In vivo imaging identifies temporal signature of D1 and D2 medium spiny neurons in cocaine reward

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The reinforcing and rewarding properties of cocaine are attributed to its ability to increase dopaminergic transmission in nucleus accumbens (NAc). This action reinforces drug taking and seeking and leads to potent and long-lasting associations between the rewarding effects of the drug and the cues associated with its availability. The inability to extinguish these associations is a key factor contributing to relapse. Dopamine produces these effects by controlling the activity of two subpopulations of NAc medium spiny neurons (MSNs) that are defined by their predominant expression of either dopamine D1 or D2 receptors. Previous work has demonstrated that optogenetically stimulating D1 MSNs promotes reward, whereas stimulating D2 MSNs produces aversion. However, we still lack a clear understanding of how the endogenous activity of these cell types is affected by cocaine and encodes information that drives drug-associated behaviors. Using fiber photometry calcium imaging we define D1 MSNs as the specific population of cells in NAc that encodes information about drug associations and elucidate the temporal profile with which D1 activity is increased to drive drug seeking in response to contextual cues. Chronic cocaine exposure dysregulates these D1 signals to both prevent extinction and facilitate reinstatement of drug seeking to drive relapse. Directly manipulating these D1 signals using designer receptors exclusively activated by designer drugs prevents contextual associations. Together, these data elucidate the responses of D1- and D2-type MSNs in NAc to acute cocaine and during the formation of context-reward associations and define how prior cocaine exposure selectively dysregulates D1 signaling to drive relapse.

Dopamine neurons in awake behaving animals (20, 21). Here, we combine Ca2+ imaging and fiber photometry with conditioned place preference (CPP) to establish the patterns of activity of D1- and D2-type MSNs in NAc during formation of reward–context associations and to determine how these patterns are dysregulated.

Significance

Strong associations between cocaine and the environmental contexts where cocaine is administered thought to drive relapse. The nucleus accumbens (NAc) encodes these cue–reward associations and here we determined how cocaine alters the ability of cells in NAc to respond to drug-associated environmental stimuli to drive drug seeking. Using fiber photometry calcium imaging we define the specific population of cells, dopamine D1 receptor-expressing neurons, that encodes information about drug associations and show that these cells can be manipulated to attenuate the strength of drug associations and prevent relapse. Together, these data define a basic circuit mechanism underlying drug–context associations and suggest that pharmacotherapeutic agents aimed at D1-type neurons may help to promote sustained abstinence in cocaine abusers.


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by prior chronic exposure to cocaine. We then causally establish the role of altered firing of a given cell type to key aspects of context–reward associations by use of chemogenetic approaches.

**Results**

**Biphasic Ca^{2+} Responses in NAc to Cocaine-Associated Contextual Cues.** To examine real-time activity of NAc neurons in vivo during associative learning for drug rewards, we injected wild-type C57BL/6J mice with AAV-GCaMP6f—which infects all types of neurons but not nonneuronal cells—into NAc core and recorded Ca^{2+} transients through an optic fiber (20) while mice were trained for CPP (Fig. 1A and B). During pairing, cocaine reduced the frequency of Ca^{2+} transients in NAc (Fig. 1C and D). In a choice test 24 h after the final pairing, animals spent more time on the side that was previously paired with drug, indicating that cocaine had induced a place preference via an association between the context and the rewarding effects of cocaine (Fig. 1E). Robust spikes in Ca^{2+} activity were recorded immediately preceding entry into the drug-paired, but not saline-paired, chamber (Fig. 1F and G).

Further, firing was suppressed while the mouse remained in the drug-paired chamber (Fig. 1H and J).

**Specific Contribution of D1 and D2 MSNs to Learning Cocaine-Associated Cues.** These changes in NAc neuronal activity presumably reflect altered firing of MSNs, which comprise >95% of all NAc neurons. To determine the MSN subtypes that mediate the two distinct phases of NAc activity during context–reward associations, we injected AAV-DIO-GCaMP6f into NAc of mice expressing Cre-recombinase in either D1 or D2 MSNs to induce GCaMP6f expression specifically in each cell type (Fig. S1). At baseline, D2 MSNs displayed greater than fivefold higher Ca^{2+} transient frequency than D1 MSNs (Fig. 2C–E). Expression levels of GCaMP6f were not different between the neuron subtypes, indicating that the effect is physiological and not an artifact of the mouse line or viral expression (Fig. S1A and B). Because MSNs make up the vast majority of NAc neurons, the most parsimonious explanation of these findings is that D2 MSNs are more active at baseline, although one important caveat is that D2 receptors are also expressed by certain NAc interneurons. Single cocaine injections, during training, increased D1 MSN Ca^{2+} transient frequency, while reducing D2 frequency (Fig. 2A). During the subsequent choice test (Fig. 2B), entry into the drug-paired context similarly increased D1 and decreased D2 firing frequency (Fig. 2C and D), eliciting the same physiological response as cocaine had previously, suggesting that these contextual cues acquire rewarding properties (22). D1 and D2 MSNs displayed distinct temporal profiles of firing; increased D1 MSN activity immediately preceded entry into the drug-paired context (Fig. 2C and Movie S1), whereas D2 activity was suppressed only after entry (Fig. 2D and Movie S2). It is possible that D1 activity drives the motivation to enter a paired compartment, with the reduced activity of D2 cells after entry driving the motivation to remain in that compartment. Further, these data suggest that the biphasic neural signature of global NAc neuronal activity (Fig. 1H and J) is mediated by the two distinct populations of MSNs. Additionally, during choice testing, D1 MSN peak amplitude in the 5 s preceding entry into the drug-paired context correlated with time spent in the drug-paired side (Fig. 2E), suggesting that D1, but not D2 (Fig. 2F), signaling specifically drives expression of place preference.

**Cocaine Pretreatment Alters D1 MSN Signaling in Association with Reduced Extinction.** Animals were injected for 7 d with 10 mg/kg i.p. cocaine in their home cage followed by 7 d of withdrawal (Fig. S2) and then baseline MSN activity was recorded by CPP (Fig. 3A). Prior cocaine administration selectively increased baseline D1 MSN activity without affecting D2 activity (Fig. S3). Further, although cocaine administration did not alter initial CPP strength (Fig. 3B and C) it impaired later extinction of the place preference, indicating a latent potentiation of the cocaine–context association (Fig. 3C and D). This effect associated with heightened D1 activity (Fig. 3E) preceding drug-paired chamber entry that uniquely persisted throughout extinction in cocaine-preexposed animals (Fig. 3F). Cocaine pretreatment did not alter the magnitude of D2 MSN responses to the drug-associated contexts during choice testing, extinction, or reinstatement (Fig. 3G). These findings suggest that cocaine experience alters associative learning processes by selectively enhancing D1 MSN activity, to potentiate the association between drugs and cues and promote relapse during periods of abstinence.

**Cocaine Pretreatment Facilitates Reinstatement of Conditioned Place Preferences.** Previous work has shown that prior cocaine exposure induces time-dependent increases in cocaine seeking (6, 23). We found that a subthreshold challenge dose of cocaine [5 mg/kg i.p.; i.e., a dose insufficient to induce preference in cocaine-naive animals (24)] induced reinstatement of CPP only in animals with a history of prior chronic cocaine exposure and that the reinstated preference exceeded the initial preference for the drug-paired chamber (Fig. 3D). This increased preference was accompanied by an augmented spike in D1, but no change in D2

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**Fig. 1.** Biphasic responses of NAc neurons to cocaine-associated cues. (A) Timeline of the experimental design. (B) Viral expression of AAV-GCaMP6f and placement of the fiber-optic probe in NAc core. (C) Representative Ca^{2+} traces from a single animal over pairing sessions. Data are represented as the percent change in fluorescence over the mean fluorescence (ΔF/F). (D) Peak analysis of Ca^{2+} imaging traces. Cocaine reduces the number of events [Student’s t test; t(5) = 3.48, P < 0.05, n = 6]. (E) (Left) Animals formed a preference for the chamber associated with cocaine [Student’s t test; t(5) = 4.04, P < 0.01, n = 6]. (Right) Heat maps showing time spent in each area of the chamber. (F) Representative traces demonstrating the temporal profile of NAc activity around paired and unpaired chamber entry. (G) Quantification of the peak amplitude of the Ca^{2+} signal in the five seconds preceding paired and unpaired chamber entry [Student’s t test; t(5) = 4.38, P < 0.01, n = 6]. (H) Representative trace showing the change in event frequency when the animal is in the paired or unpaired chamber. (I) Quantification of event frequency [Student’s t test; t(5) = 2.68, P < 0.05, n = 6]. *P < 0.05, **P < 0.01.
Fig. 2. Specific contribution of D1 and D2 NAc MSNs to learning cocaine-associated cues. (A) Representative Ca^{2+} traces and peak analysis from a D1-Cre (Left, green) and D2-Cre (Right, blue) mouse showing higher activity of D2-MSNs under baseline conditions. Cocaine increases D1 events [Student’s t test; t(3) = 5.84, P < 0.05, n = 4] and decreases D2 events [Student’s t test; t(4) = 2.92, P < 0.05, n = 4] in NAc. (B) (Left) Animals formed a conditioned preference [Student’s t test; t(21) = 3.96, P < 0.001, n = 22]. (Right) Heat maps showing time spent in each area of the CPP chamber. (C) Top Representative heat map of D1-MSN-mediated Ca^{2+} signaling during successive entries into the drug-paired chamber. (Bottom) Averaged D1 traces (also see Movie S1). Quantification of peak amplitude of Ca^{2+} events 5 s around entry [Student’s t test; t(10) = 2.61, P < 0.05, n = 11]. (D) Top Heat map of D2-MSN-mediated Ca^{2+} signaling during successive entries into the drug-paired chamber. (Bottom) Averaged D2 traces (also see Movie S2). Quantification of peak amplitude of Ca^{2+} events [Student’s t test; t(9) = 2.70, P < 0.05, n = 10]. (E) Correlation analysis showing the relationship between CPP and the amplitude of D1 events preceding drug-paired chamber entry (r = 0.63, P < 0.05, n = 11). (F) Correlation analysis showing no relationship with D2 events (r = −0.15, P = not significant, n = 10). **P < 0.05, ***P < 0.001.

D1 MSN Activity Is Required for the Acquisition and Expression of Cocaine Conditioned Place Preferences. To confirm this causal role of D1 MSNs in associative learning, we expressed the inhibitory designer receptors exclusively activated by designer drugs (DREADD), hM4Di, selectively in D1 MSNs and administered clozapine-N-oxide (CNO; 5 mg/kg i.p.) before cocaine pairing to inhibit D1 activity (Fig. 4). CNO administration at this dose inhibited D1-specific Ca^{2+} activity (Fig. 4B). When CNO was administered 1 h before each cocaine pairing it blocked the cocaine-induced increases in D1 activity (Fig. 4 C and D). Further, it blocked both the preference for the drug-paired chamber as well as the spike in D1 MSN activity that preceded entry into the drug-paired chamber (Fig. 4 E–G), showing that this signal is indeed required for associative learning for drug rewards.

To assess whether the D1 signal is also causal for the decision to enter the drug-paired chamber, we administered CNO on choice test day (Fig. 4H). CNO administration completely abolished the temporally specific increase in D1 MSN activity that is associated with entry into the drug-paired chamber as well as the expression of a place preference (Fig. 4 I–K). Further, when animals were examined in a choice test 2 wk later, long after CNO had cleared, preference scores remained down (Fig. 4K), suggesting that inhibiting D1 firing in the presence of the associated context is sufficient to extinguish the association indefinitely. This could reflect enhanced extinction learning or blockade of reconsolidation. Regardless, the effect was specific for D1 MSNs: Inhibiting D2 MSN activity during the choice test had no effect on cocaine place preference (Fig. S6).

Discussion

Results of the present study demonstrate distinct patterns of D1 and D2 MSN signaling in NAc during cocaine reward learning, extinction, and reinstatement and provide fundamentally new insight into the circuit basis of drug–cue associations and drug seeking. We show that D2 MSNs exhibit manifold higher activity than D1 MSNs in NAc at baseline, that acute cocaine administration enhances D1 and suppresses D2 MSN activity, and that cocaine-induced facilitation of D1 MSN activity is required for formation of cocaine–context associations. Further, temporally precise, cell-type-specific signaling encodes contextual information about cocaine experiences such that increased D1 activity precedes entry into a drug-paired context, with decreased D2 activity occurring only after entry. Further, prior chronic exposure to cocaine impairs extinction of contextual associations by preventing the concomitant extinction of D1 MSN signaling that precedes drug-paired context entry. Inhibiting this D1 signal by DREADD-induced D1 MSN inhibition blocked the expression of conditioned preference, confirming that D1 signaling is the critical mediator of...
drug-context learning. More importantly, reducing the activity of D1 MSNs is sufficient to block (i.e., extinguish) the expression of preference, an effect that persists for weeks. Together, these data elucidate cell-type-specific engrams that contribute to the encoding of cocaine reward, and a D1 MSN-mediated mechanism by which cocaine exposure retards extinction of drug–cue associations to promote relapse.

The precise temporal profile of the encoding of cue–reward associations in MSNs provided here now enables elucidation of the circuit-wide signaling that controls these responses to drive reward- and reinforcement-related behaviors. The majority of work outlining the neural networks underlying cue–reward associations for both natural and drug rewards has shown that the ventral tegmental area (VTA) and NAc exhibit temporally specific signaling in response to unexpected rewards (25). However, once animals have learned the association between cues and reward availability the neural response transfers to the reward-predictive cue, thus guiding behavior toward environments that will result in obtaining that reward (26). Studies using in vivo pharmacological approaches have demonstrated differential roles of NAc D1 and D2 receptors in drug conditioning by use of selective receptor agonists or antagonists, further supporting a role for both dopamine and D1 and D2 MSN subtypes in associative learning (27). Whereas this work has focused on the VTA-to-NAc dopamine circuit, tracking postsynaptic responses in NAc MSNs is particularly important because they integrate information not only from VTA dopamine neurons but also from numerous glutamatergic projections (28, 29). From a network perspective, D1 and D2 MSNs receive inputs from several regions known to encode and store information about context or context–drug associations such as the prefrontal cortex, basolateral amygdala, and hippocampus (30). Although dopamine plays an integral role in controlling motivated behaviors, its effects are largely modulatory, and it is the glutamatergic inputs that provide the excitatory drive for these circuits. Thus, interactions between dopamine and glutamate are critical in the case of drugs of abuse where dopaminergic involvement changes over the course of drug administration. Initially,

Fig. 3. Chronic cocaine administration alters D1 MSN signaling in association with reduced extinction and facilitated reinstatement of CPP. (A) Experimental timeline. (B) Animals form a preference for the drug-paired chamber [two-way ANOVA; F (1, 88) = 14.17, P < 0.001, n = 14]; cocaine pretreatment does not change CPP. (C) Heat maps showing time spent in each chamber. (D) Chronic cocaine administration impairs extinction and facilitates cocaine-primed reinstatement [two-way ANOVA; F (1, 147) = 20.08, P < 0.0001; n = 20]. Data plotted as percent change from the original choice test. During extinction test 1, cocaine pretreated animals increased their preference (one-sample t test; t22 = 2.56, P < 0.05). During reinstatement, cocaine-pretreated animals reinstated above their original choice test values (one-sample t test; t22 = 2.09, P < 0.05). (E) Representative traces averaged over entries showing increased amplitude of D1 MSN Ca2+ activity at paired chamber entry during extinction. (F) Peak amplitude analysis. Saline-treated animals extinguish D1 MSN responses; cocaine-pretreated animals do not [two-way ANOVA; F (1, 17) = 4.492, P < 0.05, n = 10]. (G) Representative traces averaged over entries showing increased amplitude of D1 MSN Ca2+ activity at paired chamber entry during cocaine-primed reinstatement. (H) Peak amplitude analysis over trials. Cocaine-pretreated animals reinstate D1 MSN responses, whereas saline-pretreated animals do not [two-way ANOVA; F (1, 12) = 5.262, P < 0.05, n = 6, 9]. (I) Representative D1 MSN traces from a cocaine-treated (Left) and saline-treated (Right) animal during reinstatement. Acute effects of the cocaine challenge are augmented in cocaine-pretreated animals, but only when the animal is in the paired chamber. (J) Peak analysis: cocaine effects are enhanced in cocaine-pretreated animals in the previously drug-paired context [two-way ANOVA; F (1, 4) = 22.16, P < 0.01, n = 3]. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.
D1 MSN activity is required for the expression of cocaine CPP. (A) Confocal image showing hM4Di and GCaMP6f coexpression in NAc. (B) CNO reduces D1-mediated Ca\(^{2+}\) activity. (C) Representative D1 Ca\(^{2+}\) traces from mCherry (red) and hM4Di (black) animals following cocaine (10 mg/kg i.p.). (D) Peak analysis of D1 MSNs. CNO reduces the frequency of events [Student’s t test; (F) = 4.87, \(P < 0.01, n = 6\)]. (E) Animals were injected with CNO (5 mg/kg i.p.) before pairing sessions. (F) Representative traces averaged over entries. (G) Time spent in each area of the CPP chamber. (H) Animals form a preference for the drug-paired chamber, with CNO reducing the time spent in the drug-paired chamber [two-way ANOVA; (J) = 4.55, \(P < 0.05, n = 5, 10\)]. (I) Animals were injected with CNO before choice test. (J) Representative traces averaged over entries showing increased amplitude of D1 MSN Ca\(^{2+}\) activity at paired chamber entry. (K) Time spent in each area of the CPP chamber. (L) Animals form a preference for the drug-paired chamber, with CNO reducing the time spent in the drug-paired chamber. The reduced preference in hM4Di animals remained 2 wk after the initial choice test [two-way ANOVA; (L) = 5.32, \(P < 0.05, n = 5, 10\)]. *\(P < 0.05\), **\(P < 0.01\).

Prior chronic cocaine exposure not only inhibits extinction of place conditioning but also facilitates reinstatement of place conditioning. Similar dysregulated associative learning concerning environmental stimuli and rewards occurs in human drug addicts, and an inability to extinguish previously formed associations is thought to contribute to pathological drug seeking as well as relapse (6, 7). Our findings provide a neural mechanism for the deficits in extinction and enhanced reinstatement, whereby cocaine treatment (i) dysregulates the ability of D1 MSNs to respond to context information and update information when the association no longer occurs and (ii) enhances the effects of challenge doses of cocaine on D1 MSN signaling only when animals are in the drug-paired context. These data show that, in addition to the contextual cues eliciting a temporally specific response, the context in which drug is administered can greatly enhance the pharmacological effects of the drug, as has been demonstrated in many other settings (32, 33). Enhancement of the pharmacological effects of drugs is likely one mechanism by which the presentation of drug-paired cues can increase the motivation to administer cocaine.

Our data highlight the important role played by D1 MSNs in NAc core in establishing context–reward associations and in controlling the strength of these associations after cocaine exposure. This prominent influence of D1 MSNs in cocaine action is consistent with previous studies that have shown that chronic cocaine exposure alters D1 MSNs selectively via both changes in dendritic morphology and function as well as changes in gene transcription and epigenetic mechanisms that last long into the abstinence period (34–36). Further work is needed to determine whether similar patterns of D1 MSN activity in NAc core as shown here are also
observed in NAc shell and to establish the function of cocaine-induced changes in D2 MSNs that have also been demonstrated. Here we show that regulation of associative learning, and its dysregulation by cocaine, is driven primarily through alterations in D1 MSNs in NAc, which both impair the extinction of previously learned associations and enhance reinstatement following abstinence. The magnitude of D1 firing to reward-associated cues may play a critical role in determining the perceived value of a reward and bias decision making toward outcomes most likely to result in obtaining that reward. Further, we show that inhibiting D1 MSNs in NAc is sufficient to prevent preference for contexts previously paired with a drug reward, an effect that lasts indefinitely. These findings suggest that therapies targeted selectively to D1 MSNs may help to normalize D1 activity following a history of cocaine use and prevent relapse. Additionally, the causal role of temporally specific cue-elicited D1 signaling in the expression of learned associations demonstrates the importance of this cell type in the development of fundamental associative learning processes. Disruption of associative learning is a hallmark of diverse psychopathologies that span developmental stages. Demonstrating the causal role of NAc D1 MSN signaling in mediating these processes provides fundamental insight to guide the development of novel targeted therapeutic interventions not only for addiction but also for a wide range of psychiatric disorders.

Methods
See SI Methods for more detailed explanations.

Experimental Subjects. D1-Cre and D2-Cre BAC transgenic mice on a C57BL/6J background were obtained from N. Heintz, P. Greengard, C. Gerfen, and National Institute of Neurological Disorders and Stroke/Genes Expression System. Rockefeller University/National Institute of Mental Health. C57BL/6J wild-type mice were obtained from The Jackson Laboratory (SN: 000664). All experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee at Icahn School of Medicine at Mount Sinai.

Stereo tactic Virus Injection and Cannula Implantation. AA.VV:CaMKIIa-GCaMP6F-WPRE (for pan-neuronal recordings), AA.VV:EF1a-DIO-GCaMP6F-WPRE (for cell-type-specific recording), or AA.VV:Syn-DIO-hmDi-mcherry [University of North Carolina at Chapel Hill (37), for DREADD inhibition studies] was infused into NAc core. Chronically implantable optical fibers (Doric Lenses) were positioned above the injection site.

Fiber Photometry Ca²⁺ Imaging. Fiber photometry uses the same fiber to both excite and record from GCaMP in real time. The system used two light-emitting diodes at 490 and 405 (Thor Labs), reflected off dichroic mirrors (Semrock, FF495), and coupled into a 400-μm 0.48 N.A. optical fiber (BFH48-600; Thorlabs). Analysis of the resulting signal was performed with custom-written MATLAB software.

Behavioral Testing. CPP was performed in a rectangular apparatus consisting of two side chambers with different contextual cues. Mice were paired with cocaine in one of the chambers and saline in the other chamber. After pairing, during a choice test, mice were allowed to freely explore the entire apparatus and preference for each chamber was determined. During extinction animals were placed into the chamber with free access. For reinstatement, animals were injected with 5 mg/kg cocaine i.p. immediately before being placed into the chamber with free access. For DREADD experiments, CNO (5 mg/kg) was administered 1 h before the session.

Statistics. For groups of three or more, one-way ANOVA was run. For groups of two a two-tailed Student’s t test was run. Significance was set at P < 0.05 for all tests. All data are expressed as mean ± SEM.

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