Adenosine A₁ and Dopamine D₁ Receptor Regulation of AMPA Receptor Phosphorylation and Cocaine-Seeking Behavior

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AMPA (α -amino-3-hydroxy-5-methylisoxazole-4-propionate glutamate receptor) stimulation in the nucleus accumbens (NAc) is critical in cocaine seeking. Here, we investigate the functional interaction between D₁ dopamine receptors (D1DR) and AMPARs in the NAc, and explore how A₁ adenosine receptor (A1AR) stimulation may reduce dopamine-induced facilitation of AMPARs and cocaine seeking. All animals were trained to self-administer cocaine and were tested for reinstatement of cocaine seeking following extinction procedures. The role of AMPARs in both AMPA- and D1DR-induced cocaine seeking was assessed using viral-mediated gene transfer to bi-directionally modulate AMPAR activity in the NAc core. The ability of pharmacological AMPAR blockade to modulate D1DR-induced cocaine seeking also was tested. Immunoblotting was used to determine whether stimulating D1DR altered synaptic AMPA GluA1 phosphorylation (pGluA1). Finally, the ability of an A1AR agonist to modulate D1DR-induced cocaine seeking and synaptic GluA1 receptor subunit phosphorylation was explored. Decreasing AMPAR function inhibited both AMPA- and D1DR-induced cocaine seeking. D1DR stimulation increased AMPA pGluA1^{S845}. Administration of the A1AR agonist alone decreased synaptic GluA1 expression, whereas coadministration of the A1AR agonist inhibited both cocaine- and D1DR-induced cocaine seeking and reversed D1DR-induced AMPA pGluA1^{S845}. These findings suggest that D1DR stimulation facilitates AMPAR function to initiate cocaine seeking in D1DR-containing direct pathway NAc neurons. A1AR stimulation inhibits both the facilitation of AMPAR function and subsequent cocaine seeking, suggesting that reducing AMPA glutamate neurotransmission in direct pathway neurons may restore inhibitory control and reduce cocaine relapse.

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INTRODUCTION

Research in humans and animal models demonstrates that the mesolimbic dopamine system is an important neural circuit involved in drug-seeking behaviors (Anderson and Pierce, 2005; Shalev *et al*, 2002). The mesolimbic dopamine system consists of dopamine cells in the ventral tegmental area that project to medium spiny neurons in the nucleus accumbens (Swanson, 1982). Activation of this pathway through stress exposure, drug-associated cues and pharmacological stimuli induces relapse to cocaine seeking (Shaham *et al*, 2003). For example, administration of

cocaine rapidly increases both dopamine and glutamate release in the nucleus accumbens (NAc). Stimulation of α -amino-3-hydroxy-5-methylisoxazole-4-propionate glutamate receptors (AMPA) on NAc neurons is both necessary and sufficient to induce drug seeking, whereas stimulation of dopamine receptors is also sufficient to induce drug seeking (Bachtell *et al*, 2005; Cornish *et al*, 1999; Cornish and Kalivas, 2000; Kalivas and Duffy, 1990; Schmidt *et al*, 2006). Here, we explore how these receptor types interact in the NAc to impact cocaine seeking.

Dopamine receptors are differentiated into subclasses by their pharmacology, metabotropic signal transduction, and anatomical localization (Kebabian *et al*, 1972; Sibley *et al*, 1993). Dopamine D₁ receptors (D1DR) are primarily expressed on substance P/dynorphin-expressing direct pathway neurons where they couple to stimulatory G proteins and increase the activity of the cyclic adenosine monophosphate-protein kinase A (PKA) signaling pathway (Harrison *et al*, 1990; Lu *et al*, 1998; Sibley *et al*, 1998).

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Stimulation of D1DR increases PKA phosphorylation at serine 845 on the GluA1 AMPAR subunit (Chao *et al*, 2002b), increases the membrane expression of GluA1-containing AMPARs (Chao *et al*, 2002a; Ferrario *et al*, 2011; Mangiavacchi and Wolf, 2004), and enhances AMPAR currents in striatal neurons (Cepeda *et al*, 1993; Price *et al*, 1999; Snyder *et al*, 2000). Thus, there is overwhelming support that D1DR stimulation facilitates AMPAR function. We therefore hypothesized that D1DR-induced AMPAR facilitation is necessary for cocaine seeking. In this study, we test the necessity of AMPARs in D1DR-induced cocaine seeking. First, we overexpress a GluA1 subunit with a dominant-negative mutation in the pore region (GluA1^{Q582E}) that renders AMPARs dysfunctional (Bachtell *et al*, 2008; Dingledine *et al*, 1999; Shi *et al*, 2001). Second, we utilize pharmacological AMPA (($\pm$)- α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid hydrate) blockade to non-selectively reduce AMPAR neurotransmission.

Identifying strategies to offset D1DR facilitation of AMPARs may provide insight into the factors contributing to relapse and reveal novel therapeutic approaches. We have recently shown that stimulating adenosine receptors in the NAc reverses the expression of behavioral sensitization to cocaine (Hobson *et al*, 2012). Adenosine is a purine nucleoside and neuromodulator that functionally antagonizes dopamine signaling in the NAc through stimulation of A₁ or A_{2A} adenosine receptors (Ferre *et al*, 1992; Filip *et al*, 2012). Importantly, A₁ adenosine receptors (A1AR) are colocalized with D1DR on medium spiny neurons where they oppose D1DR activity, in part, by inhibiting activity in the cAMP-PKA pathway (Ferre *et al*, 1994, 1999; Yabuuchi *et al*, 2006). Therefore, we hypothesized that stimulation of A1ARs may dampen the extent to which D1DR stimulation facilitates AMPAR function, and ultimately cocaine seeking. In this study, we identify whether A1AR stimulation alone affects synaptic GluA1 subunit expression and phosphorylation, and determine its effects on D1DR-induced PKA GluA1 phosphorylation and cocaine seeking.

MATERIALS AND METHODS

Animals

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

Drugs

The D1DR agonist, SKF 81297 (($\pm$)-6-chloro-PB hydrobromide), AMPA, and the AMPA antagonist, CNQX (6-cyano-7-nitroquinoxaline-2,3-dione disodium salt hydrate), were obtained from Sigma Aldrich (St Louis, MO). The A1AR agonist, CPA (N⁶-cyclopentyladenosine), and presynaptic 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), were obtained from Tocris Bioscience (Ellisville, MO). Cocaine hydrochloride was obtained from Sigma-Aldrich (St Louis, MO). All drugs were dissolved in sterile-filtered physiological (0.9%) saline or physiological PBS (pH 7.4) except SCH 442416, which was suspended in a solution of 5% dimethyl sulfoxide, 5% Tween-80, and 90% ddH₂O.

Surgery

Jugular catheters and intracranial cannulae were sequentially implanted during the same surgery under halothane anesthesia (1–2.5%), as described elsewhere (O'Neill *et al*, 2012; Supplementary Methods). To maintain catheter patency, catheters were flushed daily with 0.2 ml of heparinized (20 IU/ml) bacteriostatic saline containing gentamycin sulfate (0.33 mg/ml). Intracranial guide cannulae were implanted into the medial portions of NAc core (A/P: +1.7; M/L: $\pm$ 1.5; D/V: – 5.7 from bregma; Paxinos and Watson, 1998) based on findings demonstrating SKF 81297 administration into the medial core and shell are most effective in inducing cocaine seeking (Bachtell *et al*, 2005; Schmidt *et al*, 2006).

Microinfusions and histology

Drug microinfusions (0.5 μ l per side over 100 s) were delivered through bilateral 33-G microinjector extending 1 mm beyond the guide, as described elsewhere (O'Neill *et al*, 2012; Supplementary Methods). Herpes simplex viral vectors encoding GluA1 (GluA1^{WT}), a pore-dead mutant (GluA1^{Q582E}), and LacZ (expressing β -galactosidase as a control) were produced and administered as described previously (Carlezon *et al*, 1997; Neve *et al*, 1997; Sutton *et al*, 2003). Viral vectors were infused 2–3 days after extinction training and reinstatement testing occurred 24–96 h after virus was infused to the NAc, consistent with the previously established time course of viral expression (Bachtell *et al*, 2008; Sutton *et al*, 2003). Following behavioral testing (except when tissue was used for immunoblotting, see below), localization of infusion sites was determined by infusing 0.5 μ l of cresyl violet dye in anesthetized animals and analyzed in 0.8-mm-thick coronal slices under a dissecting microscope.

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 lever-press training with sucrose pellets and recovery from surgery, animals self-administered cocaine (0.5 mg/kg/100 μ l, intravenously) under an FR1 reinforcement schedule (FR1:TO20) over 15 daily 4-h sessions (see Supplementary Methods). After a minimum of 15 cocaine self-administration sessions, animals returned to the operant conditioning chambers for extinction training (6 daily 4-h sessions) where 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. Reinstatement testing began after 5 days after extinction training and was run in sessions initiated with a 2-h extinction period, followed by a 2-h reinstatement test period (see Supplementary Methods).

Experiment 1: Effects of GluA1 Modulation on AMPA-Induced Reinstatement

Animals were counterbalanced according to cocaine self-administration/extinction responding and administered a herpes simplex viral vector encoding GluA1^{WT}, GluA1^{Q582E}, or LacZ. At 24 h after the viral infusion, cocaine seeking was measured following an intra-NAc microinfusion of AMPA (vehicle, 0.2, or 0.4 nM per side) as described above. Each rat was tested across the three treatment doses in a randomized order.

Experiment 2: Effects of GluA1 Modulation on Intra-NAc D1DR-Induced Reinstatement

Animals were counterbalanced according to cocaine self-administration/extinction responding and administered a herpes simplex viral vector encoding GluA1^{WT}, GluA1^{Q582E}, or LacZ. At 24 h after the viral infusion, cocaine seeking was measured following an intra-NAc microinfusion of SKF 81297 (vehicle, 1.0, or 3.0 μ g per side) as described above. Each rat was tested across the three treatment doses in a randomized order.

Experiment 3: Effects of AMPAR Blockade on D1DR-Induced Reinstatement

Following cocaine self-administration/extinction training, rats were tested for D1DR-induced cocaine seeking following an intra-NAc microinfusion of SKF 81297 (vehicle or 3.0 μ g per side) as described above. An intra-NAc microinfusion of CNQX (vehicle or 0.1 nM per side) was administered as a pre-treatment 5 min before the SKF 81297 microinfusion.

Experiment 4: Effects of A1AR Stimulation on Cocaine-Induced Reinstatement

Following cocaine self-administration/extinction training, rats were tested for cocaine seeking following a systemic priming injection of cocaine (15 mg/kg, intraperitoneally or vehicle). The effects of A1AR stimulation were evaluated with a pre-treatment of CPA (0.0, 0.03, or 0.1 mg/kg, intraperitoneally). We also assessed the effects of CPA on sucrose reinstatement in animals that were trained to self-administer sucrose and extinguished (see Supplementary Methods).

Experiment 5: Effects of A1AR Stimulation on Synaptic GluA1 Subunit Expression and Phosphorylation

Animals were trained to self-administer cocaine and extinguished. At 24 h following reinstatement testing, an intra-NAc microinfusion of CPA (vehicle or 1.5 μ g per side)

was administered. At 24 min following the infusion, 12-G tissue punches from 1.0-mm-thick slices were collected from the infusion and dorsal control sites. Tissue punches were processed for synaptoneurosomal fractionation and analyzed for synaptic GluA1 expression (tGluA1) and phosphorylation (protein kinase C (PKC)/calcium/calmodulin-dependent kinase (CaMK) site: GluA1^{S831}; PKA site: GluA1^{S845}) using immunoblotting. See Supplementary Methods for detailed procedures.

Experiment 6: Effects of Adenosine Receptor Modulation on D1DR-Induced Reinstatement

Following cocaine self-administration/extinction training, rats were tested for cocaine seeking following an intra-NAc microinfusion of vehicle, SKF 81297 (3.0 μ g per side), or the coadministration of SKF 81297 and CPA (3.0 and 1.5 μ g per side, respectively). In a separate set of animals, we tested the effects of a presynaptic adenosine A_{2A} receptor antagonist (SCH 442416) that facilitates the activity of presynaptic A1AR on glutamate terminals on intra-NAc D1DR-induced reinstatement (Orzu *et al*, 2011a, b). SCH 442416 (vehicle or 1.0 mg/kg, intraperitoneally) was administered as a pre-treatment to intra-NAc SKF 81297 (3.0 μ g per side).

Experiment 7: Effects of A1AR Stimulation on D1DR-Induced GluA1 Phosphorylation

Animals were trained to self-administer cocaine and extinguished. At 24 h following reinstatement testing, rats received an intra-NAc microinfusion of vehicle, SKF 81297 (3.0 μ g per side), or the coadministration of SKF 81297 and CPA (3.0 and 1.5 μ g per side, respectively). At 30 min following the infusion, 12-G tissue punches from 1.0-mm-thick slices were collected from the infusion and dorsal control sites. Tissue punches were processed for synaptoneurosomal fractionation and analyzed for synaptic GluA1 expression and phosphorylation using immunoblotting. See Supplementary Methods for detailed procedures.

Data Analysis

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 (drug-paired lever responses) were analyzed using a one or two-way ANOVA. Drug treatment and viral treatments were used as between factors where appropriate. Protein quantification data was analyzed using a one-way ANOVA or *t*-test, as appropriate. Significant interactions were followed up with simple main effects analyses (one-way ANOVA) and *post hoc* tests (Bonferroni's comparisons). Significant main effects were followed up with appropriate *post hoc* tests. Statistical significance was set at $p < 0.05$ for all tests.

RESULTS

Bi-Directional Modulation of AMPA-Induced Cocaine Seeking

Rats self-administered cocaine for at least 15 days (mean daily intake during last week = 47.27 ± 1.84 mg/kg) and

Figure 1 Bi-directional regulation of GluA1-containing AMPARs (α -amino-3-hydroxy-5-methylisoxazole-4-propionate glutamate receptors) in the nucleus accumbens alters AMPA (($\pm$)- α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid hydrate)-induced cocaine seeking. (a) Average number of cocaine infusions in the last five 4-h sessions during the cocaine self-administration phase. (b) Extinction training over 6 daily 4-h sessions. (c) Histology plate illustrating the infusion sites for the virus delivery and AMPA microinfusions. (d) Overexpression of GluA1^{WT} in the nucleus accumbens enhances, whereas overexpression of the pore-dead mutant GluA1^{Q582E} reduces cocaine seeking following nucleus accumbens infusions of AMPA relative to LacZ control. Hourly distribution of lever presses induced by vehicle (e) and 0.4 nmol AMPA (f) compared with extinction. $N = 7$ –12 per group *Bonferroni's test significant from LacZ, $p < 0.05$.

lever pressing was subsequently extinguished in six daily extinction sessions (Figure 1). Figure 1d shows that overexpression of GluA1^{WT} in the NAc potentiated, whereas expression of GluA1^{Q582E} in the NAc inhibited AMPA-induced cocaine seeking (Group $\times$ AMPA dose interaction, $F_{4,50} = 3.76$; $p = 0.0095$; Group, $F_{2,50} = 9.20$; $p = 0.0010$; AMPA dose, $F_{2,50} = 30.50$; $p = 0.0001$). Analysis of the interaction between GluA1 expression and AMPA dosage determined that GluA1^{WT} significantly increased, whereas GluA1^{Q582E} significantly decreased lever pressing induced by the high dose of AMPA (0.4 nM per side).

AMPA Stimulation is Necessary for D1DR-Induced Cocaine Seeking

Rats self-administered cocaine for at least 15 days (data not shown), lever pressing was extinguished, and viral vectors were infused. Microinfusions of the D1DR agonist, SKF 81297, into the NAc produced a significant dose-dependent increase in cocaine seeking (Figure 2a; $F_{2,93} = 27.73$; $p = 0.0001$). Overexpression of GluA1^{WT} in the NAc had no effect on D1DR-induced cocaine seeking, whereas overexpression of GluA1^{Q582E} significantly reduced D1DR-induced cocaine seeking compared with LacZ-expressing controls ($F_{2,93} = 5.56$; $p = 0.0052$). Pharmacological blockade of AMPARs with an NAc pre-treatment of the AMPA antagonist, CNQX, produced similar effects on

D1DR-induced cocaine seeking. Figure 2b shows that pre-treatment with CNQX significantly reduced D1DR-induced cocaine seeking (pre-treatment $\times$ SKF 81297 interaction, $F_{1,36} = 4.18$; $p = 0.0483$; SKF 81297, $F_{1,36} = 13.71$; $p = 0.0007$; pre-treatment, $F_{1,36} = 3.809$; $p = 0.0588$). These findings corroborate observations that degrading AMPAR function with GluA1^{Q582E} overexpression impairs D1DR-induced cocaine seeking and suggest that AMPAR stimulation is necessary for D1DR-induced cocaine seeking.

Stimulating A1AR Impairs Cocaine-Primed Cocaine Seeking

Stimulating NAC A1AR reverses the expression of cocaine sensitization (Hobson *et al*, 2012). We therefore tested whether stimulation of A1ARs would have analogous effects on cocaine seeking elicited by a cocaine prime. Figure 3a illustrates that A1AR stimulation significantly reduced cocaine seeking elicited by a systemic cocaine priming injection. An ANOVA across CPA pre-treatment dose produced significant main effects of cocaine ($F_{2,18} = 6.926$; $p = 0.0059$). We also tested the effects of CPA administration on sucrose seeking. We observed no significant effects of CPA administration on the reinstatement of sucrose seeking (Figures 3b; $t_{12} = 1.173$, $p = 0.2635$). These studies suggest that A1ARs may

Figure 2 AMPAR (α -amino-3-hydroxy-5-methylisoxazole-4-propionate glutamate receptors) function in the nucleus accumbens is necessary for D₁ dopamine receptors (D1DR) agonist-induced cocaine seeking. (a) Overexpression of pore-dead mutant GluA1^{Q582E} in the nucleus accumbens reduces cocaine seeking following nucleus accumbens microinfusions of SKF 81297 (($\pm$)-6-chloro-PB hydrobromide), whereas overexpression of GluA1^{WT} has no effect compared with LacZ control. $N = 8$ –18 per group, * Bonferroni's test: significant from LacZ controls, $p < 0.05$. (b) Histology plate illustrating the infusion sites for virus and SKF 81297 microinfusions. (c) Pre-treatment with a nucleus accumbens microinfusion of the AMPA antagonist, CNQX (6-cyano-7-nitroquinoxaline-2,3-dione disodium salt hydrate) (0.1 nM per side), reduces D1DR agonist (SKF 81297)-induced cocaine seeking. $N = 10$ per group, *Bonferroni's test: significant from 0.0 SKF 81297 per vehicle, $p < 0.05$. (d) Histology plate illustrating the infusion sites for CNQX/SKF 81297 microinfusions.

represent a viable target to offset signaling cascades induced by D1DR stimulation.

Stimulating A1AR Decreases Synaptic Expression of AMPA Glutamate Receptor Subunit, GluA1

We next tested how stimulation of A1AR influences the basal synaptic levels of the AMPA GluA1 subunit and its phosphorylation. Significant enrichment of PSD-95 (postsynaptic density protein 95) and total GluA1 (tGluA1) in the synaptic fraction (P2) and enrichment of β -tubulin in the cytosolic fraction (S2) was observed in our synaptonuclear preparation (Supplementary Figure S1). A microinfusion of CPA into the NAc following cocaine self-administration and extinction significantly reduced synaptic expression of tGluA1 levels by approximately 30% (Figures 3c; $t_{10} = 5.764$, $p = 0.0002$). The NAc microinfusion of CPA did not produce any statistical differences in synaptic pGluA1^{S831} or pGluA1^{S845} (Figure 3c), or in the ratio of phosphorylated to total GluA1 in synaptic fractions (Figure 3d). Similar analyses performed in the cytosolic fractions and tissue collected from regions dorsal of the infusion site revealed no significant effects in GluA1 regulation, suggesting that the changes were restricted to

Figure 3 Stimulation of A₁ adenosine receptors (A1ARs) reduces cocaine-induced cocaine seeking and the expression of synaptic GluA1 subunits. (a) Systemic administration of the A1AR agonist, CPA (N⁶-cyclopentyladenosine), significantly reduced cocaine seeking induced by 15 mg/kg cocaine, intraperitoneally. *Bonferroni's test: significant from control, $p < 0.05$. (b) Reinstatement of sucrose seeking over a 2-h test session was not significantly altered by the administration of the A1AR agonist, CPA. (c) Nucleus accumbens microinfusions of the A1AR agonist, CPA (1.5 μ g per side), decreased the synaptic expression of total GluA1 (tGluA1) protein, but had no effect on calcium/calmodulin-dependent kinase II/ protein kinase C (CaMKII/PKC)-mediated phosphorylation (pGluA1^{S831}) or protein kinase A (PKA)-mediated phosphorylation (pGluA1^{S845}) when normalized to PSD-95 (postsynaptic density protein 95). (d) Stimulation of A1AR with nucleus accumbens microinfusions of CPA also had no effect on CaMKII/PKC-mediated phosphorylation (pGluA1^{S831}) or PKA-mediated phosphorylation (pGluA1^{S845}) when normalized to tGluA1. Representative immunoblot bands are depicted below the corresponding group. All changes are reported as % of vehicle control. $N = 6$ per group.

the synaptic fractions surrounding the infusion site (Supplementary Table S1).

Stimulating A1AR Impaired D1DR-Induced Cocaine Seeking and GluA1^{S845} Phosphorylation

Figure 4 illustrates that localized A1AR stimulation significantly reduced D1DR-induced cocaine seeking produced by a microinfusion of SKF 81297. A one-way ANOVA across treatment groups reveals a significant main effect ($F_{2,17} = 9.620$; $p = 0.0020$) where SKF 81297 alone significantly increased cocaine seeking ($p < 0.01$) and CPA coadministration blunted it ($p < 0.05$). In addition, microinfusion of SKF 81297 also elicited cocaine seeking that was unaltered by intra-accumbens administration of the

Figure 4 Stimulation of A₁ adenosine receptors (A₁ARs) in the nucleus accumbens inhibits D₁ dopamine receptors (D₁DR) agonist-induced cocaine seeking and offsets D₁DR agonist-induced protein kinase A (PKA)-mediated phosphorylation of synaptic GluA1. (a) Nucleus accumbens microinfusions CPA (N⁶-cyclopentyladenosine) (1.5 µg per side) significantly reduced cocaine seeking elicited by a nucleus accumbens microinfusion of SKF 81297((±)-6-chloro-PB hydrobromide). Enabling presynaptic A₁AR stimulation with the presynaptic 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), had no effect on D₁DR agonist-induced cocaine seeking. *N* = 5–9 per group, *Bonferroni's test: significant from control, *p* < 0.05; #Bonferroni's test: significant from 0.0 CPA, *p* < 0.05. (b) Total GluA1 levels are not altered following any of the treatments. (c) Stimulation of D₁DR receptors with a nucleus accumbens microinfusion of SKF 81297 significantly increased PKA-mediated GluA1 phosphorylation (pGluA1^{S845}) that was reversed by the coadministration of CPA (1.5 µg per side). (d) Stimulation of D₁DR receptors with a nucleus accumbens microinfusion of SKF 81297 had no effect on calcium/calmodulin-dependent kinase II/protein kinase C (CaMKII/PKC)-mediated GluA1 phosphorylation (pGluA1^{S831}); however, the combined treatment of CPA (1.5 µg per side) and SKF 81297 (3.0 µg per side) microinfused into the nucleus accumbens significantly increased CaMKII/PKC-mediated pGluA1^{S831}. Representative immunoblot bands are depicted below the corresponding group. All changes are reported as % of vehicle control. *N* = 9–12 per group, * Bonferroni's test: significant from control, *p* < 0.05; #Bonferroni's test: significant from 0.0 CPA, *p* < 0.05. PSD-95, postsynaptic density protein 95; tGluA1, total GluA1.

presynaptic adenosine A_{2A} receptor antagonist, SCH 442416 (Figure 4; $F_{2,17} = 4.632$; *p* = 0.0271).

In contrast to reduced synaptic expression of tGluA1 levels with CPA infusions alone, coadministration of CPA with SKF 81297 failed to alter tGluA1 levels ($F_{2,30} < 1$; NS). However, microinfusion of SKF 81297 significantly increased synaptic pGluA1^{S845} relative to vehicle controls, whereas coadministration of CPA with SKF 81297 reversed this effect ($F_{2,30} = 5.00$; *p* = 0.0139). Interestingly, the coadministration of CPA and SKF 81297 significantly increased synaptic pGluA1^{S831} relative to vehicle control, while treatment with SKF 81297 alone had no significant effect on synaptic pGluA1^{S831} ($F_{2,15} = 7.37$; *p* = 0.0072). Similar analyses performed in the cytosolic fractions and tissue collected from regions dorsal of the infusion site revealed no significant changes in GluA1 expression or phosphorylation, suggesting that the changes were restricted to the synaptic fractions surrounding the infusion site (Supplementary Table S2).

DISCUSSION

Chronic administration of cocaine is known to modulate AMPA glutamate transmission in the NAc and AMPAR

stimulation is both necessary and sufficient for several cocaine-mediated behaviors (Cornish et al, 1999; Cornish and Kalivas, 2000). Our data suggest that D₁DR stimulation recruits AMPARs and facilitates AMPA glutamate neurotransmission to induced cocaine seeking (Figure 5). First, we found that up- and downregulation of the AMPAR subunit, GluA1, produces a bi-directional effect on AMPA-induced drug seeking. We found that downregulation of AMPAR function using both pharmacological and genetic strategies attenuate cocaine seeking induced by a microinfusion of the D₁DR agonist, SKF 81297 into the NAc. We also show that D₁DR stimulation increases PKA phosphorylation of the GluA1 AMPAR subunit that likely facilitates AMPA glutamate transmission by enhancing AMPAR trafficking and contributes to enhanced cocaine seeking upon D₁DR stimulation (Cepeda et al, 1993; Chao et al, 2002a; Ferrario et al, 2011; Price et al, 1999; Snyder et al, 2000). Finally, we utilized A₁AR stimulation to impair synaptic AMPAR expression and D₁DR-mediated GluA1^{S845} phosphorylation, which also inhibits both cocaine-primed and localized D₁DR-induced drug seeking in the NAc.

These studies suggest a functional interaction between D₁DR and AMPA glutamate receptors where D₁DR stimulation provides a feed-forward enhancement in AMPA glutamate transmission in the NAc that is necessary for

Figure 5 Schematic illustrating the proposed model by which D₁ dopamine receptors (D1DRs) facilitate AMPAR (α -amino-3-hydroxy-5-methylisoxazole-4-propionate glutamate receptor) function to produce reinstatement. In this model, a mechanism by which D1DR stimulation produces cocaine seeking is the facilitation of AMPAR activity. (1) Stimulation of D1DR facilitates AMPAR trafficking through initial protein kinase A (PKA)-mediated phosphorylation (S845) that directs AMPARs to extrasynaptic sites. (2) Prolonged activity at D1DR increases L-type Ca^{2+} channel activity and promotes calcium/calmodulin-dependent kinase II/protein kinase C (CaMKII/PKC) phosphorylation (S831) providing additional facilitation of AMPARs by redistributing them to synaptic sites (Anderson *et al*, 2008). (3) Stimulation of A₁ adenosine receptor (A1AR) impairs the ability of D1DR stimulation to initiate PKA-mediated phosphorylation through opposing influences on PKA activity. Functionally, this inhibits the initial step by which D1DRs enhance AMPAR activity. VGCC, voltage-gated Ca^{2+} channels.

cocaine seeking (Figure 5). Previous work has demonstrated that dopamine D1DR stimulation and PKA activity facilitates AMPAR function through AMPAR redistribution (Ehlers, 2000; Newpher and Ehlers, 2008; Wolf, 2010). For example, stimulation of D1DR increases PKA-mediated phosphorylation of the GluA1^{S845} AMPAR subunit and is associated with increased synaptic trafficking of AMPARs (Chao *et al*, 2002a, b; Ferrario *et al*, 2011; Mangiavacchi and Wolf, 2004). These phosphorylation mechanisms provide an explanation for D1DR-induced enhancements in AMPAR currents in striatal neurons (Cepeda *et al*, 1993; Price *et al*, 1999; Snyder *et al*, 2000). Our studies suggest that D1DR stimulation in cocaine self-administering animals increases PKA phosphorylation at GluA1^{S845} and that AMPAR function is necessary for D1DR-induced cocaine seeking. Thus, we conclude that D1DR stimulation amplifies AMPAR transmission to elicit cocaine seeking.

We observed increases in PKA phosphorylation at GluA1^{S845}, but not in the CaMKII/PKC phosphorylation at GluA1^{S831} following D1DR stimulation. It is important to note that phosphorylation at both the PKA and CaMKII/PKC serine residues are critical for AMPAR trafficking and function (Derkach *et al*, 2007). It has been postulated that PKA phosphorylation provides the initial 'priming' where AMPARs are redistributed to extrasynaptic sites and CaMKII/PKC phosphorylation facilitates synaptic membrane insertion (Boehm *et al*, 2006; Esteban *et al*, 2003; Hayashi *et al*, 2000; Oh *et al*, 2006). Phosphorylation of GluA1^{S845} in homomeric GluA1-containing AMPARs increases the open probability of AMPARs, whereas phos-

phorylation of GluA1^{S831} increases the channel conductance of homomeric GluA1 AMPARs by enhancing the coupling efficiency between glutamate binding and channel opening (Derkach *et al*, 1999).

A recent study observed that cocaine administration in cocaine self-administering animals increased phosphorylation of GluA1^{S831} phosphorylation, but not phosphorylation of GluA1^{S845} (Anderson *et al*, 2008). Interestingly, the enhancements in GluA1^{S831} phosphorylation were inhibited by D1DR antagonism, suggesting that cocaine-induced elevations in dopamine enhance GluA1^{S831} phosphorylation through a D1DR mechanism. Theoretically, the phosphorylation at these different serine residues produces similar functional consequences (ie, facilitation of AMPAR trafficking and function). Although others have found that both serine residues are phosphorylated concurrently immediately after a self-administration session (Edwards *et al*, 2007), it is possible that systemic cocaine administration and NAc D1DR stimulation produce similar phosphorylation events that are temporally distinct. A local microinfusion may produce a more rapid receptor stimulation to a very discrete population of neurons, whereas systemic or self-administered cocaine may have more delayed and diffuse effects on the accumbens receptor stimulation. Nonetheless, it appears clear that facilitation of AMPARs by D1DR stimulation is a critical element of D1DR-induced cocaine seeking (Figure 5).

Interestingly, the results of these studies conflict with previous work demonstrating that downregulation of AMPAR function enhanced, whereas upregulation of AMPAR function diminished cocaine- and dopamine D₂-induced reinstatement following systemic administration (Bachtell *et al*, 2008). Accumulating evidence suggests that the direct and indirect pathway medium spiny neurons contribute to specific aspects of behavior (Lobo and Nestler, 2011). For instance, D1DR are expressed in the direct pathway neurons that facilitate reward learning (Kravitz *et al*, 2012; Lobo *et al*, 2010). In contrast, activation of indirect pathway neurons that contain D₂ dopamine receptors is associated with a resilience to compulsive cocaine use (Bock *et al*, 2013). Thus, we hypothesize that increasing GluA1 activity in D1DR neurons may promote D1DR-induced reinstatement, whether through D1DR-induced increases in pGluA1 and surface AMPAR expression or via a parallel mechanism for excitation of this pathway (ie, facilitation of voltage-gated Ca^{2+} channels, etc). Conversely, increasing GluA1 levels in D₂ neurons opposes D₂ reinstatement by opposing G_{i/o} inhibitory cellular effects including activation of K⁺ channels or possible de-phosphorylation of GluA1 and reduced surface expression. Thus, strategies to reduce AMPA glutamate transmission in direct pathway D1DR-containing neurons (while increasing AMPA input to indirect pathway neurons) represent interesting therapeutic targets.

The advantageous colocalization of postsynaptic A1AR with D1DR in direct pathway neurons and their functional antagonism of D1DR actions uniquely positions them to offset D1DR-induced GluA1 phosphorylation that facilitates AMPA glutamate transmission and subsequently cocaine seeking. Previous work demonstrates that cocaine administration attenuates A1AR agonist-stimulated [³⁵S]GTP γ S binding, decreases membrane expression of the A1AR, and

decreases the formation of A1AR-D1DR heteromeric receptors (Toda *et al*, 2003)). Functionally, these cocaine-induced changes may promote cellular effects of D1DR stimulation (ie, AMPAR facilitation) and simultaneous stimulation of A1AR may aid in restoring the functional balance between A1AR and D1DR receptors. We demonstrate that A1AR stimulation alone in the NAc reduces the expression of synaptic GluA1, an effect observed with stimulation of other receptors coupled to inhibitory G proteins (Ferrario *et al*, 2011). When A1ARs are stimulated concurrently with D1DRs, both D1DR-induced PKA GluA1^{S845} phosphorylation and D1DR-induced cocaine seeking are inhibited, presumably through opposing actions on intracellular signaling cascades (Cao *et al*, 2006; Ferre *et al*, 1994, 1999, 1998). However, A1AR stimulation also diminishes D1DR affinity and enhances D1DR desensitization (Cao *et al*, 2006, 2007; Ferre *et al*, 1996), both of which would also effectively decrease the stimulation of intracellular signaling cascades culminating in reduced GluA1^{S845} phosphorylation.

Our results strongly suggest that the effects of A1AR stimulation in the NAc are mediated by postsynaptic receptors on medium spiny neurons. However, it is possible that presynaptic expression of A1AR has a role in the effects of the A1AR agonist CPA. Presynaptic A1AR and A_{2A} adenosine receptors are localized to glutamate terminals in the NAc where they provide tonic inhibition and phasic excitation on synaptic glutamate release, respectively (Ferre *et al*, 2008). Stimulation of A1AR could presumably inhibit synaptic glutamate release and contribute to the decrease in D1DR-induced cocaine seeking or the D1DR-induced phosphorylation of GluA1^{S845}. We determined if presynaptic adenosine receptors played a role in the inhibition of D1DR-induced cocaine seeking by administering SCH 442416, a presynaptic A_{2A} adenosine receptor antagonist. SCH 442416 administration facilitates presynaptic A1AR inhibitory actions on glutamate terminals, as demonstrated by its ability to reduce cortically evoked glutamate release in the striatum (Orzu *et al*, 2011b). Importantly, administration of SCH 442416 does not result in any characteristic postsynaptic effects of A_{2A} antagonists, such as increased locomotion or enhanced drug seeking (O'Neill *et al*, 2012; Orzu *et al*, 2011a, b; RKB, SCL, and CEO, unpublished observations). We observed no change in D1DR-induced cocaine seeking with SCH 442416 administration, suggesting that postsynaptic A1AR stimulation likely mediates the reversal in D1DR-induced changes in cocaine seeking and GluA1 phosphorylation.

Collectively, our results suggest that D1DR and A1AR bidirectionally regulate AMPAR subunit phosphorylation in the NAc that is critical for eliciting cocaine seeking. We speculate that these interactions are occurring in a cell-specific manner where D1DR amplify AMPA neurotransmission in the direct pathway neurons that subsequently drive cocaine seeking. In addition, A1AR stimulation opposes D1DR amplification of AMPA neurotransmission in the same population of direct pathway neurons to reduce this excitatory drive and restore inhibitory control. These findings illuminate the potential for A1AR stimulation as an effective strategy for reversing cocaine-induced alterations in striatal signaling that may underlie the persistent susceptibility to relapse.

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DISCLOSURE

The authors declare no conflict of interest.

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