Published in final edited form as:

Behav Brain Res. 2014 November 1; 274: 84–94. doi:10.1016/j.bbr.2014.07.047.

The Effects of Nucleus Accumbens μ -opioid and Adenosine 2A Receptor Stimulation and Blockade on Instrumental Learning

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Abstract

Prior research has shown that glutamate and dopamine receptors in the nucleus accumbens (NAcc) core are critical for the learning of an instrumental response for food reinforcement. It has also been demonstrated that μ -opioid and adenosine A2A receptors within the NAcc impact feeding and motivational processes. In these experiments, we examined the potential roles of NAcc μ -opioid and A2A receptors on instrumental learning and performance. Sprague-Dawley rats were food restricted and trained to lever press following daily intra-accumbens injections of the A2A receptor agonist CGS 21680 (at 0.0, 6.0, or 24.0 ng/side), the A2A antagonist pro-drug MSX-3 (at 0.0, 1.0, or 3.0 μ g/side), the μ -opioid agonist DAMGO (at 0.0, 0.025, or 0.025 μ g/side), or the opioid receptor antagonist naltrexone (at 0.0, 2.0 or 20.0 μ g/side). After five days, rats continued training without drug injections until lever pressing rates stabilized, and were then tested with a final drug test to assess potential performance effects. Stimulation, but not inhibition, of NAcc adenosine A2A receptors depressed lever pressing during learning and performance tests, but did not impact lever pressing on non-drug days. Both μ -opioid receptor stimulation and blockade inhibited learning of the lever-press response, though only naltrexone treatment caused impairments in lever-pressing after the task had been learned. The effect of A2A receptor stimulation on learning and performance were consistent with known effects of adenosine on effort-related processes, whereas the pattern of lever presses, magazine approaches, and pellet consumption following opioid receptor manipulations suggested that their effects may have been driven by drug-induced shifts in the incentive value of the sugar reinforcer.

Keywords

adenosine; mu-opioid receptor; nucleus accumbens; learning; motivation

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1. Introduction

The nucleus accumbens (NAcc) has been demonstrated to be an important neural center for both appetitive goal-seeking behavior as well as consummatory behavior directed at food or drugs of abuse. Among its multiple roles in regulating reward seeking and consumption, the NAcc is important for the learning of an operant response that leads to reinforcement. Inhibition of protein synthesis within the core of the NAcc impairs the learning (but not performance) of a lever-press response for food [1], suggesting that protein synthesis within the NAcc is required for the formation of long-term memories within an appetitive instrumental task. Manipulations of individual neurotransmitters within the NAcc effectively block the learning of an operant task. For instance, microinjections of NMDA receptor antagonists into the NAcc core prior to training sessions blocked the acquisition of a lever-press response for a food reward without adversely affecting locomotor or feeding behavior [2]. It has been suggested that one role of the NAcc in mediating goal-seeking behavior is through the facilitation of Pavlovian to instrumental transfer. Indeed, disruption of dopamine signaling in the NAcc blocks the acquisition and performance of Pavlovian approach behavior [3].

Though the roles of NAcc glutamate and dopamine in mediating appetitive learning have been well characterized, several other closely related receptor systems have received considerably less attention. Specifically, the μ -opioid and adenosine 2A (A2A) receptors have been associated with glutamate and dopamine receptor signaling. Functional associations between NMDA and μ -opioid receptors occur at colocalization sites within NAcc dendrites and axons, as well as at synaptic and appositional contacts between NMDAR1-containing axons and MOR-containing dendrites [4]. Similarly, heteromerization of dopamine D2 and A2A receptors are seen frequently throughout striatal regions [5]. These heteromers have been demonstrated *in vitro* to regulate NMDA-mediated excitation of NAcc neurons [6], suggesting that alteration of these receptor systems could functionally alter behaviors known to be sensitive to NMDA and DA manipulations. Given the known role for glutamate and dopamine in instrumental learning tasks, the interactions of A2A and μ -opioids with glutamate and dopamine systems in the nucleus accumbens suggest that these receptors may also play important roles in operant learning.

Prior investigations have reported that systemic manipulations of opioid and adenosine receptors influence general learning processes. For instance, μ -opioid receptor knock-out mice show impairments in learning and subsequent performance on tests of spatial memory compared with control mice [7], while systemic blockade of opioid activity during learning inhibits appropriate responding to a food reward after its value has been altered [8]. Additionally, systemic administration of caffeine, a non-selective adenosine receptor antagonist, enhances performance in a water maze task in rodents [9], likely by affecting memory retention rather than acquisition [10]. Both A2A and μ -opioid receptors are distributed within striatal pathways, including within the NAcc [11-15]. Consistent with the potential role for these receptors in regulating instrumental learning, there is evidence that links these receptors in the ventral striatum to the regulation of food-motivated behavior and reward processes. For instance, injections of A2A receptor agonists into the NAcc reduced lever pressing for a preferred food reward and promoted a switch to enhanced consumption

of a freely available but less preferred food [16]. Furthermore, intra-accumbens microinjections of the A2A antagonist prodrug MSX-3 increased consumption of a freely available high fat diet [17]. Such data suggests that NAcc A2A receptors regulate effort expenditure even within highly motivated behaviors. With regard to the opioid systems of the striatum, multiple laboratories have demonstrated that intra-NAcc injections of a μ -opioid agonist increase consumption of a high fat diet in sated animals [18-23], indicating a role for opioid receptors in promoting food intake beyond homeostatic levels. Additionally, microinjections of opioid receptor agonists directly into the NAcc increased the degree of effort rats were willing to exert in order to gain access to a food reward [24]. Thus, there is both physiological and behavioral data to suggest that μ -opioid and A2A receptors within the NAcc are important in regulating food-directed reward-seeking behaviors.

To date, there has not been an examination of the potential role for NAcc A2A or μ -opioid receptors in an instrumental learning task. Given that the NAcc is important for performance of highly motivated behavioral tasks and possesses dense populations of A2A and μ -opioid receptors that functionally and structurally interact with NMDA and DA receptors known to be important in learning processes, it is reasonable to suggest that these neurotransmitters may play a role in mediating learning processes related to instrumental behavior. The goal for these experiments was to examine the effects of A2A or μ -opioid activation or blockade of the NAcc core on the learning of a lever-pressing operant response for a sugar reward. Independent groups of rats were trained to lever press for sugar pellets following intra-NAcc injections of a vehicle solution or increasing doses of an A2A receptor agonist, an A2A receptor antagonist, a μ -opioid receptor agonist, or an opioid receptor antagonist. We report here that manipulations of both adenosine and opioid systems impacted learning and (in some cases) performance of the operant response. Additionally, given prior reports that the manipulation of adenosine receptor activity may moderate the effects of opioid receptor activation on food intake [17, 25], we further examined whether antagonism of A2A receptors would modulate the learning impairment that was observed following μ -opioid receptor stimulation within the same appetitive learning task.

2. Methods

2.1 Subjects

Adult male Sprague-Dawley Rats (Harlan, Madison, WI), approximately 275-300 g at experiment onset, were acclimated to dual housing in clear plastic cages. The colony room was maintained at ~21°C under a 12-hr light-dark cycle (lights on at 7 am). Standard rat chow and water were available ad libitum during acclimation to the laboratory and surgical recovery. All procedures were conducted in accordance to National Institutes of Health animal care guidelines and approved by the Wake Forest University Animal Care and Use Committee.

2.2 Surgery

Rats were anesthetized with a Ketamine-Xylazine cocktail (100 mg/kg-10mg/kg). Standard aseptic surgical procedures were followed to bilaterally implant stainless steel guide cannulas (23 gauge) above the NAcc core (flat skull; 1.3 mm anterior and 1.7 mm lateral to

bregma, 5.0 mm ventral to skull surface). Guide cannulas were secured to the skull with jeweler's screws and dental acrylic. Rats recovered for at least seven days before gradual food restriction to 90% of their ad libitum weight. Food restriction was accomplished by providing each rat a ration of 8 g/day of standard rat chow (Prolab RMH3000, Lab Diet, St. Louis, MO, 3.46 kcal/g) for six days or until the target weight was reached. During the last two days of this initial food restriction, 2 g of sugar pellets (Bio-Serv; Frenchtown, New Jersey) were added to the home cage to reduce the novelty of the taste of sugar. Rats were then maintained at this 90% weight by providing 10-16 g/day of chow, as needed.

2.3 Drugs and Microinfusion Procedure

The adenosine 2A agonist CGS 21680 (Tocris Biosciences), the A2A antagonist pro-drug prodrug 3-(3-hydroxypropyl)-8-(*m*-methoxystyryl)-7-methyl-1-propargylxanthine phosphate disodium salt (MSX-3; Sigma), selective μ -opioid receptor agonist D-Ala², N-MePhe⁴, Gly-ol]-enkephalin (DAMGO; Tocris Biosciences), and nonselective opioid receptor antagonist naltrexone (Sigma) were dissolved in 0.9% sterile saline. Drug solutions were mixed at the beginning of each experiment and stored in aliquots either at +4°C (naltrexone) or -20 °C (DAMGO, MSX-3, CGS 21680) until used.

For all experiments, rats were habituated to the injection procedures for two days prior to the start of the experimental injections. Each rat received two mock injections on successive days. On the first mock injection day, 10 mm injectors were lowered to the bottom of the guide cannulas and the microinfusion pump was activated with no solution present. On the second mock injection day, 12.5 mm injectors were lowered to the target site and the pump was again activated with no solutions present. For drug and vehicle solution infusions, 12.5 mm injectors were lowered to the target site. These cannulas were connected to a syringe via polyethylene tubing and 0.5 μ L of solution was delivered at a rate of 0.5 μ L/80 sec. Following a 1 min period for diffusion of the drug away from the injector tips the injectors were removed and wire occlusion stylets replaced into the cannulas. Rats were returned to their home cage for approximately 5 min before they were transferred to the testing apparatus.

2.4 Instrumental Apparatus

Behavioral testing took place in six Med Associates (St. Albans, Vermont) experimental operant chambers. The chambers were outfitted with a house light which provided constant low-light illumination, a food magazine with an IR eyebeam, and a retractable lever. During each 15 min session, timestamps for nosepokes into the food magazine, lever presses, and reward deliveries were recorded on a standard PC computer.

2.5 Learning Paradigm

The learning paradigm was adapted from previous reports examining the neural systems involved in appetitive learning [26-28]. Briefly, following surgical recovery and food restriction, rats received three days of magazine training in the operant chambers in which they were presented with 45 mg sugar pellets into the food magazine but were not exposed to the lever. On the first day, rats were exposed to random presentations of noncontingent pellets on an average of one pellet every 15 sec (RT-15). On the two subsequent days of

habituation, rats received mock injections as described above prior to the start of the session. For these two successive days rats were exposed to random presentations of noncontingent pellets on an average of one pellet every 30 sec (RT-30). All magazine training and subsequent instrumental learning sessions lasted 15 min.

After the acclimation period, rats received five days of injections of drug or vehicle into the NAcc prior to being placed in the operant chamber with access to a lever that produced sugar delivery (with-drug acquisition phase). For the first two days of drug injections rats were concurrently run on an FR-1/RT-30 schedule, such that each lever press resulted in the delivery of one sugar pellet in addition to the RT-30 schedule. The use of the RT-30 schedule during the first two sessions served two purposes: 1) to maintain approximately equal exposure of the treatment groups to the sugar reinforcer during the early learning period, and 2) to ensure a high level of arousal and exploration. In our experience, the RT-30 actively facilitates sampling of the lever, which increases early exposure to the concurrent FR-1 schedule and results in faster and more consistent learning across rats. On the third day of operant training, the RT-30 schedule was removed, and rats pressed initially on an FR-1 schedule. After 50 sugar presentations, the program switched to a random ratio schedule where each lever press had a 0.5 probability of resulting in the presentation of a sugar pellet (RR-2 schedule). The purpose of the RR-2 schedule was to facilitate heightened rates of responding and to reduce the impact of satiety on lever pressing during the 15 min session.

On subsequent training days (6+, no drug training days), rats were placed directly into the operant chambers without microinfusions in order to allow all groups to complete learning of the task (between 10 and 14 days). Once stable rates of lever pressing were established, rats were re-randomized into drug conditions (matching all groups on the original drug condition) and given a final drug injection day in order to assess the effects of the drugs on lever pressing performance.

2.6 Experimental Groups

Four separate groups of rats were used to examine the effects of adenosine and opioid agonists and antagonists on the learning of an instrumental task.

2.6.1 Experiment 1 & 2: The effects of an adenosine 2A agonist or antagonist on instrumental learning—In Experiment 1, learning was measured following stimulation of NAcc adenosine 2A receptors with CGS 21680. Rats ($N = 20$) were randomly assigned to receive 0.0 ng/0.5 μ L/side ($n = 6$; control group), 6.0 ng/0.5 μ L/side ($n = 7$), or 24.0 ng/0.5 μ L/side ($n = 7$) of CGS 21680. After 11 days of training, rats were matched based on previous drug experience and randomized across the same drug doses to assess lever pressing performance. In Experiment 2, learning was measured after infusions of the A2A antagonist prodrug MSX-3 into the NAcc. Rats ($N = 21$) were randomly assigned into the following drug conditions for the first five days of learning: 0.0 μ g/0.5 μ L/side ($n = 7$; control group), 1.0 μ g/0.5 μ L/side ($n = 7$), and 3.0 μ g/0.5 μ L/side ($n = 7$). After 10 days of training, rats were re-randomized to drug condition for a performance test. Doses of CGS 21680 and MSX-3 were chosen to be consistent with prior reports examining the effects of the drugs on operant responding, effort, and/or food intake [16, 17, 29-32].

2.6.2 Experiments 3 & 4: The effect of a μ -opioid agonist or antagonist on instrumental learning

Experiment 3 tested the effects of μ -opioid receptor stimulation of the NAcc on instrumental learning and performance. Rats ($N = 20$) were randomly assigned into to receive 0.0 $\mu\text{g}/0.5\mu\text{L}$ ($n = 6$), 0.025 $\mu\text{g}/0.5\mu\text{L}$ ($n = 7$), and 0.25 $\mu\text{g}/0.5\mu\text{L}/\text{side}$ ($n = 7$) across the first five days of training. The rats then trained without prior drug microinfusions for 13 additional days, after which animals were matched based on rates of lever pressing and previous drug dose and randomized across the same doses to assess lever pressing performance. In Experiment 4, learning was measured following infusions of the opioid antagonist naltrexone into the NAcc. Rats ($N = 21$) were randomly assigned into the following drug conditions for the first five days of learning: 0.0 $\mu\text{g}/0.5\mu\text{L}$ ($n = 7$), 2.0 $\mu\text{g}/0.5\mu\text{L}$ ($n = 8$), and 20.0 $\mu\text{g}/0.5\mu\text{L}/\text{side}$ ($n = 6$). After 10 days of training, rats received a final drug injection to assess performance, with dosage randomly assigned as described above. Doses of DAMGO and naltrexone were chosen based on prior examinations in this laboratory and others that have examined the effects of intra-NAcc opioid receptor manipulation on food intake and motivation [23, 33-39].

2.7 Histology

After conclusion of each experiment, rats were deeply anesthetized with an overdose of sodium pentobarbital and underwent transcardial perfusion with a 0.9% NaCl solution followed by a 10.0% buffered formalin solution. Brains were extracted and stored in a 10% sucrose formalin solution until they were frozen and sliced into 60 μm sections using a cryostat. The brain slices were stained with cresyl violet and the location of the injector tips identified using light microscopy. As can be seen in Figures 1 and 2, injector tips were positioned within the nucleus accumbens core and at the transition zone between the core and the ventral or medial ventral shell. Rats were excluded from analysis if injectors were not bilaterally placed into the nucleus accumbens, if the injector tips extended past the transition zone into the ventral shell, or if necrosis surrounded the injection site. Of 106 animals tested, 88 animals were included in the analysis presented below.

2.8 Data Analysis

For each experiment, the lever press and nosepoke data were analyzed using separate mixed 2-way ANOVAs across the with-drug acquisition phase (Days 1-5) and the subsequent no-drug training days (Days 6+), with drug dose as the between-groups factor, and days as the within-subject factor. If significant effects were found between drug groups or in the drug $\times$ day interaction term, one-way ANOVAs were used to compare differences between drug groups on days of interest. Separate repeated measures ANOVA analyses were performed to compare lever pressing and nosepoking behavior between the final testing day and the performance test. If that test was significant, an additional one-way ANOVA was performed on the performance test day in order to identify significant differences between drug groups. Post hoc pairwise tests utilizing Tukey's HSD were performed, as appropriate.

3. Results

3.1 Experiment 1: A2A receptor stimulation on instrumental learning

3.1.1 Effect of Nacc A2A stimulation on lever pressing—Stimulation of NAcc core A2A receptors with CGS 21680 resulted in a significant drug x day interaction on lever pressing behavior across days over the with-drug acquisition phase ($F(8,68) = 3.814, p = .001$) and a significant main effect of drug treatment ($F(2,17) = 4.409, p = .029$) (see Figure 3A). Follow up ANOVAs and post hoc analyses utilizing Tukey's HSD demonstrated that a significant between group differences occurred on the fourth ($F(2,17) = 4.060, p = .036$) and fifth ($F(2,17) = 8.344, p = .003$) days of training. These differences were driven by lower rates of lever pressing of rats who received microinjections of 24.0 ng/0.5 μ L/side prior to training compared to rats who received vehicle injections day four ($p = .029$) and day five ($p = .003$). Lever pressing during the six subsequent no-drug training days was not affected by previous with-drug training. There was neither a significant main effect of drug ($F(2,21) = 1.722, p = .208$) nor a significant drug x day interaction ($F(10,85) = 1.854, p = .063$).

3.1.2. Effect of NAcc A2A stimulation on nose poking—There was a significant drug x day interaction across treatment groups on nose poking behavior ($F(8, 68) = 2.159, p = .042$) during the with-drug training days, although the main effect of drug did not achieve significance ($F(2,17) = 2.82, p = .088$; see Figure 3A). The interaction effect was driven by significant between group differences on the fourth ($F(2,17) = 3.602, p = .05$) and fifth ($F(2,17) = 5.593, p = .014$) days of training. Further post hoc analyses revealed that animals who received 24.0 ng/0.5 μ L/side prior to training nose poked significantly fewer times than animals who received vehicle injections on both day four ($p = .043$) and day five ($p = .020$), and that on the fifth day animals who received 6.0 ng/0.5 μ L/side also nose poked significantly fewer times than animals who received vehicle injections ($p = .03$). On subsequent no-drug training days, previous acquisition training under drug conditions did not influence nose poking behavior. There were no significant main effects of previous drug group on nose poke behavior ($F(2,17) = 0.019, p = .982$), nor was there a significant drug x day interaction ($F(10,85) = 1.167, p = .324$).

3.1.3 Effect of NAcc A2A stimulation on instrumental performance—

Performance testing was conducted on the 12th day of operant training. Rats were matched by initial drug dose group and re-randomized into new drug dosage groups ($n_{vehicle} = 8$, $n_{6.0\text{ ng}/0.5\mu\text{L}/side} = 6$, $n_{24.0\text{ ng}/0.5\mu\text{L}/side} = 6$). There was a significant effect of treatment day (final no-drug training day vs. performance test, $F(1,17) = 6.089, p = .025$, but no significant main effect of drug treatment on lever pressing, $F(2, 17) = 1.881, p = .183$, compared with baseline performance lever pressing (see Figure 3A). The drug X day interaction effect was also not significant ($F(2,17) = 1.493, p = .253$). As can be seen in Figure 3A, the significant decrease in lever pressing during the performance test was driven by a decline in lever pressing by the two drug treatment groups on the performance day, as the animals that received the vehicle injections did not reduce their lever-pressing as compared to the prior testing day. A similar pattern was observed on nose-poking, with significant effects of both drug ($F(2,17) = 6.910, p = .006$) and day ($F(2,17) = 7.145, p < .016$). Microinfusions of 24.0

ng/0.5µL/side of CGS 21680 significantly decreased nose poking compared to animals who received vehicle microinjections ($p = .002$; Figure 3A).

3.2 Experiment 2: A2A receptor blockade on instrumental learning

3.2.1 Effect of NAcc A2A blockade on lever pressing—Blockade of NAcc core A2A receptors with the A2A antagonist pro-drug MSX-3 caused a significant drug X day interaction over the with-drug training block ($F(8,72) = 3.360, p = .003$) although there was not a significant main effect of drug treatment ($F(2,18) = 1.141, p = .341$; Figure 3B). Follow-up analyses showed that this significant difference was driven by significant between group differences on Day 1 ($F(2,18) = 5.225, p = .016$). Specifically, rats who received vehicle injections pressed significantly less than rats who received 1.0 µg/0.5µL/side of MSX-3 ($p = .012$) on the first day of training. However, this between-group difference disappeared by the second drug training day, after which there were no differences in lever pressing between the drug groups. Prior drug treatment had no effects on lever pressing during the no-drug training days (drug effect: ($F(2,18) = 0.086, p = .918$; day X drug interaction ($F(8,72) = 1.400, p = .221$).

3.2.2 Effect of NAcc A2A blockade on nose poking—Blockade of NAcc A2A receptors during initial training did not significantly affect nose poking behavior on the drug treatment days (see Figure 3B). The number of nose pokes elicited by the rats across did not differ as a function of drug dose, $F(2, 18) = 0.216, p = .808$, nor was there a significant interaction between drug treatment and day of training ($F(8,72) = 0.661, p = .724$). Although there was no main effect of prior drug treatment during the five days of no-drug training ($F(2,18) = .449, p = .645$), there was a significant day X drug interaction effect ($F(8,72) = 2.353, p = .026$). It is unclear what drove this significant interaction, as post-hoc analyses did not reveal a significant effect of treatment group on nose pokes for any individual training day (all p -values $> .10$).

3.2.3 Effect of NAcc A2A blockade on instrumental performance—On the 11th day of training, rats were re-randomized into new drug groups for performance assessment ($n_{vehicle} = 5, n_{1.0 \mu g/0.5 \mu L/side} = 7, n_{3.0 \mu g/0.5 \mu L/side} = 9$). Microinfusions of the adenosine 2A receptor antagonist MSX-3 did not affect performance on the lever pressing task compared to baseline rate of lever pressing. There was no significant main effect of drug, $F(2, 18) = 1.253, p = .309$, nor was there a significant drug x day interaction, $F(2, 18) = 0.786, p = .471$. Additionally, microinjections of MSX-3 did not affect rate of nose poking behavior during the performance assessment; there was no significant effect of drug, $F(2, 18) = .1712, p = .209$ on nose poking, nor was there a significant drug x day interaction, $F(2, 18) = 0.196, p = .824$ (see Figure 3B).

3.3 Experiment 3: Mu-opioid receptor activation on instrumental learning

3.3.1 Effect of µ-opioid receptor activation on lever pressing—Microinjections of the µ-opioid agonist DAMGO into the NAcc significantly and dose-dependently diminished lever pressing behavior over the with-drug acquisition phase and during the subsequent no-drug training phase without affecting lever pressing during the performance assessment phase (see Figure 4A).

During the initial with-drug acquisition of lever pressing, there was a significant between-subjects effect of drug ($F(2, 17) = 23.705, p < .001$) and a significant drug x day interaction ($F(8, 68) = 2.944, p = .007$). One-way ANOVAs on individual training days revealed significant between-group differences in lever pressing on all with-drug acquisition training days. Follow up analyses using Tukey's HSD show that rats who received .025 µg/0.5µL/side and .25 µg/0.5µL/side lever pressed significantly less than rats who received a vehicle injection on the first ($p = .001; p < .001$, respectively) and second ($p = .003; p < .001$, respectively) days of exposure to the lever. On the third day of acquisition there were significant differences between all three groups (Tukey's HSD, $p < .05$), demonstrating that the effect was dose-dependent. On the fourth and fifth days, rats who received the low dose of DAMGO increased their lever pressing to control levels, while rats who received .25 µg/0.5µL/side were still impaired in lever pressing performance compared to rats who received both .025 µg/0.5µL/side ($p = .049$ and $p = .027$ for Day 4 and Day 5 respectively) and vehicle injections ($p = .003, p = .005$ for Day 4 and Day 5 respectively),

Microinjections of DAMGO during the initial acquisition phase also resulted in between-group differences on the subsequent no-drug training days ($F(2,17) = 4.101, p = .035$), though there was no drug X day interaction effect ($F(14, 119) = 0.726, p = .745$). The drug effect appears to have been the result of a general trend for decreased lever pressing throughout the no-drug training period for rats who received .025 µg/0.5µL/side of DAMGO during training. This group significantly differed from the vehicle group on the final day of training ($p = .01$).

3.3.2 Effect of µ-opioid receptor activation on nose poking—Microinjections of DAMGO prior to training on the lever-pressing task also affected the number of nose pokes the rats made into the food chamber during the training session. During the with-drug acquisition phase, there was a significant drug x day interaction on nose poking ($F(8,68) = 2.256, p = .033$), though the main effect of drug did not achieve significance ($F(2,17) = 0.779, p = .475$). The interaction was likely driven by the differential pattern of nose poking between the drug and control group across two of the training days; injections of DAMGO tended to increase the number of nose pokes on the second training day compared to the control group, and this pattern reversed on the third day of training such that the 0.25 µg group nose poked significantly less than the control group ($p = .017$). There were no significant effects of prior drug treatment on nose pokes during the subsequent no-drug training days (drug effect: $F(2,17) = 0.535, p = .595$; day X drug interaction: $F(14, 119) = 0.933, p = .526$).

3.3.3 Effect of µ-opioid receptor activation on instrumental performance—On the 14th day of training, rats were re-randomized to determine new drug groups for the performance assessment day ($n_{\text{vehicle}} = 7, n_{.025 \text{ µg/0.5µL/side}} = 7$, and $n_{.25 \text{ µg/0.5µL/side}} = 6$). There was not a significant effect of drug treatment on either lever pressing or nose poking across the final training day and the performance test (all p values $> .05$; see Figure 4A).

3.4 Experiment 4: Opioid receptor blockade on instrumental learning

3.4.1 Effect of opioid receptor blockade on lever pressing—Microinjections of the opioid antagonist naltrexone into the NAcc prior to training resulted in a significant drug x day interaction for lever pressing over the with-drug acquisition block ($F(8,72) = 7.309$, $p < .001$) and a significant between-subject effect of drug treatment ($F(2,18) = 4.073$, $p = .035$) (see Figure 4B). The differences between drug injection group during the initial acquisition of the lever press occurred during the fourth and fifth days of training ($F(2,18) = 5.578$, $p = .013$; $F(2,18) = 7.396$, $p = .005$, respectively). On those days, rats that received 20.0 $\mu\text{g}/0.5\mu\text{L}/\text{side}$ of naltrexone lever pressed significantly less than rats who received a vehicle injection ($p = .01$ and $p = .003$, respectively). There also was a significant drug x day interaction for no-drug training days ($F(8,72) = 2.453$, $p = .021$; see Figure 4B). This was driven by a significant effect of drug condition on training day 6 ($F(2,18) = 6.139$, $p = .009$). Post hoc analyses revealed that rats who received 20.0 $\mu\text{g}/0.5\mu\text{L}/\text{side}$ pressed significantly fewer times than rats who received a vehicle injection ($p = .008$) on that day.

3.4.2 Effect of opioid receptor blockade on nose poking—Microinjections of naltrexone during the with-drug acquisition days affected the number of nose pokes rats made into the feeding chamber ($F(2,18) = 4.880$, $p = .020$), though there was not a significant interaction between drug and day of training ($F(8,72) = 1.367$, $p = .225$). Significant between group differences emerged on the second day of training ($F(2,18) = 5.006$, $p = .019$), the third day of training ($F(2,18) = 3.877$, $p = .040$), and the fifth day of training ($F(2,18) = 7.295$, $p = .005$). These effects were driven by a significantly lower frequency of nose poking by rats who received 20.0 $\mu\text{g}/0.5\mu\text{L}/\text{side}$ of naltrexone compared to rats who received vehicle injections on those days of training. During the no-drug training phase, there was a significant drug x day interaction for nose pokes ($F(8,72) = 2.206$, $p = .037$) and a significant effect of naltrexone treatment on nose poking ($F(2,18) = 4.530$, $p = .026$). These effects were driven by between group differences on the seventh and eighth days of no-drug training ($F(2,18) = 6.180$, $p = .009$; $F(2,18) = 5.762$, $p = .012$, respectively). On those days, rats who had received 20.0 $\mu\text{g}/0.5\mu\text{L}/\text{side}$ of naltrexone during initial acquisition training nose poked significantly more than rats who received a vehicle injection ($p < .05$ for both days); see Figure 4B), though group differences were no longer significant on or after day 9.

3.4.3 Effect of opioid receptor blockade on instrumental performance—On the 11th day of training, rats within each group were randomized again to determine new dose groups for the performance assessment ($n_{\text{vehicle}} = 6$, $n_{2.0 \mu\text{g}/0.5\mu\text{L}/\text{side}} = 8$, $n_{20.0 \mu\text{g}/0.5\mu\text{L}/\text{side}} = 7$). There was a significant effect of drug treatment on lever pressing on the performance test compared to the baseline day of training ($F(2, 18) = 8.017$, $p = .003$; drug X day interaction: $F(2, 18) = 3.634$, $p = .047$). These effects were driven by a decline in lever pressing following naltrexone injection on the performance test, as microinfusions of 20.0 $\mu\text{g}/0.5\mu\text{L}/\text{side}$ of naltrexone significantly decreased lever pressing compared to 2.0 $\mu\text{g}/0.5\mu\text{L}/\text{side}$ and vehicle injections on the day of the drug test (drug X day interaction: $F(2,18) = 8.017$, $p = .003$; see Figure 4B). Additionally, there was a significant effect of drug treatment on number of nose pokes during the performance test ($F(2, 18) = 4.834$, $p = .021$; drug X day interaction: $F(2, 18) = 0.502$, $p = .614$). Post hoc analyses indicated that rats who received

20.0 µg/0.5µL/side nose-poked significantly fewer times than rats who received 2.0 µg/0.5µL/side ($p = .018$), though neither drug group nose-poked significantly differently from the control group (see Figure 4B).

3.5 A2A receptor antagonism does not recover DAMGO-inhibited acquisition of the lever press

Previous experiments have demonstrated that NAc adenosine has functional interactions with opioid receptor signaling. Blockade of NAc A2A receptors blocks heroin-induced reinstatement of heroin self-administration in rats [40], but also potentiates µ-opioid induced increases in the intake of a high fat diet [17]. Thus, we examined whether or not blockade of A2A receptors alongside activation of µ-opioid receptors would recover the impairment in lever pressing seen after activation of NAc µ-opioid receptors. A separate group of rats ($N = 6$) were trained in the same lever-pressing task, but were administered a cocktail of 0.25 µg DAMGO + 3.0 µg MSX-3/0.5µL/side into the NAcc prior to the with-drug acquisition training sessions. Co-administration of an A2A receptor antagonist and a µ-opioid agonist did not significantly recover lever pressing behavior over the with-drug acquisition block compared to rats who received microinjections of 0.25 µg DAMGO/0.5µL/side alone (see Figure 4A). There was no significant main effect of drug treatment (DAMGO vs. DAMGO + MSX-3; $F(1,11) = 0.541$, $p = .477$) nor was there a significant drug x day interaction ($F(4,44) = 0.399$, $p = .808$). Comparing the two groups to each other during the subsequent no-drug training days, there was a significant drug x day interaction ($F(5,55) = 2.804$, $p = .025$) that resulted from a slower acquisition of lever pressing for the combined drug group on days 6 and 7. The main effect of drug did not achieve significance ($F(1,11) = 4.672$, $p = .054$). Despite these effects on lever pressing, co-administration of MSX-3 and DAMGO did not significantly impact nose-poking behavior compared to DAMGO-only microinjections into the NAc. There were no significant main effects or drug x day interactions for nose-poking across either the with-drug acquisition period ($F(1,11) = 0.008$, $p = .929$; $F(4,44) = .1.224$, $p = .314$, respectively) or the no-drug training period ($F(1,11) = 0.416$, $p = .532$; $F(5,55) = 1.070$, $p = .387$, respectively).

4. Discussion

The purpose of these experiments was to examine the respective roles of the adenosine 2A and µ-opioid receptors on the learning and performance of an instrumental lever-pressing response for sugar reinforcement. Here, we demonstrate for the first time that adenosine 2A receptor activation, µ-opioid receptor activation, and µ-opioid receptor blockade of the NAcc significantly impaired the learning of a lever press for a sugar reward, though the data suggest that the effects of A2A and µ-opioid receptor blockade may be due to general performance deficits (see below). The following sections interpret these effects within the context of observed drug-induced performance deficits following learning (in some cases), as well as what is currently known about NAcc adenosine and opioid receptors in the regulation of learning and motivated behavior.

4.1 The role of nucleus accumbens A2A receptors in instrumental learning

Administration of 24.0 ng/side of the A2A receptor agonist CGS 21680 prior to training significantly impaired lever pressing compared to controls by the fourth day of training, an effect that was continued into the fifth day of the with-drug acquisition period. However, on the first day of no-drug training, these same animals no longer differed from vehicle-injected animals. This suggests that although rats lever pressed fewer times than controls during the with-drug acquisition period, they nonetheless learned the contingencies of the task, as they both lever pressed and nose poked at the same level as the control animals once the drug was removed. Furthermore, after learning had been established, microinfusions of the A2A receptor agonist CGS 21680 decreased both lever pressing and nosepoking. Together, these data suggest that the effects of A2A receptor stimulation were likely due to effects on some parameter of performance, rather than a specific effect on learning *per se*. Although this is the first study to examine the specific role for the A2A receptor within the NAcc on learning, these results are consistent with systemic treatments of adenosine receptor agonists on operant responding in well-trained rats [41].

Two possible causes for the impairment in learning and performance observed here include drug-induced locomotor deficits or shifts in effort- and/or arousal-related mechanisms following A2A receptor stimulation of the NAcc. Systemic and i.c.v. injections of adenosine agonists have been shown to cause behavioral sedation or locomotor inhibition in food-restricted rats [16, 32, 42, 43]. Prior studies in which CGS 21680 has been injected into the NAcc have reported reductions in ambulatory activity in mice and rats following drug treatment at doses higher than that used here [17, 44]. NAcc A2A receptor stimulation has been shown to shift behavior from lever pressing for a preferred food to the consumption of a freely available but less preferred food without causing overt sedation or drowsiness [16] at doses of CGS 21680 identical to those used in the present study. Although we did not directly measure the effects of A2A receptor agonism on locomotor activity, rats that received the highest dose of drug during the performance test still pressed and nosepoked at a high rate ($M = 187$ and $M = 167$ times, respectively), suggesting that locomotor activity was not blocked to the extent of disabling exploratory behavior and operant responding.

Prior research has demonstrated that intra-NAcc infusions of A2A receptor agonists decreases the amount of effort rats will exert in order to access a reward [16, 32], which suggests that adenosine 2A receptors in the NAcc regulate effort-related motivation and behavior. Adenosine 2A receptors frequently form heterodimers with dopamine D2 receptors [45-47], and similar shifts in behavior from lever-pressing to free food seeking have also been demonstrated with intra-accumbens infusion of the D2 antagonist haloperidol [48]. The learning impairment demonstrated in the current study may thus have been the consequence of A2A receptor blockade depressing effort-related processes through its regulation of dopamine function in the NAcc.

In contrast to the effects of NAcc receptor stimulation on instrumental learning and performance, rats with A2A receptor antagonism showed initial levels of lever pressing that were higher than that of its control group; animals that received 1.0 μ g of MSX-3 lever pressed more than the vehicle injected rats on the first day of acquisition. However, there is

reason to be skeptical of this apparent learning “enhancement,” as the control group in this task lever pressed fewer times on the first day of learning than any of the other control groups in these experiments ($M = 10.4$, as compared to 42.0, 58.8, and 25.7, for experiments 1, 3, and 4 respectively). Furthermore, on the second day of training all groups were lever pressing equivalently and there were no effects of drug on nose-poking. Thus, although a significant drug $\times$ time interaction was found on lever pressing throughout training, it is unlikely that this represents a meaningful enhancement of learning following the blockade of striatal A2A receptors.

Although blockade of A2A receptors within the ventral striatum in Experiment 4 did not affect learning or performance of this appetitive instrumental learning task, other studies have demonstrated memory preserving effects of systemic adenosine receptor antagonism. For example, systemic adenosine receptor blockade has demonstrated preservation of learning and memory functions which normally show an age-related decline [49] or which were impaired through sleep deprivation [50]. It is possible that antagonism of adenosine receptors preserves functions associated with long term memory retrieval (particularly under conditions that promote memory impairment), rather than enhances mechanisms of memory formation and consolidation. As these studies were not designed to examine the mechanism by which adenosine receptor blockade may enhance or rescue memory under these conditions, one avenue for future research may be to determine if the NAcc is involved in these known effects.

Thus, activation, but not blockade, of adenosine 2A receptors within the ventral striatum resulted in impairments in performance during the learning of an instrumental learning task. This impairment was most likely through mechanisms associated with effort-related motivation and/or arousal, rather than a selective deficit of learning-related plasticity within the NAcc.

4.2 The role of nucleus accumbens μ -opioid receptors in instrumental learning

Mu-opioid receptor activation decreased lever pressing across the with-drug acquisition period in a dose-dependent manner. However, no deficits were observed during the performance test, suggesting that the DAMGO impaired some aspect of learning processes over the first five with-drug acquisition days, without affecting general locomotor or motivational behaviors that might otherwise impair lever pressing. Interestingly, concurrent nose-poking behavior across the with-drug acquisition period was largely preserved between drug doses across the days of training. This is notable; in experiments utilizing similar or identical experimental procedures, instrumental learning deficits have been consistently paired with simultaneous decreases in nose-pokes following injections of an NMDA receptor antagonist into the NAcc core [2], co-infusions of low doses of an NMDA/D1 receptor antagonists [51], or injections of an muscarinic acetylcholine receptor antagonist into the NAcc [28]. Such decreases were also observed in the current experiments following A2A receptor stimulation or opioid receptor blockade. However, microinjections of DAMGO largely preserved nose-poking behavior, despite the fact that rats did not receive as many reinforcers as the control rats due to fewer lever presses. This deviant pattern may give insight into the DAMGO-induced impairment of instrumental learning. Consistent with their

known roles in promoting food intake [36, 52-54], stimulation of NAcc μ -opioid receptors prior to operant training may have shifted actions towards consummatory behavior rather than appetitive goal-seeking behavior (such as exploration and the resultant lever press). Within the present learning paradigm, it is possible that the random time interval schedule of pellet delivery that was maintained on the first two days of the learning phase of the experiment (to ensure exploration in the operant chamber) may have interacted with the drug to shift behavior away from the lever and towards the food/food magazine. Under the influence of DAMGO, exposure to the sugar pellet reinforcer outside of the context of the lever press may have preferentially shifted the behavior of the rats away from behaviors associated with actively seeking the reinforcer (such as lever pressing) and toward behaviors directly associated with consumption of the sugar pellet (such as nose-poking). The removal of the random time component of the reinforcement schedule on the third day of with-drug acquisition training coincided with a decline in nose-poking behavior across all groups, and most particularly for those rats that received 0.25 μ g/side of DAMGO (which resulted in a significant drug X day interaction effect; see Figure 3A). Thus, the learning impairment observed may have been due to a shift in the food-seeking strategy of the animals under the influence of DAMGO. That DAMGO did not subsequently impact performance may reflect the fact that during the performance test, rats were already aware of the environmental contingencies that would earn them food, and no free pellets were given to tempt them to shift away from the lever.

In contrast to the effects of μ -opioid receptor stimulation, blockade of NAcc opioid receptors with 20.0 μ g/side of naltrexone (Experiment 4) significantly decreased *both* lever pressing and nose-poking behavior over the with-drug acquisition period. Lever-pressing was also inhibited on the performance test at the highest dose given. Additionally, although it was not quantified, rats receiving the 20 μ g dose of naltrexone often failed to eat all of the sugar pellets that had been earned during their operant session, despite having performed the lever pressing task and approaching the food hopper. Such a decrease is consistent with lower rates of palatable food intake shown in other paradigms following NAcc opioid receptor blockade [35]. It has been proposed that activity of opioid receptors within the ventral striatum modulate the positive affect elicited by consumption of palatable foods, while blockade of opioid signaling decreases the rewarding pleasure associated with a stimulus [52, 55]. The pattern of reduced lever pressing observed across training and during the performance test in these experiments may be secondary to a decline in the motivational salience of the sugar pellet during both learning and the final drug test.

In conclusion, our data suggest that both μ -opioid receptor activation and blockade within the NAcc decrease lever pressing behavior during the learning of an instrumental task, though for different reasons, as indicated by different patterns of nose-poking and the presence of a performance deficit in lever pressing following opioid receptor blockade. Opioid receptor antagonism may have caused this impairment by altering the hedonic value of the reinforcer, while μ -opioid receptor activation may have altered motivational and attentional resources away from the target lever in favor of approaching the food magazine.

4.3 Examination of a functional interaction between μ -opioid and A2A receptor systems in instrumental learning

In these experiments, we demonstrated that independent manipulation of the adenosine and opioid receptor systems both impaired acquisition of an instrumental task for a sugar reward. It is of interest then that interactions between the adenosine and opioid signaling system have been demonstrated in both drug addiction models [40] and food intake paradigms [17]. Striatal adenosine receptor antagonism reduces the impairment in neural firing rates induced by both adenosine and morphine exposure [56]. Thus, we examined if A2A receptor blockade could rescue the impairment in lever pressing seen after μ -opioid receptor activation during the with-drug acquisition period by co-administering an A2A receptor antagonist prodrug with a μ -opioid receptor agonist into the NAcc prior to behavioral testing. However, we did not find a significant difference in either lever pressing or nose-poking behavior in rats who received both drugs compared to rats who only received microinjections of a μ -opioid agonist during the drug training days. Although this examination of striatal opioid-adenosine was limited in scope and it is possible that such interactive effects may not be seen within the short timeframe of this learning procedure, these data do not support a role for A2A receptor blockade in mediating the learning impairment that follows NAcc μ -opioid receptor activation.

4.4 Concluding Remarks

The NAcc has been recognized for its role in the translation of “motivation to action” since Mogenson's seminal work in 1980 [57]. Since that time, the roles of multiple neurotransmitter systems within the NAcc have been characterized, demonstrating important roles for glutamate [58-60], GABA [61-63], dopamine [64], opioids [36, 52-54], acetylcholine [28, 65-67], and serotonin [68-71] receptors on appetitive and consummatory behaviors directed towards food. Though both adenosine and opioid systems of the NAcc have previously been shown to impact food intake, this is the first examination of the potential roles for A2A and μ -opioid receptors of this region on the learning of an operant response reinforced by food. Manipulations of both receptor types affected learning, albeit with behavioral patterns suggestive of different motivational mechanisms. NAcc adenosine 2A receptor activation impaired learning and instrumental performance in a manner consistent with prior demonstration of its role in effort-directed behavior [16, 30, 32], and may influence learning through effects on dopamine signaling within the ventral striatum. Both stimulation and blockade of NAcc μ -opioid receptors impaired learning, in a manner consistent with their potential role in driving consummatory processes. Stimulation may have shifted rats towards the procurement of food at the hopper (and away from the lever), while opioid receptor blockade with naltrexone appears to have reduced the perceived value of the food reinforcer itself (as evidenced by a reduction in the consumption of the earned food pellets). These experiments add to the growing literature aimed at understanding the role of individual neurotransmitter systems and their potential interactions within the NAcc core in regulating the initial learning and subsequent performance of food-motivated behavior.

Acknowledgments

These experiments were supported by the Wake Forest University Department of Psychology and by a grant from the National Institute of Drug Abuse (R15 DA030618). We would like to thank Peagan Lin for her technical assistance and support during this project.

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Research Highlights

- Nucleus accumbens adenosine and μ -opioid receptors impact motivational processes
- We examined A2A and μ -opioid receptor manipulations on instrumental learning
- Nucleus accumbens A2A receptor stimulation blocked instrumental performance
- Nucleus accumbens μ -opioid receptor stimulation impaired learning of lever-pressing
- Mu-opioid receptor blockade suppressed both learning and performance of the task

Experiment 1:
CGS 21680

Experiment 2:
MSX-3

Figure 1.

Representative photomicrographs and histological placements of microinjector tips within the NAcc for Experiments 1 (left panel) and Experiment 2 (right panel). Numbers at the top right of each section represent millimeters anterior to bregma. Open circles represent vehicle dose injection sites, gray circles represent injection sites for rats who received 6.0 ng/0.5µL/side of CGS 21680 (Experiment 1, left) or 1.0 µg/0.5µL/side of MSX-3 (Experiment 2; right), and black circles represent injection sites for rats who received 24.0 ng/0.5µL/side of CGS 21680 (Experiment 1, left) or 3.0 µg/0.5µL/side of MSX-3 (Experiment 2; right). This

figure represents placements/doses for the drug training phase; the same rats were re-randomized into drug groups during performance testing.

**Experiment 3:
DAMGO**

**Experiment 4:
Naltrexone**

Figure 2.

Representative photomicrographs and histological placements of microinjector tips within the NAcc for Experiment 3 (left panel) and Experiment 4 (right panel). Numbers at the top right of each section represent millimeters anterior to bregma. Open circles represent vehicle dose injection sites, gray circles represent injection sites for rats who received 0.025 µg/0.5µL side of DAMGO (Experiment 3, left) or 2.0 µg/0.5µL/side of naltrexone (Experiment 4; right), and black circles represent injection sites for rats who received 0.25 µg/0.5µL of DAMGO (Experiment 3, left) or 20.0 µg/0.5µL/side of naltrexone (Experiment 4; right).

A. Effects of A2A receptor agonist CGS 21680 on learning and performance.

B. Effects of A2A receptor antagonist MSX-3 on learning and performance.

Figure 3.

Effects of adenosine receptor stimulation or blockade on the learning and performance of an instrumental learning task. Stimulation of NAcc A2A receptors with 24.0 ng/0.5µL/side of CGS 21680 inhibited the acquisition of lever press and nose poking behavior across the first five days of instrumental learning. Furthermore, drug treatment tended to reduce both lever pressing (A, left panel) and nose pokes (A, right panel) during the performance test (PT), which was performed after the rats had learned the task. In a separate group of animals, injections of the A2A antagonist prodrug MSX-3 significantly increased lever pressing on the first acquisition day (compared to control); thereafter no further effect of MSX-3 injections were seen for either lever pressing or nose poke behavior (B). FTD = Final training day; * $p < .05$, ** $p < .01$ for main drug effects; single and double crosses demark $p < .05$ and $p < .01$ for drug x time interaction effect, respectively. Stars above or below the data for a given day on the line graph denote significant drug group differences from the vehicle control group on that day, as assessed by Tukey's HSD ($p < .05$).

A. Effects of the μ -opioid receptor agonist DAMGO on learning and performance.

B. Effects of opioid receptor antagonist naltrexone on learning and performance.

Figure 4.

Effects of μ -opioid receptor stimulation or blockade on the learning and performance of an instrumental learning task. Activation of NAcc μ -opioid receptors with DAMGO decreased lever pressing (Figure 4A) during learning. Rats who had received 0.025 $\mu\text{g}/0.5\mu\text{L}$ also lever pressed significantly fewer times than controls on Day 13, even after eight days of drug-free training. Mu-opioid receptor stimulation caused a significant drug X day interaction effect on nosepeking (see text). Following learning of the response, there was no effect of DAMGO on either lever pressing (A) or nosepeking (B) during the final performance assessment. Furthermore, blockade of NAcc opioid receptors with naltrexone decreased both lever pressing (A) and nosepeking (B) during instrumental learning and during task performance. Statistical symbols as for Figure 3.