Nucleus accumbens modulation of reinstatement

RUNNING HEAD: Nucleus accumbens modulation of reinstatement

Nucleus accumbens dopamine and mu-opioid receptors modulate the reinstatement of food-seeking behavior by food-associated cues

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Abstract

The high attrition rates for dietary interventions aimed at promoting a healthier body mass may be caused, at least in part, by constant exposure to environmental stimuli that are associated with palatable foods. In both humans and animals, conditioned stimuli (CSs) that signal reward availability reliably reinstate food- and drug-seeking behaviors. The nucleus accumbens (NAcc) is critically involved in the cue-evoked reinstatement of food-seeking, but the role of individual neurotransmitter systems within the NAcc remains to be determined. These experiments tested the effects of intra-accumbal pharmacological manipulations of dopamine (DA) D$_1$ and D$_2$ receptors, mu-opioid receptors, or serotonin (5-HT) receptors on cue-evoked relapse to food-seeking. Rats were trained to lever press for sucrose pellets and the concurrent presentation of a light-tone CS. Once training was complete, lever-pressing was extinguished in the absence of either sucrose or CS presentation. Once each rat had reached extinction criterion, they received two reinstatement sessions in which lever pressing was renewed by response-contingent presentation of the CS. Prior to each reinstatement test, rats received NAcc microinfusions of saline or the selective D$_1$ receptor antagonist SCH 23390, the D$_2$ receptor antagonist raclopride, the mu-opioid receptor agonist [D-Ala$_2$, N-MePhe$_4$, Gly-$\beta$-ol]-enkephalin (DAMGO), or 5-HT hydrogen maleate. Compared to saline test days, intra-accumbens infusions of SCH 23390 (1 μg/0.5 μL), raclopride (1 μg/0.5 μL), or DAMGO (0.25 μg/0.5 μL) effectively blocked the cue-evoked reinstatement of food-seeking. In contrast, stimulation of serotonin (5-HT) receptors by 5-HT hydrogen maleate (5 μg/0.5 μL) had no effect on cue-induced reinstatement. These novel data support roles for NAcc DA D$_1$, D$_2$, and mu-opioid receptors in the cue-evoked reinstatement of food seeking.

Keywords: Nucleus accumbens, motivation, reinstatement, dopamine, opioids, serotonin.
1. Introduction

One of the most salient issues in the present health media is the rising rate of obesity. Currently, it is estimated that at least 34% of adults in the United States are obese (i.e., BMI≥30). This rate is disconcerting due to the fact that obesity is linked to a plethora of health problems, including cardiovascular disease, type II diabetes, sleep apnea, and infertility [1]. Unfortunately, dietary changes aimed at a lower and healthier body mass are notoriously difficult to maintain. Many weight loss interventions fail to produce body weight reductions that last longer than 4 to 5 years [2]. For instance, only 2% of women in a longitudinal study were able to maintain weight loss achieved during young adulthood (i.e., age 18 to 30) until the age of 50 [3]. One potential factor influencing the failure to maintain weight loss may be high rates of dietary relapse. Considering the consistent bombardment of food-associated cues in modern society (e.g., food commercials, fast-food signs, etc.), dietary maintenance may be undermined by exposure to a plethora of stimuli associated with palatable food intake.

Numerous human and animal studies have indicated that cues associated with reward availability have incentive properties and can effectively reinstate food and drug-seeking behaviors [4-9]. Reward-associated conditioned stimuli (CS)\(^1\) can activate mnemonic circuitry and evoke motivated approach behaviors based upon the learned incentive value of the previously experienced reward [10-12]. Evidence in support of this contention is present in studies of both drug and food-related cravings. Human neuroimaging studies have demonstrated that cocaine users have enhanced glucose metabolism in memory circuits (e.g., prefrontal cortices, temporal lobe) related to self-reports of cocaine craving following exposure to cocaine

\(^1\) Abbreviations: Conditioned stimulus (CS); Nucleus accumbens (NAcc); Dopamine (DA); Serotonin (5-HT); [D-Ala\(^2\), N-MePhe\(^4\), Gly-ol]-enkephalin (DAMGO); Variable Ratio (VR); Fixed-Interval (FI); Pavlovian-Instrumental Transfer (PIT).
cues versus neutral cues [13]. Based upon this and other evidence, several theories of drug addiction emphasize a role for learned incentives in drug craving [11, 14]. Along similar lines, chronic dieters show an enhanced desire to seek out and consume specifically those foods associated with a particular CS following its presentation (e.g., enhanced desire to specifically consume pizza when exposed to the smell of freshly baked pizza), while craving for non-cued foods (e.g., fresh-baked cookies) is unaltered. This evidence indicates that food-associated cues do not merely increase the motivation to consume any available food, and that incentive learning is critically involved in food craving [15]. Given the powerful role for conditioned stimuli in directing appetitive behaviors, it is of interest in the midst of the present obesity epidemic to determine the neural substrates that are involved in driving the relapse to food-seeking behavior by associated conditioned stimuli.

Prior research has firmly established a role for mesolimbic reward circuitry, including the nucleus accumbens (NAcc), in the reinstatement of both food and drug-seeking behaviors [5, 6, 16]. Anatomically, the NAcc is well-positioned to transfer relevant cued reward associations into motivated behavioral output. Neural structures involved in encoding and retrieving the affective mnemonic trace of a CS, such as the hippocampus, prefrontal cortex, and amygdala, all have converging inputs to the NAcc [17, 18]. In turn, the NAcc projects to motor output structures in the basal ganglia, such as the ventral pallidum [19]. Recent evidence suggests that the NAcc plays a critical role in directing behavior based upon the learned incentive value of environmental cues. For instance, pharmacological inactivation of the NAcc disrupts lever pressing behavior in the presence of discriminant stimuli associated with natural reinforcer availability [9]. Furthermore, a recent study by Floresco, McLaughlin, and Haluk [5] demonstrated that inactivation of the NAcc core attenuated the reinstatement of lever-pressing in
extinguished rats when the lever presses resulted in the presentation of cues that had previously been associated with food reinforcement. In contrast, similar inactivation of the NAcc shell invigorated instrumental responding in the reinstatement condition.

This evidence strongly suggest that the NAcc is critical for directing the seeking of natural rewards in the presence of discrete cues that predict reinforcer availability. However, it remains to be determined which neurotransmitter systems within NAcc are specifically involved in the cue-evoked reinstatement of food-seeking. Multiple neurotransmitters within the ventral striatum are known to modulate the appetitive and consummatory phases of food and drug-motivated behaviors. For instance, antagonism of NAcc D₁ or D₂ receptors reliably attenuates drug-seeking [16, 20] and reduces the effort that rats will expend to obtain a preferred food [9, 21]. Also, rodent models of motivated behaviors indicate that μ-opioid receptor stimulation within the NAcc robustly increases the intake of preferred diets [22, 23] and is involved in precipitating cocaine and alcohol craving and relapse [24, 25]. Drugs that impact serotonergic function affect food intake when given systemically, and recent reports have shown that individual serotonin receptors within the NAcc modulate the intake of standard rat chow under deprived conditions, as well as food intake in response to palatable diet presentation in sated rats [26, 27]. Thus, any or all of these neurotransmitters within the NAcc may play a role in modulating the reinstatement of food-seeking behavior in the presence of food-associated CSs.

In these experiments, we examined the effects of manipulating NAcc dopaminergic receptors, mu-opioid receptors, or serotonin receptors on cue-induced reinstatement of food seeking behavior. Individual groups of food restricted rats were trained across seven days to lever press for sucrose pellets, which were delivered concurrently with the presentation of a light-tone stimulus. Once training was complete, rats received daily extinction sessions, during
which lever presses resulted in neither sucrose delivery nor the light/tone CS. Once lever-pressing behavior had lowered to extinction criterion, rats were given two reinstatement sessions, during which lever pressing elicited the presentation of the light/tone CS. Across the two reinstatement sessions, individual groups of rats received NAcc injections of either saline or the D₁ receptor antagonist SCH23390 (Experiment 1), the D₂ receptor antagonist raclopride (Experiment 2), the mu-opioid receptor agonist DAMGO (Experiment 3), or the serotonin receptor agonist 5-HT hydrogen maleate (Experiment 4). The effects of each drug treatment on cue-induced reinstatement of lever-pressing behavior were then assessed.

2. Material and Method

2.1. Subjects and Housing

Male Sprague-Dawley rats (Harlan, Madison, WI) were dually housed in transparent polycarbonate cages with wire covers in a temperature and light controlled vivarium (21 °C, 12-hr light-dark cycle, lights on/off- 7am/7pm). In order to minimize stress, animals were handled daily upon arrival. Behavioral testing was conducted during the light phase. All experiments were conducted in accordance with NIH animal care guidelines and were approved by the Wake Forest University Animal Care and Use Committee.

2.2. Surgery

After the animals for each experiment were acclimated to the housing environment for a period of one week, rats underwent cannula placement surgery. The rats were anesthetized with a Ketamine-Xylazine cocktail (90 mg/kg-9 mg/kg) and standard aseptic surgical procedures were followed to implant stainless steel indwelling guide cannulas (23 gauge) bilaterally above the medial NAcc, near the transitional zone between the shell and core (flat skull surgery, A-P: 1.7 mm anterior to bregma, M-L: ± 1.4 mm, D-V: -7.5 mm from the top of skull). The cannulas
were affixed to the skull with self-curing dental acrylic adhered to three stainless steel jeweler’s screws. Wire stylets were placed in the guide cannulas to prevent occlusion.

2.3. Apparatus

Four standard operant chambers (Med-Associates, St. Albans, VT, USA) were utilized for the present experiments. Each was enclosed in a sound-attenuating chamber equipped with ventilation fans. The chambers were fitted with a house light and two retractable levers on each side of the central food receptacle. Two identical 100-mA stimulus lights were located just above each lever. A programmable speaker, positioned on the wall opposite to the food receptacle, presented auditory stimuli.

2.4. Cue-evoked reinstatement of food-seeking paradigm

Rats were allowed to recover from surgery for a period of one week before they were gradually food restricted until reaching approximately 90% of their ad libitum body weight. Two days prior to the beginning of magazine training, rats were habituated to the sucrose pellets (45 mg; BioServ) by supplementing their daily food ration with 2 g of the pellets.

The following reinstatement procedure was adapted from the experimental protocol utilized by Floresco et al. [5]. All experimental procedures were conducted during the beginning of the light phase. Magazine training consisted of two days (Days 1 and 2) of 30 minute random time 60s training sessions in which sucrose pellet delivery was presented at an average of 60s between deliveries, in the absence of any levers or stimulus presentations. The day following the magazine training period (i.e., Day 3), both levers were inserted into the chambers for operant training and rats received 20 min training sessions where lever presses on the active lever resulted in the presentation of a combined CS (light/tone)-US (sucrose pellet). An inactive lever with no programmed consequences was presented on the opposite side of the food receptacle.
Left/right positioning of the active lever was counterbalanced across animals in all experimental groups. Active lever presses on the first operant training day were reinforced on a fixed-ratio-1 schedule in which each lever press resulted in the presentation of a 5s light-tone cue (the light above the stimulus lever was illuminated, in conjunction with the presentation of a 80 dB, 3 kHz tone). Exactly 0.5s following the onset of the CS cue, a sucrose pellet primary reinforcer was delivered to the magazine. On the fourth day of training, rats were further acclimated to the procedure on a fixed-ratio 2 schedule in which every two lever presses resulted in the same cue-reward presentation. Active lever presses during the cue presentation were recorded, but not reinforced nor counted toward ratio requirements. Days 5-9 consisted of a variable-ratio (VR) 5 reinforcement schedule superimposed upon a fixed-interval (FI) 20 schedule of reinforcement. This VR-5, FI-20 schedule resulted in the first CS-pellet delivery on an average of 5 active lever presses. After this initial reinforcer was earned, a 20s time out period initiated in which lever presses resulted in no consequences. Following this rest interval, the VR5 schedule resumed until another reinforcer was earned and the schedule repeated. Consistent with prior reports [5], this procedure led to reliably robust responding on the active lever by the end of the 7 day operant training period.

Following the last day of VR-5 training, rats underwent daily 20-min extinction sessions in which lever presses resulted in no programmed consequences. A rat was considered to have reached extinction criterion (and therefore ready for reinstatement sessions) when they had lever pressed less than 10% compared to the last day of VR-5 training on the previously active lever. Rats typically took 3-5 days to reach this extinction criterion. One day after each rat’s active lever pressing behavior was extinguished, they were subjected to the first of two, 20-min reinstatement sessions separated by a period of 24 hrs. This consisted of the renewed
presentation of the light-tone CS following the first lever press on the previously active lever. Further responding on the previously active lever resulted in the CS presentation on a VR-5 schedule. There were no time out periods for CS availability on these test days. Additionally, no sucrose was delivered following the light-tone presentations for the purpose of specifically assessing cue-evoked motivation to lever press, as opposed to motivation activated by availability of the reward itself. The number of lever presses on both active and inactive levers was recorded for subsequent analysis.

2.5. **Cue-induced reinstatement microinfusion procedures.**

These experiments were conducted with four separate cohorts of animals, each of which was tested with a single drug treatment. Immediately prior to the two reinstatement sessions, rats received an intra-accumbens infusion of drug or the vehicle solution (sterile saline), the order of which was counterbalanced between rats across the two test days. To examine the potential role of dopamine (DA) D₁ receptors on cue-induced reinstatement, rats in Experiment 1 (n = 9) received injections of the vehicle or the D₁ antagonist SCH 23390 (Tocris; 1 μg/0.5 μL/side). Rats in Experiment 2 (n = 13) were tested following intracranial injections of saline or the D₂ receptor antagonist raclopride (Tocris; 1 μg/0.5 μL/side). Subjects in Experiment 3 (n = 10) received counterbalanced infusions of saline or the μ-opioid agonist [D-Ala², N-MePhe⁴, Gly-ol]-enkephalin (DAMGO) (Sigma; 0.25 μg/0.5 μL/side) into the NAcc. Examination of the possible role of serotonin was done in Experiment 4 (n = 7), and rats received intra-accumbens saline or 5-HT hydrogen maleate (Sigma; 10 μg/0.5 μL/side) across reinstatement test days. All drugs were dissolved in sterile saline and pH-balanced to the vehicle solution when necessary. Injections were given through 12.5-mm, 30-gauge injector cannulae connected with polyethylene tubing to a microdrive pump (Harvard Apparatus, South Natick, MA). Solutions were injected
in a volume of 0.5 μL at a rate of 0.32 μL per minute, followed by a one minute rest period in which injectors were left in place to allow drug to diffuse away from the cannula. As described previously [27], rats received two days of habituation to microinjection procedures during extinction training, at a disparate time of day to ensure that the procedures were not predictive of reward unavailability.

Previous studies have determined that the drugs and drug doses are effective in modulating reward-motivated behaviors when injected into the NAcc [20, 23, 28]. Dopaminergic D1 and D2 receptors were blocked with SCH 23390 and raclopride, respectively, to assess the role of both DA receptor subtypes and to allow direct comparison with prior reports examining the effects of these agents on conditioned reinforcement and cue-induced relapse to drug-seeking [20, 21, 29-31]. To examine the possible role of mu-opioid and 5-HT receptors in cue-induced reinstatement, DAMGO and 5-HT hydrogen maleate were administered for Experiments 3 & 4 at doses previously shown to affect food intake or conditioned reinforcement, respectively [22, 28, 32, 33].

2.6. Data Analysis

Responding on the sucrose paired lever was compared across the final day of extinction training and both reinstatement sessions. Active lever responding during each of the 20 minute sessions was divided into five 4-min epochs so that patterns of active lever responding over time within each session could be examined. Reward-paired lever responses were analyzed for each experiment using a two-way repeated measures ANOVA with treatment day (i.e., last day of extinction, drug Reinstatement-drug treatment day, or Reinstatement-vehicle day) and time (i.e., 4 min epochs) as the within-subjects factors. To ensure any observed drug effects were not due to non-specific alterations of operant activity, the proportion of inactive lever presses relative to
total lever presses was also compared between drug treatment versus saline reinstatement sessions using paired t-tests for each experiment. Total inactive lever presses were also analyzed with repeated-measures ANOVAs comparing responses across the final extinction day and the two reinstatement sessions. Post-hoc pairwise comparisons utilized Tukey’s HSD procedures, as appropriate.

2.7. Histology

Once experiments were completed, rats were anesthetized with sodium pentobarbital and perfused intracardially with a 0.9% buffered NaCl solution followed by a 10.0% formalin solution. The fixed brains were extracted and placed in a 10.0% sucrose formalin solution overnight. Brains were then mounted, frozen in a cryostat, and sliced into 60-μm sections. Nissl bodies were stained with cresyl violet and cannula tip locations were determined using light microscopy and charted according to Paxinos and Watson [34]. Data from five subjects determined to have cannula placements outside the region of interest were excluded from analyses.

3. Results

3.1. Experiment 1: The Effects of D₁ Receptor Antagonism on the Cue-Evoked Reinstatement of Food-Seeking

Reinstatement was operationally defined as an enhancement of lever presses on the previously active lever compared to the last day of extinction training. Saline reinstatement days were those trials in which rats received intra-accumbens infusions of the saline vehicle prior to reinstatement testing. Reinstatement-drug treatment days were defined as those trials in which the ligand of interest for each experiment was infused into the NAcc prior to cue-evoked
reinstatement tests. The order of the reinstatement trials (i.e., vehicle versus drug) was counterbalanced across subjects.

In the first experiment, inhibition of D₁ receptors with SCH 23390 effectively suppressed the reinstatement of food-seeking in the presence of the CS (see Figure 2A). A within-subjects ANOVA indicated a significant effect of day (i.e., last extinction day, saline reinstatement day, or SCH 23390 reinstatement day; \( F(2, 16) = 6.54, p = .008 \)) on overall active lever pressing behavior. Post-hoc analyses utilizing a Tukey’s HSD procedure indicated that the significant effect was due to an effective reinstatement of food-seeking behavior by the CS on the saline reinstatement day when compared to the final day of extinction training \((p < .05)\). In contrast, active lever pressing behavior following intra-accumbens infusions of the 1 μg/0.5 μL dose of the D₁ receptor antagonist SCH 23390 did not differ from the final day of extinction. There was no significant day (last extinction day, reinstatement-vehicle day, or reinstatement-drug day) x time (4 min epochs) interaction on active lever pressing behavior, \( F(8, 64) = .55, p = .82 \).

The differences in reinstatement following intra-accumbens drug or saline infusions were not likely due to a failure to discern the active lever in reinstatement sessions following infusions of SCH 23390. Following both manipulations, rats similarly focused their lever-pressing behavior on the previously active lever, as indicated by the similar low average proportions of inactive lever presses to overall lever presses for both drug \((M = .08, SD = .08)\) and saline \((M = .10, SD = .11)\) reinstatement sessions, \( t(8) = .58, p = .58 \). However, inhibition of D₁ receptors of the NAcc reduced lever pressing on the inactive lever relative to both the final extinction day and the saline reinstatement day \([F(2,16) = 10.8, p = .001]\).

3.2. Experiment 2: The Effects of D₂ Receptor Antagonism on the Cue-Evoked Relapse to Food-Seeking
Within-subjects ANOVA analyses indicated a significant main effect of day on the reinstatement of active lever-pressing behavior, $F(2, 24) = 4.12, p = .03$. Post-hoc analyses indicated that this effect was largely driven by the significant potentiation of reward-directed operant behavior in the presence of the CS on the saline reinstatement test day compared to the last day of extinction training ($p < .05$). In contrast, $D_2$ receptor antagonism blunted cue-evoked reinstatement (see Figure 2B). Active lever pressing in the reinstatement session following intra-accumbal raclopride infusions did not significantly differ from the last day of extinction ($p > .05$). The test for a day x time interaction did not reach significance criterion, $F(8, 96) = 1.37, p = .22$.

$D_2$ receptor antagonism also reduced lever pressing on the inactive lever, as compared to extinction and the saline reinstatement days [$F(2,24) = 6.44, p = .006$]. However, as in the first experiment, the raclopride manipulation did not appear to suppress food-seeking due to a failure to retrieve the appropriate lever-reward association. Rats focused their lever pressing behavior largely toward the active lever following both saline and raclopride infusions into the NAcc ($M = .12$, $SD = .08$ and $M = .14$, $SD = .14$, respectively) and the proportion of inactive lever presses to overall lever presses did not significantly differ between these manipulations, $t(12) = .75, p = .47$.

### 3.3. Experiment 3: The Effects of mu-opioid Receptor Agonism on the Cue-Evoked Reinstatement of Food-Seeking

A within-subjects ANOVA indicated a significant main effect of day on overall active lever pressing behavior, $F(2, 18) = 5.67, p = .012$. In addition, there was a significant day x time interaction, $F(8, 72) = 4.50, p < .001$. As with the previous two experiments, the CS elicited reinstatement of active lever responding on the saline infusion test day ($p < 0.05$; see Figure 3A).
However, there were no significant differences in active lever pressing between the last day of extinction testing and the DAMGO reinstatement day \( (p > .05) \).

The observed suppression of the reinstatement of active lever pressing by the reward-paired CS on the DAMGO reinstatement day did not appear to be due to a failure to retrieve the associative memory linking the active lever and sucrose pellet delivery. As indicated by the low average proportions of inactive lever presses to overall lever presses, rats primarily responded on the lever associated with sucrose pellet delivery during both saline \( (M = .08, SD = .04) \) and DAMGO \( (M = .22, SD = .41) \) reinstatement sessions, and these proportions did not significantly differ, \( t(9) = 1.14, p = .28 \). Neither did mu-opioid receptor stimulation have an effect on the total number of inactive lever presses \[ F(2,18) = 1.30, p = .30 \].

### 3.4. Experiment 4: The Effects of Intra-accumbens 5-HT infusions on the Cue-Evoked Reinstatement of Food-Seeking

Stimulation of NAcc 5-HT receptors with a 10 μg/0.5 μL infusion of 5-HT hydrogen maleate in the NAcc prior to reinstatement testing did not affect the cue-evoked relapse to food-seeking for subjects in experiment 4 (see Figure 3B). A within-subjects ANOVA indicated the absence of a significant main effect of day, \( F(2, 12) = 1.82, p = .21 \) and no significant day x time interaction, \( F(8, 48) = .36, p = .94 \). However, the CS was effective in reinstating active lever responding during the first exposure to the reinstatement testing situation (see inset, middle panel of Figure 3B). A separate repeated-measures ANOVA comparing active lever pressing activity with test day order (i.e., final extinction day, first day of reinstatement testing, or the second reinstatement day) as a within-subjects factor was significant, \( F(2, 12) = 10.62, p = .002 \). Tukey’s HSD Post-hoc analyses indicated that the significant effect of test day order was driven by an enhancement of active lever pressing during the first reinstatement session compared to the
The final day of extinction training \((p < .05; \text{see inset in the second panel of Figure 3B})\). The increase in lever pressing elicited by the food-paired cue was extinguished by the second reinstatement session regardless of 5-HT receptor stimulation, as active lever pressing did not significantly differ from the last day of extinction testing \((p > .05)\).

Drug infusions did not disrupt the memory for the association between the active lever and reward delivery. Rats remained focused towards the previously active lever, as indicated by the low average proportions of inactive lever presses to total lever presses for both saline \((M = .11, SD = .08)\) and 5-HT hydrogen maleate \((M = .15, SD = .18)\) reinstatement days, \(t(6) = .59, p = .58\). Furthermore, total inactive lever presses were similar across the final extinction session and the two reinstatement tests \([F(2,12) = 2.74, p = .11]\).

4. Discussion

The present set of experiments is the first to characterize the effects of DA, \(\mu\)-opioid, and 5-HT receptor involvement within the NAcc on cue-evoked reinstatement of food-seeking behavior. Consistent with earlier reports that support a role for the NAcc in promoting food-seeking behavior in response to conditioned stimuli [5, 9], we found that intra-accumbens antagonism of dopaminergic D\(_1\) receptors with SCH 23390, or D\(_2\) receptors with raclopride, blocked the CS-initiated reactivation of lever pressing during reinstatement testing. Additionally, intra-accumbens \(\mu\)-opioid receptor activation also attenuated cue-evoked relapse to food-seeking. Stimulation of 5-HT receptors within the NAcc did not alter reinstatement. Together, these data extend our understanding of the neurobiology of appetitive behavior by demonstrating that dopaminergic and \(\mu\)-opioid receptors within the NAcc mediate the ability for food-paired cues to reactivate food-seeking behaviors in a food reinstatement paradigm.
That the blockade of both DA D₁ and D₂ receptors within the NAcc impaired the reactivation of appetitive behavior is consistent with other demonstrations that NAcc DA modulates incentive motivation in the presence of cues predictive of reward availability. For instance, stimulation of DA receptors within the NAcc enhances lever-pressing in a secondary reinforcement paradigm, in which hungry rats are initially trained to make a classically conditioned association between a CS and food delivery and then are subsequently trained to lever press for access to the CS alone [35-37]. Similarly, cues that have been classically conditioned to predict reward presentation will invigorate responding when they are presented concurrently to rats that are lever-pressing on an instrumental schedule of reinforcement (Pavlovian-Instrumental Transfer, or PIT). Lesions of the NAcc attenuate PIT [38], and dopaminergic signaling within the NAcc appears to be critical for this effect. PIT can be augmented by treatments that increase NAcc DA output [39] or blocked following the antagonism of NAcc D₁ or D₂ receptors [40]. The current data also provide a possible neural locus of effect for a recent report in which systemic D₂ receptor antagonism attenuated the reinstatement of food seeking in the presence of contextual stimuli previously associated with sucrose pellet [41]. It is of note that the reinstatement paradigm utilized in these experiments effectively reactivates reward-seeking behaviors in the absence of primary reinforcement, thus parsing out the appetitive phase of reward procurement from the consummatory phases of food intake. The present data therefore provide converging support for a role of ventral striatum DA in regulating the salience of incentive cues during the appetitive phases of reward-directed behavior [42].

Significant inhibition of cue-induced relapse to food-seeking was also seen following mu-opioid receptor stimulation with DAMGO. This was surprising, as activation of mu-opioid
receptors of the NAcc increases feeding behavior in rats when food is freely available, and has been suggested to do so by enhancing the hedonic value of preferred food [22, 32, 33]. While stimulation of mu-opioid receptors of the NAcc does increase the effort that rats will expend to obtain sucrose reinforcement [43], this is not the first report that has failed to show enhanced responding to reward-associated cues following NAcc mu-opioid stimulation in the absence of primary food reinforcement. Previously, Cunningham and Kelley [44] reported no effects of NAcc opiate receptor activation on lever responding in a conditioned reinforcement paradigm. Likewise, the current experiments do not support a role for ventral striatum mu-opioid receptors in enhancing the incentive value of reward-predictive cues. Thus, the motivational enhancement observed following DAMGO treatment of the NAcc may be limited to situations that involve access to the primary food reward, and not transfer to classically conditioned cues that have been associated with reward delivery. Perhaps in the foraging rat, intrinsic activation of mu-opioid receptors within the NAcc during the consummatory phases of feeding serves to inhibit cue-directed appetitive behaviors that would compete with the feeding response, particularly when the food involved is highly palatable and energy-dense. Thus, stimulation of mu-opioid receptors may result in a pleasurable hedonic state (akin to that which occurs during the eating of palatable diets) that suppresses the expression of further appetitive (seeking) behaviors in the favor of consummatory (feeding) processes.

In the current experiments, D1 or D2 receptor blockade reduced lever pressing on the inactive lever, as compared to that seen on the vehicle injection day. However, in both experiments 1 and 2, presses on the active lever following drug treatment were equivalent to that of the final day of extinction, suggesting that the rats were capable of lever-pressing at appropriate behavioral levels. It should be noted that manipulations of both dopaminergic and
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opioid systems of the NAcc have been shown to alter locomotor activity [44-46]. However, prior reports suggest that it is unlikely that motor impairments per se account for the lever pressing reductions observed in the current experiments. Intra-accumbens infusions of SCH 23390 (at doses equivalent to those used here) do not affect operant behavior on rich (FR5) schedules of food-reinforcement, although they do reduce lever pressing under more demanding schedules [20, 31]. Similarly, prior studies utilizing alcohol and cocaine self-administration paradigms support a selective role for DA antagonism in modulating reward-related effortful behavior. NAcc microinjections of 1 or 2 micrograms SCH 23390 decrease cocaine self-administration under demanding progressive ratio (PR) schedules but increase cocaine self-administration under low, FR schedules of reinforcement [29]. Additionally, antagonism of both D1 and D2 receptors selectively decreases the self-administration of ethanol while leaving responding for water at baseline levels [30]. Together, these data support a specific role for DA receptors in altering incentive motivation rather than affecting baseline levels of activity and consummatory behaviors [21, 29, 31]. The current data suggest that this effect of NAcc dopaminergic receptor inhibition may generalize as well to cues that predict reinforcement, supporting a theorized role for DA in enhancing the salience of incentive stimuli in the presence of reward-predictive cues [42]. With regard to mu-opioid receptor stimulation, although similar treatments within the NAcc cause biphasic effects on locomotion that is characterized by an early inhibition and a later enhancement of locomotor activity [44, 46], there is no evidence that DAMGO injections impaired lever pressing behavior in this study. Stimulation of mu-opioid receptors did not change active lever pressing as compared to the final extinction day or inactive lever pressing compared to the vehicle treatment condition. Furthermore, at the dose used here, DAMGO infusions into the NAcc significantly increase (by 200%) the amount of palatable diet a
rat will consume within 30 minutes of drug treatment [46, 47], providing further support that the effects of this treatment on reinstatement was not caused by gross locomotor impairment.

In contrast to the effects of DA and opiate treatments, infusion of 5-HT hydrogen maleate into the NAcc prior to reinstatement did not affect reinstatement in the present study. This is consistent with previous reports that have shown that 5-HT injections into the NAcc fail to selectively impact lever pressing in conditioned reinforcement paradigms [28, 48]. However, activation of serotonin receptors may play a more important role in regulating dopaminergic circuits that are upregulated due to drug-enhanced dopaminergic tone within the NAcc. Specifically, intra-accumbens 5-HT dampens the response-potentiating effects of NAcc infusions of d-amphetamine in conditioned reinforcement paradigms [28]. Recent research has demonstrated that DA levels within the NAcc increase in response to CS presentations that predict food reward [49], but perhaps such fluctuations are below the threshold of modulation by NAcc 5-HT infusions. Together, this evidence suggests an important dissociation between possible 5-HT receptor involvement in modulating reward-seeking in the presence of drug versus natural rewards. Specifically, striatal 5-HT may attenuate drug-seeking behavior while leaving appetitive motivation for natural reinforcement intact. However, modulation of motivated behaviors by NAcc 5-HT may depend on the specific receptor subtype stimulated. For instance, the intra-accumbens pharmacological stimulation of 5-HT\textsubscript{1A} receptors decreases food intake and food-directed locomotor behavior for up to 2 hours in a free-feeding paradigm while the stimulation of 5-HT\textsubscript{6} receptors results in an increase in consumption [27]. In light of this evidence, perhaps the global stimulation of NAcc 5-HT receptor subtypes in the present experiment may mask the function of any one particular serotonin receptor class. Alternatively, it may be the case that the use of the endogenous ligand (as done here) in the absence of d-
amphetamine permitted rapid uptake of the drug, ameliorating an effect that would be apparent at a different drug dose or if 5-HT were co-administered with another drug that blocked its uptake.

5. Conclusions

These data are the first to demonstrate unique roles for different neurotransmitter systems within the NAcc in a cue-evoked relapse to food-seeking paradigm. The results from these experiments extend previous demonstrations that food-paired cues can exert a powerful influence on appetitive behaviors involved in food craving and dietary relapse [15]. Additionally, we provide evidence that dopaminergic and opioidergic signaling in the NAcc regulate appetitive motivation following renewed exposure to food-associated cues. These data are in line with the plethora of research implicating DA involvement in drug craving and seeking behaviors [16, 42, 50] and corroborate claims that similar mechanisms are involved in both compulsive drug intake and maladaptive feeding patterns [51, 52]. The finding that mu-opioid receptor stimulation with DAMGO suppressed food-seeking is also of interest. The observed blunting of cue-evoked reinstatement following intra-accumbal DAMGO infusions may reflect a role for endogenous NAcc mu-opioid receptor stimulation in inhibiting appetitive seeking behaviors at times when it benefits the organism to maintain ongoing consummatory behavior, such as after the discovery of a particularly dense (and palatable) caloric food source. While there was no observable effect on cue-evoked reinstatement following manipulations of 5-HT receptors within the NAcc, future research may wish to explore individual receptor subtypes within this paradigm as extant evidence suggests that specific 5-HT receptors within mesolimbic circuitry modulate reward-directed behavior [4, 27, 53, 54]. With obesity rates at historically high prevalence, additional research utilizing this and similar paradigms is necessary to determine the neural mechanisms that regulate appetitive behaviors as they are affected by environmental cues, toward the goal of
determining pharmacological and behavioral strategies aimed at resisting dietary relapse and promoting the maintenance of a healthy body mass.
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Figure Captions.

Figure 1. NAcc injector tip placements for the animals included in the analysis for each experiment. Drawings adapted from Figures 10, 12, & 13 of *The Rat Brain in Sterotaxic Coordinates*, 4th ed., G. Paxinos and C. Watson, copyright 1998.

Figure 2. Antagonism of NAcc D₁ (A) or D₂ (B) receptors attenuates the cue-induced reinstatement of food-seeking. The left panels depict the average number of active and inactive lever presses for the five days of FI20-VR5 training for both experiments. The central panels show total average active and inactive lever responding on the final extinction day and two reinstatement days. The panels on the right show the responding on the active lever across the 20 minute sessions, as subdivided into four-minute epochs. Crosses († and ††) demark a significant effect of drug across the three test days on active lever pressing (at p < .05 and p < .01, respectively). Stars (*) on the bar graphs indicate a significant increase in lever pressing on the saline reinstatement day when compared to the final extinction day (as determined by Tukey HSD); number signs (#) indicate a significant decrease in lever pressing activity as compared to that observed on the saline reinstatement day.

Figure 3. Stimulation of mu-opioid receptors (A), but not 5-HT receptors (B), attenuates cue-evoked reinstatement of food-seeking. The left panels depict the average number of active and inactive lever presses for the five days of FI20-VR5 training for both experiments. The central panels show total average active and inactive lever responding on the final extinction day and two reinstatement days. The inset in the center panel of B shows that the 5-HT-treated animals significant reinstated lever-pressing behavior on the first test session, regardless of drug
treatment. The panels on the right show the responding on the active lever across the 20 minute sessions, as subdivided into four-minute epochs. Crosses (††) demark a significant effect of drug across the three test days on active lever pressing (at p < .01). Double crosses (‡‡) indicate a significant day X time interaction effect (p < .01). Stars (*) indicate a significant increase in lever pressing on the saline reinstatement day when compared to the final extinction day (as determined by Tukey HSD).
Figure 1

Experiment 1: SCH 23390 (N = 9)

Experiment 2: Raclopride (N = 13)

Experiment 3: DAMGO (N = 10)

Experiment 4: 5-HT (N = 7)
Figure 2
Figure 3