Stimulation of Adenosine Receptors in the Nucleus Accumbens Reduces Dopamine Receptor-Induced Cocaine Seeking by Antagonizing Dopamine-Mediated Modulation of Glutamate Transmission

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Abstract:

Cocaine addiction is a brain disorder that affects millions of people and generates enormous social and economic costs to society. The continuous potential for relapse during periods of withdrawal makes treatment of cocaine addiction especially difficult. These studies investigate the molecular mechanisms mediating relapse behavior in a rodent model of cocaine addiction. Recent studies have demonstrated that AMPA glutamate receptor-dependent synaptic plasticity in the nucleus accumbens (NAc) core plays a critical role in cocaine seeking. It is unclear, however, how dopamine receptor signaling interacts with NAc AMPA receptors to mediate this behavior. Here, we investigate the interaction between dopamine and AMPA receptors in the nucleus accumbens and explore how adenosine receptor stimulation may counteract dopamine receptor-induced cocaine seeking and modulation of AMPA receptor trafficking. Specifically, we tested whether stimulating adenosine A_1 or A_{2A} receptors in the NAc could reduce dopamine D_1 or D_2, respectively, receptor-induced reinstatement. We also determined whether stimulating A_1 or A_{2A} receptors in the NAc would reduce D_1 or D_2 receptor-induced changes in synaptic GluR1 phosphorylation using a synaptoneurosome preparation and subsequent immunoblot analyses. As a correlate to these studies, we used viral-mediated gene transfer to determine the effects of expressing a $GluR1^{S845A}$ phosphomutant (Ser 845 $\rightarrow$Ala) in the NAc on cocaine, D_1, and D_2-induced reinstatement. Our results demonstrate that stimulation of A_1 receptors decreases D_1-induced cocaine seeking and $GluR1^{S845}$ phosphorylation. Stimulation of A_{2A} receptors both increases $GluR1^{S845}$ phosphorylation and decreases D_2-induced cocaine seeking and GluR1 internalization. Interestingly, overexpressing the $GluR1^{S845A}$ phosphomutant in the NAc significantly reduced D_2, but not D_1 or cocaine-induced

reinstatement. These findings suggest that adenosine receptors act to offset dopamine receptor-mediated modulation of AMPA receptor trafficking that drives cocaine seeking.

Introduction:

Treatment of cocaine addiction is especially difficult because of the constant potential for relapse in addicts during periods of abstinence. Research in humans and animal models has shown that the mesolimbic dopamine system is a key neural circuit involved in drug seeking behaviors (Shaham et. al, 2003, Shalev et. al, 2002). The mesolimbic dopamine system consists of dopamine cells in the ventral tegmental area that project to the nucleus accumbens (NAc) among other forebrain targets. Activation of this pathway contributes to the initial euphoria associated with drug taking, while re-activation of this pathway through stress exposure, drug-associated cues, and pharmacological stimuli plays a role in drug relapse during abstinence (Shaham et. al, 2003). Identifying molecular mechanisms that contribute to mesolimbic dopamine system re-activation during abstinence will provide insight into the dysregulation of neurotransmitter systems that occurs during addiction. Furthermore, characterization of the signaling events relevant to relapse may elucidate novel targets to offset drug-induced perturbations to these systems.

Two prominent rodent models of cocaine addiction are locomotor sensitization and the self-administration/reinstatement procedure (Robinson and Berridge, 2008, Shaham et. al, 2003). In the locomotor sensitization model, rodents exhibit enhanced (sensitized) locomotor responses to repeated daily injections of cocaine. Enhanced locomotor activity persists after periods of drug withdrawal whereby subsequent administration of cocaine or other pharmacological stimuli induces expression of sensitized responses to the treatment (Thomas et. al, 2008). It is thought that the development and expression of locomotor sensitization reflects neurobiological perturbations in the mesolimbic dopamine system resulting from continuous injections of cocaine (Thomas et. al, 2008, Kalivas et. al, 1998).

In the self-administration/reinstatement model, rodents are trained to perform a lever-press response to receive intravenous infusions of cocaine (DeVries et. al, 1999). Rodents are placed in an operant conditioning chamber for daily self-administration sessions, during which active (drug-paired) lever presses are reinforced with cocaine infusions. Both active and inactive lever presses are recorded, but inactive lever presses have no effect. Following approximately 3 weeks of daily self-administration sessions, rodents then undergo extinction training to abolish lever-pressing behavior. During this phase, rodents are placed in the operant conditioning chamber for daily extinction sessions during which responses at the previously drug-paired (active) lever no longer produce cocaine reinforcement. After lever responses are significantly abolished, rodents are placed back in the operant conditioning chamber where drug seeking (active lever pressing) is reinstated by drug-paired cues, cocaine injection, or other pharmacological stimuli. Reinstatement of cocaine seeking models the persistent potential for relapse in addicts, and indicates that dysregulation of the mesolimbic dopamine pathway persists long into periods of abstinence from drug use (DeVries et. al, 1999, Shaham et. al, 2003). In these studies, we seek to enhance understanding of how the neurotransmitters glutamate, dopamine, and adenosine interact within the NAc to regulate cocaine sensitization and cocaine seeking behavior.

The initial rewarding properties of cocaine are largely due to blockade of dopamine, norepinephrine, and serotonin transporters, causing increased extracellular concentrations of these neurotransmitters (Ritz et. al, 1990). Increased dopamine neurotransmission in the NAc is critical to the development and expression of behavioral sensitization, the acquisition of cocaine self-administration, and reinstatement of cocaine seeking (Di Chiara, 1995, Koob, 2009, Self and Nestler, 1998). Five subtypes of dopamine receptors are stimulated by cocaine-induced enhancements in dopamine neurotransmission, including the D_1-like receptor family consisting

of D_1 and D_5 receptors, and the D_2-like receptor family consisting of D_2, D_3, and D_5 receptors (Missale et. al, 1998, Sibley et. al, 1993). D_1 receptors are metabotropic receptors that enhance adenylyl cyclase activity through $G\alpha_S$ coupling (Missale et. al, 1998), leading to increased cyclic adenosine monophosphate (cAMP) formation and protein kinase A (PKA) activity (Sibley et. al, 1993). Conversely, D_2 receptors are coupled to $G\alpha_{i/o}$ proteins and inhibit adenylyl cyclase, cAMP formation, and PKA activity (Sibley et. al, 1993). In rodent models of cocaine sensitization and self-administration, stimulation of either dopamine D_1 or D_2 receptors in the NAc core is sufficient to induce expression of cocaine sensitization as well as cocaine seeking following extinction training (Bachtell et. al, 2005, DeVries et. al, 1999, Self et. al, 1996, Dias et. al, 2004, Ujike et. al, 1990). It has become increasing clear, however, that dopamine receptors play a modulatory role on glutamate neurotransmission in the NAc.

Glutamate is the major excitatory neurotransmitter of the central nervous system and is critical to learning and memory processes (Anggono and Huganir, 2008). Glutamate input into the NAc from the hippocampus, prefrontal cortex, and amygdala is involved in cocaine sensitization and reinstatement of drug seeking (Kalivas et. al, 2005, Kalivas and Hu, 2006). Glutamate input is mediated by (±)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors, which are ligand-gated ion channels that cause rapid, post-synaptic depolarization (Anggono and Huganir, 2008). AMPA receptors play a critical role in multiple forms of synaptic plasticity that underlie learning and memory (Anggono and Huganir, 2008, Malenka and Nicoll, 1999). Long-term potentiation (LTP) is a model of synaptic plasticity where trafficking of AMPA receptors to the postsynaptic density increases synaptic strength and excitatory glutamate neurotransmission (Malenka and Nicoll, 1999). Long-term depression (LTD), on the other hand, is also a model of synaptic plasticity where internalization of AMPA

receptors from the cell surface decreases synaptic strength and excitatory glutamate neurotransmission (Traynelis et. al, 2010). AMPA receptor-based plasticity alters the excitability of NAc neurons throughout cocaine administration (Hyman et. al, 2006), and acute stimulation of AMPA receptors in the NAc following chronic cocaine administration induces both locomotor sensitization and reinstatement of cocaine seeking (Cornish et. al, 1999, Cornish and Kalivas, 2000, Bachtell et. al, 2008). Cocaine administration during withdrawal produces locomotor sensitization and induces reinstatement, both of which are also mediated by AMPA receptor stimulation (Cornish et. al, 1999, Pierce et. al, 1996, Bell et. al, 2000). Thus, administration of cocaine during withdrawal produces a rapid increase in glutamate release in the NAc that enhances AMPA-mediated glutamate transmission and is thought to be critical for locomotor sensitization and cocaine-induced reinstatement (Bell and Kalivas, 1996, 2000, Bachtell et. al 2008). Recent studies suggest that cocaine modulates AMPA-receptor trafficking through phosphorylation of the GluR1 subunit, and that AMPA-receptor trafficking in the NAc is necessary for cocaine-induced reinstatement and expression of cocaine sensitization (Anderson et al., 2008, Bachtell et. al, 2008). However, the contribution of metabotropic dopamine receptor signaling to AMPA-receptor trafficking in the NAc is unclear, especially in the modulation of cocaine-related behaviors.

Recent work suggests that dopamine and glutamate receptors interact to produce behavioral changes, although the mechanisms of these interactions are not clear. Thus, modulating AMPA-receptor activity in the NAc by viral gene transfer alters behavioral responses to cocaine as well as dopamine receptor agonists (Bachtell et al., 2008a, Bachtell et al., 2008b). AMPA receptors containing the GluR1 subunit are especially prone to activity-dependent receptor trafficking. The GluR1 subunit contains multiple phosphorylation sites that regulate GluR1-containing AMPA

receptor trafficking and insertion into post-synaptic spines (Anggono and Huganir, 2008, Lee et. al, 2003, Lee et. al, 2010). Of particular interest are two serine residues, 845 and 831 ($GluR1^{S845}/^{S831}$) that are phosphorylated by PKA and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), respectively (Lee et. al, 2010, Malenka and Nicoll, 1999). Since D_1 receptor stimulation increases, while D_2 receptor stimulation decreases PKA activity, $GluR1^{S845}$ phosphorylation in the NAc may play a critical role in AMPA-receptor based plasticity that directly regulates the expression of cocaine sensitization and cocaine seeking. Identifying strategies to offset glutamate signaling and dopamine receptor modulation of glutamate signaling in the NAc will provide greater insight into the factors contributing to relapse as well as reveal novel therapeutic targets.

Adenosine is a purine nucleoside and neuromodulator that antagonizes dopamine signaling in the NAc through stimulation of adenosine A_1 or A_{2A} receptors expressed on NAc medium spiny GABAergic neurons (MSNs; Ferre et. al, 1992, 1999, Filip et. al, 2012, Svenningson, 1999b). MSNs comprise approximately 90-95% of all NAc neurons and exist in two main populations characterized by distinct peptide expression, receptor expression and projection targets (Aubert et. al, 2000, Steiner and Gerfen, 1998). MSNs of the direct pathway express dynorphin, postsynaptic D_1 and A_1 receptors and project primarily to the ventral tegmental area, while MSNs of the indirect pathway express enkephalin, postsynaptic D_2 and A_{2A} receptors and project to the ventral pallidum (Lu et. al, 1998). Co-localization of A_1 receptors with D_1 receptors allows adenosine to regulate dopamine signaling within direct pathway MSNs, since stimulation of A_1 receptors activates inhibitory G-proteins that oppose D_1-induced activation of adenylyl cyclase and cAMP formation (Ferre et. al, 1997, 1999, Dunwiddie and Masino, 2001). Similarly, stimulation of A_{2A} receptors on indirect pathway neurons opposes D_2 receptor

signaling by activating stimulatory G-proteins that enhance adenylyl cyclase activity and cAMP formation (Schiffman et. al, 2007, Svenningson et. al, 1999a, Tozzi et. al, 2007, Dunwiddie and Masino, 2001, Ferre et. al, 1994).

There has been recent interest in the unique contributions that these two populations of MSNs contribute to drug addiction. For example, it is thought that repeated cocaine administration produces enhanced signaling through the direct pathway neurons that promotes drug seeking, while simultaneous reducing activity in the indirect pathway neurons that oppose drug seeking (Lobo and Nestler, 2011). Therefore, we are interested in exploring how NAc A_1 receptors may reduce the extent to which D_1 receptor-mediated enhancements of the direct pathway drive drug-seeking behaviors. We are also interested in how NAc A_{2A} receptors may reduce D_2 receptor-mediated inhibition of the indirect pathway to restore inhibitory control. We have recently demonstrated that stimulation of NAc A_1 or A_{2A} receptors blocks the expression of cocaine sensitization, suggesting that adenosine receptor stimulation might reduce dopamine receptor-mediated reinstatement of cocaine seeking (Hobson et. al, 2012). The purpose of these studies was to 1) characterize how both D_1- and D_2-receptor signaling interacts with AMPA receptor activity in the NAc to regulate reinstatement of cocaine seeking in rats, 2) determine whether stimulation of adenosine receptors offsets dopamine receptor-mediated reinstatement, and 3) determine whether adenosine receptor stimulation offsets dopamine receptor-mediated changes in GluR1 phosphorylation.

Methods:

Animals and Housing Conditions

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.

General Locomotor Sensitization Procedure

Locomotor activity was recorded in plexiglass chambers (San Diego Instruments, San Diego, CA, USA) measuring 16 × 16 × 15 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. Animals were initially habituated to the locomotor testing chambers for 2-h the day prior to start of the sensitization procedure. Cocaine (15 mg/kg, i.p.) injections were performed daily for one week and horizontal locomotor activity was assessed in the activity monitoring chambers. Locomotor sensitization criteria was defined as Day 7 cocaine-induced activity > Day 1 cocaine-induced locomotor activity.

Surgery

For all self-administration experiments, jugular catheters and intracranial cannulae were sequentially implanted during the same surgery under halothane anesthesia (1–2.5%). First, catheters were implanted into the jugular vein according to previously published procedures (O'Neill et al., 2011). 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. Catheters were flushed daily with 0.1 ml heparinized saline and rats were allowed 7 days recovery in their home cage before experimental procedures began. For experiment 3 (cocaine sensitization), only intracranial cannulae were implanted by the same procedure described above.

Cocaine Self-Administration and Extinction 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 were 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 jugular catheter infusion) on an FR1 reinforcement schedule in daily 4-h sessions for 5-6 days per week. Cocaine infusions were delivered over 5 s concurrent with the illumination of a cue light above the active lever. The infusion and cue light were followed by a 15 s time-out period during which 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 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 (active lever) and on the inactive lever were recorded, but had no programmed drug or cue delivery.

Reinstatement Procedures

Each reinstatement session was initiated with 2 h of extinction conditions, followed by a 2 h reinstatement test period. For experiments involving viral vector infusion to the NAc, reinstatement testing occurred during the peak of viral expression (see below). Drug microinfusions were administered to the NAc after the 2-h extinction period, and were followed by the 2-h reinstatement test period (see below). Responses at both the previously drug-paired and inactive levers were recorded, but resulted in no cue or drug delivery during testing.

Microinjections and Histology

Drug microinfusions (1 μl/side over 60 s) were delivered through bilateral 33 gauge injectors extending 1 mm beyond the guides. The microinjectors were removed 1 min after the full infusion volume was given to ensure absorption into the tissues. In these experiments, reinstatement was assessed over repeated sessions and animals received a maximum of four treatments in a randomized/counter-balanced order. Herpes simplex viral (HSV) vectors encoding mutant GluR1^{S845A}, and LacZ (expressing ß-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, which is consistent with the previously established time course of viral expression (Bachtell et al., 2008, Sutton et al., 2003). Following behavioral testing (excluding experiments involving tissue collection for immunoblotting, see below), localization of infusion sites was determined by infusing 0.5 μl of cresyl violet dye in anesthetized animals and analyzed in 0.8 mm thick coronal slices under a dissecting microscope.

Experiment 1: Effects of A2A stimulation on cocaine naïve animals

This experiment tested the effects of acute systemic administration of the A_{2A} agonist CGS 21680 (0.03 mg/kg, i.p.) on cocaine naïve animals. Injections of CGS 21680 or saline were administered 30 minutes prior to sacrifice and tissue collection. Tissue punches were homogenized as whole cell lysates (no fractionation) for subsequent immunoblotting analyses (see below).

Experiment 2: Effects of A_{2A} stimulation on cocaine challenge in cocaine sensitized animals

This experiment tested the effects of a systemic cocaine challenge (15 mg/kg, i.p.) in the presence or absence of the A_{2A} agonist CGS 21680 (0.03 mg/kg, i.p.) in cocaine sensitized animals. After cocaine locomotor sensitization and one week of withdrawal (see above), animals received a pretreatment of vehicle or CGS 21680 followed by a challenge of vehicle or cocaine. Thirty minutes after the challenge treatment, animals were sacrificed and tissues were collected. Tissue punches were homogenized as whole cell lysates (no fractionation) for subsequent immunoblotting analyses (see below).

Experiment 3: Effects of A_{2A} stimulation on a quinpirole (D_2 agonist) challenge in cocaine sensitized animals

This experiment tested the effects of intra-NAc administration of quinpirole (3.0 μg/side) in the presence or absence of the A_{2A} agonist CGS 21680 (2.5 ng/side) in cocaine sensitized animals. After cocaine locomotor sensitization and one week of withdrawal (see above), animals received an intra-NAc pretreatement of vehicle or CGS 21680 followed by an intra-NAc treatment of vehicle or quinpirole. Pre-treatments were administered 5 minutes prior to the treatment of quinpirole or saline. Thirty minutes after treatment, animals were sacrificed and infusion sites were verified under a dissection microscope. Tissue punches were then collected

and homogenized as whole cell lysates (no fractionation) for subsequent immunoblotting analyses (see below).

Experiment 4: Effects of A_{2A} stimulation on D_2-induced reinstatement and GluR1 Phosphorylation and Expression

This experiment tested the effects of intra-NAc adenosine A_{2A} receptor stimulation on intra-NAc D_2-induced reinstatement. Following a 2-hr extinction period, intra-NAc infusions of vehicle, the D_2 agonist quinpirole **(**3.0 μg/side**)**, CGS 21680 (2.5 ng/side) or the co-infusion of quinpirole and the A_{2A} agonist CGS 21680 were administered. A 2-hr reinstatement test was performed immediately following the intra-NAc infusion. Twenty-four hrs following the reinstatement test, the same intra-NAc treatment was administered, and tissue from the infusion site was collected 30 min post-treatment for synaptoneurosome fractionation and subsequent immunoblotting analyses (see below).

Experiment 5: Effects of A_1 stimulation on D_1-induced reinstatement and GluR1 Phosphorylation and Expression

This experiment tested the effects of intra-NAc adenosine A_1 receptor stimulation on intra-NAc D_1-induced reinstatement. Following a 2-hr extinction period, intra-NAc infusions of vehicle, the D_1 agonist SKF 81297 **(**3.0 μg/side**)**, or the co-infusion of SKF 81297 (3.0 μg/side) and the A_1 agonist CPA **(**1.5 μg/side**)** were administered. A 2-hr reinstatement test was performed immediately following the intra-NAc infusion. Twenty-four hrs following the reinstatement test, the same intra-NAc treatment was administered, and tissue from the infusion site was collected 30 min post-treatment for synaptoneurosome fractionation and subsequent immunoblotting analyses (see below).

Experiment 6: Effects of Intra-NAc GluR1^{S845A} Phosphomutant on D_1, D_2, and cocaine -induced reinstatement

This experiment tested the effects PKA-phospho mutant GluR1^{S845A}, and LacZ viral expression in the NAc on intra-NAc dopamine D_1-mediated reinstatement (SKF 81297), intra-NAc dopamine D_2-mediated reinstatement (quinpirole), and cocaine reinstatement. Following extinction training animals were counterbalanced according to cocaine self-administration/extinction responding and administered an HSV vector encoding GluR1^{S845A} or LacZ. Twenty-four hrs following HSV infusion, reinstatement was induced by infusing SKF 81297 (3.0 μg/side) into the NAc, infusing quinpirole (3.0 μg/side) into the NAc, or by cocaine injection (15 mg/kg, i.p.). Treatments occurred after the 2-hr extinction period, and were immediately followed by the 2-hr reinstatement period. Each rat was tested across the three treatments in a randomized order.

Tissue collection, Synaptoneurosome Preparation, and Immunoblotting

For all experiments involving immunoblotting, rats underwent rapid decapitation and brain dissection. Brains were sliced into 1.0 mm thick coronal sections using ice-cold razor blades and a brain matrix (Braintree Scientific Inc, Braintree, MA). For experiments 1 and 2 (no intracranial cannulae), the brain regions of interest were collected using bilateral 12-gauge tissue punches. For experiments 3-5, the infusion site was first verified under a dissecting microscope, and bilateral 12-gauge tissue punches were performed directly surrounding the infusion site. Infusion sites located outside the NAc were excluded from behavioral and immunoblot analyses. Tissue punches from non-infused dorsal striatum were also collected as a site control. For experiments 1-3 (no fractionation), tissue punches were immediately homogenized by sonication in lysis buffer.

For experiments 4 and 5, tissue punches were mechanically minced with a glass dounce homogenizer in 50µL homogenization buffer (HB: 1M Tris, 1M Sucrose, 0.5M EDTA, 0.25M EGTA, 0.5M NaF, 1M benzamidine, 100mM AEBSF) containing phosphatase and protease inhibitor cocktails (Sigma-Aldrich, St. Louis, MO). The tissue was then subjected to a synaptoneurosome preparation protocol previously described (Cortese et al., 2011). Once the synaptic (P2) fraction was obtained and homogenized in HB (same as above, but containing 1x STE buffer), synaptic enrichment of the P2 fraction was confirmed by probing for GluR1 and post-synaptic density protein 95 (PSD95), common synaptic markers. Protein content was quantified using the Lowry protein (BioRad, Hercules, CA, USA).

Samples were prepared under reducing conditions in 4x Laemmli buffer and heated at 70°C for 10 minutes. Protein samples (15µg) were loaded onto 4–12% NuPage Bis-Tris SDS-polyacrylamide gels (Invitrogen, Grand Island, NY) and transferred onto Immobilon-FL PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked in 5% bovine serum albumin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) in tris-buffered saline with 0.1% tween (TBST) for 1 hour at room temperature. Membranes were incubated with primary antibody (see below) at 4°C overnight followed by 3 × 15 minute TBST washes. Membranes were then incubated with appropriate secondary antibodies at room temperature for 1 hour, washed and detected by enhanced chemiluminescence (Super Signal West Dura, Thermo Scientific) for experiments 1-3, or by a Licor Odyssey infrared scanning device for experiments 4 and 5. All images were converted to gray-scale and quantified using Odyssey densitometry software. The following primary antibodies were all obtained from Millipore and used at dilutions of 1:1000, except where noted: Tubulin isoform beta III (MAB1637, 1:2500), Total GluR1 (MAB2263), Phospho-GluR1-Serine 831 (04-823), Phospho-GluR1-Serine 845

(AB5849), and PSD95 (MABN68). Primary antibodies for Total ERK (p44/42 MAPK, Cat. #4695, 1:2000) and Phospho-ERK Thr202/Tyr204 (Phospho-p44/42 MAPK, Cat. #9106, 1:2000) were obtained from Cell Signaling. Secondary antibodies conjugated to horseradish peroxidase were purchased from Millipore and used at a dilution of 1:25,000 in 5% non-fat dried milk/TBST. Secondary antibodies conjugated to infrared dyes were purchased from Licor (Lincoln, NE) and were used at a dilution of 1:15,000 in 5% BSA/TBST. For multiple detections of the same blot, blots were stripped using Restore Western Blot Stripping Buffer (Pierce, Rockford, IL) for 20 minutes and washed 3 × 15 minutes in TBST and subjected to standard immunoblotting conditions.

Drugs

The D_1 receptor agonist, SKF 81297 ((±)-6-Chloro-PB hydrobromide), the D_2-selective agonist, quinpirole ((−)-quinpirole hydrochloride), and cocaine hydrochloride were obtained from Sigma Aldrich (St. Louis, MO). The A_1 receptor agonist, CPA (*N6*-cyclopentyladenosine) and the A_{2A} agonist, CGS 21680, were obtained from Tocris Bioscience (Ellisville, MO). All drugs were dissolved in sterile-filtered physiological (0.9%) saline or physiological PBS (pH 7.4).

Data analysis

The numbers of animals in each experimental group ranged from 8 to 16 and are reported for each experiment in the table/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 (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). Immunoblotting data from experiment 1 was analyzed with an

unpaired t-test. Immunoblotting data for all other experiments was analyzed by one-way ANOVA with treatment group as the factor. Significant interactions were followed up with *post hoc* tests (Dunnett's comparisons). Statistical significance was set at $p < 0.05$ for all tests.

Results

Experiment 1: Acute A_{2A} receptor stimulation has no effect on phosphorylation of GluR1 or ERK in the striatum of cocaine naïve animals

Adenosine receptors can regulate dopamine receptor signaling in both dorsal motor and ventral reward striatal pathways (Ferre et. al, 1992, 1997). We recently demonstrated that pharmacological stimulation of A_{2A} receptors in the NAc inhibits sensitized locomotor responses and cocaine mediated reinstatement of cocaine seeking (O'Neill et. al, 2011, Hobson et al. 2012). We therefore sought to determine the mechanisms involved in these behavioral changes. We first explored how acute A_{2A} stimulation would increase protein kinase A-mediated phosphorylation of GluR1 in the striatum of cocaine naïve animals. We also measured phosphorylation of extracellular-signal-regulated kinase (ERK, or p42/44 MAP kinase) to determine whether A_{2A} receptors might activate the mitogen-activated-protein kinase pathway, a canonical signaling pathway often activated by G-protein coupled receptors (Zhai et. al, 2008). We collected tissue 30 minutes after a single injection of the A_{2A} agonist CGS 21680 (0.03 mg/kg, i.p.) or saline and analyzed pGluR1^{S845} and pERK in the NAc core, NAc shell, and the dorsal striatum. Table 1 shows that administration of CGS 21680 had no effect on either pGluR1^{S845} or phospho-ERK in any of the brain regions analyzed. These findings are not surprising since intra-NAc A_{2A} stimulation did not affect acute locomotor sensitivity to cocaine, suggesting that behaviorally relevant doses of the A_{2A} agonist CGS 21680 may not significantly effect striatal signaling in cocaine naïve animals (Hobson et. al, 2012).

Table 1: Effects of Acute CGS 21680 on Cocaine Naïve Animals

Protein	Brain Region	Saline (n=4)	CGS (n=4)	Statistics
pERK / tERK	NAc Shell	100.0 ± 6.22	90.7 ± 3.87	t(14)=1.28, p=0.22
	NAc Core	100.0 ± 2.84	101.5 ± 5.14	t(14)<1, NS
	DS	100.0 ± 3.07	96.2 ± 4.65	t(14)<1, NS
pGluR1^{S845} / tGluR1	NAc Shell	100.0 ± 1.92	103.2 ± 4.42	t(14)<1, NS
	NAc Core	100.0 ± 2.05	101.6 ± 3.32	t(14)<1, NS
	DS	100.0 ± 1.17	94.8 ± 2.93	t(6)=1.66, p=0.15

CGS 21680 (0.03 mg/kg, i.p.) was administered 30 minutes prior to sacrifice and tissue collection. Values are % (mean ± sem) of saline control for phospho/total protein ratio. NAc= Nucleus Accumbens, DS= Dorsal Striatum, NS= Not significant. pERK residues are T202 and Y204.

Experiment 2: A_{2A} receptor stimulation does not inhibit cocaine-induced GluR1^{S845} phosphorylation in the striatum of cocaine-sensitized animals

We have recently demonstrated that intra NAc A_{2A} stimulation, either by CGS 21680 or by elevating concentrations of endogenous adenosine, blocks both cocaine and D_2-induced expression of cocaine sensitization (Hobson et. al, 2012). We therefore tested whether A_{2A} receptor stimulation might offset cocaine-induced changes in signaling within the NAc core and shell of cocaine-sensitized animals. Following 7 daily injections of cocaine and 7 days of withdrawal, we administered a challenge injection of either saline or cocaine (15 mg/kg, i.p.) following a pretreatment of either vehicle or CGS 21680 (0.03 mg/kg, i.p.). We collected tissue 30 minutes after treatment and analyze pGluR1^{S845} and pERK in the NAc core and shell. Figure 1a shows that the average locomotor activity of the animals is greater on Day 7 than on Day 1, indicating behavioral sensitization ($t_{30} = 1.26$, $p = 0.022$). A significant main effect of treatment on pGluR1^{S845} in the NAc core was observed ($F_{3,14} = 4.49$, $p = 0.027$) where cocaine increased pGluR1^{S845} approximately 25% relative to saline controls in the NAc core (Figure 1b, p =

0.0654). This effect was not altered by a pretreatment of CGS 21680 ($p < .05$). CGS 21680 alone had no effect on pGluR1^{S845} in the NAc core as was observed in the cocaine-naïve animals. Figure 1c shows that no treatment had any significant effect on pGluR1^{S845} in the NAc shell ($F_{3,15}$=0.38, $p = 0.77$). Table 2 shows that no treatment had any significant effect on pERK in either NAc shell or core.

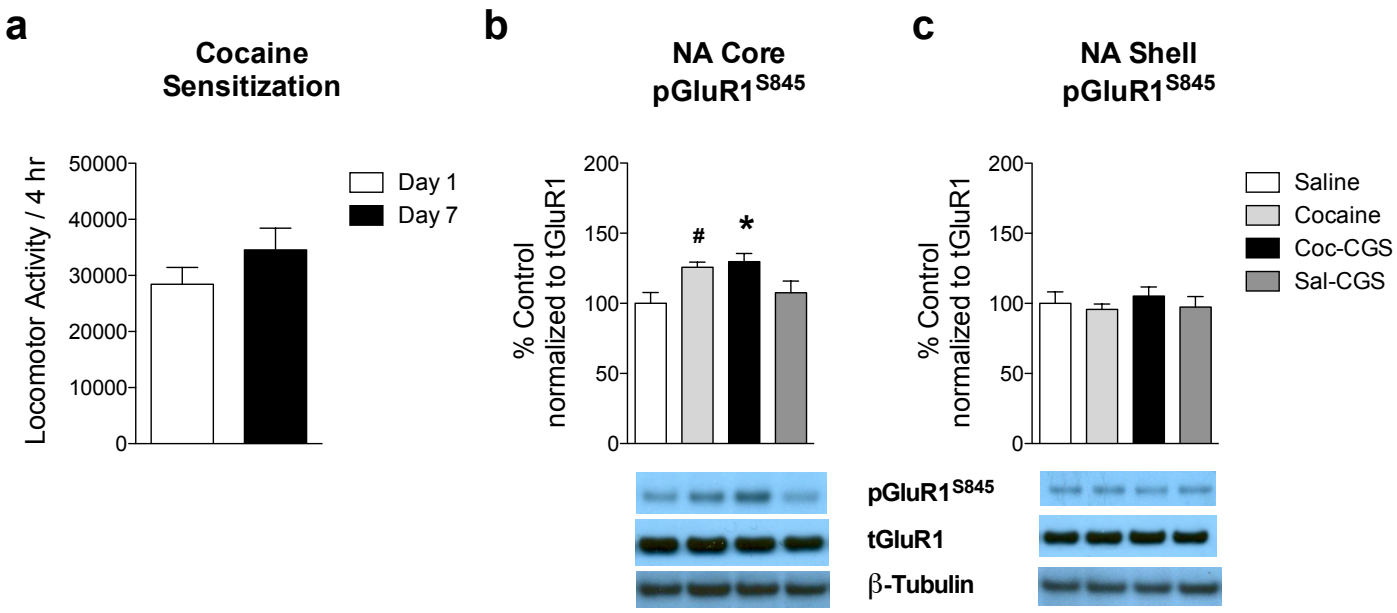

Figure 1: Intra-NAc core administration of the adenosine A2A agonist CGS 21680 does not alter cocaine-induced GluR1S845 phosphorylation in cocaine-sensitized animals.
(a) Animals display locomotor sensitization over 7 days of repeated cocaine injections. (b) Systemic administration of cocaine increased pGluR1^{S845} in the NAc core regardless of pre-treatment with the A_{2A} CGS 21680 or vehicle. (c) None of the treatments administered caused significant changes in pGluR1^{S845} in the NAc shell. *Significant from Sal/Sal ($p < .05$ Bonferroni's post-test), #Significant trend from Sal/Sal ($p = .0654$ Bonferroni's post-test)

Table 2: Effects of Acute Cocaine/CGS 21680 on Cocaine Sensitized Animals

Protein	Brain Region	Sal/Sal (n=4)	Coc/Sal (n=4)	Coc/CGS (n=4)	Sal/CGS (n=4)	Statistics
pERK / tERK	NAc Shell	100.0 ± 5.11	112.2 ± 8.00	118.8 ± 4.34	99.59 ± 6.81	F(3,12)=2.30, p=0.130
	NAc Core	100.0 ± 4.18	107.7 ± 3.62	104.5 ± 5.14	94.85 ± 8.64	F(3,10)=1.09, p=0.397
pGluR1^{S845} / tGluR1	NAc Shell	100.0 ± 8.18	95.72 ± 3.89	105.2 ± 6.48	97.50 ± 7.37	F(3,12)=0.385, p=0.766
	NAc Core	100.0 ± 7.80	^125.8 ± 3.63	*129.7 ± 5.89	107.6 ± 8.33	F(3,11)=4.49, p=0.027

^Significant trend from Sal/Sal (p=.0654), *Significant from Sal/Sal, p<.05
CGS (0.03 mg/kg, i.p.) or saline was administered as a pre-treatment 5 minutes prior to cocaine (15 mg/kg, i.p.) or saline. Sacrifice and tissue collection occurred 30 minutes after cocaine or saline treatment. Values are reported as % of saline control for phospho/total protein ratio. NAc= Nucleus Accumbens. pERK residues are T202 and Y204.

Experiment 3: Intra-NAc A_{2A} receptor stimulation increases GluR1^{S845} phosphorylation in cocaine-sensitized animals

Cocaine increases extracellular dopamine that nonselectively stimulates both D_1 and D_2 receptors (Ritz et. al, 1990). We hypothesized that cocaine-induced increases in NAc core pGluR1^{S845} are likely mediated by the $G\alpha_S$-coupled D_1 receptors present in direct pathway medium spiny neurons. Since adenosine A_{2A} receptors are coupled to $G\alpha_S$ proteins and oppose D_2-induced inhibition of cAMP signaling on the postsynaptic terminals of indirect pathway medium spiny neurons (Ferre et. al, 1997), it seems that A_{2A} receptor stimulation might inhibit cocaine sensitization and cocaine seeking by counteracting D_2-signaling within these cells. Therefore, we tested whether intra-NAc A_{2A} stimulation might offset D_2-induced changes in pGluR1^{S845} or pERK in the NAc core of cocaine-sensitized animals using. Figure 2a shows that the average locomotor activity of the animals is greater on Day 7 than on Day 1, indicating

behavioral sensitization (t_{32}=3.23, p < .01). A significant effect of intra-NAc core treatment on pGluR1^{S845} ($F_{3,26}$ = 4.33, p = 0.013) and pERK ($F_{3,26}$ = 7.35, p = 0.001) was observed.

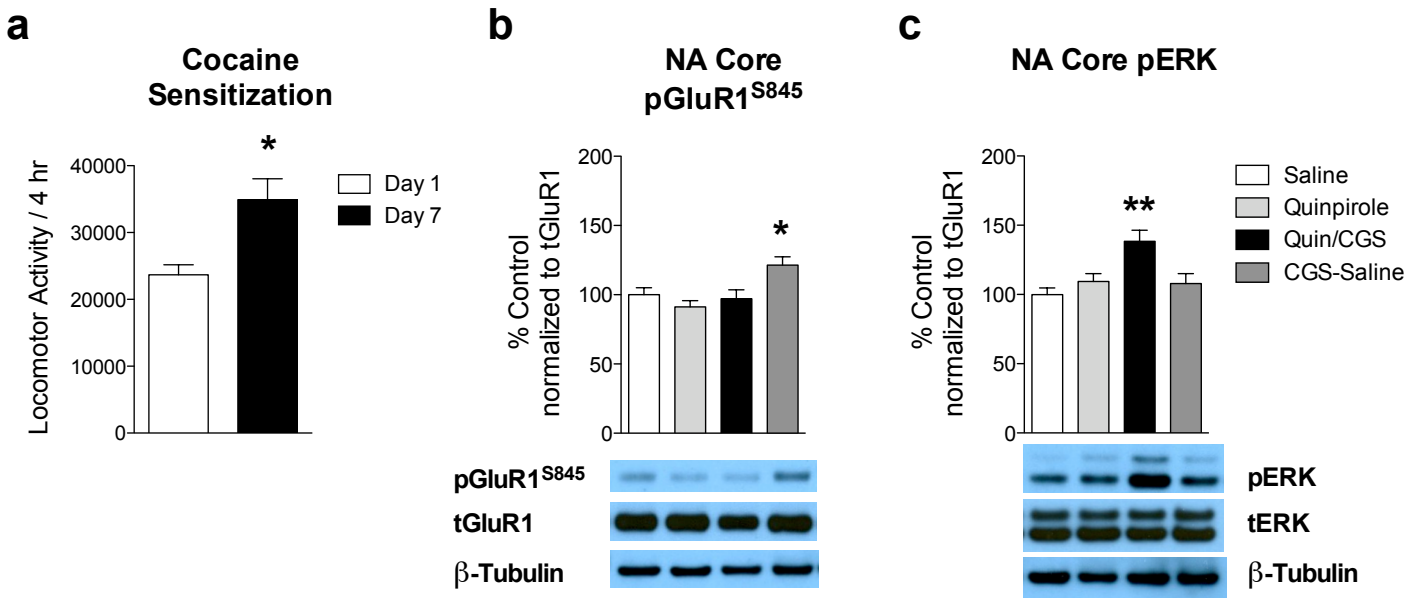

Figure 2: Intra-NAc core administration of the adenosine A2A agonist CGS 21680 increases GluR1S845 phosphorylation in cocaine-sensitized animals.
(a) Animals display locomotor sensitization over 7 days of repeated cocaine injections. (b) Intra-NAc core infusion of the A_{2A} agonist CGS 21680 significantly increases pGluR1^{S845} in the NAc core. (c) Intra-NAc core pre-treatment with the A_{2A} agonist CGS 21680 followed by infusion of the D_2 agonist quinpirole significantly increases pERK in the NAc core. Neither agonist alone has a significant effect on pERK. *Significant from Sal/Sal (p < .05 Bonferroni's post-test), **Significant from Sal/Sal (p < .01 Bonferroni's post-test)

Intra-NAc core administration of CGS 21680 alone increased pGluR1^{S845} approximately 30% relative to saline controls (Figure 2b, p < .05). Intra-NAc quinpirole produced a small decrease in pGluR1^{S845}, although this was not statistically significant (t_{16} = 1.273, p = 0.22). Infusion of both quinpirole and CGS 21680 had no effect on pGluR1^{S845} (p > .05). Figure 2c shows neither quinpirole nor CGS 21680 alone had a significant effect on pERK relative to saline control (p > .05). Infusion of both quinpirole and CGS 21680 increased pERK approximately 35% (p < .05).

Experiment 4A: Stimulation of A_{2A} receptors impairs D_2-induced cocaine seeking

We have previously shown that stimulation or blockade of NAc A_{2A} receptors inhibits or enhances, respectively, cocaine-seeking behavior elicited by systemic administration of the D_2 agonist quinpirole (O'Neill et. al, 2011). In order to identify the potential mechanism by which intra-NAc A_{2A} receptor stimulation acts to inhibit D_2-mediated reinstatement, we administered infusions of quinpirole (3.0 μg/side) or saline in the presence of either CGS 21680 (2.5 ng/side) or vehicle into the medial NAc core prior to reinstatement testing. As expected, intra-NAc quinpirole significantly induced reinstatement, which was blocked by simultaneous A_{2A} receptor stimulation (Figure 3). A significant interaction between lever and treatment ($F_{3,51} = 4.20$; $p = 0.0099$) and a main effect of treatment ($F_{3,51} = 8.42$; $p = 0.0001$) were observed. Post-hoc tests (Bonferroni's) revealed animals that received quinpirole alone significantly reinstated ($p < .001$), while those that received quinpirole and CGS 21680 did not reinstate compared to control animals ($p > .05$).

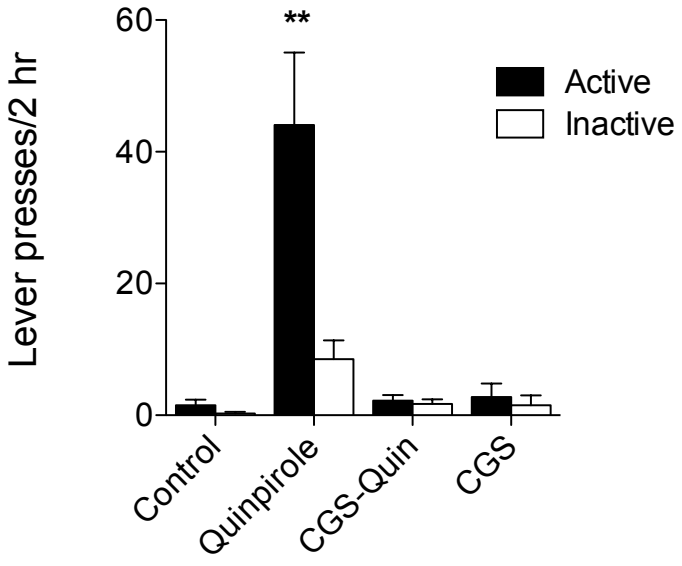

Figure 3: Intra-NAc core administration of the adenosine A2A agonist blocks D2 agonist-induced reinstatement of cocaine seeking. Intra-NAc core infusion of the D_2 agonist quinpirole significantly induces reinstatement, while co-administration of the A_{2A} agonist CGS 21680 blocks this effect. CGS 21680 alone has no effect on active lever responding.
** Significant from Sal/Sal ($p < .01$ Bonferroni's post-test)

Experiment 4B: Stimulation of A_{2A} receptors increases GluR1^{S845} phosphorylation and modestly impairs D_2-mediated GluR1 Internalization

Previous studies have shown that modulation of AMPA receptor function by overexpressing wild-type or dominant negative GluR1 in the NAc core modulates cocaine sensitization and reinstatement of cocaine seeking induced by cocaine and a D_2 agonist (Bachtell et. al, 2008). Since intra-NAc core A_{2A} stimulation blocks intra-NAc core D_2-mediated reinstatement (Figure 3), we sought to determine whether A_{2A} receptor stimulation in the NAc core might alter D_2-induced alterations in GluR1 expression and/or phosphorylation. One day after reinstatement testing, we administered infusions of quinpirole and/or CGS 21680 as described in experiment 4A. Thirty minutes after treatment, animals were sacrificed and tissues from the NAc core (infusion site) and dorsal striatum were subjected to a synaptoneurosome fractionation protocol to isolate a synaptic fraction (P2) and a cytosolic fraction (S2). As shown in Figure 4, the synaptoneurosome preparation produces an enrichment of PSD 95 and total GluR1 (tGluR1) in the P2 fraction, while the S2 fraction is enriched in ß-tubulin. Tissue samples were immunoblotted to analyze PKA phosphorylation at serine 845 (pGluR1^{S845}) and CaMKII/PKC phosphorylation at serine 831 (pGluR1^{S831}). Phosphorylation at these residues has been shown to be critical for activity-dependent AMPA receptor synaptic trafficking (Malenka and Nicoll, 1999, Lee, 2006, Shepherd and Huganir, 2007).

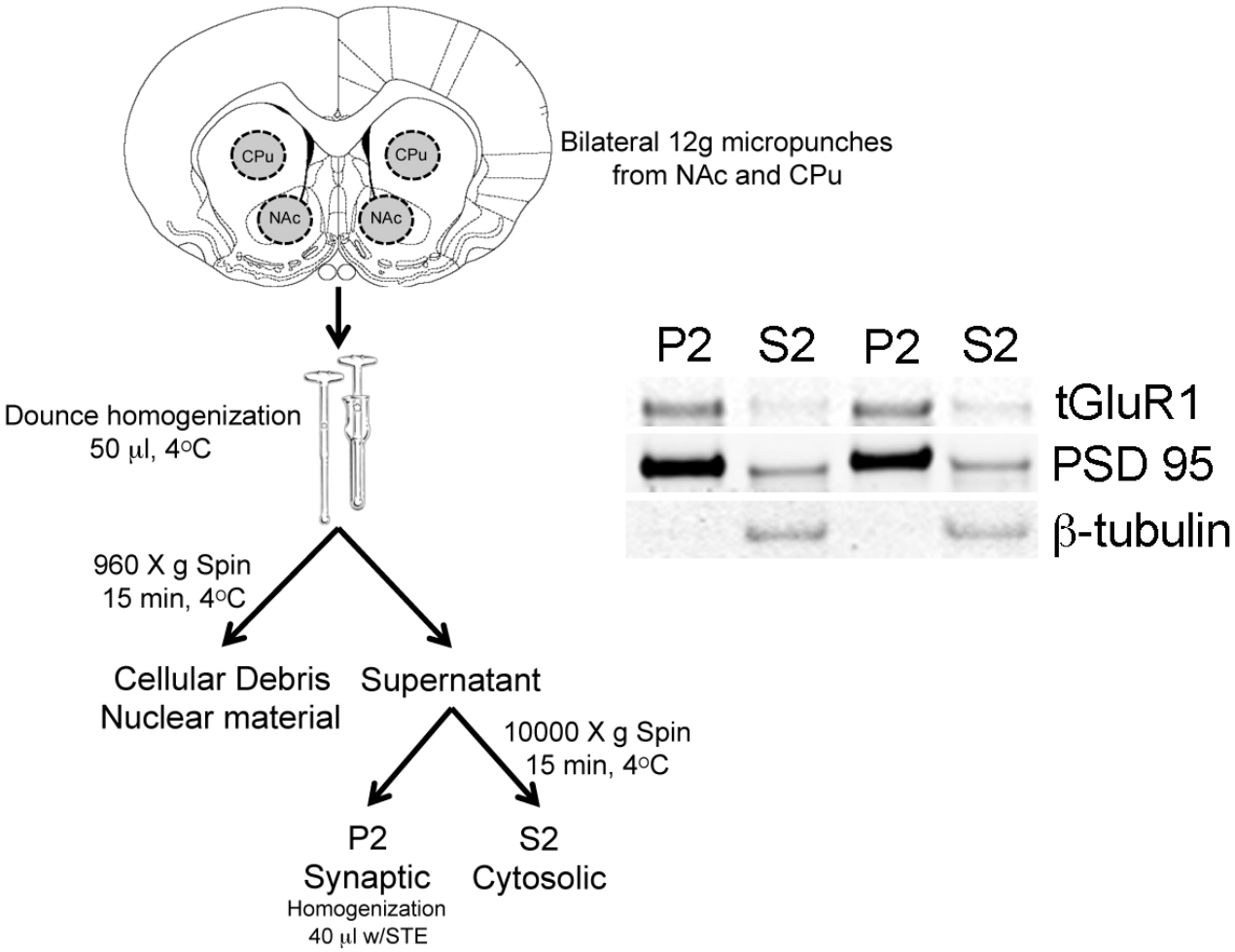

Figure 4: Synaptoneurosome fractionation protocol and representative immunoblot of tissue from NAc core and dorsal striatum.
15 µg of protein from P2 (synaptic) and S2 (cytosolic) fractions were loaded into each lane. Note that synaptic proteins (tGluR1 and PSD 95) are enriched in the P2 fraction, while cytosolic protein (β-tubulin) is enriched in the S2 fraction. For all subsequent experiments, PSD 95 and β-tubulin were used as loading controls for the P2 and S2 fractions, respectively.

A significant effect of treatment on tGluR1 expression levels ($F_{3,28} = 5.67$, $p = 0.0036$) and pGluR1^{S845} ($F_{3,28} = 7.08$, $p = 0.0011$) in the NAc core P2 fraction was observed. Figure 5 shows representative immunoblots of the NAc core P2 fraction. Quinpirole alone decreased expression of tGluR1 by 35% ($p < .05$), while quinpirole and CGS 21680 together decreased tGluR1 by only 23%, which was not significantly different from control ($p > .05$). The differences between quinpirole alone and the combination of quinpirole and CGS 21680 were also not significantly

different ($p > .05$). CGS 21680 alone had no effect on tGluR1 ($p > .05$). Quinpirole had no effect on pGluR1^{S845} alone or in the presence of CGS 21680 ($p > .05$), while CGS 21680 increased pGluR1^{S845} by 47% ($p < .01$). No significant effect of treatment was observed for pGluR1^{S831} in the NAc core P2 fraction ($F_{3,28} = 2.28$, $p = 0.1011$). Quinpirole produced approximately 30% increases in pGluR1^{S831} when administered alone or in the presence of CGS 21680, however, these effects were not statistically significant ($p > .05$). Similar analyses in the NAc core S2 fraction and dorsal striatum P2 fraction showed no differences in tGluR1 levels or GluR1 phosphorylation (Table 3).

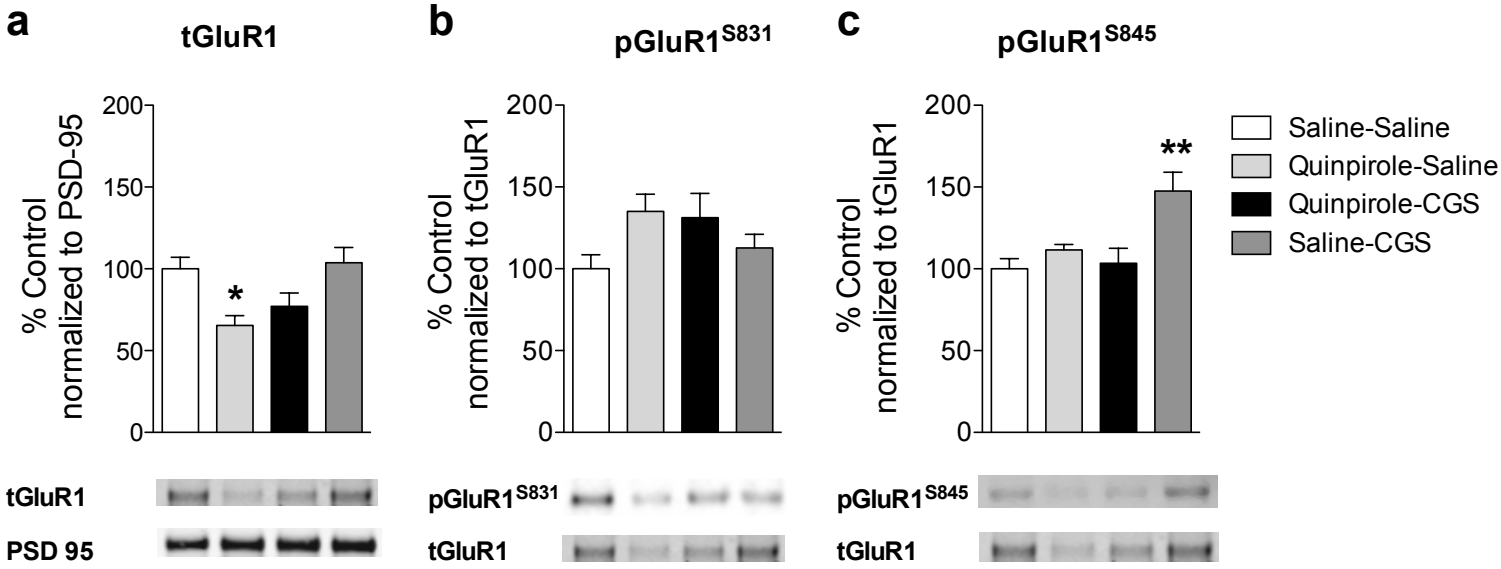

Figure 5: Intra-NAc core administration of the adenosine A2A agonist CGS 21680 increases GluR1S845 phosphorylation and modestly blocks D2 agonist-induced internalization of synaptic GluR1.
(a) Intra-NAc core infusion of the D_2 agonist quinpirole alone significantly decreases tGluR1 levels in the P2 fraction. Quinpirole does not significantly decrease tGluR1 levels in the P2 fraction when co-administered with the A_{2A} agonist CGS 21680 (b) Intra-NAc core infusion of the D_2 agonist quinpirole alone or together with the A_{2A} agonist CGS 21680 modestly increases pGluR1^{S831} in the P2 fraction, however these effects are not statistically significant. (c) Intra-NAc core infusion of the A_{2A} agonist CGS 21680 significantly increases pGluR1^{S845} in the P2 fraction. *Significant from Sal/Sal ($p < .05$ Bonferroni's post-test), **Significant from Sal/Sal ($p < .01$ Bonferroni's post-test)

Table 3: Effects of Acute Intra-NA Core Quinpirole/CGS 21680 on Cocaine Self-Administering Animals

Protein	Brain Region	Sal/Sal (n=4)	Quin/Sal (n=4)	Quin/CGS (n=4)	Sal/CGS (n=4)	Statistics
tGluR1 / ß-Tubulin	NAc Core S2	100.0 ± 19.2	90.5 ± 10.09	111.5 ± 12.38	108.2 ± 19.5	F(3,21)=0.385, p=0.765
tGluR1 / PSD 95	DS P2	100.0 ± 11.0	138.9 ± 30.39	87.1 ± 3.76	113.5 ± 15.8	F(3,11)=1.25, p=0.338
pGluR1^{S831} / tGluR1	DS P2	100.0 ± 2.66	99.5 ± 6.98	98.9 ± 6.60	99.9 ± 4.10	F(3,12)=0.009, p=0.999
pGluR1^{S845} / tGluR1	DS P2	100.0 ± 3.70	104.1 ± 13.46	108.3 ± 11.75	110.1 ± 9.23	F(3,12)=0.195, p=0.898

CGS 21680 (2.5 ng/side) and/or quinpirole (3.0 μg/side) were bilaterally infused in 1 μl of saline over a period of 1 minute. Sacrifice and tissue collection occurred 30 minutes after intracranial infusions treatment. Values are reported as % of saline control for phospho/total protein ratio. NAc= Nucleus Accumbens, DS= Dorsal Striatum.

Experiment 5A: Stimulation of A_1 receptors impairs D_1-induced cocaine seeking

Adenosine A_1 receptors are coupled to inhibitory G proteins and oppose D_1-induced enhancements in cAMP signaling on the postsynaptic terminals of direct pathway medium spiny neurons in the NAc (Ferre et. al, 1997, Dunwiddie and Masino, 2001). Thus, adenosine A_1 receptors may be a prime target for inhibiting D_1-mediated behaviors. We have recently shown that, like A_{2A} receptor stimulation, stimulation of A_1 receptors in the NAc also reverses the expression of cocaine sensitization (Hobson et al., 2012). Preliminary evidence suggests that A_1 receptor stimulation also reduces reinstatement of cocaine seeking (unpublished observations). To test the effects of A_1 receptor stimulation on D_1-induced reinstatement, we administered intra-NAc infusions of the D_1 agonist SKF 81297 (3.0 μg/side) alone or in concert with the A_1 receptor agonist, CPA (0.1 μg/side). Figure 6 shows that A_1 receptor stimulation significantly reduced D_1-induced reinstatement. A significant interaction ($F_{2,22} = 8.38$; $p = 0.0313$) and main effects of treatment ($F_{2,22} = 62.00$; $p < 0.0001$) and of lever ($F_{1,22} = 6.12$; $p = 0.0232$) were

observed. Post-hoc analysis (Bonferroni's) revealed animals that received SKF 81297 alone significantly reinstated ($p < .01$), while those that received SKF 81297 and CPA did not ($p > .05$).

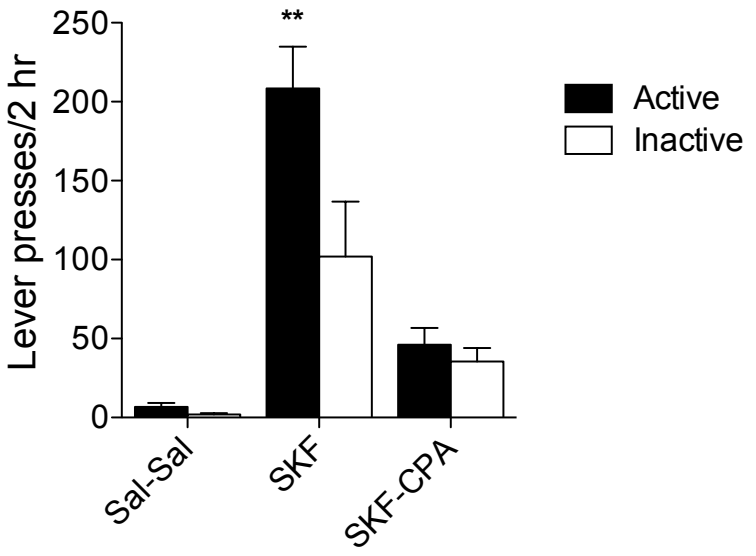

Figure 6: Intra-NAc core administration of the adenosine A1 agonist CPA blocks D1 agonist-induced reinstatement of cocaine seeking.
Intra-NAc core infusion of the D_1 agonist SKF 81297 significantly induces reinstatement, while co-administration of the D_1 agonist SKF 81297 and the A_1 agonist CPA has no significant effect. ** Significant from Sal/Sal ($p < .01$ Bonferroni's post-test)

Experiment 5B: Stimulation of A_1 receptors offsets D_1-mediated GluR1 Phosphorylation

We have previously shown that AMPA receptor function is necessary for D_1-mediated reinstatement (unpublished observations) and A_1 receptor stimulation inhibits D_1-mediated reinstatement (Figure 6). We therefore hypothesized that A_1 receptor stimulation offsets D_1-induced PKA activity that contributes to GluR1-containing AMPA receptor trafficking. To test this hypothesis, we collected tissue from the NAc core and dorsal striatum 30 minutes after intra-NAc core infusion of SKF 81297 or the combination of CPA and SKF 81297 (treatments same as in Experiment 5A and given 24 hrs after reinstatement testing). Tissue samples were processed as in Experiment 4B (synaptoneurosome preparation) and immunoblotted to analyze GluR1 expression and phosphorylation as in Experiment 4B.

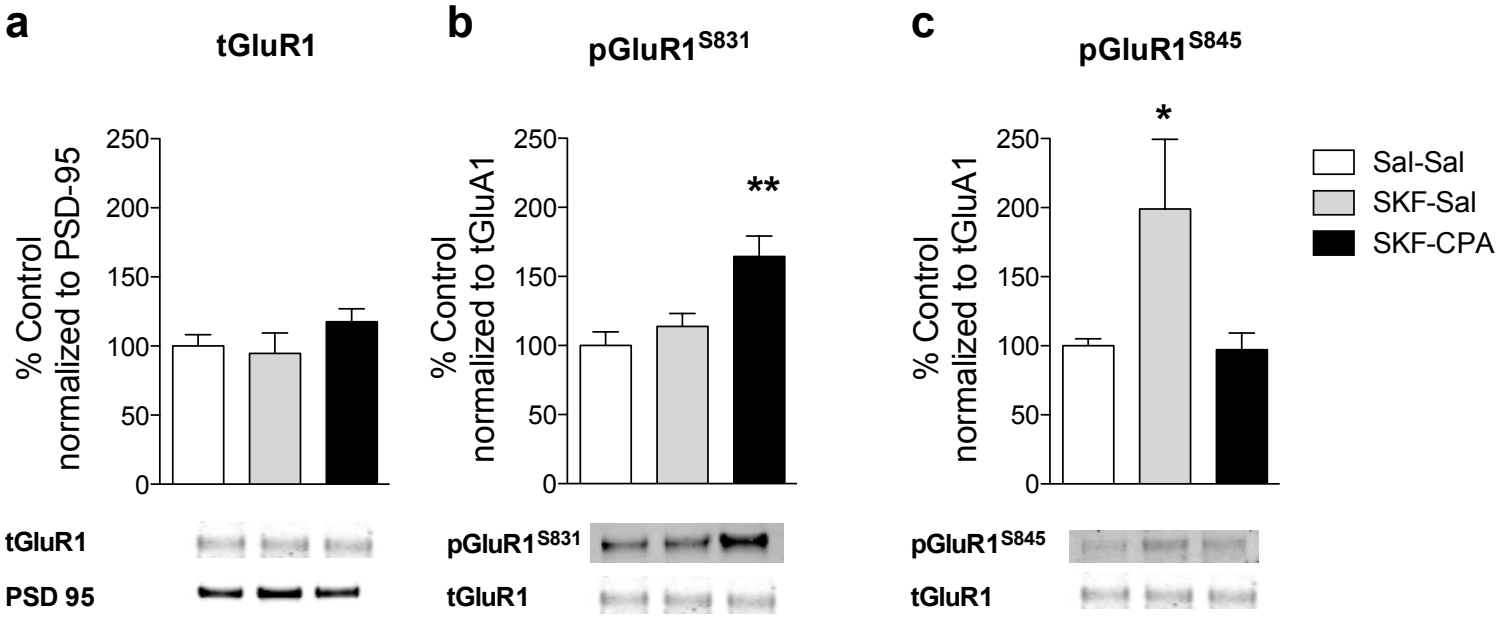

Figure 7: Intra-NAc core administration of the adenosine A1 agonist CPA blocks D1 agonist-induced phosphorylation of GluR1S845.
(a) Intra-NAc core infusion of the D_1 agonist SKF 81297 alone or in the presence of the A_1 agonist CPA has no effect on tGluR1 levels in the P2 fraction. (b) Intra-NAc core infusion of the D_1 agonist SKF 81297 and the A_1 agonist CPA together significantly increases pGluR1^{S831}, while SKF 81297 alone has no effect in the P2 fraction. (c) Intra-NAc core infusion of the D_1 agonist SKF 81297 significantly increases pGluR1^{S845} in the P2 fraction, which is reversed by co-administration of the A_1 agonist CPA. *Significant from Sal/Sal ($p < .05$ Bonferroni's post-test), **Significant from Sal/Sal ($p < .01$ Bonferroni's post-test)

Figure 7 shows representative immunoblots of the NAc core P2 fraction of samples collected 30 minutes after vehicle, SKF 81297, or SKF 81297/CPA co-administration. There was no significant difference between groups in tGluR1 levels in the NAc core P2 fraction ($F_{2,28} = 1.13$; $p = 0.34$). A significant effect of treatment was observed for pGluR1^{S845} ($F_{2,29} = 4.09$; $p = .0273$) and pGluR1^{S831} ($F_{2,28} = 8.73$; $p = 0.0011$) in the NAc core P2 fraction. Treatment of SKF 81297 alone increased pGluR1^{S845} by 99% ($p < .05$), while this effect was completely reversed by co-administration of CPA ($p > .05$). Treatment with CPA/SKF 81297 together increased pGluR1^{S831} by 65% ($p < .01$), while treatment with SKF 81297 alone had no significant effect on pGluR1^{S831} ($p > .05$). Similar analyses in the NAc core S2 fraction and dorsal striatum P2 fraction showed no differences in tGluR1 levels or GluR1 phosphorylation (Table 4).

Table 4: Effects of Acute Intra-NA Core SKF 81297/CPA on Cocaine Self-Administering Animals

Protein	Brain Region	Sal/Sal (n=6)	SKF/Sal (n=5)	SKF/CPA (n=5)	Statistics
tGluR1 / ß-Tubulin	NAc Core S2	100.0 ± 14.38	105.8 ± 3.62	110.0 ± 9.69	F(3,21)=0.385, p=0.765
tGluR1 / PSD 95	DS P2	100.0 ± 6.95	120.7 ± 5.32	105.4 ± 10.53	F(2,12)=1.836, p=0.216
pGluR1^{S831}/ tGluR1	DS P2	100.0 ± 5.35	105.7 ± 5.47	124.0 ± 15.56	F(2,12)=1.56, p=0.249
pGluR1^{S845} / tGluR1	DS P2	100.0 ± 2.39	123.1 ± 20.23	95.45 ± 17.18	F(2,13)=1.021, p=0.387

SKF 81297 (3.0 μg/side) or SKF and CPA (1.5 μg/side) were bilaterally infused in 1 μl/side over 1 minute. Sacrifice and tissue collection occurred 30 minutes after intracranial infusions treatment. Values are reported as % of saline control for phospho/total protein ratio. NAc= Nucleus Accumbens, DS= Dorsal Striatum.

Experiment 6: Expression of mutant GluR1^{S845} inhibits D_2, but not D_1 or cocaine-induced reinstatement

Recent work has shown that GluR1-containing AMPA receptor trafficking plays a critical role in cocaine seeking (Bachtell et al., 2009, Anderson et al., 2008), and that this trafficking may be modulated by NAc core dopamine receptor signaling (Anderson et al., 2008, Figures 4B and 5B). Based on these results, we hypothesized that D_1 or D_2 receptor-induced reinstatement may be mediated by increases or decreases, respectively, in synaptic GluR1 that result from changes in pGluR^{S845}. To directly test the involvement of GluR1^{S845} phosphorylation in reinstatement, we tested the effects of overexpressing the GluR1 subunit with a serine to alanine mutation at serine 845 (GluR1^{S845A}) in the NAc core, on cocaine, D_2, and D_1-induced cocaine seeking. Following extinction of lever pressing, herpes simplex virus (HSV) vectors expressing either GluR1^{S845A} or Lac Z (ß-gal expressing control) were infused into the NAc core. Two to

four days following administration of viral vectors, cocaine seeking was induced by systemic administration of cocaine (15 mg/kg, i.p.) or by intra-NAc infusion of the D_1 agonist SKF 81297 (3.0 μg/side) or the D_2 agonist quinpirole (3.0 μg/side). Figure 8 shows that GluR1^{S845A} expression in the NAc had no effect on cocaine-induced reinstatement ($t_{10} = 0.25$; $p = 0.81$). For intra-NAc elicited reinstatement, a significant effect of virus was observed ($F_{1,20} = 8.92$; $p = 0.0073$). GluR1^{S845A} significantly reduced quinpirole-induced cocaine seeking compared to LacZ expressing controls ($t = 2.69$; $p < .05$), while GluR1^{S845A} only slightly reduced SKF 81297-induced reinstatement ($t = 1.54$; $p > .05$).

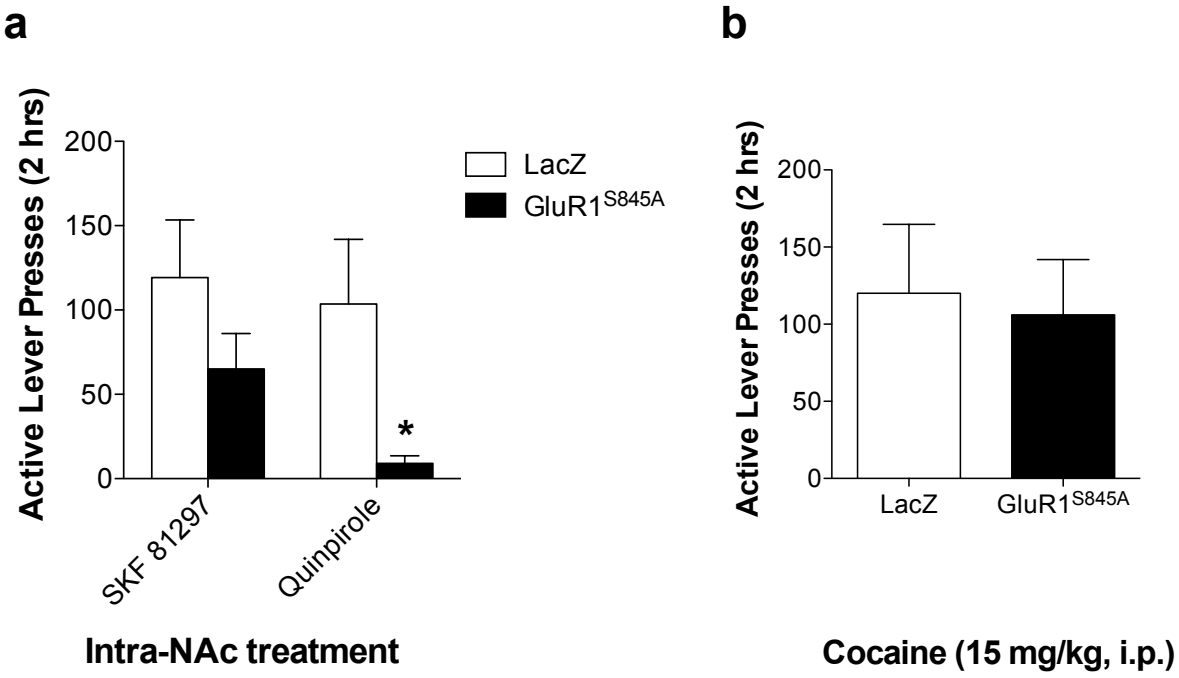

Figure 8: Intra-NAc core expression of GluR1 mutant GluR1^{S845A} blocks quinpirole-induced reinstatement.
(a) Expression of GluR1^{S845A} significantly blocked D_2 agonist-induced reinstatement. GluR1^{S845A} slightly reduced D_1 agonist-induced reinstatement, however, this effect was not statistically significant. (b) Expression of GluR1^{S845A} has no effect on cocaine-induced reinstatement. *Significant from LacZ ($p < .05$ Bonferroni's post-test)

Discussion:

Recent studies have shown that stimulation of adenosine receptors inhibits multiple cocaine-related behaviors such as locomotor sensitization and reinstatement (Filip et. al, 2006, Bachtell et. al, 2009) but the molecular mechanisms underlying these effects remain unclear. In these studies, we investigated how adenosine A_1 and A_{2A} receptor stimulation might offset dopamine D_1 and D_2 receptor signaling, respectively, in NAc neurons to regulate cocaine-seeking behavior. Given the importance of AMPA receptor-based plasticity in both cocaine- and dopamine agonist-induced locomotor sensitization and reinstatement (Bachtell et. al, 2008a, Cornish et. al, 1999, Cornish and Kalivas, 2000), we specifically investigated whether dopamine and adenosine receptors might exert opposing actions on PKA-mediated phosphorylation of GluR1^{S845}.

Emerging evidence suggests that adenosine receptors may play a critical role in neurological conditions involving hyper- and hypo-dopaminergic signaling in the striatum, such as Parkinson's disease, Huntington's disease, and psychostimulant addiction (Ferre et. al, 1997, 1999). However, several reports indicate that stimulation of adenosine receptors does not significantly alter behavioral responses to enhanced dopamine signaling in healthy, drug naïve animals (Filip et. al, 2006, Poleszak and Malec, 2002). For example, we recently demonstrated that stimulation of A_1 or A_{2A} receptors in the NAc core blocks the expression of cocaine sensitization, but has no effect on cocaine-induced locomotor activity in cocaine naïve animals (Hobson et. al, 2012). Consistent with these results, we found that systemic administration of the A_{2A} agonist CGS 21680 has no effect on either pGluR1^{S845} or pERK in the striatum. However, in cocaine-sensitized animals, intra-NAc core administration of CGS 21680 significantly increased pGluR1^{S845}. These results suggest that repeated exposure to cocaine might cause perturbations

enabling adenosine receptor stimulation to affect intracellular signaling and cellular excitability in the NAc core.

Although it is clear that stimulation of NAc core adenosine receptors can modulate behaviors associated with repeated cocaine exposure, a clear mechanism for these effects has not been established. Here, we pharmacologically stimulated adenosine receptors to investigate adenosine receptor-mediated signaling and potential changes in GluR1^{S845} phosphorylation during cocaine-induced expression of locomotor sensitization. Based on the distinct expression of D_1 and D_2 receptors on NAc MSNs, and their opposing actions on PKA activity, we hypothesized that cocaine-induced non-selective stimulation of dopamine receptors simultaneously causes increases in pGluR1^{S845} in D_1-expressing cells of the direct pathway and decreases in pGluR1^{S845} in D_2-expressing cells of the indirect pathway. This notion is supported by findings suggesting that repeated cocaine administration enhances the excitability of direct pathway neurons, while decreasing the excitability of indirect pathway neurons (Lobo and Nestler, 2011). We found that cocaine significantly increased pGluR1^{S845} in whole cell lysates from the NAc core of cocaine-sensitized animals, an effect that was not reversed by A_{2A} receptor stimulation. Since D_2 receptors inhibit, while D_1 receptors enhance PKA activity, cocaine-induced increases in pGluR1^{S845} most likely occur in direct pathway neurons. This idea is consistent with the fact that stimulation of A_{2A} receptors, localized on D_2-expressing indirect pathway neurons, does not reverse cocaine-induced pGluR1^{S845}. It seems that in the present experiment, D_1-mediated increases in pGluR1^{S845} occurring in direct pathway neurons mask our ability to observe any D_2 or A_{2A} receptor-mediated changes in pGluR1^{S845} occurring in indirect pathway neurons.

To directly test the hypothesis that A_{2A} receptor-mediated reversal of D_2-induced deficits in pGluR1^{S845} plays a role in blocking cocaine and D_2 agonist-induced expression of locomotor sensitization, we directly stimulated D_2 and/or A_{2A} receptors in the NAc core of cocaine-sensitized animals. Quinpirole alone produced a modest, non-significant decrease in pGluR1^{S845} in whole cell lysates from the NAc core, while CGS 21680 alone significantly increased pGluR1^{S845}. These results correlate with the behavioral effects of A_{2A} receptor stimulation on D_2 agonist-induced expression of locomotor sensitization and support the idea that NAc core A_{2A} receptors offset D_2-induced deficits in PKA activity (Hobson et al, 2012). Furthermore, the fact that intra-NAc CGS 21680 induces pGluR1^{S845} suggests that A_{2A} receptors may restore cocaine-induced deficits in cellular excitability of indirect pathway neurons. Interestingly, co-administration of quinpirole and CGS 21680 significantly increased pERK in the NAc core of cocaine-sensitized animals, while either agonist alone produced slight, non-significant increases. This result may be caused by additive activation of the MAP kinase pathway by the beta/gamma subunits of both G-protein coupled receptors, which are known to modestly increase MAP kinase signaling independently (Zhai et. al, 2008).

We recently demonstrated that stimulation of NAc core A_{2A} receptors blocks reinstatement induced by systemic administration of both cocaine and a D_2 agonist (O'Neill et. al, 2011). To determine whether pGluR1^{S845} is also relevant to the inhibitory effects of A_{2A} receptor stimulation on reinstatement, we explored whether changes in pGluR1^{S845} during reinstatement testing might parallel those observed during expression of sensitization. Our results show that reinstatement induced by stimulation of NAc core D_2 receptors was reversed by A_{2A} receptor stimulation, consistent with our hypothesis that A_{2A} receptors oppose D_2 signaling within NAc core indirect pathway MSNs. D_2 receptor stimulation significantly decreased synaptic levels of

total GluR1 in the NAc core. This effect was modestly blunted by simultaneous A_{2A} stimulation. Again, A_{2A} stimulation alone significantly increased pGluR1^{S845} in the NAc core. Recent work suggests that in addition to regulating trafficking to the cell surface, GluR1^{S845} phosphorylation is critical for maintaining the stability of GluR1-containing AMPA receptors within the postsynaptic density (Lee et al., 2004, 2006). Thus, D_2 receptor stimulation may act to reduce excitatory neurotransmission in indirect pathway neurons by diminishing PKA-mediated phosphorylation and facilitating removal of synaptic AMPA receptors. Stimulation of NAc core A_{2A} receptors, on the other hand, may act to offset D_2-induced deficits in pGluR1^{S845}, internalization of AMPA receptors, and decreased excitability of indirect pathway MSNs, ultimately blocking cocaine and D_2-mediated reinstatement.

A recent study proposed a similar, but distinct mechanism of cocaine reinstatement involving D_1 receptor-mediated enhancements of GluR1 trafficking to the postsynaptic density of direct pathway MSNs (Anderson et. al, 2008). Functionally, enhanced AMPA receptor trafficking in direct pathway neurons would promote behavioral activation, such as the expression of cocaine sensitization and cocaine reinstatement. Consistent with the idea that offsetting D_1-induced PKA activity might inhibit GluR1^{S845} phosphorylation that is necessary for reinstatement, we recently showed that intra-NAc core A_1 receptor stimulation blocks the expression of sensitization to cocaine (Hobson et. al, 2012). Indeed, intra-NAc core A_1 receptor stimulation inhibits D_1-induced reinstatement and reverses D_1-induced increases in pGluR1^{S845}. These results are supported by findings suggesting that AMPA receptor activity is necessary for D_1-induced reinstatement (unpublished observations). Taken together, these findings suggest that stimulation of NAc core A_1 receptors inhibits D_1-induced reinstatement through a potential mechanism

where A_1 receptors inhibit D_1-induced increases in pGluR1^{S845}, thereby reducing D_1-mediated enhancements in synaptic AMPA receptors and excitability of direct pathway neurons.

However, there are several discrepancies between our results and those previously reported by Anderson et. al (2008). Most striking is the fact that Anderson et. al did not observe an increase in NAc pGluR1^{S845} following systemic administration of 10 mg/kg cocaine. We observed a 25% increase in pGluR1^{S845} following 15 mg/kg cocaine. While the difference in dosing may be important here, other findings are still discrepant. For example, they report a cocaine-induced increase in pGluR1^{S831}, which was attributed to activation of CaMKII, an effect blocked by D_1 receptor antagonism. These results are seemingly at odds with our findings that both cocaine and D_1 receptor stimulation increased pGluR1^{S845}, while D_1 receptor stimulation alone did not increase pGluR1^{S831}. Since phosphorylation of GluR1 serines 845 and 831 are both thought to mediate insertion of AMPA receptors into the postsynaptic density (Malenka and Nicoll, 1999, Lee et. al, 2004), these differing results seem to support the same basic mechanism. However, it appears that systemic administration of cocaine, which influences multiple neurotransmitter systems throughout the brain, causes different cellular responses compared with local infusion of a D_1 agonist into the NAc. Thus, this methodological difference may account for some of the discrepancy between our findings. Future studies will shed light on the specific role of GluR1 serines 845 and 831 in cocaine and D_1-mediated reinstatement.

It is also interesting to note that in our studies, simultaneous stimulation of A_1 and D_1 receptors significantly increased pGluR1^{S831}. Unfortunately, we did not treat animals with A_1 agonist alone, so we cannot determine whether this effect is mediated by A_1 stimulation alone or by combined stimulation of A_1 and D_1 receptors. It is possible that this effect, like our finding that simultaneous stimulation of D_2 and A_{2A} receptors increases pERK, is mediated by additive

activation of the MAP kinase pathway by both G-protein coupled receptors (Zhai et. al, 2004). Another possibility is that the increased pGluR1^{S831} is a compensatory response due to the inhibition of GluR1^{S845} phosphorylation produced by A_1 receptor stimulation. This idea is supported by studies of phosphomutant transgenic mice with mutations at GluR1 serine 845, 831, or both (Lee et. al, 2010). Mice mutant for both residues cannot develop LTP, while mutation of either residue alone has no significant effect on the development and maintenance of LTP, suggesting that these residues can compensate for each other (Lee et. al, 2010). However, others have shown that PKA activity is necessary for CaMKII driven insertion of AMPA receptors into synapses (Esteban et. al, 2003). This may explain why the putatively compensatory GluR1^{S831} phosphorylation in animals treated with the A_1 agonist was not able to compensate for pGluR1^{S845} in facilitating D_1-induced reinstatement behavior.

These studies also provide insight into our finding that expression of the GluR1^{S845A} phosphomutant in the NAc core significantly inhibited D_2, but not D_1 or cocaine-induced reinstatement. Interestingly, mice lacking GluR1^{S845}, but not GluR1^{S831}, show deficits in the development of LTD (Lee et. al, 2010). Other studies also show that dephosphorylation of GluR1^{S845} is critical for internalization of AMPA receptors in LTD (Lee et. al, 2006, Shepherd and Huganir, 2007). Since we hypothesize that D_2-induced reinstatement is caused by decreased excitability of indirect pathway neurons, expression of the GluR1^{S845A} phosphomutant may inhibit the extent to which D_2 receptor signaling can cause internalization AMPA receptors, thereby blocking the ability of D_2 receptors to cause reinstatement. Conversely, since we hypothesize that D_1-induced reinstatement is driven by increased excitability of direct pathway neurons, expression of the GluR1^{S845A} phosphomutant may not inhibit the ability of D_1 receptors to enhance synaptic AMPA receptors because GluR1^{S31} is either already involved in this process

(Anderson et. al, 2008) or able to compensate for GluR1^{S845} (Lee et. al, 2010). A lack of full compensation might explain why GluR1^{S845A} expression produced a nonsignificant decrease in D_1-induced reinstatement. However, it is difficult to explain why expression of GluR1^{S845A} had no effect on cocaine-induced reinstatement. It is also difficult to determine how much GluR1^{S845A} is present in the NAc core relative to endogenous GluR1, and the extent to which GluR1^{S845A} is actually incorporated into functional AMPA receptors. The lack of cell-type specificity in the expression of GluR1^{S845A} further complicates interpretation of these results. Future studies using viral constructs whose expression is driven by D_1 or D_2 promoters may provide a more elegant experimental strategy to test our hypotheses about the role of GluR1^{S845} in direct and indirect pathway MSNs.

While our results strongly suggest that the behavioral and biochemical effects of adenosine receptor stimulation in the NAc are mediated by postsynaptic receptors on MSNs, it is possible that A_1 and A_{2A} present on presynaptic glutamate terminals may play a role in our findings. The majority of these presynaptic glutamate terminals expressing both A_1 and A_{2A} receptors project onto direct pathway neurons (Ciruela et. al, 2006, Hettinger et. al, 2001, Orru et. al, 2011a), therefore enabling adenosine to regulate glutamate release and subsequent activation of the direct pathway (Ferre et. al, 2008, Rodrigues et. al, 2005, Orru et. al, 2011b). Stimulation of presynaptic A_{2A} receptors increases glutamate release onto direct pathway neurons and enhances their activity (Ciruela et. al, 2006, Corsi et. al, 1999, 2000). Since both of these events are known to induce reinstatement (Bachtell et. al, 2008, Cornish and Kalivas, 2000), it seems unlikely that presynaptic A_{2A} receptors play a significant role in our experiments. Stimulation of presynaptic A_1 receptors, however, decreases glutamate release and decreases the activation of the direct pathway (Ciruela et. al, 2006, Corsi et. al, 1999, 2000). Both of these actions would presumably

inhibit D_1-induced reinstatement (Self et. al, 1996, Bachtell et, al, 2008a, 2008b, unpublished observations). Although our phosphorylation data strongly suggest a postsynaptic site of action, it is possible that intra-NAc infusion of the A_1 agonist CPA may simultaneously inhibit both presynaptic glutamate release and postsynaptic D_1 signaling. Recent studies have identified an A_{2A} receptor antagonist that selectively targets presynaptic receptors, thereby inhibiting glutamate release and direct pathway activation without targeting postsynaptic A_{2A} receptors (Ferre et. al, 2008). Future studies using this compound will help clarify whether presynaptically mediated inhibition of glutamate release plays a significant role in the A_1 agonist-mediated reversal of D_1-induced reinstatement.

Collectively, our results suggest that adenosine receptors act in a cell-specific manner to oppose striatal dopamine receptor signaling that drives expression of cocaine sensitization and reinstatement of drug seeking. Specifically, we demonstrate that stimulation of adenosine A_1 or A_{2A} receptors in the NAc core is sufficient to offset D_1 or D_2-induced cocaine seeking, respectively. These effects may be dependent upon dopamine and adenosine receptors' opposing actions on GluR1 phosphorylation that regulates AMPA receptor trafficking and cellular excitability within direct and indirect pathway neurons. Our findings also provide further evidence that adenosine receptors are critical regulators of dopamine receptor signaling in diseases involving dysregulation of the basal ganglia and/or dopamine pathways. Finally, the results of these studies illuminate the potential for adenosine receptor stimulation as an effective strategy for reversing cocaine-induced alterations in striatal signaling that may underlie an addicts' persistent susceptibility to relapse. Future research will provide further insight into the complex interactions between glutamate, dopamine, and adenosine signaling in the striatum. This work may eventually lead to the development of novel strategies to restore balance in striatal

output pathways, which will provide relief to millions of patients suffering from neurological diseases that currently remain untreatable.

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