Loss of Plasticity in the D2-Accumbens Pallidal Pathway Promotes Cocaine Seeking

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Distinct populations of D1- and D2-dopamine receptor-expressing medium spiny neurons (D1-/D2-MSNs) comprise the nucleus accumbens, and activity in D1-MSNs promotes, whereas activity in D2-MSNs inhibits, motivated behaviors. We used chemogenetics to extend D1-/D2-MSN cell specific regulation to cue-reinstated cocaine seeking in a mouse model of self-administration and relapse, and found that either increasing activity in D1-MSNs or decreasing activity in D2-MSNs augmented cue-induced reinstatement. Both D1- and D2-MSNs provide substantial GABAergic innervation to the ventral pallidum, and chemogenetic inhibition of ventral pallidal neurons blocked the augmented reinstatement elicited by chemogenetic regulation of either D1- or D2-MSNs. Because D1- and D2-MSNs innervate overlapping populations of ventral pallidal neurons, we next used optogenetics to examine whether changes in synaptic plasticity in D1- versus D2-MSN GABAergic synapses in the ventral pallidum could explain the differential regulation of VP activity. In mice trained to self-administer cocaine, GABAergic LTD was abolished in D2-, but not in D1-MSN synapses. A μ opioid receptor antagonist restored GABA currents in D2-, but not D1-MSN synapses of cocaine-trained mice, indicating that increased enkephalin tone on presynaptic μ opioid receptors was responsible for occluding the LTD. These results identify a behavioral function for D1-MSN innervation of the ventral pallidum, and suggest that losing LTD_{GABA} in D2-MSN, but not D1-MSN input to ventral pallidum may promote cue-induced reinstatement of cocaine-seeking.

Key words: accumbens; cocaine; GABA; LTD; pallidum; relapse

Significance Statement

More than 90% of ventral striatum is composed of two cell types, those expressing dopamine D1 or D2 receptors, which exert opposing roles on motivated behavior. Both cell types send GABAergic projections to the ventral pallidum and were found to differentially promote cue-induced reinstatement of cocaine seeking via the ventral pallidum. Furthermore, after cocaine self-administration, synaptic plasticity was selectively lost in D2, but not D1 inputs to the ventral pallidum. The selective impairment in D2 afferents may promote the influence of D1 inputs to drive relapse to cocaine seeking.

Introduction

The striatum is composed of D1- and D2-dopamine receptor-expressing medium spiny neurons (D1- and D2-MSNs) that

canonically project through distinct circuits. Thus, D1-MSNs innervate the ventral mesencephalon via the “direct” pathway and D2-MSNs innervate the globus pallidus via the “indirect” pathway (Gerfen and Surmeier, 2011). Contrasting this view, the nucleus accumbens, which occupies the ventromedial quadrant of the rodent striatum (Zahm and Heimer, 1988), has abundant D1- and D2-MSN innervation of the ventral portion of the globus pallidus, the ventral pallidum (VP) (Lu et al., 1998; Kupchik et al., 2015).

The nucleus accumbens and its projection to the VP regulates the expression of behavioral responding to motivationally relevant stimuli, including addictive drugs (Koob and Volkow, 2010; Floresco, 2015; Laurent et al., 2015), and the advent of D1- and D2-Cre transgenic mice has allowed investigators to selectively probe the role of D1- and D2-MSNs in regulating behavior (Lobo

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and Nestler, 2011; Kravitz et al., 2012; Britt and Bonci, 2013). Activating D1-MSNs promotes the execution of motivated behaviors, such as sucrose consumption and use of addictive drugs, whereas stimulating D2-MSNs has the opposite effect (Lobo et al., 2010; Bock et al., 2013; Francis et al., 2015). Also, cocaine exposure increases the strength of synaptic inputs onto D1-MSNs, which has been linked to enhanced motivation to use and seek cocaine (Pascoli et al., 2011, 2014; Bock et al., 2013). These studies in transgenic mice are generally interpreted to indicate distinct behavioral functions for D1-MSN direct projections to the ventral mesencephalon and the D2-MSN indirect projections to the VP. However, because both D1- and D2-MSNs strongly innervate the VP, we hypothesized that cue-induced cocaine seeking is regulated by both D1- and D2-MSN innervation of the VP. To test this hypothesis, we used a chemogenetic strategy using designer receptors exclusively activated by designer drugs (DREADDs) coupled to either Gs (rM3D) or Gi (hM4D), to mimic the G-protein signaling of dopamine onto D1- or D2-receptors, respectively (Armbruster et al., 2007; Farrell et al., 2013; Goto et al., 2015; Roth, 2016). To test the propensity of discrete stimuli to elicit drug seeking behavior, we used a mouse model of cocaine self-administration and extinction where the animal can press a lever to obtain an intravenous delivery of drug over the course of multiple days, after which this behavior is extinguished in subsequent sessions without drug availability. In this model, returning drug-paired cues to each lever press reinvigorates drug-seeking behavior (Fuchs et al., 2003; Panlilio and Goldberg, 2007). Combining these methods, we found that both the D1 and D2 inputs to the VP regulate cocaine seeking, with activity in the D1 pathway facilitating and D2 pathway reducing cued reinstatement.

Because both D1- and D2-MSN terminals release GABA onto the same VP neurons (Kupchik et al., 2015), we investigated whether differences in synaptic plasticity might underlie these behavioral effects using an optogenetic strategy in *D1-* and *D2-Cre* mice. After extinction of cocaine self-administration, there was a loss of GABAergic LTD (LTD_{GABA}) in synapses of the D2, not D1, pathway to the VP, and this loss was likely mediated by elevated inhibitory tone on presynaptic μ opioid receptors (MORs) on D2 pathway afferents, which occluded the induction of LTD_{GABA} . Together, our data show a behavioral function for the D1 pathway to the VP, and that impaired synaptic plasticity in D2 pathway synapses may predispose toward enhanced reinstatement of cocaine seeking via the D1-MSN innervation of the VP.

Materials and Methods

Animals. *D1-* and *D2-Cre* BAC transgenic lines were obtained from N. Heintz, P. Greengard (Rockefeller University), C. Gerfen (National Institute of Mental Health), and NINDS/GENSAT (www.gensat.org). These transgenic mice express Cre recombinase under control of the dopamine D1 receptor promoter (*Drd1a*, line FK150, RRID:MMRRC_029178-UCD) or dopamine D2 receptor promoter (*Drd2*, line ER44, RRID:MMRRC_017263-UCD). Mice were bred in house and periodically outbred with wild-type C57BL/6J (The Jackson Laboratory). Before experimental procedures, mice were group housed, fed *ad libitum*, and maintained in a humidity- and temperature-controlled environment. All experiments occurred during the dark phase and were conducted in accordance with the National Institute of Health's *Guidelines for the care and use of laboratory animals*. All procedures were approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina.

Surgery. Mice (20–30 g) of either sex were anesthetized with isoflurane (induction 3%–5% v/v, maintenance 1%–2% v/v) and implanted with

an indwelling jugular catheter connected to a head-mounted entry port as described previously (Griffin and Middaugh, 2003). In brief, the neck of the mouse was incised and the tip of the catheter was inserted ~5 mm from the clavicle between 10 and 12 mm into the vein. The catheter was sutured in place, and the entry port was secured to the head of the mouse using dental cement. Cephazolin (200 mg/kg, i.v.) was administered acutely to prevent infection, and carprofen (5 mg/kg, s.c.) and topical antibacterial ointment were given postoperatively for 2–3 d following surgery. Catheters were flushed twice daily with heparinized (100 units/ml) normal saline.

For viral microinfusions, microinjection needles (33 Ga; Plastics One) were connected to a microinjection pump and injections (300 nl) were delivered at a rate of 50 nl/min. Afterward injectors were left in place for 10 min to allow for diffusion of the virus and then slowly retracted. To achieve cell-type specific expression, double-floxed inverted orientation (DIO) transgenes were introduced into Cre recombinase-expressing neurons using AAV viral vectors (AAV2-hSyn-DIO-hM4D-mCherry; titer $\sim 4.6 \times 10^{12}$; AAV2-hSyn-DIO-rM3D-mCherry, titer $\sim 6.5 \times 10^{12}$; AAV2-EF1a-hChR2(H134R)-eYFP, titer $\sim 6.0 \times 10^{12}$) infused in the nucleus accumbens (in mm from bregma: anteroposterior: 1.5; medio-lateral: ± 1.2 ; dorsoventral: -4.4). Additionally, a nonfloxed vector was infused into the VP (in mm from bregma: anteroposterior: 0.2; medio-lateral: 1.5; dorsoventral: -4.9 ; AAV2-hSyn-HA-hM4D-IRES-mCitrine, titer $\sim 2.6 \times 10^{12}$). All vectors were generated by the University of North Carolina Vector Core. Following surgery, mice were single housed and given at least 3 d of recovery before the start of behavioral experiments, and a minimum of 3 weeks was used between surgery and slice electrophysiology or behavioral testing to allow for expression of viral vectors.

Cocaine self-administration, extinction, and reinstatement. Following surgery, catheters were maintained by flushing twice daily with heparinized saline throughout the procedure, and catheter patency was checked at the end of the self-administration phase for all mice by using the short-acting barbiturate Brevital (0.2 mg i.v.). Mice in which apparent signs of hypotonia were not observed within 3 s were excluded from the study. Mice were trained for 10–15 d on a fixed ratio 1 schedule of reinforcement during daily 2 h cocaine self-administration sessions until they reached criterion of at least 3 d of 15 cocaine infusions. During the first 3–6 sessions, mice were mildly food restrained (to 90%–95% of their free feeding weight) to stimulate exploration of the operant box. During each session, presses on the active lever resulted in the infusion of cocaine (~ 0.75 mg/kg/infusion, in sterile saline) paired with a compound stimulus (2 s tone + light), followed by a 20 s time-out period during which cocaine was unavailable. Yoked saline control mice received saline infusions and cue presentations in the same manner, independent of their own lever responding. Following self-administration, mice were extinguished for at least 10 d until criterion was reached (2 successive days < 20 active lever presses/d) and given cue-induced reinstatement tests in counterbalanced order with at least 2 d of extinction training in between. Before each test mice were injected with CNO (1 mg/kg, i.p., Tocris Bioscience, #4936) or vehicle (sterile saline) and returned to their home cage for 30 min. Cue-induced reinstatement tests lasted 2 h and responding on the active lever produced the tone + light cue previously paired with cocaine on the same 20 s fixed interval time-out schedule. After self-administration, mice for electrophysiological experiments were run for at least 10 daily extinction session, and recordings were performed 24 h after the last session.

Sucrose self-administration, extinction, and reinstatement. Following stereotaxic surgery, mice were mildly food deprived ($\sim 90\%$ – 95% of free feeding weight) and trained to self-administer sucrose pellets (14 mg, BioServ) on a fixed ratio 1 schedule of reinforcement wherein active lever pressing resulted in the delivery of a sucrose pellet in a food receptacle and presentation of the same compound (light + tone) cue as for the cocaine studies. All mice self-administered sucrose for at least 12 d followed by 10 d of extinction training. Afterward mice received an injection of CNO (1 mg/kg, i.p.) or vehicle (sterile saline) and were returned to their home cage for 30 min before reinstatement testing. During cue-induced reinstatement, active lever presses resulted in the presentation of the compound stimulus that previously signaled sucrose delivery. During cue- and pellet-primed reinstatement in the first 10 min of the ses-

sion, every 2 min a pellet was delivered noncontingently, followed by another noncontingent pellet delivered every 20 min during the rest of the session.

Slice preparation and whole-cell patch-clamp recording. Fresh VP slices (220 μm ; VT100S Leica vibratome) were collected into a vial containing aCSF as follows (in mM: 126 NaCl, 1.4 NaH_2PO_4 , 25 NaHCO_3 , 11 glucose, 1.2 MgCl_2 , 2.4 CaCl_2 , 2.5 KCl, 2.0 sodium pyruvate, 0.4 ascorbic acid, bubbled with 95% O_2 and 5% CO_2) and a mixture of 5 mM kynurenic acid and 50 μM D-APV. Slices were kept at 22°C–24°C until they were used for recordings, and were constantly perfused with oxygenated aCSF heated to 32°C (TC-344B, Warner Instruments). Excitatory synaptic transmission was blocked with 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM). Neurons were visualized with a Zeiss AxioScope 2 FS plus microscope with a 40 $\times$ objective. Recordings of synaptic currents were performed in voltage-clamp configuration at a membrane potential of -80 mV (Multiclamp 700B, Molecular Devices). Glass microelectrodes (1.5–2.5 $\text{M}\Omega$ tip resistance) were prepared using a PC-10 vertical puller (Narishige) and filled with internal solution as follows (in mM: 68 KCl, 65 D-gluconic acid potassium salt, 7.5 HEPES potassium, 1 EGTA, 1.25 MgCl_2 , 10 NaCl, 2.0 MgATP , and 0.4 NaGTP ; pH 7.2–7.3, 275 mOsm). Data were acquired at 10 kHz and filtered at 2 kHz using Axograph X software (Axograph Scientific). IPSCs were evoked optogenetically, and the stimulation intensity was set to evoke an IPSC with amplitudes within the dynamic range of each neuron (i.e., 30%–70% of maximal IPSCs, 200–900 pA). The light pulse was produced by a 460 nm LED (Mightex) that was transmitted on the slice through the microscope objective. For the LTD experiments, a high-frequency stimulation (HFS) protocol was delivered electrically via a bipolar stimulation electrode positioned 200–300 μm dorsomedial to the recorded cell. The HFS protocol consisted of three trains separated by 20 s, each delivering 100 stimulations at 100 Hz. The intensity (100–600 μA) was set to elicit IPSCs of the same amplitude as for optically induced IPSCs from each neuron. Recordings of synaptic transmission were started 10 min after invading the cell to allow for internal solution diffusion to remote dendrites. Membrane capacitance and input resistance were calculated automatically from a -2 mV pulse (Axograph X software, Axograph Scientific). Recordings with unstable series resistance or with a series resistance >20 $\text{M}\Omega$ were discarded.

Histology. Mice were transcardially perfused with ice-cold phosphate buffer (0.1 M, pH 7.4), followed by 4% PFA solution. After extraction, brains were postfixed in 4% PFA solution overnight and then transferred to 20% sucrose in PBS for an additional 24 h. Brains were frozen on dry ice and sliced on a microtome at 35 μm . Slices were blocked in PBS containing 5% normal goat serum, 2.5% BSA, and 0.25–0.5% Triton X-100, and incubated in primary antibody overnight (mouse anti-NeuN 1:1000, Millipore #MAB377, RRID:AB_2298772; rabbit anti-dsRed, 1:1000, Clontech #632496, RRID:AB_10013483; rabbit anti-DARPP-32, 1:1000, Abcam #ab18551, RRID:AB_2284277; chicken anti-GFP, 1:500, Abcam #ab13970, RRID:AB_300798; rabbit anti-met-enkephalin, 1:1000, Immunostar #20065, RRID:AB_572250) followed by 2 h incubation at room temperature in Alexa-conjugated secondary antibodies (Thermo Fisher, 1:1000). Images were acquired as stacks using a SP6 confocal microscope (Leica Microsystems), deconvolved (AutoQuant X2, MediaCybernetics), and visualized in Imaris (Bitplane).

Statistical analysis. All data are represented as mean $\pm$ SEM. Statistical analyses were performed using Prism version 6.0 (GraphPad). Two-way repeated-measures ANOVA, one-way ANOVA, and paired and unpaired Student's *t* tests were used for electrophysiological and behavioral data as specified in Results. One-sample *t* test was used for normalized IPSC comparisons. Statistical significance was set at 0.05.

Results

Cocaine self-administration and functional expression of genetic constructs in D1- and D2-Cre mice

D1- and D2-Cre mice underwent 12 d of cocaine self-administration or yoked-saline delivery followed by at least 10 d of extinction training (2 h each day; Fig. 1A). Infusion of cocaine was paired with the presentation of a compound light/tone cue.

Figure 1. Cocaine self-administration and viral infection with optogenetic and chemogenetic transgenes in D1-Cre and D2-Cre mouse lines. **A**, D1-Cre ($n = 27$) and D2-Cre ($n = 29$) mice were trained to self-administer cocaine followed by extinction training. Note the burst of active lever responding on day 1 of extinction. **B**, Coronal slice showing representative Gi-DREADD hM4D expression in the accumbens core, and a higher-magnification inset showing that expression colabeled with the MSN marker DARPP-32. Left scale bar, 300 μm . Right scale bar, 50 μm . ac, Anterior commissure. Arrows indicate dorsoventral (DV) and mediolateral (ML) orientation.

Figure 2. Chemogenetic inhibition of D1 and D2 terminals abolished optical eIPSC in VP neurons. **A**, Sagittal sections showing Gi-DREADD and ChR2 expression in the projection from the accumbens to the VP, in particular to the dorsolateral VP subcompartment (dlVP) and to a lesser extent the ventromedial subcompartment (vmVP). Scale bar, 300 μm . ac, Anterior commissure. Arrow and letters indicate anteroposterior (AP) and dorsoventral (DV) orientation. **B**, Electrophysiological demonstration that optically evoked eIPSCs from D1 and D2 projections were inhibited by bath applying CNO (1 μM). D1 projection ($n = 6$), D2 projection ($n = 5$), and Control ($n = 4$) cells from non-DREADD-infected D1- and D2-MSN.

Figure 3. Augmented cocaine seeking produced by activating D1 MSNs depends on activity in the VP. *D1-Cre* mice were injected either with Gs-DREADD rM3D in the accumbens core or with a combination of Gs-DREADD in the accumbens core and Gi-DREADD hM4D in the VP. **A**, Middle, Low-magnification section of Gi DREADD expression in the VP. Left, Higher magnification with colabeled immunostaining for met-enkephalin, a histological marker for the VP. Scale bar, 300 μ m. ac, Anterior commissure; cc, corpus callosum; ic, internal capsule; lv, lateral ventricle; BST, bed nuclei of the stria terminalis; DBH, horizontal nucleus of the diagonal band; LH, lateral hypothalamus; SI, substantia innominata. Arrows indicate dorsoventral (DV) and mediolateral (ML) orientation. **B**, Example of accumbens terminals in the dorsolateral VP expressing mCherry (red)-labeled Gs-DREADD surrounded by mCitrine-labeled Gi DREADD (green) infected VP neurons (for coronal example of accumbens core injection site, see Fig. 1B). Scale bars: left, 300 μ m; right, 60 μ m. CPu, Caudate-putamen; dlVP, dorsolateral part of the VP. **C**, Bath application of CNO (1 μ M) hyperpolarized VP neurons infected with Gi-DREADD. **D**, Average active lever, inactive lever presses, and cocaine infusions earned over last 3 d of self-administration (top) and active and inactive lever pressing over last 2 d of extinction training (bottom) for *D1-Cre* mice. **E**, Stimulation of Gs-DREADD in D1-MSNs augmented cue-induced reinstatement, which was reversed by the simultaneous inhibition of the VP. Numbers in all bars indicate animals. * $p < 0.05$, Tukey *post hoc* test. **F**, Summary of average active lever, inactive lever presses during self-administration, and extinction data for *D2-Cre* mice infected with Gs-DREADD in accumbens D2-MSNs. **G**, Stimulating Gs-DREADD in D2-MSNs had no effect on cue-induced reinstatement of cocaine seeking.

There was no difference in lever pressing between the two transgenic mouse strains and both strains distinguished between the active and inactive lever after 3 d of self-administration (Fig. 1A). Both strains showed a marked extinction burst, evidenced by an increase in active lever pressing during the first extinction session compared with the average last 3 d of self-administration (Fig. 1A; *D1-Cre*: $t_{(25)} = 3.681$, $p = 0.001$; *D2-Cre*: $t_{(28)} = 4.342$, $p < 0.001$). All mice were microinjected with AAV vectors carrying transgenes for the DIO Gs-coupled receptor rM3D (Gs-DREADD; Fig. 1B), and the DIO or nonfloxed Gi-coupled hM4D receptor (Gi-DREADD; see Figs. 2A, 3A, B, 4B). Alternatively, for electrophysiological studies mice were infected with an AAV carrying a DIO channel rhodopsin (ChR2) (Fig. 2A). Microinjections were targeted to the nucleus accumbens core adjacent to the anterior commissure and showed limited spread into the medial and ventral accumbens shell. Expression of the transgenes was localized to MSNs as indicated by DARPP-32 counterstaining (Fig. 1B) (Bertran-Gonzalez et al., 2008).

To demonstrate the efficacy of these genetic reagents in modulating activity in the accumbens-pallidal pathway, separate groups of drug-naïve *D1-* and *D2-Cre* mice were infected with a combination of DIO Gi-DREADD and ChR2 vectors. Both constructs expressed robustly in the D1 and D2 projections to the VP (Fig. 2A). Optical stimulation (460 nm) evoked eIPSCs in neurons of the dorsolateral VP, and bath application of the Gi-DREADD ligand CNO (1 μ M) abolished these eIPSCs from both D1 and D2 pathway projections onto VP neurons demonstrating significant terminal silencing by Gi-DREADD (Fig. 2B; two-way ANOVA: effect of pathway $F_{(2,12)} = 51.48$, $p < 0.001$, drug $F_{(1,12)} = 240.7$, $p < 0.001$, and interaction $F_{(2,12)} = 54.09$, $p < 0.001$).

Activating D1-MSNs augmented reinstatement of cocaine seeking via projections to the VP

Activation of D1-MSNs potentiates the execution of motivated behaviors, whereas activation of D2-MSNs reduces them (Lobo and Nestler, 2011; Smith et al., 2013). Dopamine exerts its effects on D1-MSNs by stimulating the Gs signaling cascade, which enhances the excitability of these neurons when in a relatively depolarized up state, without directly altering membrane potential (Surmeier et al., 2007). In line with this, activation of the Gs-DREADD does not change the resting membrane potential of infected neurons (Nakajima et al., 2016). The nucleus accumbens of *D1-Cre* mice was transduced with the DIO Gs-DREADD rM3D and the nonfloxed Gi-DREADD hM4D into the VP to allow simultaneous activation of D1-MSN and inhibition of VP neurons (Fig. 3A,B). To test the functionality of the Gi-DREADD in the VP, infected neurons were patched in current clamp while CNO (1 μ M) was bath applied to measure changes in membrane potential. CNO significantly hyperpolarized VP neurons infected with the Gi-DREADD (Fig. 3C; ΔV_m mean $\pm$ SEM: -4.03 ± 1.44 ; $t_{(7)} = 2.80$, $p = 0.027$ comparing min 1–5 with baseline). Activating D1-MSNs with the Gs-DREADD during cue-induced reinstatement increased cocaine seeking (Fig. 3D,E), and this was reversed by the simultaneous inhibition of VP neurons with Gi-DREADD (one-way ANOVA: $F_{(2,19)} = 5.48$, $p = 0.013$). In contrast, activating D2-MSNs in *D2-Cre* mice infected with Gs-DREADD using CNO did not reveal any effect during cue-induced reinstatement (Fig. 3F,G; $t_{(6)} = 0.834$, $p = 0.436$). These data demonstrate that activating D1-MSNs is sufficient to potentiate cocaine seeking, most likely via their projections to the VP.

Inhibiting D2-MSNs augmented reinstatement of cocaine seeking via projections to the VP

D2-Cre mice were injected with the inhibitory DIO Gi-DREADD targeted to the nucleus accumbens core and trained to self-administer cocaine. In addition, some *D2-Cre* mice received a second microinjection with nonfloxed Gi-DREADD in the VP (Fig. 4*A,B*). Following cocaine self-administration and extinction, mice underwent a test for cue-induced reinstatement after pretreatment with either vehicle or CNO (1 mg/kg, i.p.). Inhibition of D2-MSNs with the Gi-DREADD augmented cue-induced reinstatement (Fig. 4*C,D*). The augmented cocaine seeking was reversed by simultaneous inhibition of neurons in the VP with the Gi-DREADD (one-way ANOVA: $F_{(2,19)} = 15.71$; $p < 0.001$). In contrast, inhibiting D1-MSNs did not affect cue-induced cocaine seeking ($t_{(5)} = 1.106$, $p = 0.319$; Fig. 4*E,F*). These data show that inhibiting D2-MSNs potentiates reinstatement, which depends on the D2 projection to the VP.

Activating D1-MSNs did not alter reinstatement of sucrose seeking

In addition to its critical role in drug seeking, the accumbens core is also involved in food seeking behavior (Peters and Kalivas, 2006; Floresco et al., 2008), and sucrose consumption (Francis et al., 2015). These studies suggest that the bidirectional effect of D1- versus D2-MSNs extends to non-drug-related motivated behaviors. We explored the generalizability of our findings to food reward by chemogenetically activating D1- and D2-MSNs transfected with the DIO Gs-DREADD during the reinstatement of sucrose seeking. Following sucrose self-administration and extinction, in a manner analogous to that of cocaine (Fig. 5*A,C*), mice were pretreated with CNO or vehicle, and tested for cue-induced reinstatement of sucrose seeking. In contrast with cocaine-seeking, no effects of CNO were observed in either *D1-Cre* mice (Fig. 5*B*; $t_{(10)} = 0.58$, $p = 0.572$) or *D2-Cre* mice (Fig. 5*D*; $t_{(10)} = 0.250$, $p = 0.811$). This was surprising given the studies showing that selective D1-MSN stimulation reinforces behavior (Lobo et al., 2010; Ferguson et al., 2011; Kravitz et al., 2012), and indicates that while the cue-cocaine association can be regulated by accumbens D1- and D2-MSNs, the cue-sucrose association might be independent of the accumbens. One possibility is that previous studies showing a role for the accumbens used food, not food-paired conditioned cues to motivate animals. To explore the possibility that food availability is critical, *D1-Cre* mice infected with DIO Gs-DREADD were re-extinguished for at least 2 d after the cue-induced reinstatement session and tested for reinstatement elicited by both cues and noncontingent sucrose delivery. Under these conditions, a marked potentiation of reinstatement by CNO was evident (Fig. 5*B*; $t_{(9)} = 3.352$, $p = 0.009$). In *D2-Cre* mice infected with DIO Gs-DREADD, CNO did not potentiate reinstatement induced by both cues and sucrose pellets (Fig. 5*D*; $t_{(6)} = 0.721$, $p = 0.498$).

Cocaine self-administration selectively altered synaptic plasticity in the D2 pathway while sparing plasticity in the D1 pathway to the VP

Both D1- and D2-MSN projections from the nucleus accumbens core robustly innervate the VP (Lu et al., 1998; Francis et al., 2015), and at least 50% of dorsolateral VP neurons are innervated by both MSNs (Kupchik et al., 2015). Because activity in VP neurons was necessary for the chemogenetic regulation of cocaine seeking in both *D1-* and *D2-Cre* mice, we next investigated how parallel innervation of the VP by distinct D1- and D2-MSNs could contribute to cocaine seeking. We postulated that cocaine

self-administration might differentially alter GABAergic synaptic transmission in these pathways in a manner that facilitates reinstated behavior. To evaluate the synaptic physiology of the D1 and D2 pathways to the VP, *D1-* and *D2-Cre* mice received microinjections into the nucleus accumbens core with an AAV the DIO ChR2 transgene (Kupchik et al., 2015), resulting in the labeling of ChR2-expressing terminals in the VP (Fig. 2*A*). Following cocaine self-administration and extinction, whole-cell patch recordings were obtained from dorsolateral VP neurons, and GABAergic eIPSCs were evoked by application of blue light pulses (0.5–1 ms duration) (Fig. 6*A,B*). Using an electrical HFS protocol previously shown to induce LTD_{GABA} in the accumbens-pallidal pathway in rats (Kupchik et al., 2014), we induced LTD_{GABA} in synapses from both the D1 and D2 pathway onto VP neurons of yoked saline (D1: $n = 7$; D2: $n = 8$) and drug-naïve (D1: $n = 2$; D2: $n = 1$) mice. No differences were observed between these two groups; data were thus pooled for analyses. The HFS protocol induced LTD_{GABA} (Fig. 6*C*; two-way ANOVA: effect of time $F_{(19,304)} = 2.97$, $p < 0.001$), and no differences were observed between LTD_{GABA} in D1 and D2 pathway eIPSCs. Comparing baseline to the average eIPSC over 6–15 min after inducing LTD_{GABA} revealed significant LTD in both D1 ($t_{(8)} = 2.82$, $p = 0.023$) and D2 pathways (Fig. 6*D*; $t_{(8)} = 3.47$, $p = 0.008$). By contrast, in VP neurons from cocaine self-administering and extinguished mice, HFS failed to induce LTD_{GABA} in the D2 pathway (Fig. 6*F*; two-way ANOVA: effect of pathway $F_{(1,11)} = 12.83$, $p = 0.004$, and interaction $F_{(19,209)} = 2.925$, $p < 0.001$, but no effect of time). Average post-HFS eIPSC amplitude compared with baseline revealed that this effect was specific to the D2 pathway (Fig. 6*G*; $t_{(5)} = 3.81$, $p = 0.013$), whereas LTD_{GABA} in the D1 pathway remained intact ($t_{(6)} = 1.54$, $p = 0.176$).

We next assessed the paired pulse ratio (PPR) after LTD_{GABA} induction to assess whether this LTD_{GABA} was presynaptic. The PPR was increased for both D1 and D2 pathways in control mice (Fig. 6*E*; $t_{(7)} = 2.65$, $p = 0.033$ and $t_{(8)} = 5.11$, $p < 0.001$ for D1 and D2 pathway terminals, respectively). Similarly, in cocaine-extinguished mice, the PPR was increased after inducing LTD_{GABA} in D1-MSNs (Fig. 6*H*; $t_{(3)} = 3.14$, $p = 0.025$). However, the failure to induce LTD_{GABA} in D2 pathway afferents of cocaine-extinguished mice was associated with no change in the PPR (Fig. 6*H*; $t_{(6)} = 1.29$, $p = 0.245$). Together, these PPR data indicate that the ability to induce LTD_{GABA} is likely due to a decrease in presynaptic transmitter release probability (Bonci and Williams, 1997).

Persistent activation of MOR accounts for the loss of LTD in the D2 pathway to the VP

Enkephalin is colocalized with GABA in D2-, but not D1-expressing, MSNs (Zahm et al., 1985; Lu et al., 1998) and modulates neurotransmission in the accumbens-pallidal pathway (Chrobak and Napier, 1993). These effects are likely mediated by presynaptic MOR located on MSN terminals within the VP (Olive et al., 1997; Kupchik et al., 2014). Consistent with recent findings that GABAergic synapses in the VP undergo a MOR-dependent LTD after the electrically stimulated release of enkephalin (Kupchik et al., 2014), LTD_{GABA} was blocked in both D1 and D2 pathways to the VP of cocaine-naïve mice by bath application of the MOR antagonist CTOP (50 nM) (Fig. 7*A,B*; two-way ANOVA: no effect of pathway, time, or interaction). Recordings from both yoked saline (D1: $n = 6$, D2: $n = 3$) and naïve mice (D1: $n = 3$, D2: $n = 3$) were pooled for this analysis because no differences in eIPSCs were observed. Thus, the loss of LTD_{GABA} in the D2 pathway may result from a persistent increase

Figure 4. Augmented cocaine seeking produced by inhibiting D2 MSNs depends on activity in the VP. **A**, *D1*- and *D2*-Cre mice were infected with Gi-DREADD hM4D in the accumbens alone or a combination of Gi-DREADD in the accumbens and Gi-DREADD in the VP. **B**, Sagittal image showing coexpression of mCherry-labeled Gi-DREADD-expressing neurons in the accumbens core (NAcore, red) and mCitrine-labeled Gi-DREADD-expressing neurons in the VP (green). Scale bar, 200 μ m. IPAC, Interstitial nuclei of the posterior limb of the anterior commissure; CPu, caudate-putamen; dVP, dorsolateral part of the VP. NAshell, nucleus accumbens shell. Arrows indicate anteroposterior (AP) and dorsoventral (DV) orientation. **C**, Average responding on the active lever and inactive lever, as well as infusions earned over the last 3 d of self-administration and 2 d of extinction in *D2*-Cre mice infected with Gi-DREADD in the accumbens and dorsolateral VP. **D**, Accumbens D2-MSN inhibition augmented cue-induced reinstatement, which was reversed by the simultaneous inhibition of the VP. Numbers in all bars indicate animals. $*p < 0.001$, Tukey *post hoc*. **E**, Average active lever and inactive lever presses and infusions during the last 3 d of self-administration and 2 d of extinction for *D1*-Cre mice transduced with Gi-DREADD in the accumbens. **F**, Inhibition of accumbens D1-MSN had no effect on cue-induced reinstatement of cocaine seeking. Numbers in all bars indicate animals.

Figure 5. Stimulation of D1-MSNs did not affect cue-induced sucrose seeking but increased responding in the presence of both cues and noncontingent sucrose pellets. **A**, Self-administration and extinction of sucrose in *D1*-Cre mice ($n = 6$). **B**, Stimulation of Gs-DREADD rM3D in accumbens D1-MSNs with CNO did not affect cue-induced reinstatement of sucrose seeking but significantly increased reinstatement to both cues and noncontingently delivered sucrose pellets. $*p < 0.05$, vehicle compared with CNO (t test). Bars represent number of mice. **C**, Sucrose self-administration and extinction in *D2*-Cre mice ($n = 6$). **D**, Stimulation of Gs-DREADD in D2-MSNs did not affect cue-induced reinstatement of sucrose seeking in the presence or absence of noncontingent sucrose pellets. Bars represent number of mice tested.

Figure 6. Induction of LTD_{GABA} was selectively abolished in accumbens-pallidal synapses of the dorsolateral VP after cocaine self-administration. **A**, Experimental setup for slice electrophysiology experiments illustrated on a sagittal section. Channelrhodopsin injected in the accumbens of D1-Cre or D2-Cre mice was used for eliciting optical eIPSCs in VP neurons. Following baseline recordings, HFS was applied by electrically stimulating the accumbens-pallidal pathway in current clamp, after which optically evoked IPSCs were recorded from dorsolateral VP neurons. IPAC, Interstitial nuclei of the posterior limb of the anterior commissure; ac, anterior commissure; dIVP, dorsolateral part of the VP; vmVP, ventromedial part of the VP. **B**, Location of recordings in subcommissural dorsolateral VP and example of a patched VP neuron (insert right). ic, Internal capsule. **C**, Time course of optically evoked eIPSCs recorded from VP neurons induced by the selective optical stimulation of D1 and D2 terminals in yoked saline and naive mice before and after electrical HFS. Inserts (above graph), Example traces of optically evoked current traces before (gray) and after (colored) electrical HFS. * $p < 0.001$, main effect of time. **D**, Boxplots for averaged normalized eIPSC responses. * $p < 0.05$, t test comparing baseline (100%) with the average of 6–15 min after HFS in control mice. **E**, HFS increased PPR compared with baseline. Traces represent optical eIPSCs recorded from VP neurons before and after HFS. * $p < 0.05$, t test comparing prestimulation with poststimulation. **F**, Time course of optically evoked eIPSCs before and after HFS from cocaine self-administering mice. * $p < 0.001$, interaction D1 and D2 projection and time. **G**, Cocaine self-administration and extinction abolished HFS-induced LTD_{GABA} in D2 pathway synapses. * $p < 0.05$, t test comparing baseline to the average 6–15 min after HFS. **H**, Cocaine self-administration had no effect on the increase in PPR in the D1 pathway, but abolished this increase in the D2 pathway to the VP. * $p < 0.05$, t test comparing prestimulus with poststimulus. Numbers in all bars indicate cells/animals.

in MOR tone that effectively masks MOR-induced LTD in cocaine-extinguished mice. Consistent with this, bath applied CTOP had no effect on the amplitude of D1 or D2 pathway eIPSCs in the dorsolateral VP of control mice (Fig. 7C,D; two-way ANOVA: no effect of pathway, time, or interaction), but it selectively increased the amplitude of D2 pathway, but not D1 pathway eIPSCs in cocaine-extinguished mice (Fig. 7E; two-way ANOVA: effect of treatment $F_{(1,19)} = 8.276$, $p < 0.01$ and interaction $F_{(20,380)} = 2.56$, $p < 0.001$, but no effect of time). Comparing baseline to the average of min 6–15 during CTOP application showed an increase in eIPSC amplitude selectively in

D2 pathway terminals (Fig. 7F; $t_{(11)} = 3.076$, $p = 0.011$), but not in D1 pathway terminals ($t_{(8)} = 0.918$, $p = 0.385$). These results suggest that MORs are tonically activated on D2, but not D1, pathway terminals in the VP of cocaine-extinguished mice.

Discussion

In mice extinguished from cocaine self-administration, chemogenetic activation of D1-MSNs or inhibition of D2-MSNs in the nucleus accumbens core increased cue-induced reinstatement of cocaine seeking. Regardless of which MSN cell type was chemogenetically regulated to augment cued reinstatement, output to the

Figure 7. Constitutive tone onto MORs after cocaine exposure in D2 pathway synapses masks LTD_{GABA}. **A**, Bath application of the selective MOR antagonist CTOP blocked HFS-induced LTD_{GABA} in both D1 and D2 pathway synapses. Gray background fill represents application of CTOP. Arrowhead indicates application of the electrical HFS protocol. **B**, Summary of optically evoked IPSCs after HFS from control mice. CTOP blocked LTD_{GABA} in both the D1 and D2 pathway to the VP. Dashed line indicates baseline recordings averaging 100%. Traces represent typical eIPSC before (gray) and after (colored) LTD protocol application. **C**, Bath application of CTOP had no effect on brain slices from control mice in the D1 pathway or the D2 pathway to the VP, indicating no endogenous MOR tone. **D**, CTOP did not affect eIPSCs in D1 or D2 pathway synapses of control mice. Gray line and fill indicates the start of CTOP application. Traces represent typical eIPSCs before (gray) and after (colored) CTOP application. **E**, CTOP application restored eIPSC amplitude in the D2 pathway by unmasking MOR tone in the VP, without affecting eIPSC amplitude in the D1 pathway to the VP. Gray line and fill indicate the start of CTOP application. * $p < 0.001$, interaction between D1 and D2 pathway and time. **F**, Bar graphs summarizing change in eIPSC after CTOP application. * $p < 0.05$, t test comparing baseline to the average of min 6–15 during CTOP application. Traces represent typical eIPSCs before (gray) and after (colored) CTOP application. Numbers in all bars represent cells/animals.

VP was required because simultaneous inhibition of VP neurons prevented reinstatement. We hypothesized that GABAergic transmission and plasticity in one or both of these pathways to the VP would be altered by cocaine self-administration. Indeed, the ability to induce LTD_{GABA} in dorsolateral VP neurons was abolished in the D2 pathway after cocaine, whereas LTD_{GABA} was spared at D1 synapses. LTD_{GABA} in both accumbens-pallidal

projections was presynaptic and depended on MORs because CTOP blocked LTD_{GABA} in either the D1 or D2 pathway of control mice. However, cocaine use masked this plasticity by increasing endogenous MOR tone specifically on D2-MSN terminals because CTOP restored GABAergic currents in this pathway. These data indicate that D1- and D2-MSNs bidirectionally regulate cocaine seeking through projections to the VP, and that increased MOR tone decreases D2 pathway GABA transmission in the VP, thus shifting the inherent balance of the circuit in favor of the D1 pathway to facilitate cocaine seeking.

Differential roles for D1- and D2-MSNs in relapse to drug seeking

Multiple studies reveal opposing roles for D1- and D2-MSNs in cocaine-related behavior (Lobo et al., 2010; Bock et al., 2013; Pascoli et al., 2014). For instance, D2-MSNs display reduced activity during expression of a cocaine conditioned place preference, whereas D1-MSN activity increases immediately before entry into a cocaine-paired compartment (Calipari et al., 2016). In line with these studies, we found that activating D1-MSNs increased cue-induced cocaine seeking. In addition to the dorso-lateral VP, accumbens core D1-MSNs project to substantia nigra, and neither pharmacological nor optogenetic silencing of this D1 pathway affected on cocaine seeking (McFarland and Kalivas, 2001; Stefanik et al., 2013). Although systemic activation of DREADDs in multiple brain regions is not conclusive evidence of a monosynaptic pathway between D1-MSNs or D2-MSNs and the VP in driving drug seeking, it shows that the VP is a critical downstream mediator.

Our finding that inhibiting D2-MSNs also increased drug seeking in a VP-dependent manner is consistent with a previous study showing that synaptic strength onto D2-MSNs is inversely correlated with cocaine use and inhibiting D2-MSNs increases the motivation to take cocaine (Bock et al., 2013). Thus, D2-MSNs suppress cocaine seeking, and reducing activity of these cells predisposes animals to increase responding for drugs and drug-associated cues. D2-MSNs also evaluate proper response strategies (Zalocusky et al., 2016) and guide flexible behavior (Yawata et al., 2012). Thus, inhibiting their activity may render animals less prone to change their response strategy despite changes in reward contingencies.

Chemogenetic activation of Gs-coupled signaling in D1-MSNs and Gi-signaling on D2-MSNs mimics the effects of dopamine onto these neurons. Dopamine is released into accumbens core during conditioned cue presentation in abstinent cocaine self-administering animals (Phillips et al., 2003) and is necessary for cue-induced cocaine seeking in sign-tracking animals (Saunders et al., 2013). Although systemic D2 receptor agonists and intra-accumbens shell microinjections of D1 or D2 receptor agonists reinstates cocaine seeking (Self et al., 1996; De Vries et al., 2002; Schmidt et al., 2006), microinjecting a D1/D2-receptor antagonist or selective D1/D2-receptor agonists into the accumbens core does not reduce or increase, respectively, reinstated cocaine seeking (McFarland and Kalivas, 2001; Schmidt et al., 2006). Thus, dopamine likely facilitates reinstatement only in conjunction with circuit activity in glutamatergic accumbens core afferents, as evidenced by D1 receptor signaling facilitating MSN activity only when the cells are in a depolarized state (Surmeier et al., 2007).

Opposing cocaine-induced adaptations in D1- and D2-MSNs

Confirming previous studies, we demonstrated that cocaine changes MOR regulation of GABAergic transmission in the VP

(Tang et al., 2005; Kupchik et al., 2014). Although only D2-MSNs release enkephalin in the VP, both D1 and D2 synapses demonstrated MOR-dependent LTD_{GABA} in control mice. This may be caused by the spillover of enkephalin from D2-MSN terminals after the HFS protocol used to induce LTD_{GABA}, thus reflecting heterosynaptic plasticity. However, enkephalin exerts greater tonic presynaptic inhibition on D2-MSN terminals after cocaine use because CTOP only increased eIPSC amplitude in D2, not D1, pathway synapses. Thus, homosynaptic tonic stimulation of presynaptic MORs on D2-MSN terminals occurs in the VP after cocaine use, and this occludes LTD selectively in D2-MSN synapses. These findings demonstrate cell-type specificity of MOR plasticity in the accumbens-pallidal pathway and add to a growing literature on MOR-dependent LTD in various neural systems (Wamsteeker Cusulin et al., 2013; Atwood et al., 2014).

A recent study found that repeated cocaine injections change plasticity in both D1- and D2-MSN projections to the ventromedial VP (Creed et al., 2016). Although a similar loss of LTD in the D2 pathway was observed, a striking loss of LTP was found for the D1 projection. This bidirectional loss of plasticity in the D1 and D2 pathways was associated with cocaine-induced sensitization and withdrawal-induced anhedonia, respectively. Moreover, D2 pathway plasticity was mediated by presynaptic δ opioid receptors. The distinctions between our studies may arise because we electrically stimulated the entire projection to induce plasticity and then optogenetically interrogated the circuit for D1- and D2-MSN involvement, whereas Creed et al. (2016) both induced plasticity and interrogated the circuit selectively stimulating D1- and D2-MSN afferents. Also, our studies examined two distinct subcircuits. Although only ~50% of dorsolateral VP neurons are functionally innervated by D1-MSNs (Kupchik et al., 2015), apparently nearly all ventromedial VP neurons receive D1-MSN innervation from accumbens shell (Creed et al., 2016). Also, although Creed et al. (2016) focused exclusively on ventromedial VP neurons projecting to the VTA, our recordings did not discriminate among dorsolateral VP neurons, and the different topographies of the dorsolateral and ventromedial VP efferent projections indicate the two studies recorded from largely non-overlapping circuits (Zahm, 1989; Bell et al., 1995).

Collectively, these findings indicate that cocaine use reorganizes the balance between D1- and D2-MSN inputs to VP by enhancing enkephalin tone on D2 pathway synapses. The shift in balance of accumbens-pallidal output in favor of the D1 pathway is parsimonious with electrophysiological studies showing overall strengthened glutamatergic inputs onto accumbens D1-MSNs after contingent (Bock et al., 2013; Pascoli et al., 2014) and non-contingent cocaine or heroin exposure (Pascoli et al., 2011; MacAskill et al., 2014; Hearing et al., 2016), as well as increased expression of genes involved in synaptic plasticity (e.g., *Egr3* and Δ FosB) in D1-MSNs and decreased expression in D2-MSNs (Grueter et al., 2013; Lobo et al., 2013; Chandra et al., 2015).

How can D1 and D2 MSN afferents in the VP produce bidirectional behavior?

Given that the D1 and D2 pathways are GABAergic and synapse on substantially overlapping VP neurons (Kupchik et al., 2015), possibly even targeting the same dendritic segment (Pickel et al., 2012), it is surprising that opposite behaviors are engendered by the two MSN populations. This indicates a level of nuanced regulation beyond simple inhibition of VP neurons by GABA released from MSNs. How both D1 and D2 pathway GABAergic synapses in the VP encode opposite behaviors is unknown, but different neuropeptides coreleased with GABA in D1- versus D2-MSN terminals are likely

candidates in the differential encoding of information between these pathways. For instance, enkephalin from D2 pathway terminals inhibits the presynaptic release of GABA into the VP (Kupchik et al., 2014), and thereby disinhibits VP neurons to augment cocaine reinstatement (Tang et al., 2005). Thus, blocking MORs in the VP inhibits reinstatement of cocaine and alcohol seeking (Tang et al., 2005; Perry and McNally, 2013). MOR signaling in the VP also mediates hedonic responses (Smith and Berridge, 2005), which may contribute to drug seeking.

Another mechanism by which D1- and D2-MSN inputs to the VP could elicit opposite effects on cocaine seeking is through targeting VP neurons that project to different downstream areas, such as the medial thalamus and ventral mesencephalon (Haber et al., 1985; Groenewegen et al., 1993; Tripathi et al., 2013). However, dorsolateral VP neurons retrogradely labeled from these two major output structures did not respond differently to D1- and D2-MSN input (Kupchik et al., 2015).

A final possibility is that D1 and D2 pathways project to different neuronal subtypes in the VP. A proportion of VP cells are glutamatergic, which could drive opposite downstream effects from those elicited by GABAergic neurons. For example, the projection from ventromedial VP to the ventral tegmental area contains both GABAergic and glutamatergic efferents and is critical for cocaine seeking (Geisler et al., 2007; Mahler et al., 2014). Thus, the heterogeneous firing patterns observed in VP neurons during cocaine seeking may arise from heterogeneity in neuronal subtypes and subpopulations (Root et al., 2013).

In conclusion, our results demonstrate of a functional role for D1- and D2-MSN projections from the nucleus accumbens to the VP in cue-induced reinstatement of cocaine seeking, and show a differential loss of synaptic plasticity in D2 versus D1 pathways to the VP after cocaine self-administration. These data set the stage for future studies addressing the cell type-specific circuitry of the D1 and D2 pathways to the VP in the regulation of drug seeking.

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