocaine seeking.

Interestingly, presynaptic adenosine A2A receptor blockade had no effect on cocaine seeking alone, but blunted systemic cocaine-primed reinstatement. Additionally, infusion of either AMPA or cocaine into the mPFC induced reinstatement that was eliminated by presynaptic adenosine A2A receptor blockade. However, intra-NAc AMPA
and cocaine induced cocaine seeking was unaffected by pretreatment with SCH 442416, the presynaptic adenosine A<sub>2A</sub> receptor antagonist. This suggests that inhibition of presynaptic adenosine A<sub>2A</sub> receptors located on glutamate terminals inhibits cocaine seeking presumably through a negative regulation of glutamate signaling from the mPFC.

Human studies have indicated that cocaine craving is associated with increased activity of the PFC (Kilts et al., 2001; Volkow et al., 1999a). Similarly, animal cocaine self-administration studies have suggested that glutamate signaling from the mPFC to the nucleus accumbens is necessary for cocaine-primed reinstatement (McFarland et al., 2003; Park et al., 2002; Rebec and Sun, 2005). Dopamine receptor stimulation, via intracranial infusion of cocaine, in the mPFC increases cortical glutamate release to the NAc and also plays a necessary role in cocaine-primed reinstatement (Park et al., 2002). Thus in our experiments, cocaine seeking induced by a mPFC infusion of cocaine, and likely AMPA, is dependent on glutamate release in the NAc. Previous studies have shown that antagonism of presynaptic adenosine A<sub>2A</sub> receptors is able to decrease glutamate release into the striatum (Orru et al., 2011a) and if this is the case in the NAc, SCH 442416 is likely preventing glutamate release driven by mPFC stimulation. This explanation is further supported by the fact that reinstatement driven by intra-NAcc infusion of AMPA or cocaine, which is not dependent on mPFC glutamate release, was not decreased (affected) by blockade of presynaptic adenosine A<sub>2A</sub> receptors with SCH 442416.
It is possible that our effects are due to blockade of adenosine A\textsubscript{2A} receptors expressed outside of the striatum since administration of KW 6002 and SCH 442416 was systemic. However, this seems unlikely given the fact that adenosine A\textsubscript{2A} receptors are mainly expressed in the striatum, and that previous work from our lab has shown intra-NAc antagonism of postsynaptic adenosine A\textsubscript{2A} receptors produces increases in cocaine seeking (O’Neill \textit{et al}, 2012). Additionally, systemic administration of SCH 442416 but not KW6002 decreases glutamate release in the striatum in response to electrical stimulation of cortex (Orru \textit{et al}, 2011a). Furthermore, genetic deletion studies support the idea that conditional knockout of forebrain adenosine A\textsubscript{2A} receptors, which would include presynaptic adenosine A\textsubscript{2A} receptors, decrease sensitivity to psychostimulants (Bastia \textit{et al}, 2005; Shen \textit{et al}, 2008), while a conditional knockout of striatal adenosine A\textsubscript{2A} receptors enhances sensitivity to psychostimulants (Shen \textit{et al}, 2008).

Although it is not clear why SCH 442416 binds only presynaptic A\textsubscript{2A} receptors, in vitro binding assays show that SCH 442416 has a decreased affinity for adenosine A\textsubscript{2A} receptors when cells are co-transfected with dopamine D\textsubscript{2} receptors. Thus, it appears that the presence of dopamine D\textsubscript{2} receptors inhibits it’s ability to block adenosine A\textsubscript{2A} receptors, possibly due to the formation of A\textsubscript{2A}-D\textsubscript{2} heteromeric receptor complexes (Orru \textit{et al}, 2011a). This is not the case for the postsynaptic adenosine A\textsubscript{2A} receptor antagonist, KW 6002 (Orru \textit{et al}, 2011a). Because dopamine D\textsubscript{2} receptors and adenosine A\textsubscript{2A} receptors are highly colocalized on MSNs of the indirect pathway within the striatum (Fink \textit{et al}, 1992; Svenningsson \textit{et al}, 1999b), SCH 442416 is unlikely to
have any direct effect on signaling within these neurons. Despite the unidentified mechanism for the binding of SCH 442416 to presynaptic adenosine A$_{2A}$ receptors and KW 6002 to postsynaptic adenosine A$_{2A}$ receptors, the opposing effects on cocaine seeking confirm that these antagonists are targeting different populations of adenosine A$_{2A}$ receptors.

Together these findings support previous studies that revealed differential effects of pre- and postsynaptic adenosine A$_{2A}$ receptor antagonism (O’Neill et al, 2014; Orru et al, 2011b). This work also validates previous findings indicating that adenosine receptors modulate cocaine related behaviors (Bachtell et al, 2009; Ferre et al, 2007; Hobson et al, 2012; Hobson et al, 2013; O’Neill et al, 2012) and may be a viable target for future pharmacotherapies. These findings are novel because they reveal the effects of adenosine A$_{2A}$ receptor blockade on reinstatement mediated by mPFC activation in comparison with cocaine seeking induced in the NAc itself. Future studies should examine the cellular mechanisms that underlie the bi-directional effects of presynaptic compared to postsynaptic adenosine A$_{2A}$ receptor blockade.
Chapter 4: Persistent reduction of cocaine seeking by pharmacological manipulation of adenosine A\textsubscript{1} and A\textsubscript{2A} receptors during extinction training in rats

Abstract

Adenosine receptor stimulation and blockade have been shown to modulate a variety of cocaine-related behaviors. These studies identify the direct effects of adenosine receptor stimulation on cocaine seeking during extinction training and the persistent effects on subsequent reinstatement to cocaine seeking. Rats self-administered cocaine on a fixed ratio one schedule in daily sessions over 3 weeks. Following a 1-week withdrawal, the direct effects of adenosine receptor modulation were tested by administering the adenosine A\textsubscript{1} receptor agonist, N6-cyclopentyladenosine (CPA, 0.03 and 0.1 mg/kg), the adenosine A\textsubscript{2A} agonist, CGS 21680 (0.03 and 0.1 mg/kg), the presynaptic adenosine A\textsubscript{2A} receptor antagonist, SCH 442416 (0.3, 1, and 3 mg/kg), or vehicle prior to each of six daily extinction sessions. The persistent effects of adenosine receptor modulation during extinction training were subsequently tested on reinstatement to cocaine seeking induced by cues, cocaine, and the dopamine D\textsubscript{2} receptor agonist, quinpirole. All doses of CPA and CGS 21680 impaired initial extinction responding; however, only CPA treatment during extinction produced persistent impairment in subsequent cocaine- and quinpirole-induced seeking. Dissociating CPA treatment from extinction did not alter extinction responding or subsequent reinstatement. Administration of SCH 442416 had no direct effects on extinction responding but produced dose-dependent persistent impairment of cocaine- and
quinpirole-induced seeking. These findings demonstrate that adenosine A$_1$ or A$_{2A}$ receptor stimulation directly impair extinction responding. Interestingly, adenosine A$_1$ receptor stimulation or presynaptic adenosine A$_{2A}$ receptor blockade during extinction produces lasting changes in relapse susceptibility.
Introduction

Chronic cocaine use alters the signaling of numerous neurotransmitters throughout the brain, and these changes are thought to underlie the compulsive drug seeking that characterizes addiction (Koob and Volkow, 2010). Addicts are susceptible to relapse even after prolonged abstinence from cocaine, suggesting that drug-induced changes persist and contribute to drug relapse (Nestler, 2001). Relapse is modeled in rodents using the drug self-administration/reinstatement paradigm where rats are initially trained to perform an operant response to acquire a drug reinforcer. Rats then undergo extinction training resulting in newly learned contextual relationships, culminating in progressive decreases in responding in the previously drug-associated context (Bouton, 2004). Following extinction training, reinstatement can be induced by a priming injection of the previously self-administered drug, stress, or the reintroduction of a drug-associated cue (Shalev et al, 2002). This model has been used to identify pharmacotherapies that directly reduce reinstatement of drug seeking (Schmidt et al, 2010; Shalev et al, 2002; Uys and LaLumiere, 2008). However, recent studies have begun examining the effects of pharmacotherapies administered during abstinence or extinction training with the goal of finding treatments that produce enduring reductions in relapse susceptibility (Reichel et al, 2011; Zhou and Kalivas, 2008).

Adenosine is a ubiquitous neuromodulator that influences a variety of neurotransmitters through its activity at two adenosine receptor subtypes expressed in the brain. Adenosine A1 and adenosine A2A receptors are G protein-coupled receptors that produce inhibitory and stimulatory cellular effects, respectively (Svenningsson et al, 2003).
Recent work from our lab and others has demonstrated that both adenosine A₁ receptors and adenosine A₂A receptors are capable of modulating numerous cocaine-related behaviors. For example, stimulation of both adenosine A₁ receptors and adenosine A₂A receptors either systemically or in the nucleus accumbens blocks the expression of cocaine sensitization (Filip *et al*, 2006; Hobson *et al*, 2012). Blockade of adenosine A₂A receptors, on the other hand, enhances both expression and development of cocaine sensitization (Filip *et al*, 2006; Hobson *et al*, 2012). Similarly, stimulation of either adenosine A₁ receptors or adenosine A₂A receptors blocks the expression of cocaine conditioned place preference (Poleszak *et al*, 2002a). In a self-administration paradigm adenosine A₂A receptor stimulation attenuates acquisition of cocaine self-administration (Knapp *et al*, 2001), while antagonism enhances responding for cocaine on a progressive ratio schedule of reinforcement (Doyle *et al*, 2012). Finally, stimulation of both adenosine A₁ receptors and adenosine A₂A receptors suppresses cocaine reinstatement, while blockade of adenosine A₂A receptors enhances cocaine seeking (Bachtell *et al*, 2009; Hobson *et al*, 2013; O'Neill *et al*, 2012).

In this study, we expand upon the role of adenosine receptors in cocaine seeking by stimulating adenosine receptor subtypes during extinction training. We assess the direct effect of adenosine A₁ receptor or adenosine A₂A receptor stimulation on cocaine seeking during extinction and the persistent effects on subsequent reinstatement induced by cocaine-associated cues and drug priming. We hypothesize that targeting adenosine receptors during the extinction phase of the self-administration/reinstatement
model will enhance the effects of extinction and produce lasting inhibitory effects on relapse susceptibility.

**Materials and Methods**

*Animals and housing conditions*

Male Sprague–Dawley rats (Charles River, Wilmington, MA) initially weighing 250–300 g were individually housed with food and water available *ad libitum*. All experiments were conducted during the light period of a 12 hr light/dark cycle in accordance with the guidelines established by the Institutional Animal Care and Use Committee at the University of Colorado at Boulder.

*Drugs*

The adenosine A$_{2A}$ receptor agonist, CGS 21680, was purchased from Tocris Bioscience (Ellisville, MO). The adenosine A$_1$ receptor agonist, CPA (N$_6$-cyclopentyladenosine), presynaptic adenosine A$_{2A}$ receptor antagonist, SCH 442416(2-(2-Furanyl)-7-[3-(4-methoxyphenyl)propyl]-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine), dopamine D$_2$ receptor agonist, quinpirole ((−)-quinpirole hydrochloride), and cocaine hydrochloride were obtained from Sigma-Aldrich (St Louis, MO). All drugs, except SCH 442416, were dissolved in sterile-filtered physiological (0.9%) saline. The presynaptic A2AR antagonist, SCH 442416, was dissolved in 33% DMSO and 66% ddH$_2$O.
**Cocaine Self-Administration Procedure**

Self-administration procedures were performed in operant conditioning chambers (Med-Associates, St Albans, VT) equipped with two response levers and an infusion pump system. Animals were initially trained to lever press for sucrose pellets to facilitate acquisition of cocaine self-administration (O’Neill et al, 2012). After lever-press training, animals were fed *ad libitum* for at least 24 hr before catheter implantation surgery, and for the duration of the study. Surgery and cocaine self-administration procedures were similar to those described in O’Neill et al, 2012. Rats were implanted with jugular catheters under halothane anesthesia (1–2.5%). Rats were allowed 3–7 days recovery in their home cage before experimental procedures began. During this time, catheters were flushed daily with 0.1 ml heparinized saline. Animals showing signs of post-surgical distress were administered (S)-(+)–ketoprofen (5 mg/kg, s.c.), a non-steroidal anti-inflammatory analgesic (Carabaza et al, 1996). After recovery, animals were allowed to self-administer intravenous cocaine (0.5 mg/kg/100 μl injection) on an FR1 reinforcement schedule in daily 4 h sessions for 5–7 days per week. Cocaine injections were delivered over 5 s concurrent with the illumination of a cue light above the active lever and were followed by a 15 s time-out period (TO 20 s) when the house light remained off and responding produced no consequence. Inactive lever responses produced no consequence throughout testing. After a minimum of 15 cocaine self-administration sessions, animals remained in their home cages for 6 days of forced abstinence.
Effects of adenosine receptor stimulation and blockade on extinction responding

On days 7–12 following self-administration, animals returned to the operant conditioning chambers for 6 daily 4 h extinction training. Responses on the lever previously paired with cocaine injections during self-administration (active lever) and on the inactive lever were recorded, but had no programmed drug or cue delivery. The effect of adenosine receptor stimulation on responding on both levers was tested in animals counterbalanced for cocaine intake. Five minutes prior to the initiation of the extinction sessions, animals were treated with vehicle, the adenosine A<sub>1</sub> receptor agonist, (CPA: 0.03 mg/kg or 0.1 mg/kg, ip), the adenosine A<sub>2A</sub> receptor agonist, (CGS 21680: 0.03 mg/kg or 0.1 mg/kg, ip), or the presynaptic adenosine A<sub>2A</sub> receptor antagonist (SCH 442416: 0.3 mg/kg, 1 mg/kg or 3 mg/kg, ip). Doses of the adenosine agonists/antagonist were chosen based on previous findings illustrating their effects on lever responding in the absence of locomotor suppression or generalized effects on operant behavior (Bachtell et al, 2009; Hobson et al, 2012; Orru et al, 2011a).

Effects of temporally dissociating adenosine A<sub>1</sub> receptor stimulation from extinction training

In order to determine whether the effects of adenosine A<sub>1</sub> receptor stimulation during extinction on subsequent reinstatement are due to an enhancement of extinction learning we decided to temporally dissociate the adenosine A<sub>1</sub> receptor receptor stimulation from extinction training. This way we can assess the necessity of extinction training in the effectiveness of the adenosine A<sub>1</sub> receptor agonist in decreasing
subsequent reinstatement. All cocaine self-administration and extinction procedures were the same except that animals were treated with vehicle or CPA (0.1 mg/kg, ip) 4 h after the end of each 4 h extinction session with the first treatment administered 4 h after the first extinction session. We chose to administer the adenosine A₁ receptor agonist 4 h post extinction based on the pharmacokinetics of CPA (Mathot et al, 1993) and other experiments examining similar effects (Hammond et al, 2012; Mickley et al, 2012).

Reinstatement procedures

The enduring effects of the adenosine agonist/antagonist treatments administered during extinction training were also tested on reinstatement responding over the subsequent 4 days following extinction training using a repeated testing paradigm. Thus, all animals were tested for cue-, cocaine-, and dopamine D₂ agonist-induced reinstatement. All reinstatement tests consisted of a 4 h reinstatement session comprised of a 2 h of extinction phase followed by a 2 h reinstatement test phase. Cue-induced reinstatement was initiated by 5 non-contingent saline infusions paired with the illumination of the cocaine cue light (5 s) administered every 2 min over the first 10 min of the reinstatement phase. Throughout the reinstatement phase, responding at the previously active lever resulted in a 5 s cue light illumination and saline infusion. Responding at the inactive lever resulted in no cue or infusion. Cocaine-induced reinstatement was initiated by a systemic injection of cocaine (15 mg/kg, i.p.) 5 min prior to the reinstatement phase. Dopamine D₂ agonist-induced reinstatement was initiated
by a systemic injection of quinpirole (0.3 mg/kg, s.c.) 5 min prior to the reinstatement phase. Quinpirole was used to induce reinstatement because previous studies have shown that dopamine D$_2$ receptors play a key role in mediating cocaine-related behaviors (Bachtell et al., 2005; De Vries et al., 2002; Fontana et al., 1993; Graham et al., 2007; Khroyan et al., 2000; Merritt and Bachtell, 2013; Self et al., 1996). Thus, dopamine D$_2$ receptor stimulation produces behavioral cross-sensitization in animals receiving repeated cocaine and produces robust reinstatement responding in animals extinguished from cocaine self-administration. Responding at both levers was recorded, but resulted in no cues or reward delivery.

**Data Analyses**

The numbers of animals in each experimental group ranged from 6 to 22. Extinction data for animals receiving systemic injections of either adenosine A$_1$ receptor agonist or adenosine A$_{2A}$ receptor agonist 5 min prior to extinction training were analyzed by a two-way mixed-design ANOVA with lever and treatments (vehicle, adenosine A$_1$ receptor agonist or adenosine A$_{2A}$ receptor agonist) as the factors. Extinction data for animals receiving systemic injections of the presynaptic adenosine A$_{2A}$ receptor antagonist 5 min prior to extinction training was analyzed by a separate two-way mixed-design ANOVA with lever and treatments (vehicle or adenosine A$_{2A}$ receptor antagonist) as the factors. Lever responding during reinstatement testing was analyzed by a two-way ANOVA with session (extinction or reinstatement) and extinction treatments serving as the factors. Responding on the active and inactive levers (see Supplemental Results)
during reinstatement was analyzed separately by a two-way ANOVA. Significant interactions were followed with simple main effects analyses (one-way ANOVA) and post hoc tests (Bonferroni's comparisons). Statistical significance was set at $p < 0.05$ for all tests.

**Results**

*Adenosine $A_1$ and $A_{2A}$ receptor stimulation decreases initial extinction responding*

Prior to extinction training, animals were assigned to treatment groups based on their cocaine intake over the last five self-administration sessions (Fig. 1a). Figure 1b illustrates that pretreatment with either CPA or CGS 21680 significantly decreased extinction responding on the first of six daily 4-h extinction training sessions. We observed a significant treatment X day interaction ($F_{20,280} = 1.70, p < 0.05$) and significant main effects of treatment ($F_{4,280} = 2.91, p < 0.05$) and day ($F_{5,280} = 38.94, p < 0.0001$). Subsequent analysis of the interaction revealed that pretreatment with CPA (0.3 and 0.1 mg/kg) and CGS 21680 (0.03 and 0.1 mg/kg) significantly reduced active lever responding compared to vehicle during the first extinction training session. Post hoc analysis revealed a significant reduction in lever responding of all treatment groups compared to vehicle (0.03 mg/kg CPA: $t_{280} = 4.14, p < 0.001$; 0.1 mg/kg CPA: $t_{280} = 4.38, p < 0.001$; 0.03 mg/kg CGS 21680: $t_{280} = 2.92, p < 0.05$; 0.1 mg/kg CGS 21680: $t_{280} = 4.05, p < 0.001$). Analysis of the first and second hour of active lever responding in the first extinction training session revealed a significant treatment X hour interaction ($F_{2,43} = 6.37; p = 0.0038$) and significant main effects of treatment ($F_{2,43} =$
Figure 4.1 Stimulating adenosine A$_1$ or adenosine A$_{2A}$ receptors decreases extinction responding during the first extinction session. a) Average number of cocaine infusions and active lever responses for each treatment group in each 4-h session over the last 6 days of the self-administration phase. b) Systemic administration of the adenosine A$_1$ receptor agonist, CPA or the A$_{2A}$ receptor agonist, CGS 21860, reduced extinction responding on the first day of extinction training. Asterisk indicates significant from vehicle pretreatment (t test, p < 0.05)
Figure 4.2 Temporal effects of stimulating adenosine A\textsubscript{1} or adenosine A\textsubscript{2A} receptors during extinction training a) Systemic administration of the adenosine A\textsubscript{1} receptor agonist, CPA (0.03 and 0.1 mg/kg, i.p.) or the adenosine A\textsubscript{2A} receptor agonist, CGS 21680 (0.03 and 0.1 mg/kg, i.p.), immediately prior to extinction significantly decreases active lever responses during the first 15 min of the first extinction session (left). Simple main effects analysis of the significant interaction found that a pretreatment with CPA 0.03 mg/kg ($t_{392} = 5.03$, $p < 0.001$), 0.1 mg/kg ($t_{392} = 6.26$, $p < 0.001$), and CGS 21680 0.03 mg/kg ($t_{392} = 4.74$, $p < 0.001$), 0.1 mg/kg ($t_{392} = 4.98$, $p < 0.001$) decreased active lever responding in the first 15 min of the first extinction session compared with vehicle control. During the 1\textsuperscript{st} h of the first extinction session animals pretreated with either 0.03 mg/kg ($t_{55} = 3.15$, $p < 0.05$), 0.1 mg/kg ($t_{55} = 3.58$, $p < 0.01$) CGS 21680 or 0.03 mg/kg ($t_{55} = 3.45$, $p < 0.01$) 0.1 mg/kg ($t_{55} = 4.71$, $p < 0.001$) CPA showed significantly reduced active lever pressing compared to a vehicle pretreatment (right). * Indicates all groups significant from vehicle pretreatment (t-test, $p < 0.05$). b) Administration of the adenosine A\textsubscript{1} or adenosine A\textsubscript{2A} receptor agonist also has time-dependent effects throughout extinction training. Administration of 0.03 mg/kg and 0.1 mg/kg of the adenosine A\textsubscript{1} receptor agonist, CGS 21680 or 0.1 mg/kg of the adenosine A\textsubscript{1} receptor agonist CPA decreased extinction responding in the first hour of the 3\textsuperscript{rd} extinction session (^ Significant from vehicle, t-test, $p < 0.05$). On the 4\textsuperscript{th} day of extinction training 0.1 mg/kg CGS 21680 and 0.1 mg/kg CPA significantly decreased active lever responses in the first hour of the extinction session (# Significant from vehicle pretreatment, t-test, $p < 0.05$).
Table 4.1 Statistical analyses of adenosine agonist effects during the first 2 hours of extinction sessions 1-6

<table>
<thead>
<tr>
<th>Extinction Session</th>
<th>Statistical Analysis</th>
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| 1                  | Hour: $F_{1,56} = 48.53, p < 0.0001$  
                        Treatment: $F_{4,56} = 3.76, p < 0.01$  
                        Interaction: $F_{4,56} = 5.07, p < 0.01$ |
| 2                  | Hour: $F_{1,56} = 19.77, p < 0.0001$  
                        Treatment: NS  
                        Interaction: NS |
| 3                  | Hour: $F_{1,56} = 25.77, p = 0.0001$  
                        Treatment: $F_{4,56} = 4.05, p < 0.01$  
                        Interaction: $F_{4,56} = 4.49, p < 0.01$ |
| 4                  | Hour: $F_{1,56} = 4.66, p < 0.05$  
                        Treatment: NS  
                        Interaction: $F_{4,56} = 2.61, p < 0.05$ |
| 5                  | Hour: $F_{1,56} = 45.64, p < 0.0001$  
                        Treatment: NS  
                        Interaction: NS |
| 6                  | Hour: $F_{1,56} = 40.96, p < 0.0001$  
                        Treatment: NS  
                        Interaction: NS |
and hour (F_{1,43} = 52.59; p < 0.0001). We also analyzed responding during extinction training sessions 2 through 6 in hourly intervals over the first 2 h of each extinction session (figure 4.2b) Statistical analyses are presented in Table 4.1.

Adenosine A₁ and A₂A receptor stimulation decreases initial extinction responding at the previously inactive lever

Analyses of inactive lever responding during extinction training revealed significant main effects of day (2 h: F_{5, 215} = 12.45; p < 0.0001 & 4 h: F_{5, 215} = 14.09; p < 0.0001) and treatment in 2 h sessions (F_{2, 215} = 3.76; p = 0.03), but not in 4 h sessions (F_{2, 215} = 1.58, p = 0.22). No significant interactive effects were observed (2 h: F_{10, 215} = 1.34; p = 0.21 & 4 h: F_{10, 215} = 1.14, p = 0.33).

Further analysis of inactive lever responding in 15 m intervals of the first extinction session found a significant main effect of time (F_{7, 301} = 6.19; p < 0.0001), but not significant main effects of treatment (F_{2, 301} = 1.83; p = 0.17). No significant interactive effects were observed (F_{14, 301} = 1.27; p = 0.22). The hourly comparison of inactive lever responding revealed a significant main effect of hour in extinction sessions 1 (F_{1,43} = 26.01; p < 0.0001), 2 (F_{1,43} = 10.43; p < 0.01), 3 (F_{1,43} = 5.06; p < 0.05), 5 (F_{1,43} = 22.80; p < 0.0001), and 6 (F_{1,43} = 19.16; p < 0.0001), but not in extinction session 4 (F_{1,43} = 3.21; p = 0.08). Significant main effects of treatment during the first 2 h of extinction session were observed in extinction sessions 2 (F_{2,43} = 4.09; p < 0.05) and 4 (F_{2,43} = 4.24; p < 0.05), but not in extinction sessions 1 (F_{2,43} = 1.88; p = 0.17), 3 (F_{2,43} = 0.94; p = 0.40), 5 (F_{2,43} = 0.07; p = 0.93), or 6 (F_{2,43} = 1.37; p = 0.27). No significant interactive
effects were observed in any of the extinction sessions (Session 1: $F_{2, 43} = 2.20; p = 0.12$; Session 2: $F_{2, 43} = 1.18; p = 0.32$; Session 3: $F_{2, 43} = 1.78; p = 0.18$; Session 4: $F_{2, 43} = 0.47; p = 0.63$; Session 5: $F_{2, 43} = 0.11; p = 0.89$; Session 6: $F_{2, 43} = 1.72; p = 0.19$).

Adenosine $A_1$ receptor stimulation during extinction training blunts subsequent cocaine- and quinpirole-induced reinstatement

We next assessed the persistent effects of adenosine receptor stimulation during extinction training on subsequent reinstatement testing. Figure 4.3 illustrates that the highest dose of CPA (0.1 mg/kg), but not the lower dose of CPA (0.03 mg/kg) or either dose of CGS 21680 administered during extinction training inhibited subsequent reinstatement induced by cocaine and quinpirole. None of the treatments had any effect on cue-induced reinstatement. Analysis of active lever responding during cue-induced reinstatement revealed a significant main effect of reinstatement for all animals (CPA experiment: $F_{1,39} = 72.56, p< 0.0001$; CGS experiment: $F_{1,36} = 69.59, p< 0.0001$). No treatment or treatment X reinstatement interaction effects were observed indicating that regardless of treatment during extinction, all animals reinstated similarly. Analysis of cocaine-induced reinstatement in animals treated with CPA during extinction training revealed a significant treatment X reinstatement interaction ($F_{2,39} = 3.63, p< 0.05$) and significant main effects of treatment ($F_{1,39} = 3.62, p< 0.05$) and reinstatement ($F_{1,39} = 36.17, p< 0.0001$). Subsequent analysis of the interaction revealed that rats treated with 0.1 mg/kg CPA during extinction showed reduced cocaine-induced reinstatement when compared to vehicle treated rats ($t_{39} = 3.76, p< 0.001$). Analysis of cocaine-induced
Figure 4.3 Persistent effects of stimulating adenosine A\textsubscript{1} receptors during extinction training on subsequent cocaine- and D\textsubscript{2}-agonist-induced cocaine seeking. a) Pretreatment with the adenosine A\textsubscript{1} receptor agonist, CPA, or the adenosine A\textsubscript{2A} receptor agonist, CGS 21680, during extinction training had no effect on subsequent cue-induced reinstatement. Subsequent reinstatement induced by b) cocaine and c) quinpirole was significantly blunted by administration of the adenosine A\textsubscript{1} receptor agonist, CPA (0.1 mg/kg), but not by the adenosine A\textsubscript{2A} receptor agonist, CGS 21680, administered during extinction training. Asterisks indicate significant from vehicle pretreatment during extinction training (Bonferroni’s post test, p<0.001)
reinstatement in animals treated with CGS 21680 during extinction revealed a significant main effect of reinstatement ($F_{1,36} = 42.78, p < 0.0001$), but no main effect of treatment or treatment X reinstatement interaction. Analysis of quinpirole-induced reinstatement in animals treated with CPA during extinction training revealed a significant treatment X reinstatement interaction ($F_{2,37} = 3.56, p < 0.05$) and significant main effects of treatment ($F_{1, 37} = 3.81, p < 0.05$) and reinstatement ($F_{1, 37} = 18.84, p < 0.0001$). Subsequent analysis of the interaction found that animals treated with 0.1 mg/kg CPA during extinction showed less $D_2$ agonist-induced reinstatement responding compared to vehicle animals ($t_{37} = 3.80, p < 0.001$). Analysis of quinpirole-induced reinstatement in animals treated with CGS during extinction showed a significant main effect of reinstatement ($F_{1,34} = 15.56, p < 0.001$), but no main effect of treatment or treatment X reinstatement interaction.

*Adenosine $A_1$ receptor stimulation temporally dissociated from extinction training has no effect on extinction responding or subsequent reinstatement responding*

Given the persistent effects of CPA to diminish subsequent reinstatement, we next assessed whether adenosine $A_1$ receptor stimulation temporally dissociated from the extinction training sessions would recapitulate these effects. Animals were separated into balanced treatment groups based on cocaine intake prior to extinction training (figure 4.4). Four hours after the end of each extinction training session, animals were administered either vehicle or 0.1 mg/kg CPA, the dose effective in reducing subsequent reinstatement (see above). Analysis of extinction responding at the active
Figure 4.4 Dissociating adenosine A₁ receptor stimulation from extinction training has no effect on extinction responding or subsequent reinstatement. a) Average number of cocaine infusions and active lever responses in each 4-h session over the last 5 days of the self-administration phase. b) Systemic treatment with the adenosine A₁ receptor agonist, CPA (0.1 mg/kg, i.p.), 4 h after each extinction session has no effect on extinction responding. Adenosine A₁ receptor stimulation temporally dissociated from extinction training has no effect on c) cue-, d) cocaine-, or e) dopamine D₂-agonist-induced reinstatement.
lever revealed a significant main effect of session \((F_{5,60}=24.49, p<0.0001)\), but there
was no effect of treatment or the session X treatment interaction. Following extinction
training, animals were tested for cue-, cocaine-, and quinpirole-induced drug seeking
(figure 4.4). In all reinstatement tests, there was a significant main effect of
reinstatement (cue: \(F_{1,12}=14.25, p<0.01\); cocaine: \(F_{1,12}=11.46, p<0.01\); quinpirole:
\(F_{1,12}=14.27, p<0.01\)), but there was no significant treatment or treatment X
reinstatement interaction suggesting that dissociating adenosine A_1 receptor
stimulation from the extinction training sessions is not sufficient to produce
these persistent effects on reinstatement.

*Adenosine A_1 receptor stimulation temporally dissociated from extinction training has no
effect on extinction responding or subsequent reinstatement responding at the
previously inactive lever*

Analysis of inactive lever responding during extinction training revealed no significant
differences between treatment groups \((F_{1,60} = 1.84, p = 0.1998)\) or the treatment X
session interaction \((F_{5,60} = 1.28, p = 0.2852)\), but a significant main effect of session
was observed \((F_{1,12} = 7.08; p< 0.0001)\).

Inactive lever responding was also evaluated and there were no significant main
effects of session on cue- \((F_{2,42} = 2.38; p = 0.10)\), or quinpirole-induced \((F_{2,42} = 2.38; p
= 0.10)\) reinstatement, however, analysis of cocaine-induced drug seeking revealed a
significant main effect of session \((F_{1,12} = 12.58; p = 0.004)\). No significant main effects
of treatment were detected in cue- \((F_{1,12} = 0.11; p = 0.75)\), cocaine- \((F_{1,12} = 0.13; p =
0.91), or quinpirole-induced ($F_{1,12} = 0.89; p = 0.36$) reinstatement. Additionally, no interactive effects were observed in any of the reinstatement tests (cue: $F_{1,12} = 0.21; p = 0.66$; cocaine: $F_{1,12} = 0.50; p = 0.49$; quinpirole: $F_{1,12} = 0.89; p = 0.36$).

**Adenosine $A_{2A}$ receptor blockade has no effect on extinction responding**

Prior to extinction training, animals were assigned to treatment groups based on their cocaine intake over the last five self-administration sessions (figure 4.5). Lever responding was then extinguished in six daily sessions where a pretreatment of vehicle or the adenosine $A_{2A}$ receptor antagonist, SCH 442416 (0.3, 1, or 3 mg/kg) was administered prior to each extinction training session (figure 4.5). These doses of SCH 442416 were chosen based on previous work illustrating that low doses (0.3 and 1.0 mg/kg) primarily inhibit presynaptic adenosine $A_{2A}$ receptors decreasing both locomotor activity and evoked glutamate release, while 3.0 mg/kg inhibits postsynaptic adenosine $A_{2A}$ receptors to increase locomotor activity (Orru et al., 2011a; Orru et al., 2011b). Analysis of extinction responding over the entire 4-h session revealed a significant main effect of session (4 h: $F_{5,135} = 79.04, p < 0.0001$; 2 h: $F_{5,135} = 85.74, p < 0.0001$), but there was no main effect of treatment or treatment X session interaction.

**Presynaptic adenosine $A_{2A}$ receptor blockade has no effect on extinction responding at the previously inactive lever**

Analysis of inactive lever responding during the extinction sessions revealed significant main effects of session (2 h: $F_{5,70} = 4.79; p < 0.001$ & 4 h: $F_{5,70} = 4.78; p <$
Figure 4.5 Blocking adenosine $A_{2A}$ receptors during extinction has no effects on extinction responding. a) Average number of cocaine infusions and active lever responses in each 4-h session over the last 6 days of the self-administration phase. b) adenosine $A_{2A}$ receptor antagonism by SCH 442416 (0.3, 1, or 3 mg/kg, i.p.) has no effect on extinction responding when administered immediately prior to the beginning of each 4-h extinction session.
0.001). No significant main effects of treatment (4 h: $F_{1,70} = 0.01; p = 0.95$) or significant interactive effects (4 h: $F_{5,70} = 0.38; p = 0.89$) were observed on inactive lever responding during extinction.

*Presynaptic A$_{2A}$ receptor blockade during extinction training decreases subsequent cocaine- and quinpirole-induced reinstatements*

SCH 442416 administered during extinction training dose dependently inhibited subsequent reinstatement induced by cocaine and quinpirole but had no effect on cue-induced reinstatement (figure 4.6). Analysis of active lever responding during cue-induced reinstatement revealed a significant main effect of reinstatement ($F_{1,26} = 58.12, p<0.0001$), but there was no main effect of treatment or treatment X reinstatement interaction. Analysis of active lever responding during cocaine-induced reinstatement revealed a significant treatment X reinstatement interaction ($F_{3,27} = 4.02, p<0.05$) and significant main effects of treatment ($F_{3,27} = 3.98, p<0.05$) and reinstatement ($F_{1,27} = 29.42, p<0.0001$). Post hoc analyses demonstrate that pretreatment with either 0.3 or 1.0 mg/kg SCH 442416 during extinction training significantly reduced cocaine-induced reinstatement compared to vehicle and 3 mg/kg SCH 442416 (vehicle vs 0.3 SCH 442416: $t_{27} = 2.40, p<0.05$, vehicle vs 1.0 SCH 442416: $t_{27} = 2.79, p<0.05$). Analysis of active lever responding during quinpirole-induced reinstatement revealed a significant treatment X reinstatement interaction ($F_{3,26} = 3.13, p<0.05$), and significant main effects of treatment ($F_{3,26} = 3.05, p<0.05$) and reinstatement ($F_{1,26} = 36.70, p<0.0001$) were observed. Post hoc analyses
Figure 4.6 Blocking presynaptic, but not postsynaptic, adenosine A$_{2A}$ receptors during extinction produces enduring reductions on reinstatement of cocaine seeking. a) Blocking adenosine A$_{2A}$ receptors during extinction training has no effect on subsequent cue-induced reinstatement. b) Pretreatment of SCH 442416 during extinction training impaired subsequent reinstatement of cocaine-induced seeking when administered at doses effective at blocking presynaptic adenosine A$_{2A}$ receptors (0.3 or 1 mg/kg). c) Similarly, antagonism of presynaptic adenosine A$_{2A}$ receptors during extinction also impaired subsequent cocaine seeking induced by quinpirole. Asterisks indicate significant from vehicle pretreatment (t test, p<0.05)
demonstrate that pretreatment with either 0.3 or 1.0 mg/kg SCH 442416 during extinction training significantly reduced quinpirole-induced reinstatement compared to vehicle and 3 mg/kg SCH 442416 (vehicle vs 0.3 SCH 442416: $t_{27} = 2.72, p < 0.05$ and vehicle vs 1.0 SCH 442416: $t_{27} = 2.34, p < 0.05$).

*Presynaptic $A_{2A}$ receptor blockade during extinction decreases subsequent cue-, cocaine- and $D_2$ agonist-induced reinstatement at the previously inactive lever*

Inactive lever responding from the reinstatement session was compared to inactive lever responding from the last hour of the extinction phase that occurred immediately prior. No significant main effects of session were observed for any of the reinstatement sessions (cue: $F_{1, 14} = 3.84; p = 0.07$; cocaine: $F_{1, 14} = 3.93; p = 0.07$; quinpirole: $F_{1, 14} = 2.05; p = 0.17$). For cue-induced reinstatement, a significant main effect of treatment ($F_{1, 14} = 5.90; p < 0.05$) was observed, however, no significant main effect of treatment was detected for cocaine- ($F_{1, 14} = 0.11; p = 0.75$) or quinpirole-induced ($F_{1, 14} = 0.35; p = 0.56$) reinstatement. No interactive effects were observed (cue: $F_{1, 14} = 3.03; p = 0.10$; cocaine: $F_{1, 14} = 0.20; p = 0.66$; quinpirole: $F_{1, 14} = 0.35; p = 0.56$).

**Discussion**

We have previously shown that stimulation of adenosine receptors can directly attenuate the reinstatement of cocaine seeking induced by pharmacological stimuli (Bachtell *et al*, 2009; Hobson *et al*, 2013; O’Neill *et al*, 2012). Here, we examine the effect of adenosine receptor stimulation or blockade on extinction responding and
subsequent reinstatement. Our findings reveal that stimulation of adenosine A₁ receptors or adenosine A₂A receptors inhibit initial extinction responding, which parallels previous work from our lab and others illustrating that adenosine receptor stimulation can inhibit different types of cocaine seeking including the initiation of cocaine taking (Knapp et al, 2001), as well as cue- and drug- primed reinstatement (Bachtell et al, 2009; Hobson et al, 2013; O'Neill et al, 2012). We suspect these reductions in initial cocaine seeking observed on the first day of extinction training are due to the ability of stimulation at postsynaptic adenosine A₁ receptors and adenosine A₂A receptors to antagonize activity of dopamine D₁ and D₂ receptors, respectively (Franco et al, 2007; Fuxe et al, 2007a; Tozzi et al, 2007; Yabuuchi et al, 2006). However, only adenosine A₁ receptor stimulation produced lasting reductions in cocaine- and dopamine D₂ agonist-induced cocaine seeking. These persistent effects on reinstatement were not observed when adenosine A₁ receptor stimulation was temporally dissociated from extinction training. In order to further elucidate the role of adenosine receptors in cocaine seeking we examined the effects of antagonizing presynaptic adenosine A₂A receptors, a mechanism known to facilitate presynaptic adenosine A₁ receptor activity (Orru et al, 2011a), during extinction training. While this treatment had no direct effect on extinction responding, it produced persistent decreases in cocaine- and dopamine D₂ agonist-induced cocaine seeking. Notably, antagonism of postsynaptic adenosine A₂A receptors had no effect on extinction or subsequent reinstatement. These results suggest that adenosine modulation during extinction can produce lasting effects on reinstatement.
It is important to consider the anatomical and neuronal locations of adenosine receptors when interpreting our results. Both Adenosine A₁ receptors and adenosine A<sub>2A</sub> receptors are highly localized to the NAc, caudate and putamen where they have been shown to regulate cocaine-mediated responses (Ferre <i>et al</i>, 2011; Fink <i>et al</i>, 1992; Fuxe <i>et al</i>, 2007a; Hobson <i>et al</i>, 2012; O'Neill <i>et al</i>, 2012). Presynaptic adenosine A<sub>1</sub> receptors in the striatum are located on glutamate terminals to reduce basal glutamate release (Corsi <i>et al</i>, 1997; Mahan <i>et al</i>, 1991; McCool and Farroni, 2001; Quarta <i>et al</i>, 2004; Solinas <i>et al</i>, 2002). Presynaptic adenosine A<sub>2A</sub> receptors are expressed specifically on glutamate terminals that synapse onto dopamine D<sub>1</sub> receptor-expressing GABA neurons of the direct pathway where they act to enhance glutamate release (Corsi <i>et al</i>, 1997; Martire <i>et al</i>, 2011; Orru <i>et al</i>, 2011b; Quarta <i>et al</i>, 2004; Quiroz <i>et al</i>, 2009; Rosin <i>et al</i>, 1998; Sebastiao and Ribeiro, 1996). Postsynaptic adenosine A1 receptors are expressed on the direct pathway neurons of the striatum where they oppose actions of dopamine D<sub>1</sub> receptors and decrease glutamate receptor trafficking (Fuxe <i>et al</i>, 2007a; Hobson <i>et al</i>, 2013). Postsynaptic adenosine A<sub>2A</sub> receptors colocalize with dopamine D<sub>2</sub> receptors on the indirect pathway striatal neurons where they oppose the intracellular signaling cascades of dopamine D<sub>2</sub> receptors and increase glutamate receptor trafficking (Fuxe <i>et al</i>, 2007a; Hakansson <i>et al</i>, 2006; Tozzi <i>et al</i>, 2007). Cocaine self-administration produces persistent alterations in glutamate homeostasis in the NAc and other reward-related brain areas (Baker <i>et al</i>, 2003a; Cornish <i>et al</i>, 2000; Kalivas <i>et al</i>, 2005; McFarland <i>et al</i>, 2003; Pierce <i>et al</i>, 1996; Reid and Berger, 1996). Cocaine seeking is associated with reduced basal extracellular
glutamate levels and increased release of glutamate in the NAc in response to a cocaine prime (McFarland et al, 2003). Thus, adenosine receptors in the striatum are capable of modulating both dopamine and glutamate signaling to impair cocaine seeking during extinction and reinstatement procedures. We suspect our effects are primarily mediated by adenosine receptors within the striatum, although future studies are necessary to identify the contribution of additional brain regions (see below).

Given the presynaptic and postsynaptic actions of adenosine A₁ receptors to reduce glutamate neurotransmission in the striatum, we were somewhat surprised to observe decreased cocaine seeking throughout extinction training. Previous evidence suggests that increased, not decreased, glutamate activity during extinction facilitates this new learning (Nic Dhonnchadha et al, 2010; Self et al, 2004; Sutton et al, 2003; Thanos et al, 2011a; Thanos et al, 2011b). However, adenosine A₁ receptors are expressed throughout the brain where they inhibit glutamate signaling (Mahan et al, 1991). It seems likely that stimulation of adenosine A₁ receptors in areas such as the hippocampus or basolateral amygdala are involved in decreasing extinction responding since both of these structures play a role in context-induced cocaine seeking (Cooper et al, 2006; Fuchs et al, 2005; Kalivas et al, 2003; Lasseter et al, 2010; Ramirez et al, 2009; Schmidt et al, 2005; Wells et al, 2013). Stimulation of adenosine A₁ receptors in either the hippocampus (Branisteanu et al, 1987; Poli et al, 1991) or basolateral amygdala (Heinbockel and Pape, 1999; McCool et al, 2001) inhibits the activity of these structures and we suspect that this may contribute to the direct effects decreased of adenosine A₁ receptor stimulation on extinction responding.
Adenosine A<sub>1</sub> receptor stimulation during extinction training also produced lasting decreases in cocaine- and dopamine D<sub>2</sub> agonist-induced reinstatement. The persistent effects on subsequent reinstatement testing may result from decreases in overall glutamate release in the NAc and other areas during extinction training since dissociation of adenosine A<sub>1</sub> receptor stimulation from extinction sessions did not produce the same lasting effects. This decreased glutamate release coupled with postsynaptic adenosine A<sub>1</sub> receptors that reduce glutamate signaling in direct pathways neurons may help to consolidate extinction-induced changes that impair subsequent reinstatement. We determined if presynaptic adenosine receptors played a preferential role in these persistent effects by administering several doses SCH 442416, a presynaptic adenosine A<sub>2A</sub> receptor antagonist and facilitator of presynaptic adenosine A<sub>1</sub> receptors inhibitory actions on glutamate terminals, as demonstrated by its ability to reduce cortically-evoked glutamate release in the striatum (Orru et al, 2011a). We observed that presynaptic, but not postsynaptic antagonism of adenosine A<sub>2A</sub> receptors during extinction produced lasting decreases on reinstatement, although it did not have any direct effects on extinction responding. This may be partly due to the selective presynaptic expression of adenosine A<sub>2A</sub> receptors on cortical glutamate terminals onto direct pathway neurons (Orru et al, 2011a; Quiroz et al, 2009). Only two previous studies have identified and examined the presynaptic actions of SCH 442416 (Orru et al, 2011a; Orru et al, 2011b). This work provides more evidence for SCH 442416 as a presynaptic adenosine A<sub>2A</sub> receptor antagonist at low doses since the high dose (3 mg/kg) of SCH 442416 had opposite effects on reinstatement compared to the 2 lower
doses (0.3 and 1 mg/kg). Together, these findings suggest that presynaptic adenosine $A_1$ receptor stimulation of cortical terminals may produce lasting effects on cocaine seeking when concurrent with extinction training, while postsynaptic adenosine $A_1$ receptor stimulation alone or in combination with presynaptic adenosine $A_1$ receptor stimulation reduces extinction responding directly.

Adenosine $A_{2A}$ receptor stimulation resulted in decreased cocaine seeking during the first day of extinction training, but had no effect on subsequent reinstatement responding. This initial reduction in extinction responding is likely due to mild increases in overall glutamate transmission in the NAc during extinction. Increased glutamate transmission could result from either presynaptic adenosine $A_{2A}$ receptor stimulation of glutamate terminals that synapse onto the direct pathway neurons (Corsi et al, 1997; Martire et al, 2011; Orru et al, 2011b; Sebastiao et al, 1996) or postsynaptic adenosine $A_{2A}$ receptors that offset dopamine D2 receptor inhibition of glutamate signaling in the indirect pathway neurons (Ferre et al, 2011; Mayfield et al, 1993; Mingote et al, 2008; Shindou et al, 2003). These results are comparable to the facilitation of extinction observed with the partial NMDA glutamate receptor agonist, d-cycloserine (Thanos et al, 2011a; Thanos et al, 2011b). It is unclear why adenosine $A_{2A}$ receptor stimulation does not have persistent effects akin to d-cycloserine (Paolone et al, 2009), especially because extinction appears to increase expression of adenosine $A_{2A}$ receptors, and this alteration would likely lead to decreased relapse susceptibility (Frankowska et al, 2013). This may be due to adenosine $A_{2A}$ receptor stimulation not effectively influencing
presynaptic glutamate transmission as we suspect occurs with presynaptic adenosine A₁ receptor stimulation or presynaptic adenosine A₂A receptor blockade.

It is possible that our adenosine agonists and antagonist are affecting astrocytic mechanisms of neurotransmitter release/reuptake, which could contribute to our behavioral effects. In addition to alterations in glutamate signaling, changes in GABA signaling have also been implicated in cocaine seeking (Filip and Frankowska, 2007a, 2008; Filip et al, 2007b; Frankowska et al, 2008a, b; Tang et al, 2005; Torregrossa et al, 2008; Wydra et al, 2013). In fact, cocaine self-administration appears to increase basal extracellular GABA in the accumbens and ventral pallidum (Wydra et al, 2013) and decrease GABAb receptor binding (Frankowska et al, 2008a, b). Cocaine-primed reinstatement results in increases in GABAb receptor binding (Frankowska et al, 2008a) and decreases extracellular GABA in the ventral pallidum (Tang et al, 2005; Torregrossa et al, 2008). Increasing adenosine transmission in the accumbens results in increased expression of glial glutamate transporter (GLT-1) mRNA and glutamate uptake (Wu et al, 2010), which is associated with persistent attenuation of cocaine- and cue-primed reinstatement (Knackstedt et al, 2010). It seems unlikely that our behavioral effects are due to increases in GLT-1 since blockade of adenosine A₂A receptors mimicked the effects of adenosine A₁ receptor stimulation on subsequent cocaine seeking. On the other hand, stimulation of adenosine A₁ receptors decreases GABA transport into astrocytes, while stimulation of adenosine A₂A receptors increase the uptake of GABA into astrocytes (Cristovao-Ferreira et al, 2013; Kirk and Richardson, 1994). Thus, increasing extracellular GABA through adenosine A₁ receptor stimulation
or adenosine A<sub>2A</sub> receptor blockade could countermand the GABA decrease associated with reinstatement. It is currently unclear how chronic adenosine A<sub>1</sub> receptor stimulation or adenosine A<sub>2A</sub> receptor blockade during extinction training may affect extracellular GABA levels either basally or in response to a pharmacological-prime. Future studies should investigate the role of adenosine receptor modulation on GABA transmission.

Further research is necessary to fully elucidate the role of adenosine receptors in extinction and subsequent reinstatement. All experiments presented here used systemic administration of adenosine receptor agonists and antagonists; microinjections targeting these receptors specifically in the NAc would clarify the contributions of adenosine receptors located elsewhere in the brain. Future studies should also use microdialysis to identify the effects of adenosine A<sub>1</sub> receptor and adenosine A<sub>2A</sub> receptor stimulation as well as presynaptic adenosine A<sub>2A</sub> receptor blockade on extracellular glutamate and GABA in the NAc during extinction and subsequent drug-primed reinstatement.

Together, these findings build upon evidence demonstrating that adenosine receptor stimulation negatively regulates cocaine seeking in a variety of situations. These findings are novel because they illustrate lasting effects of a pharmacological treatment administered during extinction training on drug-induced cocaine seeking. This type of phenomenon may provide the basis for realistic treatment of human cocaine addiction, where it is often not feasible to treat an acute relapse episode. Future studies should examine the mechanisms by which presynaptic adenosine A<sub>1</sub> receptor stimulation and/or presynaptic adenosine A<sub>2A</sub> receptor blockade produces these lasting effects on cocaine seeking.
Chapter 5: General Discussion

The data presented here support recent findings that distinct populations of adenosine receptors within the striatum exist (Orru et al, 2011a; Orru et al, 2011b), and targeting these receptors can differentially modulate cocaine seeking following chronic self-administration. We suspect the ability for adenosine receptors to modulate cocaine seeking in these various ways is related to their modulatory effects on dopamine and glutamate signaling within the ventral striatum. However, because we have examined only pharmacological effects on behavior the cellular mechanisms that underlie these findings remain ambiguous and much of this discussion is speculative regarding the mechanisms explanations of these findings.

Mechanisms of Adenosine Signaling Within the Ventral Striatal Microcircuit

The experiments presented in Chapter 2 show the direct effects of adenosine $A_{2A}$ receptor stimulation or blockade in the NAc on cocaine- or dopamine $D_2$ receptor agonist-induced reinstatement. Stimulation of adenosine $A_{2A}$ receptors in the accumbens blocks cocaine- and quinpirole-induced drug seeking. However, at the same dose used in the cocaine reinstatement experiments, adenosine $A_{2A}$ receptor agonism has no effect on cocaine-induced locomotor activity or sucrose reinstatement indicating the specificity of these effects on cocaine seeking. Systemic and intra-NAc blockade of adenosine $A_{2A}$ receptors induces mild reinstatement, but antagonism of NAc adenosine $A_{2A}$ receptors in combination with sub-threshold doses of cocaine and quinpirole exacerbates reinstatement.
The mechanisms underlying these effects are unclear, but it is possible that stimulation of the adenosine A$_{2A}$ receptor facilitates the formation of A$_{2A}$-D$_2$ heteromers, ultimately decreasing ligand binding at dopamine D$_2$ receptors and restoring the behavioral changes following chronic cocaine administration. It remains unclear whether heteromeric A$_{2A}$-D$_2$ receptor complexes or another interactive mechanism mediate our effects since receptors that are not in heteromeric complexes still play an antagonistic and reciprocal role in modulating cellular function (Ferre, 1997; Ferre et al., 1991a). Thus, administration of an adenosine A$_{2A}$ receptor agonist reverses the effects of a dopamine D$_2$ receptor agonist on intracellular Ca$^{2+}$ release (Yang et al., 1995) and immediate early gene expression in the striatum (Morelli et al., 1994; Svenningsson et al., 1999a). Additionally, intracellular signaling cascades of adenosine A$_{2A}$ and dopamine D$_2$ receptors (see figure 5.1) have opposite effects on cAMP production and neuronal excitability (Schiffmann et al., 2007; Svenningsson et al., 1999a; Tozzi et al., 2007). In fact, a different study from our lab, involving adenosine A$_1$ receptor stimulation counteracting dopamine D$_1$ agonist-induced cocaine seeking, found that PKA-mediated phosphorylation of AMPA receptors plays an important role in regulating reinstatement (Hobson et al., 2012).

Stimulation of adenosine A$_{2A}$ receptors activates enkephalin-containing neurons in the striatum, which form the indirect pathway (Karcz-Kubicha et al., 2006; Svenningsson et al., 1999a), while stimulation of dopamine D$_2$ receptors inhibits activity at these same neurons (Svenningsson et al., 1999a). Decreased GABA release in the ventral pallidum is associated with cocaine seeking (Tang et al., 2005), and
Figure 5.1 Populations of adenosine receptors in the NAc. Postsynaptic adenosine $A_1$ and dopamine $D_1$ receptors are colocalized on the direct pathway MSNs, while postsynaptic adenosine $A_{2A}$ and dopamine $D_2$ receptors are colocalized on the indirect pathway. Presynaptic adenosine $A_1$ receptors are present on all glutamate terminals in the NAc, but presynaptic adenosine $A_{2A}$ receptors are preferentially located on neurons that synapse onto the direct pathway.
dopamine D$_2$ receptor stimulation in the NAc results in decreased GABA in the ventral pallidum through the indirect pathway (Floran et al., 1997). Interestingly, stimulation of adenosine A$_{2A}$ receptors in the ventral striatum results in enhanced GABA input to downstream structures like the ventral pallidum (Mingote et al., 2008; Ochi et al., 2000). Together, these findings suggest that the reduction in cocaine seeking seen with adenosine A$_{2A}$ stimulation in the accumbens may be mediated by restoring cocaine-induced decreases in GABA release in the ventral pallidum. Similarly, blocking the tonic inhibition of adenosine A$_{2A}$ receptors on dopamine D$_2$ receptors allows minor stimulation of dopamine D$_2$ receptors to further decrease GABA in the ventral pallidum and potentially drive cocaine seeking behaviors.

It is worth noting that the mild reinstatement seen with MSX-3, an adenosine A$_{2A}$ receptor antagonist, may be related to combined actions at presynaptic and postsynaptic adenosine A$_{2A}$ receptors. Our studies in Chapter 3 show that KW 6002, an antagonist thought to have greater specificity to postsynaptic adenosine A$_{2A}$ receptors (Orru et al., 2011a), resulted in much more robust reinstatement responding when given alone. Also, administration of CGS 21680 possibly stimulates both pre- and postsynaptic adenosine A$_{2A}$ receptors, but it's ability to inhibit dopamine D$_2$ receptor signaling and stimulate indirect pathway neurons appears to be capable of overcoming any increase in glutamate release mediated by presynaptic receptors and administration ultimately blocks cocaine seeking.

The experiments presented in chapter 3 examine the distinct effects of pre- and postsynaptic adenosine A$_{2A}$ receptor blockade on cocaine seeking. For these
experiments we used KW 6002 and SCH 442416 because a previous study has shown that these compounds exhibit preferential post- and presynaptic profiles, respectively (Orru et al, 2011a). Similar to our previous data systemic administration KW 6002 produced strong cocaine seeking alone, and pretreatment also intensified reinstatement to a sub-threshold dose of cocaine. Conversely, systemic administration of SCH 442416 did not induce reinstatement on its own, and pretreatment dampened cocaine-induced drug seeking. We suspect that the ability for SCH 442416 to reduce cocaine seeking is due to a decrease in glutamate release in the NAc, while the ability for KW 6002 to induce and enhance reinstatement is mostly likely due to removing the tonic inhibition of adenosine A2A receptors on dopamine D2 receptors.

In order to verify this, we induced reinstatement by infusing either AMPA or cocaine into the mPFC or the NAc. A previous study has shown that cocaine infusion into the mPFC induces reinstatement that can be attenuated by blocking AMPA receptors in the NAc (Park et al, 2002), presumably infusion of AMPA into the mPFC would also result in the increased glutamate release in the NAc that generates reinstatement (McFarland et al, 2004; Torregrossa et al, 2008). Infusion of AMPA into the NAc has been shown to induce reinstatement through actions at glutamate receptors on MSNs (Cornish et al, 1999; Cornish et al, 2000; Ping et al, 2008; Suto et al, 2004), while infusion of cocaine into the NAc produces reinstatement through stimulation of dopamine receptors on both the direct and indirect pathway (Bachtell et al, 2005; Schmidt et al, 2006b). As expected, pretreatment with KW 6002 exacerbated cocaine seeking induced by infusion of AMPA or cocaine into the mPFC. Surprisingly,
reinstatement responding to an intra-NAc infusion of AMPA, but not cocaine, was enhanced by pretreatment with KW 6002. Although this was unexpected, we may be observing a ceiling effect since infusing cocaine directly into the NAc results in high rates of responding on the previously drug-paired lever. It’s also possible that allowing enhanced stimulation of dopamine D₂ receptors by adenosine A₂A receptor antagonism coupled with cocaine-induced dopamine increases shifted the animals into more stereotyped behavior since dopamine D₂ receptors are thought to be responsible for stereotypy following chronic psychostimulant use (Ujike et al., 1990). Nevertheless, this data suggests that KW 6002 does in fact act postsynaptically to enhance the inhibitory effects of dopamine D₂ receptors on indirect pathway neurons. Interestingly, pretreatment with SCH 442416 has no effect on reinstatement induced by intra-NAc infusion of AMPA or cocaine, but does block cocaine seeking stimulated by infusion of AMPA or cocaine into the mPFC. This effect supports the idea that blockade of presynaptic adenosine A₂A receptors decreases mPFC glutamate release into the NAc and consequently blocks cocaine seeking.

The differential effects of pre- and postsynaptic adenosine A₂A receptors on cocaine seeking is supported by a study showing that striatal-specific knockdown of A₂A receptors enhances locomotor activity in response to cocaine, while a forebrain-specific knockdown of adenosine A₂A receptors reduces cocaine-induced locomotor activity (Shen et al., 2008). The data presented here suggests that blockade of postsynaptic adenosine A₂A receptors located on MSNs in the NAc enhance cocaine seeking, while blockade of presynaptic adenosine A₂A receptors on glutamatergic terminals decreases
cocaine seeking. Taken together, these findings suggest that adenosine $A_{2A}$ receptors localized on the indirect pathway provide inhibitory control over cocaine seeking, whereas adenosine $A_{2A}$ receptors localized to glutamate terminals in the NAc enhance glutamate signaling to stimulate cocaine seeking.

The experiments presented in chapter 4 investigate the effects of adenosine receptor agonism and antagonism on extinction and subsequent reinstatement after chronic cocaine self-administration. Both adenosine $A_1$ and $A_{2A}$ receptor agonists administered prior to extinction training decreased responding on the previously drug-paired lever on the first day of extinction training, however this effect was minor (confined to the first 15 min of the session) and was not seen in subsequent extinction training sessions. Remarkably, adenosine $A_1$ receptor stimulation during extinction training decreased subsequent reinstatement responding to cocaine and quinpirole, but if administration of the agonist was dissociated from extinction training no effects on subsequent reinstatement were observed. Given that reinstatement was inhibited by adenosine $A_1$, but not $A_{2A}$, receptor stimulation, we suspected that modulating glutamate signaling during extinction training was most likely mediating this effect. This is because in vivo microdialysis experiments have reported little to no change in dopamine release during extinction, but large increases in glutamate (Suto et al, 2010). In fact, a more recent study has shown that increases in accumbal glutamate during extinction training correlates with cocaine expectancy (Suto et al, 2013). Adenosine $A_1$ receptors are expressed on glutamate terminals in many parts of the brain, including the NAc, where stimulation will decrease glutamate release. Because presynaptic adenosine $A_{2A}$
receptor blockade, which had no effect on extinction training, also attenuated subsequent cocaine and quinpirole-induced drug seeking it seems likely that tempering glutamate release during extinction training may provide a way to reverse striatal signaling altered by chronic cocaine use. More importantly, this method of treatment represents a more viable option for many addicts due to its persistent benefits.

Based on the findings in the experiments presented here, we suspect that the direct and indirect pathways are playing distinctly different roles in cocaine reinstatement. Because dopamine D_1 receptors are stimulatory, chronic cocaine taking results in repeated activation of the direct pathway, while simultaneously exerting inhibitory actions through dopamine D_2 receptors resulting in repeated inactivation of the indirect pathway. This is supported by studies showing increased spine density in dopamine D_1 but not D_2 neurons following chronic cocaine exposure (Kim et al, 2011; Lee et al, 2006). Additionally, increases in mini EPSCs and decreases in mini IPSCs in direct pathway neurons coupled with decreases in mini EPSCs in indirect pathway neurons has been observed (Kim et al, 2011). Chronic cocaine has also been shown to increase ΔFosB in dopamine D_1, but not dopamine D_2 neurons (Lobo et al, 2013). Interestingly, overexpression of ΔFosB in the direct pathway, but not the indirect pathway, results in enhanced excitatory synaptic strength, spine density, and behavioral responses to cocaine (Grueter et al, 2013). Due to the opposing G protein coupling and intracellular signaling cascades of dopamine D_1 and D_2 receptors (Bertran-Gonzalez et al, 2008), chronic cocaine exposure may facilitate glutamate activation of dopamine D_1 neurons while inhibiting glutamate activation of dopamine D_2 neurons (Lobo et al, 2011).
MSNs of the striatum typically exhibit hyperpolarized resting membrane potentials (Cepeda et al, 2008; Planert et al, 2013). At baseline indirect pathway neurons are more excitable than direct pathway neurons, but dopamine modulates this excitability in an opposing fashion (Cepeda et al, 2008; Lobo et al, 2011; Planert et al, 2013). Dopamine increases intrinsic excitability in dopamine D₁ neurons, but decreases intrinsic excitability in dopamine D₂ neurons (Cepeda et al, 2008; Planert et al, 2013). Dopamine D₁ receptor stimulation enhances PKA activity and alters Ca²⁺ and K⁺ channels to enhance the glutamate mediated "up-state" in these MSNs (Gerfen et al, 2011; Lobo et al, 2011; Surmeier et al, 2007). Conversely, dopamine D₂ receptor stimulation decreases PKA activity and alters Ca²⁺, Na⁺, and K⁺ channels decreasing glutamatergic reactivity and shifting these MSNs into a "down-state" (Gerfen et al, 2011; Lobo et al, 2011; Surmeier et al, 2007). This imbalance in striatal signaling may ultimately underlie the vulnerability to relapse seen in chronic cocaine users. In fact, the circuitry of the indirect pathway is such that reduced activation results in decreased GABA release in the ventral pallidum which results in exacerbated inhibition of the medial dorsal thalamus and ultimately less activation of the PFC. Impaired signaling in this circuitry (Agnoli et al, 2013; Pezze et al, 2007) may explain the lack of behavioral control observed in cocaine addicts (Coffey et al, 2003; Garavan and Stout, 2005; Kaufman et al, 2003; Verdejo-Garcia et al, 2006).

Presynaptic adenosine A₂A receptors in the NAc are preferentially expressed on glutamate terminals that synapse on to dopamine D₁ expressing neurons (see figure 5.1) (Quiroz et al, 2009). They are therefore able to selectively modulate glutamate
release to the direct pathway but not the indirect pathway. This makes them an ideal target for decreasing glutamate signaling in dopamine D₁ neurons and possibly reversing imbalanced striatal signaling resulting from chronic cocaine use. We hypothesize that the long-term decreases in relapse susceptibility that we observe in the extinction experiments presented in chapter 4 are due to a reversal of this imbalance. Extinction training increases glutamate release in the NAc, but blocking presynaptic adenosine A₂A receptors decreases glutamate release at direct pathway synapses while leaving glutamate release to indirect pathway neurons unaffected. This is ideal because increased glutamate signaling to dopamine D₂ expressing neurons will likely increase the excitability of this chronically inhibited pathway, while simultaneously decreasing excitability in the over activated dopamine D₁ neurons. Together these effects may induce plasticity that approximate pre-cocaine conditions and restore neurotransmission in the ventral striatum, or at the very least help to decrease relapse vulnerability in addicts.

Potential Mechanisms of Adenosine Signaling in Glia

Adenosine A₁ and A₂A receptors are also expressed on astrocytes and microglia. It is possible that the behavioral effects we observe are in part due to actions of the adenosine agonists and antagonists on these cells that have been implicated in cocaine addiction (Beardsley and Hauser, 2014; Vijayaraghavan, 2009). Astrocytes are particularly important in regulating extracellular neurotransmitter levels, including glutamate. Importantly, because we know that glutamate homeostasis is disrupted by
chronic cocaine exposure adenosine actions at astrocytes may play an important role in decreasing reinstatement. Overexpression of adenosine $A_1$ receptors in astrocytes has been shown to increase expression and function of EAAT2 (excitatory amino acid transporter 2) also known as GLT-1 (Wu et al., 2011). EAAT2/GLT-1 is an important regulator of extracellular glutamate, and several studies have shown increasing expression with ceftriaxone, a β-lactam antibiotic, decreases cue and cocaine-induced reinstatement restores alterations in glutamate homeostasis (Knackstedt et al., 2010; Sari et al., 2009; Trantham-Davidson et al., 2012). Therefore, it is possible that adenosine $A_1$ receptor stimulation during extinction decreases subsequent reinstatement by increasing EAAT2/GLT-1 expression. However, because dissociation of adenosine $A_1$ receptor agonism from extinction training failed to decrease subsequent cocaine seeking this seems unlikely. Stimulation of adenosine $A_{2A}$ receptors on astrocytes appears to inhibit EAAT2/GLT-1 mediated glutamate uptake, and prolonged activation of these receptors can decrease expression of these transporters (Matos et al., 2012; Nishizaki et al., 2002). If our presynaptic adenosine $A_{2A}$ receptor antagonist is binding to astrocytic adenosine receptors, it is feasible that its ability to decrease subsequent reinstatement following administration during extinction training (chapter 4) or acutely decrease reinstatement (chapter 3) are related to increased glutamate uptake by astrocytes. Still, if this were the case we would expect that administration of SCH442416 would also decrease reinstatement induced by intra-NAcc AMPA or cocaine infusion, which it has no effect on.

Microglia have also been implicated in the addictive processes of cocaine and other psychostimulants (Beardsley et al., 2014; Northcutt et al., 2015). Increased
activation of microglia can increase extracellular glutamate as well as AMPA and NMDA receptor expression (Grace et al., 2014). The effects of adenosine receptor stimulation on microglia are complex. Adenosine A₁ receptor agonism decreases morphological activation of microglia (Luongo et al., 2014), while adenosine A₂A receptors appear to increase activation of microglia (Orr et al., 2009). However, at least one study has reported that instead of increasing proinflammatory cytokine release from these activated microglia, adenosine A₂A receptors act to inhibit release of proinflammatory cytokines, like TNF-α, induced by an LPS challenge (Newell et al., 2015). It is difficult to determine whether our effects on cocaine seeking are mediated by adenosine receptors on microglia since there is conflicting data regarding the effects of adenosine A₂A receptor stimulation on microglia exist, but it is plausible that decreasing extracellular glutamate through microglial mechanisms would decrease reinstatement.

Conclusions

Cocaine addiction is a significant public health problem, and the rate of relapse in addicts is very high. Identifying mechanisms to decrease relapse susceptibility is of utmost importance. Decades of research has identified that chronic cocaine use results in significant changes in glutamate and dopamine neurotransmission in NAcc, and that these nuanced changes are responsible for persistence of drug seeking behaviors even after prolonged abstinence. The data presented here suggest that adenosine, a known modulator of dopamine and glutamate signaling, may be an ideal target for reversing striatal signaling changes caused by chronic cocaine. These effects also verify that at least two separate populations of adenosine receptors with opposing effects on cocaine-
mediated behaviors exist within the striatum. Promoting activation of indirect pathway neurons through postsynaptic adenosine A$_{2a}$ receptor stimulation decreases cocaine seeking, while blockade of these same receptors enhances cocaine seeking. Blockade of presynaptic adenosine A$_{2a}$ receptors, on the other hand, decreases activation of the direct pathway by inhibiting glutamate release preventing cocaine seeking. Perhaps most interesting of the findings presented here is that blockade of presynaptic adenosine A$_{2a}$ or A$_{1}$ receptors during extinction training can prevent subsequent reinstatement, and is likely the most feasible as a treatment option for addicts. While the experiments presented in this dissertation explore the effects of adenosine receptors on cocaine seeking the identification of the mechanisms responsible for these effects remain elusive and future studies should to explore viable mechanisms underlying the observations presented here.
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