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Adenosine A_{2A} Receptors in the Nucleus Accumbens Bi-Directionally Alter Cocaine Seeking in Rats

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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 nucleus accumbens (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 were 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*-D-ribofuranuronamidosyl)-9*H*-purin-2-yl]amino]ethyl]benzenepropanoic acid hydrochloride), and the A_{2A} receptor antagonist, MSX-3 (3,7-dihydro-8-[(1*E*)-2-(3-methoxyphenyl)ethenyl]-7-methyl-3-[3-(phosphonooxy)propyl-1-(2-propynyl)-1*H*-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.
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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 are known to mediate relapse to cocaine seeking (Shaham *et al*, 2003). The mesolimbic DA system consists of DA cells in the ventral tegmental area that project to the nucleus accumbens (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 D_1 receptors increases adenylyl cyclase activity, while DA binding at D_2 receptors decreases the activity of this enzyme (Lachowicz and Sibley, 1997). In addition, D_1 and D_2 receptors are primarily expressed on two distinct populations of NAc neurons, with D_1 receptors occurring mainly on dynorphin/substance P-expressing neurons and D_2 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 and Gerfen, 1998).

Repeated cocaine administration produces alterations in DA receptor-mediated responses. Thus, repeated cocaine administration produces cross-sensitization with DA D_2 receptor agonists (Ujike *et al*, 1990), and while D_1 and D_2 receptors are necessary for the acquisition of behavioral sensitization, only D_2 receptors are necessary for its expression (Fontana *et al*, 1993). In self-administration models, systemic and intra-accumbens stimulation of DA 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 and Pierce, 2006;

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Self *et al*, 1996), and D_2 receptors appear to mediate cue-induced relapse to cocaine seeking (Cervo *et al*, 2003; Gal and Gyertyan, 2006). Therefore, tempering 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 A_{2A} receptors that are heavily expressed in the striatum, where they are highly colocalized with 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 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, A_{2A} receptor stimulation decreases DA binding at D_2 receptors (Ferre *et al*, 1991b). A recent study has suggested that this may be mediated by heteromeric receptor complexes comprised of A_{2A} and 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 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 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 and Self, 2009). The nonspecific adenosine antagonist, caffeine, produces modest reinstatement (Green and Schenk, 2002; Worley *et al*, 1994), while specific antagonism of A_{2A} receptors enhances cocaine sensitization (Filip *et al*, 2006). It remains unclear whether these A_{2A} receptor effects on cocaine behaviors are mediated by A_{2A} receptors in the NAc. Therefore, this study examines whether adenosine receptor effects on cocaine seeking are mediated by A_{2A} receptors localized to the NAc. These experiments test the effects of intra-NAc A_{2A} receptor stimulation or blockade on cocaine seeking in animals extinguished from cocaine self-administration. Local infusions of CGS 21680 (4-[2-[[6-amino-9-(*N*-ethyl-*b*-D-ribofuranuronamidosyl)-9*H*-purin-2-yl]amino] ethyl]benzenepropanoic acid hydrochloride), a selective A_{2A} receptor agonist, and MSX-3 (3,7-dihydro-8-[(1*E*)-2-(3-methoxyphenyl)ethenyl]-7-methyl-3-[3-(phosphonooxy) propyl-1-(2-propynyl)-1*H*-purine-2,6-dione disodium salt hydrate), a phosphatase prodrug of the A_{2A} receptor antagonist MSX-2 (Hauber *et al*, 1998; Sauer *et al*, 2000), were made into the medial division of the NAc core, a site where DA 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 g were individually housed with food and water available *ad libitum*. All 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 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 (5 mg/kg), a non-steroidal 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

A_{2A} receptor agonist, CGS 21680, was purchased from Tocris Bioscience (Ellisville, MO). A_{2A} receptor antagonist, MSX-3, 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 h 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 before surgery (see above).

After recovery from surgery, 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 5–6 days per week. 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 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 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-h 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 h of extinction conditions, followed by a 2-h reinstatement test period. In most experiments, an intra-NAc pretreatment was administered before a pharmacological prime (see below), which was immediately followed by the 2-h 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} 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 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, intraperitoneally) following the extinction session. Animals in a separate group were given intra-NAc injections of MSX-3 (vehicle, 5, 10, and 20 μg per side). Immediately following the systemic treatments and 5 min after the intra-NAc microinjections, the animals underwent 2 h of reinstatement testing. Animals in both groups were tested under all conditions in a randomized order and received a maximum of four 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 A_{2A} agonist, CGS 21680 (vehicle, 0.5, 1.0, 2.5, 5.0, and 10 ng per side), 5 min before the priming injection of cocaine (vehicle or 15 mg/kg, intraperitoneally). In a separate group of animals, the effects of intra-NAc adenosine A_{2A} receptor blockade on cocaine-primed reinstatement was tested by a pretreatment of the A_{2A} antagonist, MSX-3 (5 and 10 μg per side), 5 min before a priming injection of cocaine (vehicle, 5, or 10 mg/kg, intraperitoneally).

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 DA D_2 receptor-primed relapse behavior was assessed by a pretreatment of the A_{2A} agonist, CGS 21680 (vehicle or 2.5 ng per side), administered 5 min before quinpirole treatment (0.3 mg/kg). The effect of intra-NAc adenosine A_{2A} receptor antagonism on DA D_2 receptor-primed relapse behavior was assessed by administration of a pretreatment of the A_{2A} antagonist, MSX-3 (vehicle and 10 μg per side, intra-NAc), 5 min before quinpirole treatment (vehicle, 0.1, 0.3, and 1.0 mg/kg, intraperitoneally).

Sucrose Reinstatement

Animals were trained to self-administer sucrose pellets on an FR1 : TO 20 s schedule as described above. After 15 daily sessions (50 pellets per session), animals remained in their home cages for 7 days of 'abstinence', and were then subjected to extinction training in five daily 4-h sessions. Following extinction training, animals were tested for reinstatement of sucrose seeking. A pretreatment of CGS 21680 (2.5 ng per side, intra-NAc microinfusion) was administered 5 min before sucrose reinstatement testing. Reinstatement testing was initiated by non-contingent sucrose pellet delivery in a single 2-h test immediately following 2 h 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 $16 \times 16 \times 15$ in. with 16 pairs of photobeams spaced 1 in. apart on both the *x*- and *y*-axis. All locomotor tests were performed in darkened chambers during the light phase of the light : dark cycle. At 1 week following the completion of the self-administration and reinstatement procedures, animals were habituated to the locomotor testing chambers for 2 h (1 day before cocaine-induced locomotor activity testing). On test day, animals were habituated for 1.5 h, and given a pretreatment of CGS 21680 (vehicle, 2.5, or 5 ng per side, intra-NAc microinfusion). At 5 min following the pretreatment, all animals received cocaine (15 mg/kg). Total locomotor activity was measured by the number of beam breaks during the 2-h testing period.

Histology and Microinjections

Microinjections were administered as pretreatments 5 min before 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 five treatments in a randomized/counter-balanced order. All animals did not receive all treatments owing 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 per 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 experimental group ranged from 4 to 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 two-way ANOVA with lever (within) and treatments with A_{2A} agonists/antagonist–cocaine/quinpirole (between) as the factors, unless otherwise noted. Significant interactions were followed up with simple main effects analyses (one-way ANOVA) and *post hoc* tests (Bonferroni's comparisons). Sucrose reinstatement data were analyzed by two separate two-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 one-way between-subjects ANOVA. Statistical significance was set at $p<0.05$ for all tests.

RESULTS

Intra-NAc Adenosine A_{2A} Receptor Stimulation Dose-Dependently Blocks Cocaine-Induced Reinstatement

Animals were trained to self-administer cocaine for 3 weeks (avg. intake: $X=74.0\pm3.5$) and lever responding was extinguished in daily sessions (Figures 1a and b). Figure 1c illustrates that an intra-NAc pretreatment of the adenosine A_{2A} agonist CGS 21680 dose-dependently reduces cocaine-induced drug seeking. A significant lever × treatment interaction ($F_{6,72}=8.65$; $p<0.0001$) and significant main effects of lever ($F_{1,72}=27.82$; $p<0.0001$) 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 the CGS 21680 significantly induced active lever pressing, which 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 ($F_{6,72}=2.929$; $p<0.05$).

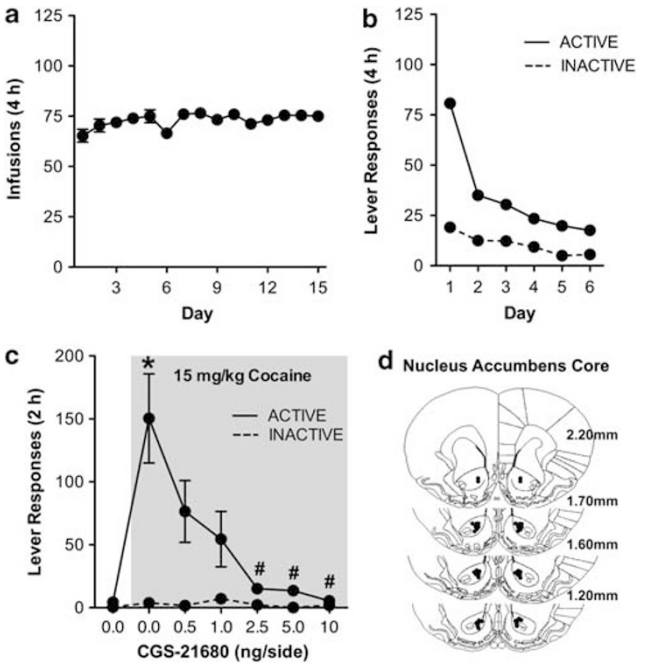

Figure 1 Intra-nucleus accumbens (NAc) administration of the adenosine A_{2A} agonist CGS 21680 dose-dependently blocked cocaine-induced reinstatement. (a) Average number of cocaine infusions in each 4 h session over the 3 week cocaine self-administration phase. (b) Extinction training was performed 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).

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 2a demonstrates that a pretreatment of CGS 21680 (2.5 ng per side) blocks quinpirole-induced reinstatement. A significant treatment × 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 A_{2A} receptors is not sufficient to completely block D_2 agonist-induced reinstatement, our findings suggest that intra-NAc stimulation of A_{2A} receptors effectively inhibits quinpirole-induced reinstatement, suggesting a localized interaction of A_{2A} and D_2 receptors within the NAc.

Effects of Intra-NAc Adenosine A_{2A} Receptor Stimulation on Cocaine-Induced Locomotor Activity

At high doses, systemic 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 of two effective doses of intra-NAc CGS 21680 (2.5 and 5 ng per side) on cocaine-induced locomotor activity. These tests were performed in a subset of animals that self-administered cocaine. Figure 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 2-h 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 per side) of CGS 21680 (Figure 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 × treatment interaction ($F_{30,160}=0.3424$, $p=0.995$).

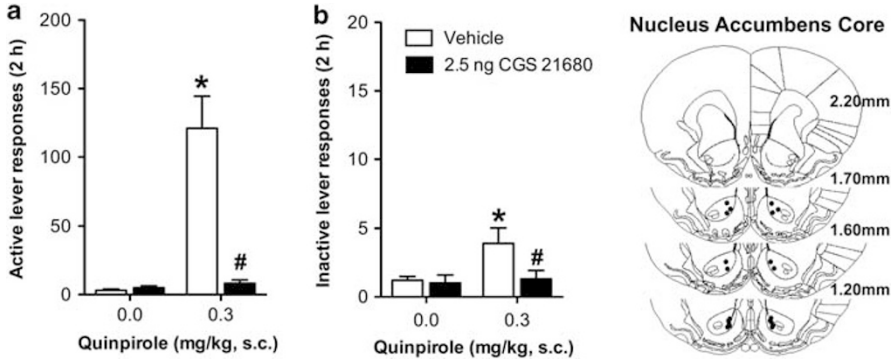

Figure 2 Intra-nucleus accumbens (NAc) administration of the adenosine A_{2A} agonist CGS 21680 blocks quinpirole-induced reinstatement. (a) An intra-NAc pretreatment of the A_{2A} receptor agonist, CGS 21680 (2.5 ng per side), was sufficient to block D_2 agonist-induced reinstatement. (b) Inactive lever 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).

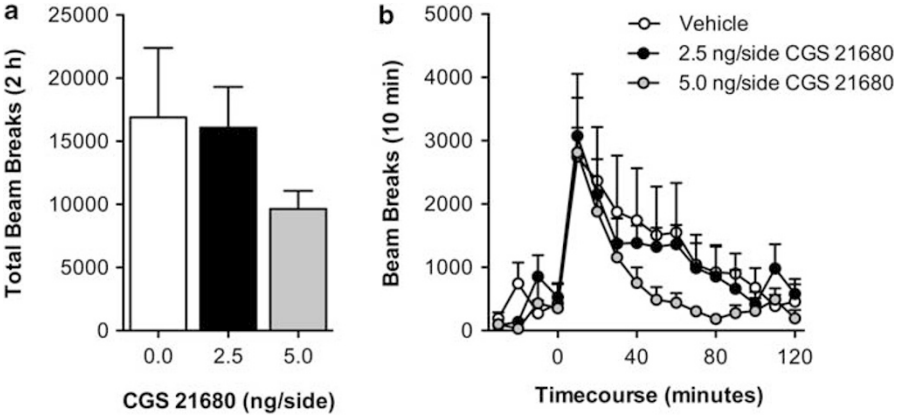

Figure 3 Adenosine A_{2A} agonist CGS 21680 does not alter cocaine-induced locomotor behavior. (a) No significant differences were observed in total cocaine-induced locomotor activity over the 2-h test period of animals between receiving a pretreatment with 0.0, 2.5 and 5.0 ng per side CGS 21680 before 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-nucleus accumbens (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.

Simple main effects analysis of treatment revealed a significant reduction in locomotor activity of the group receiving 5.0 ng per 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 A_{2A} receptor stimulation, we examined the effects of the minimally effective dose of CGS 21680 (2.5 ng per side) on reinstatement to sucrose seeking using non-contingent delivery of sucrose pellets in animals previously trained to self-administer sucrose pellets (Figure 4a). Figure 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 lever pressing (data not shown). While an intra-NAc infusion of the A_{2A} receptor agonist, CGS 21680, (2.5 ng per 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 5a illustrates that a systemic blockade of A_{2A} receptors with MSX-3 significantly increases active lever pressing in a dose-dependent manner. A significant treatment × 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 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 ($F_{2,31} = 1.666$, $p = 0.21$).

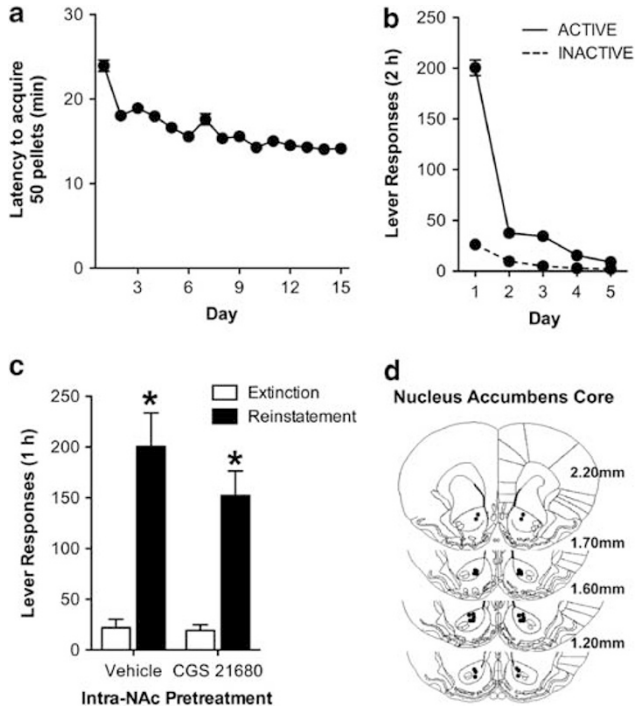

Figure 4 Sucrose reinstatement was unaffected by adenosine A_{2A} receptor agonist CGS 21680. (a) Sucrose self-administration was conducted over 3 weeks, and animals' latency to acquire 50 pellets was recorded. (b) Extinction training was performed in 5 daily sessions until active lever responding was reduced to levels comparable with inactive lever responding. (c) Significant sucrose reinstatement was observed compared with extinguished responding; however, an intra-nucleus accumbens (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 ($p < 0.0001$).

Although a systemic blockade of A_{2A} receptors resulted in a significant increase in active lever responding, the overall reinstatement produced appeared moderate compared with cocaine- and quinpirole-induced cocaine seeking. To determine if a blockade of 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 past 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 5b). A significant treatment × 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 per 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 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 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 × lever interaction

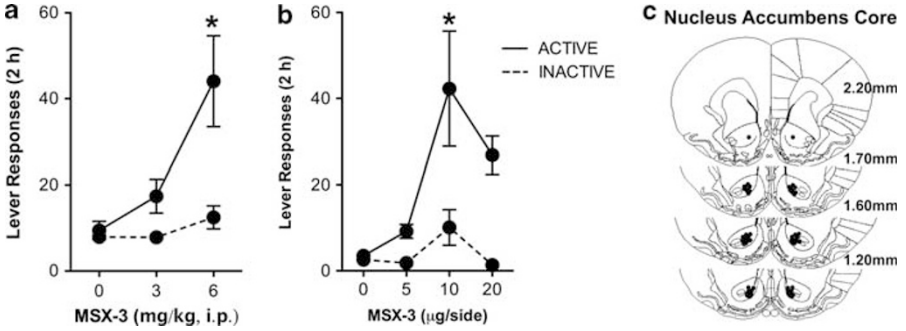

Figure 5 Systemic and intra-nucleus accumbens (NAc) blockade of adenosine A_{2A} receptors via MSX-3 produces cocaine seeking. (a) Systemic administration of MSX-3 (3 and 6 mg/kg) increased active lever responding in a dose-dependent manner. Number of animals per treatment group: saline = 13, 3 mg/kg 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).

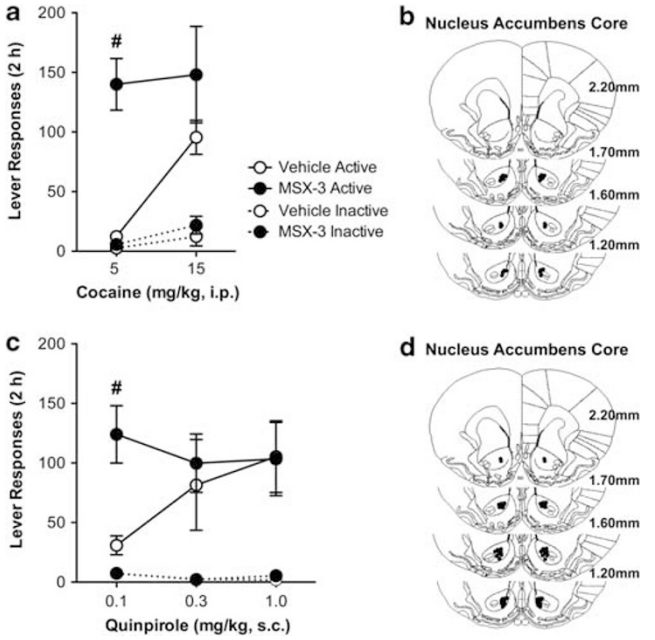

Figure 6 Intra-nucleus accumbens (NAc) blockade of adenosine A_{2A} receptors via MSX-3 potentiates reinstatement response to sub-threshold doses of cocaine and quinpirole. (a) An intra-NAc pretreatment with 10 μg per side MSX-3 potentiated active lever responding at a sub-threshold dose of cocaine (5 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.

($F_{4,58}=13.07$; $p<0.0001$) 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.

In addition, we examined the effect of A_{2A} receptor blockade on reinstatement induced by the D_2 agonist, quinpirole, to determine if removing the tonic inhibition of the A_{2A} receptor over the D_2 receptor could enhance responding to 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 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 × lever interaction ($F_{7,97}=5.86$; $p<0.0001$) and main effects of treatment ($F_{7,97}=5.863$; $p<0.0001$) and lever ($F_{1,97}=88.87$; $p<0.0001$) 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 ($F_{7,97}=5.908$; $p<0.0001$). Again, significant effects of treatment on the inactive lever were observed ($F_{7,97}=2.138$; $p<0.05$); however, subsequent *post hoc* testing revealed no significant differences between treatment groups.

DISCUSSION

We have previously shown that systemic A_{2A} receptor stimulation attenuates cocaine seeking induced by pharmacological stimuli and drug-related cues (Bachtell and Self, 2009). Here we elucidate the NAc as a primary site of action for these effects. Our findings reveal that pharmacological manipulation of adenosine A_{2A} receptors within the NAc bi-directionally alters cocaine seeking in extinguished rats. We show that intra-NAc stimulation of A_{2A} receptors attenuates cocaine seeking induced by pharmacological stimuli such as cocaine and quinpirole, suggesting that adenosine A_{2A} receptors represent a potential target for therapies aiming to curb relapse vulnerability. Because systemic and higher doses of intra-NAc A_{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 our effects are specific to cocaine, as A_{2A} stimulation did not significantly reduce sucrose seeking.

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 A_{2A} receptor exerts over the D_2 receptor allows a normally ineffectual dose of cocaine or quinpirole to induce reinstatement. Other models support this tonic inhibitory role of A_{2A} receptors in behavioral regulation. For example, a recent study demonstrated that blocking A_{2A} receptors, and hence, removing the adenosine 'brake', produces wakefulness (Lazarus *et al*, 2011). Antagonism of A_{2A} receptors also restores deficits in effort-related behaviors induced by D_2 receptor blockade (Nunes *et al*, 2010; Worden *et al*, 2009), suggesting that A_{2A} receptors are a tonic modulator of D_2 receptor expressing neurons within the striatum (Harper *et al*, 2006; Nagel *et al*, 2003). Our data provide further support that A_{2A} receptors exert tonic regulation of D_2 receptors and suggests that 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 A_{2A} receptors counteracts cocaine-mediated behaviors, while antagonism augments cocaine-mediated behaviors. Administration of an A_{2A} 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, 2002). Blockade of A_{2A} 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 A_{2A} receptors during withdrawal also has reward-related effects. Blocking A_{2A} receptors during a brain stimulation reward task reversed the elevated reward threshold produced by cocaine withdrawal, suggesting that removing the tonic activity of A_{2A} receptors enables DA signaling to restore reward deficits observed during drug withdrawal (Baldo *et al*, 1999). This explanation is supported by our findings that A_{2A} receptor blockade produces cocaine seeking by enabling DA receptor stimulation at sub-threshold doses of both cocaine and a D_2 agonist. Taken together, these findings indicate that pharmacological stimulation of A_{2A} receptors opposes the behavioral effects of cocaine, while pharmacological blockade of A_{2A} receptors enhances cocaine's effects.

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

It should be emphasized that the present experiments targeted the medial division of the NAc core, an area that 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 DA input along this medial–lateral continuum supports these functional differences in cocaine seeking. Thus, manipulations of DA 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*, 2006; Schmidt and Pierce, 2006). 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 and Gerfen, 1998). DA D_1 receptors are found mainly on dynorphin/substance P-expressing neurons that comprise the direct pathway, while 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*, 2006; Schmidt and Pierce, 2006). Thus, tempering DA signaling in the NAc is an ideal way to prevent relapse. A_{2A} receptors are colocalized with D_2 receptors, where they provide reciprocal regulation of 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*, 1998, 1999a,b).

Adenosine A_{2A} and DA 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 A_{2A} receptor stimulation to inhibit ligand binding to D_2 receptors and decrease G-protein coupling at the D_2 receptor (Ferre *et al*, 1991a; Fuxe *et al*, 1998; Hillion *et al*, 2002; Torvinen *et al*, 2005). As mentioned previously, cocaine reduces the expression of the A_{2A}–D_2 heteromer (Marcellino *et al*, 2010), which may underlie some of the changes in behavioral responses following chronic cocaine intake. It is possible that stimulation of the A_{2A} receptor facilitates the coupling of A_{2A} and D_2 receptors, ultimately 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, as receptors that are not in heteromeric complexes still play an antagonistic and reciprocal role in modulating cellular function (Ferre, 1997, 1991a). It will be critical for future studies to determine the impact of chronic cocaine intake on heteromeric A_{2A}–D_2 receptor expression and how selective pharmacological targeting of these heteromers may be relevant behaviorally.

In addition to the contribution of the A_{2A}–D_2 receptor heteromers, A_{2A} and D_2 receptors are coupled to excitatory and inhibitory G proteins, respectively. For example, stimulation of A_{2A} receptors counteracts the effects of D_2 receptor stimulation on immediate-early gene expression (Morelli *et al*, 1994; Svenningsson *et al*, 1999a) and opposes D_2 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 (eg, PKA-mediated phosphorylation targets) may play a role in the modulation of cocaine seeking. While A_{2A} receptors obviously play a significant role in modulating DA neurotransmission within the striatum, the cellular mechanisms

of our effects on cocaine seeking remain obscure. Although it is likely that both A_{2A}–D_2 heteromeric receptors and A_{2A} 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.

DA receptor stimulation in the NAc alters signaling in both the direct- and indirect-projecting subpopulations of medium spiny GABAergic neurons. The main projection target of the direct pathway is the VTA, whereas the indirect pathway targets the ventral pallidum (Aubert *et al*, 2000; Steiner and Gerfen, 1998). Stimulation of 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 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). Likewise, 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 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). Taken together, these findings suggest that the reduction in cocaine seeking seen with A_{2A} stimulation in the accumbens may be mediated by restoring cocaine (or D_2)-induced decreases in GABA release in the ventral pallidum. It is possible that this increase in GABA is a result of the ability for A_{2A} receptor stimulation to reduce the heightened sensitivity of D_2 receptors in the striatum resulting from chronic stimulant administration (Seeman *et al*, 2002). Thus, stimulation of the A_{2A} receptor functions similarly to a D_2 receptor antagonist and reverses the inhibition of GABA output from the indirect pathway of the striatum (Ferre *et al*, 1994; Floran *et al*, 1997) potentially caused by sensitized D_2 receptors. Similarly, blocking the tonic inhibition of A_{2A} receptors on D_2 receptors allows minor stimulation of D_2 receptors to further decrease GABA in the ventral pallidum and potentially drive cocaine-seeking behaviors.

A_{2A} receptors are expressed on other cell types in the NAc providing other possible explanations for our results. For example, expression of A_{2A} receptors on presynaptic glutamatergic terminals is involved in modulating striatal glutamate release and synaptic plasticity (Hettinger *et al*, 2001; Quiroz *et al*, 2009; Rodrigues *et al*, 2005). Thus, stimulation of presynaptic A_{2A} receptors increases striatal glutamate release and blockade of A_{2A} receptors produces the opposite effect (Corsi *et al*, 2000, 1999). It seems unlikely that our findings would result from A_{2A}-induced increases in glutamate release as 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 A_{2A} receptors are expressed on cholinergic interneurons (Tozzi *et al*, 2011), although this report conflicts with a previous study where 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 A_{2A} receptors and stimulation of 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 A_{2A} antagonist enhances cocaine seeking may result from reduced muscarinic activity. Recent work does not support this notion as 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 A_{2A} receptors within the NAc are having a large effect on cholinergic interneurons. Future studies will help to elucidate the interactions between A_{2A} and D_2 receptors on additional cell types in the NAc.

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 A_{2A} receptors blocks both cocaine- and quinpirole-induced drug seeking, while intra-NAc A_{2A} receptor blockade enhances cocaine seeking. While the antagonistic interaction between A_{2A} and D_2 receptors on striatal neuronal transmission is supported by these experiments, the relative contribution of heteromeric and non-heteromeric complexes is unknown. Taken together, our results suggest that interactions between A_{2A} and D_2 receptors influence striatal signaling that mediates cocaine seeking, but future studies should examine the specific cellular mechanisms by which A_{2A} stimulation reduces 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.

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DISCLOSURE

The authors declare no conflicts of interest.

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