

Nucleus Accumbens Acetylcholine Receptors Modulate Dopamine and Motivation

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Environmental reward-predictive cues can motivate reward-seeking behaviors. Although this influence is normally adaptive, it can become maladaptive in disordered states, such as addiction. Dopamine release in the nucleus accumbens core (NAc) is known to mediate the motivational impact of reward-predictive cues, but little is known about how other neuromodulatory systems contribute to cue-motivated behavior. Here, we examined the role of the NAc cholinergic receptor system in cue-motivated behavior using a Pavlovian-to-instrumental transfer task designed to assess the motivating influence of a reward-predictive cue over an independently-trained instrumental action. Disruption of NAc muscarinic acetylcholine receptor activity attenuated, whereas blockade of nicotinic receptors augmented cue-induced invigoration of reward seeking. We next examined a potential dopaminergic mechanism for this behavioral effect by combining fast-scan cyclic voltammetry with local pharmacological acetylcholine receptor manipulation. The data show evidence of opposing modulation of cue-evoked dopamine release, with muscarinic and nicotinic receptor antagonists causing suppression and augmentation, respectively, consistent with the behavioral effects of these manipulations. In addition to demonstrating cholinergic modulation of naturally-evoked and behaviorally-relevant dopamine signaling, these data suggest that NAc cholinergic receptors may gate the expression of cue-motivated behavior through modulation of phasic dopamine release.

Neuropsychopharmacology (2016) **41**, 2830–2838; doi:10.1038/npp.2016.81; published online 10 August 2016

INTRODUCTION

Environmental reward-predictive stimuli provide a major source of motivation for adaptive reward-seeking behaviors (Corbit and Balleine, 2015). But this incentive motivational value can become amplified in disordered states, allowing cues to become potent triggers for maladaptive behavior such as compulsive overeating (Johnson, 2013) or drug seeking (Robinson *et al*, 2013). Considerable evidence has implicated nucleus accumbens core (NAc) dopamine signaling in the expression of a cue's motivational value (Berridge, 2007). Modulatory mechanisms in striatal terminals can, however, regulate dopamine release. Of these, the acetylcholine receptor system has emerged as a key player (Cachope and Cheer, 2014; Cragg, 2006; Sulzer *et al*, 2016). Yet, little is known about the causal role of the NAc cholinergic system in motivated behavior and there is currently no evidence of acetylcholine receptor modulation of naturally-evoked and behaviorally-relevant dopamine.

Acetylcholine receptors are broadly distributed throughout the NAc and consist of two major subtypes: muscarinic and nicotinic. Tonically-active, cholinergic interneurons provide the primary source of acetylcholine acting at these receptors (Bolam *et al*, 1984). Nicotinic acetylcholine receptors

(nAChR) on striatal dopamine terminals (Jones *et al*, 2001) are known to be particularly relevant to the regulation of striatal dopamine signaling (Cachope *et al*, 2012; Exley *et al*, 2008a; Threlfell *et al*, 2012). Muscarinic acetylcholine receptor (mAChR) activity also contributes to dopamine modulation both via regulation of endogenous activity at nAChRs on dopamine terminals (Threlfell *et al*, 2010) and through activation of mAChRs on dopamine terminals (Foster *et al*, 2014; Shin *et al*, 2015).

Striatal cholinergic interneuron activity correlates with the presentation of reward-predictive stimuli and with motivated behavior (Morris *et al*, 2004; Nougaret and Ravel, 2015; Ravel *et al*, 2003). NAc mAChRs have been implicated in instrumental activity (Ikemoto *et al*, 1998; Pratt and Kelley, 2004), but neither their precise contribution, nor involvement in cue-motivated behavior has been determined. Therefore, here we tested the hypothesis that NAc endogenous mAChR and nAChR activity regulates expression of the motivational value of reward-predictive cues and then examined a potential dopaminergic mechanism for this influence.

To achieve this, we first evaluated the influence of NAc mAChR or nAChR blockade on cue-motivated behavior using the Pavlovian-to-instrumental transfer (PIT) task. This task assesses the motivating influence of a reward-predictive cue over the performance of an independently-trained, instrumental reward-seeking action, and thereby isolates the incentive motivational value of the cue from other processes through which cues trigger action, such as their

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Received 1 March 2016; revised 24 May 2016; accepted 25 May 2016;
accepted article preview online 31 May 2016

ability to exert discriminative control over performance through stimulus-response learning. In a second experiment, we examined the impact of nAChR or mAChR blockade on cue-evoked dopamine signaling in the NAc during PIT.

MATERIALS AND METHODS

Subjects

All procedures were conducted in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals and were approved by the UCLA Institutional Animal Care and Use Committee. Male rats served as the subjects for these experiments (Experiment 1: final $n=24$, five rats were excluded due to the misplaced cannula, three due to illness; Long Evans; 300–330 g; Charles River Laboratories, Wilmington, MA; Experiment 2: final $n=10$, an additional five rats were run through the behavioral task, but were excluded from the electrochemical analysis due to misplaced electrodes ($n=2$), or noisy electrodes and/or an inability to reliably record a significant unexpected food-pelleted-evoked dopamine response ($n=3$); Sprague Dawley; 280–320 g). Rats were group-housed and handled for 5–7 days prior to the onset of the experiment. Experimentation took place during the dark phase of the 12:12 h reverse dark:light cycle. Rats were food deprived receiving 12–14 g/day of their maintenance diet to maintain 85–90% free-feeding body weight. Free access to tap water was provided in the home cage.

Surgical Procedures

In Experiment 1, rats were bilaterally implanted with 22-gauge, stainless steel, guide cannulae (Plastics One, Roanoke, VA) targeted 1 mm above the intended NAc core infusion site (coordinates relative to Bregma: AP+1.3 mm, ML ± 1.8 , and DV -6.2). Coordinates were selected based on the previous findings demonstrating effects of cholinergic receptor antagonists on reward-related behavior (Nadal *et al*, 1998; Pratt and Kelley, 2004) and to avoid spread into the NAc shell. For Experiment 2, calibrated carbon-fiber micro-electrodes coupled with guide cannulae were implanted unilaterally into the NAc (AP: +1.3 mm; ML: ± 1.3 ; V: -7.0 from dura) along with an Ag/AgCl reference electrode in the contralateral cortex. Coordinates were selected to match our previous NAc core dopamine recording location during PIT (Aitken *et al*, 2016; Ostlund *et al*, 2014b; Wassum *et al*, 2013) and to avoid electrode place in the anterior commissure. Electrode/cannula probes were arranged such that the electrode tip was aligned to the coordinate above, with the cannula positioned more laterally parallel to the coronal suture. Standard aseptic stereotaxic procedures were used as described previously (Aitken *et al*, 2016). Following surgery, rats were individually housed and allowed to recover for 5–7 days. Placement(s) were verified using the standard histological procedures described previously (Collins *et al*, 2016); (see Supplementary Figures 1 and 2).

Behavioral Task

The PIT behavioral task procedures are similar to those described previously (Wassum *et al*, 2011, 2013). For

Experiment 1, training and testing took place in a set of 16 Med Associates (East Fairfield, VT) operant chambers described previously (Malvaez *et al*, 2015). For Experiment 2, training and testing were conducted in a set of two chambers identical to the training chambers, but outfitted with a voltammetric recording unit as described previously (Aitken *et al*, 2016; Collins *et al*, 2016).

Pavlovian conditioning. Rats first received 8 days of Pavlovian training in which one of two auditory cues (75 dB tone or white noise; counterbalanced across rats; CS⁺) was paired with noncontingent delivery of 45-mg grain pellets (Bio-Serv, Frenchtown, NJ). Each CS⁺ was presented for 2 min, during which pellets were presented on a random-time 30-s schedule. The CS⁺ was presented 6x/session with a random 2–4 min intertrial interval (mean = 3 min). The lever was never present during these sessions.

Instrumental conditioning. All rats then received 8 days of instrumental training in which lever pressing was rewarded with delivery of a single grain pellet. Each session lasted until 20 outcomes had been earned or 30 min elapsed. Rats received 1 day each of continuous, random-interval 15 s, and random-interval 30 s schedules of reinforcement, followed by 5 days on the final random-interval 60 s schedule. The CS⁺ was never present during this training.

Retraining and CS⁰ habituation. Following instrumental training, rats in Experiment 1 underwent surgery and, after recovery, were given two instrumental retraining sessions and one Pavlovian retraining session. Rats in Experiment 2 received one Pavlovian CS⁺ reminder session after instrumental training. Following this, both groups of rats received one session of habituation to the neutral control stimulus (CS⁰), which consisted of 6, 2-min presentations of the CS⁰ (opposite stimulus as the CS⁺), with a 2–4 min intertrial interval. No rewards were delivered during this session. Rats were also given 2 days of instrumental and 1 day of Pavlovian retraining in between each PIT test.

PIT test. Rats were given three total PIT tests to allow a within-subjects drug-treatment design. On the day prior to each PIT test, rats in Experiment 1 were given a single 30-min instrumental extinction session in which no cues were present, and lever pressing was unrewarded. During each PIT test, the lever was continuously available, but pressing was not reinforced. Responding was extinguished for 5 min to establish a low rate of baseline performance, after which each CS was presented four times in pseudorandom order, also without accompanying reward. Each CS lasted 2 min with a 4-min fixed intertrial interval. PIT has been robustly demonstrated in both Long Evans (Corbit *et al*, 2007; Wassum *et al*, 2011) and Sprague Dawley (Saddoris *et al*, 2011; Wassum *et al*, 2013) rats with procedures similar to those here, and the PIT effect was shown here to be similar in magnitude in the control conditions in both species.

Drugs and Infusion Procedures

In Experiment 1, immediately prior to each PIT test, rats were bilaterally infused with either the selective nAChR

antagonist mecamylamine (10 µg/side; Tocris Bioscience, Bristol, UK), the selective mAChR antagonist scopolamine (10 µg/side; Tocris), or an equivalent volume of sterile artificial cerebral spinal fluid (ACSF; Tocris). All rats were tested under each drug condition, counterbalanced for order. Doses were selected based on their ability to alter reward seeking when infused into the NAc, without causing gross motor impairments (Nadal *et al*, 1998; Nunes *et al*, 2013; Pratt and Kelley, 2004). Drugs were infused, as described previously (Wassum *et al*, 2009), into the NAc in a volume of 0.5 µl over 1 min via an injector inserted into the guide cannula fabricated to protrude 1 mm ventral to the cannula tip, using a microinfusion pump. For Experiment 2, rats were unilaterally infused with either mecamylamine (10 µg/side), scopolamine (10 µg/side), or an equivalent volume (0.5 µl/side) of ACSF via the cannula attached to the carbon-fiber microelectrode.

Fast-Scan Cyclic Voltammetry

For Experiment 2, chronically-implanted and carbon-fiber microelectrodes were used to make longitudinal, within-subject measures of dopamine concentration changes across multiple tests. These electrodes were coupled to the guide cannula, as described below, to allow infusion of pharmacological agents into the extracellular space surrounding the recording electrode. For each test session, rats were placed in the operant chamber and tethered to the voltammetric recording unit through an electrical swivel. Fast-scan cyclic voltammetry (FSCV) recordings were performed during the PIT tests as described previously (Aitken *et al*, 2016; Collins *et al*, 2016). On each test day, after stabilization of the baseline current (~20 min), rats were unilaterally infused as described above and the baseline was allowed to restabilize for 5–10 min, after which the PIT test commenced with the onset of the house light and insertion of the lever. Because bilateral drug treatment would produce a behavioral effect that would confound our neurochemical analysis (eg, a drug-induced change in the cue-evoked dopamine response could be a secondary consequence of the behavioral effects of that treatment), we used unilateral infusion to minimize such effects. For a subset of rats ($n = 7$), FSCV measurements were also collected prior to, and after the drug infusion and PIT test during the unexpected delivery of five chocolate-flavored 45-mg pellets (Bio-Serv). Behavioral performance did not differ between the subjects that received and those that did not receive food pellets prior to the test (main effect of Pellet exposure group: $F_{2,12} = 0.35$, $p = 0.564$ on lever presses during the PIT test; pellet exposure \times drug: $F_{2,12} = 0.55$, $p = 0.59$; pellet exposure \times CS: $F_{2,12} = 0.42$, $p = 0.67$).

Carbon-Fiber Microelectrode/Cannula Assembly

Carbon-fiber microelectrodes were prepared and calibrated (average calibration factor = 45.45 nM/nA, SEM = 4.13) as described previously (Clark *et al*, 2010; Collins *et al*, 2016; Wassum *et al*, 2013), with the exception that the silica shaft encasing the carbon fiber was cut to ~12 mm. Following calibration, each electrode was affixed to a 22-gauge, stainless steel guide cannula (Plastics One) with the cannula extending 3–4 mm beyond the 14-mm plastic threaded base. Injectors (Plastics One) were designed to fit these cannulae and to

protrude an additional 3–4 mm. The cannula terminated 3–4 mm above the tip of the electrode to prevent damage to the recording zone. The lower ~2/3 of the plastic base was shaved down on one side of the cannula to allow a flat space for the electrode to be attached (Figure 1a and b). The electrode was affixed to the guide cannula by two-component clear epoxy (Devcon, Danvers, MA). Using a microscope and microruler, while the epoxy was still loose the electrode position was adjusted along the shaft of the cannula such that the electrode tip was within 100–300 µm from the tip of the injector when inserted through the cannula (Figure 1b). Guide cannulae were at an ~3–5°-angle relative to the electrode.

Carbon-Fiber Microelectrode/Cannula Characterization

In vitro characterization. We calibrated *in vitro* a sample ($n = 8$) of carbon-fiber microelectrodes either alone or coupled with the cannula and injector (electrode/cannula probes) to determine whether assembly with the cannula and injector placement near electrode tip would disrupt FSCV dopamine detection. Both electrode types showed similar responses to dopamine passed by the electrode at known concentrations of 0.25, 0.5, and 1.0 µM (main effect of electrode type: $F_{1,7} = 0.78$, $p = 0.41$; dopamine concentration: $F_{3,21} = 112.0$, $p < 0.0001$; electrode type \times concentration interaction: $F_{3,21} = 0.67$, $p = 0.58$; Figure 1c).

In vivo characterization. We compared the dopamine response to unexpected food reward delivery detected at the electrode/cannula probes used in the current study (the $n = 7$ for which these measures were collected) to that detected at a sample ($n = 7$) of single carbon-fiber microelectrodes from our previous studies and found no difference (average unexpected reward dopamine response electrode only: 34.12 nM, SEM = 4.04; electrode/cannula probe: 31.71, 3.31; $t_{12} = 0.46$, $p = 0.66$; Figure 1d). Similarly, there was no difference in the dopamine response to an unexpected food reward at electrode/cannula probes prior to *vs* immediately following an infusion of ACSF (average post-vehicle response: 27.43, 2.35; $t_{12} = 1.44$, $p = 0.20$; Figure 1d). We additionally examined whether infusions in the extracellular recording space would elicit any prolonged change in FSCV dopamine detection by looking at the dopamine response to unexpected food reward delivery before any infusion had ever been made compared with the dopamine response after rats had received (on previous days) one or two infusions. In this case, we used the pretest/infusion dopamine response for each of the three tests. We detected no significant difference in reward-evoked dopamine release before any infusion relative to after one or two infusions (average dopamine response, no infusion: 36.63 nM, SEM = 5.50; after one infusion: 30.58, 3.41; after two infusions: 31.54, 3.73; main effect of test: $F_{2,6} = 0.81$, $p = 0.41$; Figure 1e).

Combined, these data show that arrangement of a carbon-fiber microelectrode with a guide cannula and infusion of liquid into the extracellular space surrounding the electrode does not disrupt FSCV dopamine detection or sensitivity.

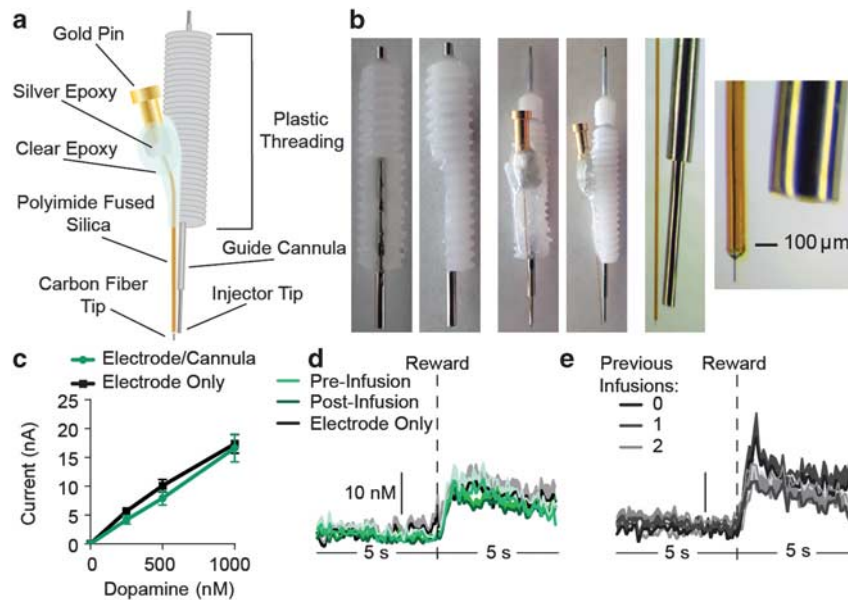


Figure 1 Design and characterization of carbon-fiber microelectrode/cannula probes. (a) Schematic of carbon-fiber microelectrode combined with a guide cannula for microinfusion in the extracellular recording space. (b) Images of guide cannulae with portion of plastic threading shaved down on one side (left) and assembled electrode/cannula probe with injector inserted through the guide cannula (middle). Right-most images show magnification of the electrode tip with the injector inserted through the guide cannula. (c) Average current response of either electrode/cannula probes or individual electrodes to known concentrations of dopamine *in vitro* in a flow-cell calibration unit. (d) Averaged (across trials and across subjects) dopamine concentration vs time traces 5 s before and after delivery of an unexpected food-pellet reward detected at either carbon-fiber microelectrodes alone, or electrode/cannula probes either before (pre-infusion) or after (post-infusion) infusion of ACSF vehicle. (e) Averaged dopamine concentration vs time around pellet delivery detected at electrode/cannula probes either before any infusion had ever been made or after rats had, on previous days, received either one or two infusions.

Data Analysis

Statistical analysis. Electrochemical data were analyzed using software written in LabVIEW (National Instruments). Data were processed with Microsoft Excel (Redmond, WA). Statistical analyses were conducted with GraphPad Prism (La Jolla, CA) and SPSS (IBM, Chicago, IL). For all hypothesis tests, the α level for significance was set to $p < 0.05$. Data were analyzed with one- and two-way ANOVAs (Geisser-Greenhouse correction), paired *t*-tests, correlation, and regression, where appropriate. Bonferroni corrected *post-hoc* comparisons and planned paired *t*-tests were used to clarify main effects and interactions.

Behavioral analysis. Lever pressing and entries into the food-delivery port were the primary behavioral output measures. During the PIT tests, these measures were counted and averaged across trials for each 2-min CS period, with behavioral output during the 2-min periods prior to each CS serving as the baseline for CS-induced changes in behavior.

Voltammetric analysis. Principal component regression, a chemometric technique that combines principal component analysis with inverse least-squares regression (Heien *et al*, 2005), was used to isolate changes in current due to dopamine and pH from the cyclic voltammetric data. We used a standard training set of dopamine, pH, and drift CVs as has been described previously (Aitken *et al*, 2016; Collins *et al*, 2016). This procedure allowed us to distinguish changes in current due to dopamine fluctuations from changes due to pH or to other electroactive substances. Individual values of

residual extraneous variance in an unknown measurement (Q) from the principal component regression analysis were compared with the average tolerance level ($Q\alpha$), which was 81.026 $Q/nA2$. On occasion, isolated transient electrical artifacts were detected, as identified by transient Q values well in excess of the $Q\alpha$ in isolated cells. Estimates of dopamine at these time points were interpolated by taking the average of the surrounding cells. Trials in which $> 10\%$ of the data points had residual values in excess of the $Q\alpha$ were omitted from the analysis. This occurred on 17.5% of all trials, which were evenly distributed between CS and drug conditions. After this analysis, all data were converted to estimated dopamine concentration via an electrode-specific, *in vitro*, pretest calibration factor.

Voltammetric data were isolated starting 30 s before the onset of the CS and ending with the offset of the 120-s CS. For each trial, the average of current across the 30-s period prior to CS onset served as the baseline for background subtraction. Maximal (ie, peak) dopamine concentration change during the first 30 s following CS onset provided a quantification of the CS onset-induced dopamine response. This has been previously demonstrated as a reliable measure of task-related and cue-evoked dopamine signaling (Aitken *et al*, 2016; Ostlund *et al*, 2014b; Wassum *et al*, 2013). Because previous data have demonstrated CS⁺-induced elevations in NAc dopamine signaling during PIT (Aitken *et al*, 2016; Ostlund *et al*, 2014b; Wassum *et al*, 2013), planned comparisons were used when comparing peak dopamine concentration during the CS⁺ vs CS⁰. The average of the background-subtracted dopamine concentration change during the entire 2-min CS period served as a

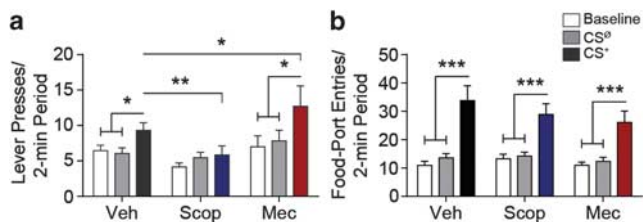


Figure 2 Effect of nucleus accumbens muscarinic and nicotinic acetylcholine receptor blockade on Pavlovian-to-instrumental transfer. Prior to each PIT test (see the 'Materials and Methods' section), rats were bilaterally infused with either scopolamine (Scop; 10 μ g), mecamlamine (Mec; 10 μ g), or ACSF vehicle (Veh) into the NAc. (a) The PIT effect. Number of lever presses during each 2-min period, averaged across trials compared between the CS-free (baseline), neutral stimulus (CS⁰), and reward-predictive cue (CS⁺) periods. (b) Conditioned food-port approach responding. Number of entries into the food-delivery port during each 2-min period, averaged across trials compared between the baseline, CS⁰, and CS⁺ periods. $n = 24$. Error bars represent ± 1 SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

measure of more prolonged CS-induced dopamine concentration changes. For both measures, dopamine concentration change estimates were averaged across the trials of the same type for each rat.

For the unexpected reward-evoked dopamine response, voltametric data were isolated for the 5 s before and after pellet delivery. For each trial, the average of current across the 5-s period prior pellet delivery served as the baseline for background subtraction, with peak dopamine concentration change in the 3 s following pellet delivery providing quantification of the response. All subjects collected each pellet within < 3 s following the delivery.

RESULTS

For Experiment 1, Pavlovian training was used to pair a 2-min auditory CS⁺ with food reward delivery. An alternate 2-min auditory stimulus was presented unpaired with reward and served as the control CS⁰. Rats were then instrumentally conditioned, in the absence of the stimuli, to lever press to earn food rewards (see Supplementary Results for training data). In the critical PIT test phase, the lever was available, though pressing was not reinforced, and each CS was presented in pseudorandom order, also without accompanying reward, to assess the motivating influence of the CS⁺ over lever-pressing activity. Changes in lever-press rate during the CS⁺ provided the measure of cue-motivated behavior (ie, expression of PIT). CS⁺-induced changes in the rate of entry into the food-delivery port were used as a measure of Pavlovian-conditioned goal-approach responses. Rats were given three PIT tests, one each following bilateral intra-NAc infusion of the nonspecific mAChR antagonist scopolamine, the nonspecific nAChR antagonist mecamlamine, or vehicle.

As can be seen in Figure 2a, blockade of NAc mAChRs and nAChRs differentially impacted motivated behavior (main effect intra-NAc drug: $F_{2,46} = 3.52$, $p = 0.04$; CS period: $F_{2,46} = 9.43$, $p = 0.0004$; CS \times drug: $F_{4,92} = 2.40$, $p = 0.056$). Demonstrating PIT, the CS⁺ elevated lever pressing relative to both the baseline and CS⁰ periods under control conditions ($p < 0.05$). Blockade of NAc mAChRs attenuated

the expression of this cue-motivated behavior ($p < 0.01$) and prevented PIT ($p > 0.05$), whereas nAChR blockade enhanced the invigorating influence of the CS⁺ ($p < 0.05$), producing robust PIT ($p < 0.001$). Acetylcholine receptor blockade predominantly influenced CS⁺-invigorated responding; neither baseline, nor CS⁰ lever-press rate were significantly altered by intra-NAc scopolamine or mecamlamine treatment ($p > 0.05$). In two separate experiments, similar effects were produced at multiple drug doses (Supplementary Figure 3).

The effects of both intra-NAc scopolamine and mecamlamine were limited to the motivating influence of the CS⁺ over instrumental behavior. Intra-NAc infusion of neither scopolamine, nor mecamlamine significantly impacted the performance of the Pavlovian-conditioned food-port approach response (main effect of CS period: $F_{2,46} = 47.35$, $p < 0.0001$; drug: $F_{2,46} = 0.55$, $p = 0.58$; CS \times drug: $F_{4,92} = 1.06$, $p = 0.38$; Figure 2b). In all cases, the CS⁺ induced a robust elevation in food-port entries relative to both the CS⁰ and baseline periods ($p < 0.001$).

These data suggest that NAc mAChRs and nAChRs have functionally important, but opposing roles in expression of the motivational value that allows reward-predictive cues to invigorate instrumental activity. Although there are other potential mechanisms for these behavioral effects, they do appear analogous to those demonstrating opposing influences of mAChR and nAChR activity on phasic striatal dopamine release. In the slice, blockade of striatal nAChRs located presynaptically on dopamine terminals (Jones *et al*, 2001) can modulate the release of dopamine in a manner dependent upon the stimulated firing pattern of dopamine neurons (Zhang and Sulzer, 2004; Zhang *et al*, 2009). Under some conditions, inactivation of these nAChRs can actually augment the release of dopamine generated by high-frequency stimulation (simulating burst firing) (Rice and Cragg, 2004). Activating mAChR autoreceptors on striatal cholinergic interneurons, which can decrease acetylcholine tone at nAChRs on dopamine terminals, can also augment terminal dopamine release to high-frequency stimulation (Shin *et al*, 2015; Threlfell *et al*, 2010). Not only is it well established that reward-predictive cues trigger burst firing in dopamine cells (Schultz, 2001), but there is growing evidence that the resulting dopamine release in the NAc mediates cue-motivated instrumental behavior (Lex and Hauber, 2008; Ostlund *et al*, 2014b; Pecina and Berridge, 2013). Combined, these data suggest that blockade of nAChR could enhance the dopamine response to reward-predictive cues, whereas antagonizing mAChRs could blunt it.

We tested this hypothesis in Experiment 2 by assessing the influence of blockade of either nAChRs or mAChRs on cue-evoked NAc dopamine signaling during PIT. To achieve this, we devised a probe consisting of a carbon-fiber microelectrode affixed to a guide cannula (see Figure 1) to allow FSCV dopamine measurement directly following the infusion of nAChR or mAChR antagonists into the extracellular space surrounding the recording electrode. Rats were unilaterally implanted with these probes in the NAc and trained as described above. These chronically-implanted microelectrode/cannula probes allowed within-subject measurement of cue-evoked dopamine responses during each of three PIT tests: one each following the unilateral infusion of scopolamine, mecamlamine, or vehicle.

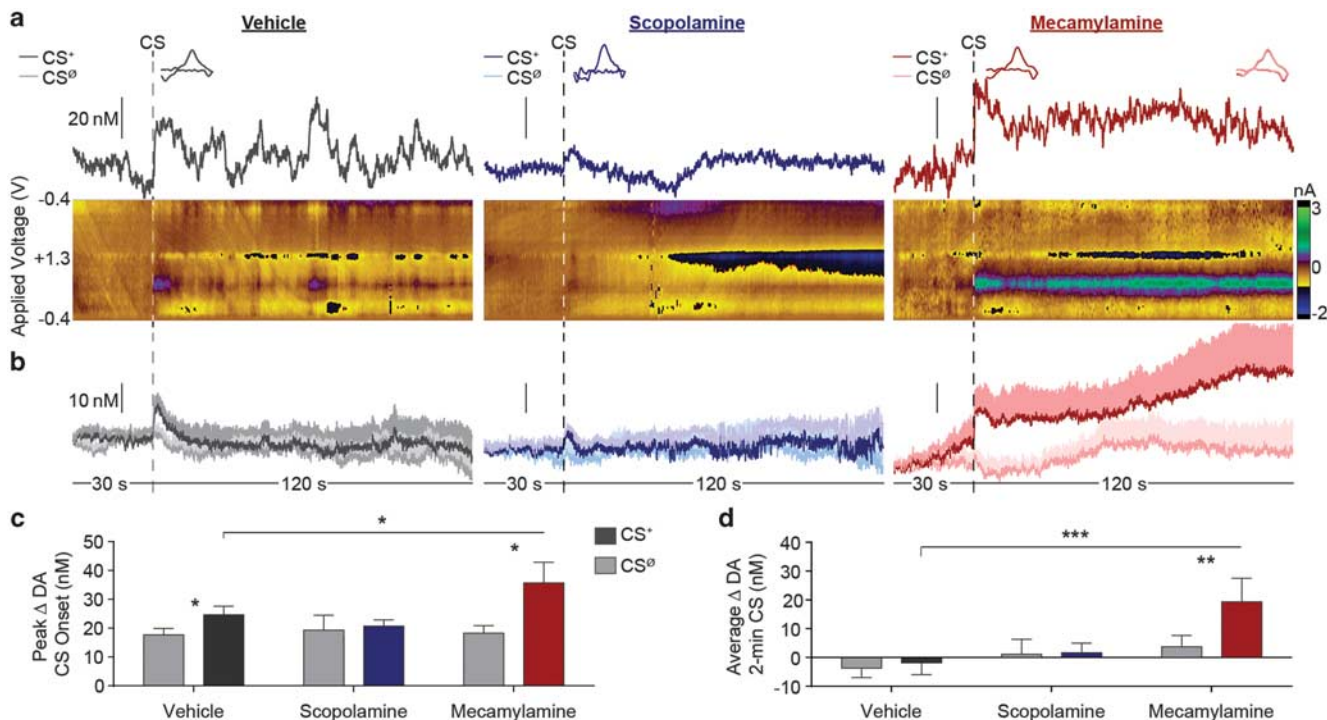


Figure 3 Effect of nucleus accumbens muscarinic and nicotinic acetylcholine receptor blockade on cue-evoked dopamine signaling during Pavlovian-to-instrumental transfer. (a) Representative example, single-trial, FSCV data 30 s before and during the entire 2-min CS⁺, during the PIT test following the unilateral infusion of ASCF-vehicle (left), scopolamine (10 μg) (middle), or mecamylamine (10 μg) (right) into the NAc recording zone. Upper plot depicts the dopamine concentration vs time trace. Inset, cyclic voltammograms identifying the detected current as dopamine, taken from within the first 30 s following CS⁺ onset. Right inset of mecamylamine condition, average of CVs taken at 1-s intervals for the duration of the CS⁺ (shading shows SEM), confirming detection of sustained dopamine throughout the CS⁺. Color plots in the lower panels show corresponding background-subtracted cyclic voltammograms as a function of the applied voltage vs time. (b) Average (across trials of the same type and across subjects) dopamine concentration vs time trace change 30 s prior to and during the entire 2-min CS⁺ or CS⁻ period. Shading reflects ± 1 between-subjects SEM. (c) Peak dopamine concentration change in the 30-s period following CS⁺ or CS⁻ onset, averaged across trials. (d) Average dopamine concentration change during 2-min CS⁺ or CS⁻ period. $n = 10$. Error bars represent ± 1 SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

As can be seen in the representative example (Figure 3a) and group-averaged dopamine concentration vs time traces (Figure 3b), under control conditions, the unexpected onset of the CS⁺ induced a NAc dopamine response that was attenuated by local blockade of mAChRs and amplified by nAChR blockade (main effect of CS period: $F_{1,9} = 6.28$, $p = 0.03$; drug: $F_{2,18} = 1.43$, $p = 0.26$; CS \times drug: $F_{2,18} = 3.55$, $p = 0.05$; Figure 3c; see Supplementary Figure 4 for effects divided by the trial). During PIT, CS⁺ onset induced an elevation in NAc dopamine concentration, relative to the CS⁻ control, in both the vehicle ($t_9 = 3.78$, $p = 0.004$) and mecamylamine ($t_9 = 2.29$, $p = 0.048$) conditions. The peak magnitude of this dopamine response was significantly larger following intra-NAc mecamylamine infusion, relative to vehicle control ($p < 0.05$, corrected for multiple comparisons). The CS⁺ was unable to significantly increase dopamine concentration following intra-NAc infusion of scopolamine ($t_9 = 0.40$, $p = 0.70$). Mecamylamine also appeared to produce a CS⁺-evoked dopamine response that was more sustained across the duration of the 2-min CS than under control conditions (main effect of CS period: $F_{1,9} = 4.33$, $p = 0.07$; drug: $F_{2,18} = 2.32$, $p = 0.13$; CS \times drug: $F_{2,18} = 4.77$, $p = 0.02$; see Figure 3d). Behavioral results from these tests are presented in Supplementary Figure 5.

These data are consistent with the hypothesis that tonic endogenous activation of nAChRs on striatal dopamine

terminals functions to blunt the release of dopamine when those cells burst fire, and that this influence can be alleviated by decreasing (mecamylamine) or enhanced by indirectly increasing (scopolamine) activity at these receptors (Cragg, 2006; Sulzer *et al*, 2016; Threlfell and Cragg, 2011). To further assess this, in a subset of subjects, we examined the influence of nAChR and mAChR blockade on the NAc dopamine response to unexpected food reward presentation, a preparation known to induce robust burst firing of midbrain dopamine cells (Schultz, 2001). NAc dopamine concentration changes were recorded during unexpected delivery of food pellet rewards for each subject both off and on drug. Off drug, unexpected reward delivery elicited a robust dopamine response (Figure 4a) and the peak magnitude of this response did not differ based on future drug treatment ($F_{2,12} = 0.75$, $p = 0.45$; Figure 4b). As can be seen in the averaged dopamine concentration vs time trace (Figure 4c) or quantification of the peak magnitude of this response (Figure 4d), blockade of NAc mAChRs attenuated the unexpected reward-evoked dopamine response ($p < 0.01$), whereas nAChR blockade augmented ($p < 0.01$) this response, relative to vehicle control (main effect of drug treatment: $F_{2,12} = 40.00$, $p < 0.0001$), mimicking the effect of those treatments on the cue-evoked dopamine response.

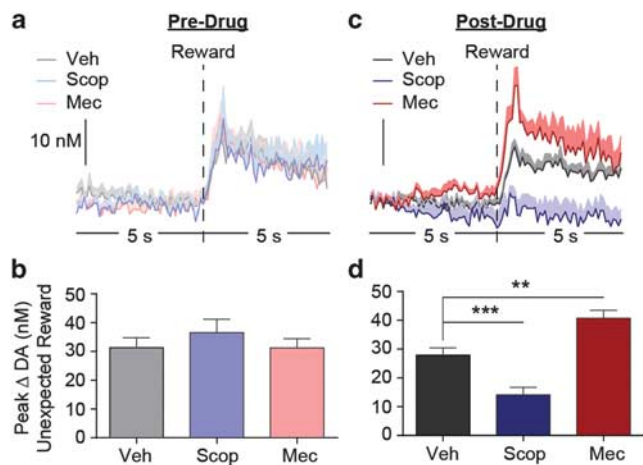


Figure 4 Effect of nucleus accumbens muscarinic and nicotinic acetylcholine receptor blockade on unexpected reward-evoked dopamine. (a and c) Averaged (across trials and across subjects) dopamine concentration vs time traces 5 s before and after delivery of an unexpected food pellet reward off drug (a) or following the unilateral infusion of either scopolamine (Scop; 10 μ g), mecamylamine (Mec; 10 μ g), or ACSF vehicle (Veh) into the NAc FSCV recording zone (c). Shading reflects ± 1 between-subjects SEM. (b and d) Peak dopamine concentration change within 3 s following unexpected food reward delivery off drug (b) or following unilateral intra-NAc infusion (d) $n = 7$. Error bars represent ± 1 SEM. *** $p < 0.01$, ** $p < 0.001$.

DISCUSSION

Here we evaluated the influence of NAc core nAChR and mAChR blockade on both cue-motivated behavior and cue-evoked dopamine signaling. Blockade of mAChRs attenuated, whereas nAChR blockade augmented the invigorating influence of a reward-predictive cue over instrumental reward-seeking activity. Providing a potential mechanism for these behavioral effects, disruption of mAChR activity was found to blunt, and nAChR inactivation to augment cue-evoked dopamine release. Similarly, NAc mAChR and nAChR inactivation attenuated, or augmented, respectively, the phasic dopamine response to unexpected reward. These data demonstrate cholinergic receptor modulation of naturally-evoked and behaviorally-relevant dopamine signaling in awake-behaving animals and point to the functional significance of cholinergic modulation of NAc dopamine signaling in mediating expression of the motivational value that allows reward-predictive cues to invigorate reward-seeking activity.

Nucleus accumbens core nAChR and mAChRs were found to have opposing roles in cue-motivated behavior. Endogenous activity at NAc nAChRs serves to limit the invigorating influence of cues over instrumental action, as evidenced by the heightened PIT effect observed following nAChR blockade. The blunted PIT effect observed following mAChR inactivation suggests endogenous NAc mAChR activity facilitates this expression of the cue's motivational value. Intra-NAc scopolamine infusion recapitulated the suppressive effect of systemic administration of this drug on PIT (Ostlund *et al*, 2014a), but interestingly, intra-NAc mecamylamine infusion showed an opposite effect to the systemic mecamylamine treatment (PIT enhancement rather than attenuation). This latter finding suggests that nAChRs may

differentially contribute to cue-motivated behavior depending on the brain region. The current results are consistent with previous work generally implicating NAc mAChRs in instrumental activity (Pratt and Kelley, 2004) and extend to show involvement of these receptors in cue-motivated behavior.

The behavioral effects of NAc nAChR and mAChR inactivation were complimented by data showing a corresponding influence of these treatments to enhance or attenuate, respectively, cue onset-evoked NAc dopamine signaling, which has been both correlated with (Aitken *et al*, 2016; Ostlund *et al*, 2014b; Wassum *et al*, 2013) and causally implicated in (Lex and Hauber, 2008; Pecina and Berridge, 2013; Wyvell and Berridge, 2000) the motivating influence of cues over reward-seeking activity. Of note, under control conditions, dopamine was found to be elevated at CS⁺ onset and to return to baseline within ~ 30 s, similar to our recent report (Aitken *et al*, 2016), but shorter-lasting than the cue-evoked dopamine response detected during PIT in the earlier work (Wassum *et al*, 2013). This discrepancy likely resulted from one of two methodological differences, the current and Aitken *et al*. (2016) studies both used a multi-test design and an operant chamber with a recessed food-delivery port, whereas the Wassum *et al*. (2013) study used a single test and an operant chamber with a protruding food-delivery port that allowed more port visibility and interaction.

There are several potential mechanisms through which blockade of acetylcholine receptor activity could have altered cue-evoked behavior and dopamine release. The effect of scopolamine could have been the result of direct action of this drug on postsynaptic M₁ mAChRs on NAc medium spiny projection neurons (Goldberg *et al*, 2012), which could indirectly modulate dopamine signaling through the regulation of midbrain dopamine cell activity. It is also possible that a component of either or both the behavioral and dopamine effects could be indirect via activity at acetylcholine receptors on NAc glutamate terminals. Indeed, cholinergic modulation of striatal dopamine release has been demonstrated, under some conditions, to be sensitive to glutamate receptor antagonists (Cachope *et al*, 2012; Wonnacott *et al*, 2000).

Data from *ex vivo* studies, demonstrating that cholinergic receptor activity can modulate dopamine signaling via direct action at dopamine terminals (Exley *et al*, 2008a; Exley and Cragg, 2008b; Threlfell *et al*, 2012), provide a parsimonious mechanism for the results here. Activating presynaptic nAChRs on dopamine terminals with endogenous acetylcholine can trigger dopamine release (Cachope *et al*, 2012; Threlfell *et al*, 2012), but *ex vivo* studies have demonstrated that this modulation depends on the activity level of dopamine cells (Zhang and Sulzer, 2004; Zhang *et al*, 2009). Nicotinic acetylcholine receptors can facilitate low probability dopamine release, but suppress dopamine release from high-frequency stimulation (mimicking burst firing) (Exley and Cragg, 2008b; Sulzer *et al*, 2016; Threlfell and Cragg, 2011). Indeed, although disrupting nAChR signaling can suppress the tonic dopamine measured by microdialysis (Lim *et al*, 2014), the higher resolution afforded by FSCV shows that it can actually augment high-frequency phasic release under some conditions (Exley *et al*, 2008a; Rice and Cragg, 2004), an effect we demonstrate here in awake-behaving animals with dopamine activity generated by

unexpected reward or reward cue presentation. Interestingly, nAChR agonists can also augment high-frequency phasic dopamine release *ex vivo* (Rice and Cragg, 2004; Zhang and Sulzer, 2004), but they do so through rapidly desensitizing the nAChRs, mimicking an antagonist-like effect (Lim *et al*, 2014; Zhang and Sulzer, 2004).

Activity at M₂/M₄ mAChR autoreceptors on NAc cholinergic interneurons (Calabresi *et al*, 1998) can also regulate terminal dopamine release to high-frequency stimulation by regulating acetylcholine tone at nAChRs on dopamine terminals (Threlfell *et al*, 2010). Recently however, activity at M₅ mAChRs on dopamine terminals has been shown to enhance striatal dopamine (Shin *et al*, 2015). The current result, demonstrating a blunting of cue- and reward-evoked dopamine release by nonselectively blocking NAc mAChR activity, could be achieved by either mechanism, and future work is needed to determine precisely how these different classes of NAc mAChRs influence behaviorally-relevant dopamine signaling.

Striatal cholinergic interneuron activity is controlled by a diverse array of afferent inputs (Lim *et al*, 2014), and cholinergic modulation of dopamine signaling has been demonstrated *ex vivo* via activation of thalamostriatal inputs (Threlfell *et al*, 2012). Cholinergic signaling is elevated in situations, such as satiety (Mark *et al*, 1992) or disadvantageous high effort or low reward conditions (Nougaret and Ravel, 2015) that discourage vigorous reward seeking. Striatal cholinergic interneurons also express receptors for insulin, a satiety and adiposity signal, and activation of these receptors increases cholinergic activity and modulates NAc dopamine signaling through a nAChR-dependent mechanism (Stouffer *et al*, 2015). Acetylcholine receptor activity could therefore provide one modulatory mechanism over cue-evoked dopamine signaling to regulate the motivating influence of cues according to their current adaptive value. This motivational influence can become excessive and/or disproportionate with need, and this is thought to contribute to overeating (Johnson, 2013) and maladaptive drug seeking (Robinson *et al*, 2013). These results therefore have implications for the understanding and treatment of compulsive overeating, addiction, and other disorders marked by maladaptive motivation.

FUNDING AND DISCLOSURE

This research was supported by a Hellman Foundation Fellowship, a UCLA Faculty Career Development award, grant DA035443 from the NIH to KMW, and grants AG045380, DK098709, and DA029035 from the NIH to SBO. The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We would like to thank Dr Scott Ng-Evans for his expert hardware and software assistance, and Drs Pamela Kennedy and Alicia Izquierdo for helpful discussions.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (<http://www.nature.com/npp>)