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Transient neuronal inhibition reveals opposing roles of indirect and direct pathways in sensitization

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Dorsal striatum is important for the development of drug addiction; however, a precise understanding of the roles of striatopallidal (indirect) and striatonigral (direct) pathway neurons in regulating behaviors remains elusive. Using viral-mediated expression of an engineered G protein–coupled receptor (hM_4D), we found that activation of hM_4D receptors with clozapine-*N*-oxide (CNO) potently reduced striatal neuron excitability. When hM_4D receptors were selectively expressed in either direct or indirect pathway neurons, CNO did not change acute locomotor responses to amphetamine, but did alter behavioral plasticity associated with repeated drug treatment. Specifically, transiently disrupting striatopallidal neuronal activity facilitated behavioral sensitization, whereas decreasing excitability of striatonigral neurons impaired its persistence. These findings suggest that acute drug effects can be parsed from the behavioral adaptations associated with repeated drug exposure and highlight the utility of this approach for deconstructing neuronal pathway contributions to behavior.

Despite the overwhelming negative consequences of drug addiction, psychostimulant abuse remains prevalent. The progression from initial drug exposure to regular use and ultimately to compulsive, habitual behavior and the loss of inhibitory control involves a series of molecular adaptations in discrete neurocircuits[1–3]. The striatum is an important site for many of the behavioral and neurobiological adaptations thought to form the core processes that mediate addiction[1–3]. The majority of striatal neurons are GABAergic medium spiny projection neurons (MSNs) that differ in their neuropeptide expression and form two major efferent pathways[4,5]. Striatopallidal MSNs contain enkephalin (ENK) and form the indirect pathway, whereas striatonigral MSNs contain dynorphin (DYN) and substance P and form the direct pathway. Many conceptual models hypothesize that these populations of MSNs oppose one another both mechanistically and functionally[6,7]. However, there is little empirical evidence to support their differential role in the control of behavior, as these cell populations are physically intermingled and morphologically indistinguishable, making selective manipulation technically elusive.

To examine the role of these striatal cell populations in the development of behaviors that occur following repeated exposure to drugs of abuse, we used viral vectors with either the *Enk* (also known as *Penk*) or *Dyn* (also known as *Pdyn*) gene promoters to target transgene expression to striatopallidal or striatonigral neurons, respectively, and an engineered GPCR ($G_{i/o}$-coupled human muscarinic M_4 designer receptor exclusively activated by a designer drug, hM_4D)[8] that is activated by an otherwise pharmacologically inert ligand, CNO[9,10] (**Supplementary Fig. 1**). Following expression in cultured neurons, administration of CNO stimulates $G_{i/o}$-coupled hM_4D receptors, activating inwardly rectifying potassium 3 (Kir3) channels, resulting in membrane hyperpolarization and transient neuronal silencing[10].

To test the cell phenotype specificity of the viral vectors, we used dual-label immunofluorescence microscopy following dorsal striatum infusion of viruses (**Supplementary Fig. 1**) that express hemagglutinin-tagged hM_4D receptors under the control of either the *Enk* promoter (p*Enk-hM$_4$D*) or the *Dyn* promoter (p*Dyn-hM$_4$D*). We found that p*Enk-hM$_4$D* was primarily expressed in ENK-containing MSNs (90% of hemagglutinin cells were ENK positive, 85 out of 94; 6% of hemagglutinin cells were substance P positive, 4 out of 70 cells; **Fig. 1a**), whereas p*Dyn-hM$_4$D* was primarily expressed in substance P–containing MSNs (95% of hemagglutinin cells were substance P positive, 109 out of 115 cells; 5% of hemagglutinin cells were ENK positive, 5 out of 97 cells; **Fig. 1b**). Similar results were obtained following infusion of promoter-specific viruses that expressed GFP (p*Enk-GFP* and p*Dyn-GFP*; **Supplementary Figs. 2a** and **3a**).

Given that striatopallidal MSNs primarily project to the globus pallidus external (GPe) and striatonigral MSNs primarily project to the substantia nigra pars reticulata (SNpr), we injected the retrograde tracer Fluoro-Gold into these brain regions and carried out dual-label fluorescent immunohistochemistry to confirm that the p*Enk* and p*Dyn* viruses yielded pathway-specific infection. We observed that p*Enk-GFP* cells colocalized with striatal Fluoro-Gold expression following infusions into the GPe, but not the SNpr (**Supplementary Fig. 2b**), whereas p*Dyn-GFP* cells colocalized with striatal Fluoro-Gold expression following infusions into the SNpr, but not the GPe (**Supplementary Fig. 3b**).

Although hM_4D receptor–based techniques have been shown to modulate activity of other neuronal types[10], their ability to affect striatal neurons has not been examined. We observed that CNO induced a hyperpolarization of the membrane potential (~7 mV, **Fig. 1c**) and reduced the input resistance of hM_4D-expressing MSNs (**Fig. 1d**,**e**), suggesting that potassium conductance (that is, Kir3-mediated current) is activated. Furthermore, CNO substantially decreased the number of evoked action potentials in hM_4D-expressing neurons. Expression of hM_4D receptors alone did not alter input resistance ($P = 0.84$) or action potential firing

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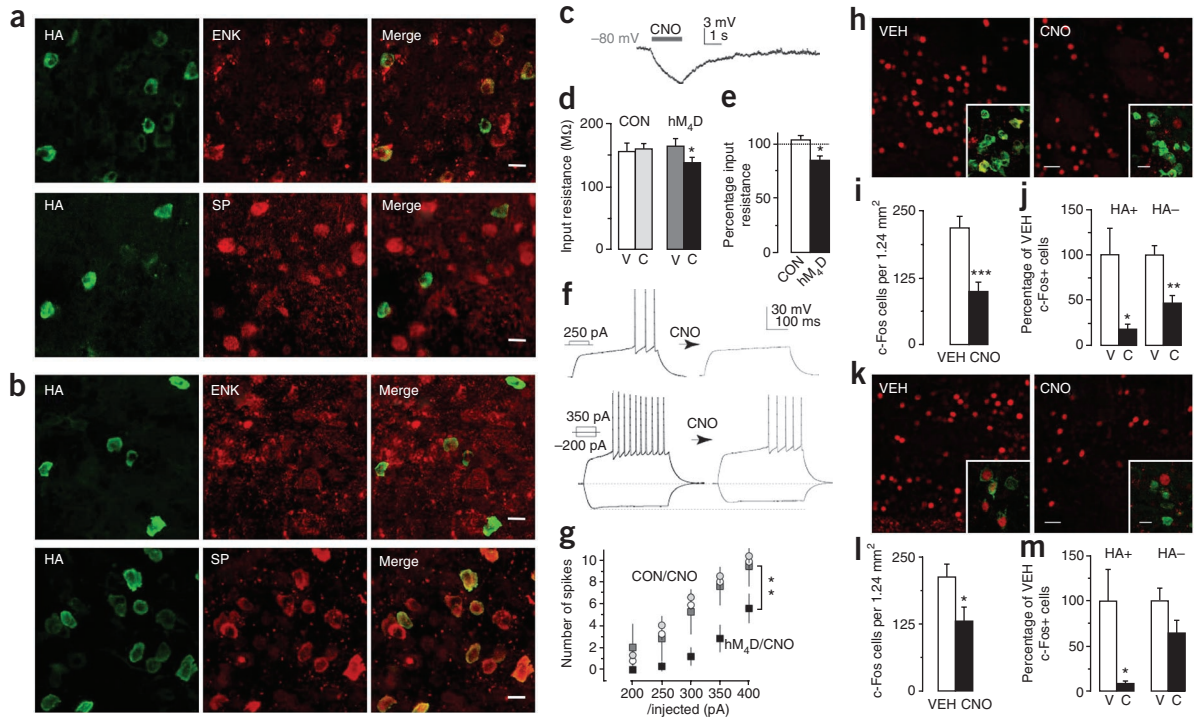

Figure 1 Transient and targeted attenuation of striatal cell signaling. (**a**) p*Enk-hM*$_4$*D* receptors were selectively expressed in striatopallidal MSNs. Green, hemagglutinin (HA); red, ENK (top) and substance P (SP, bottom); yellow, colocalization of neurons. Scale bars represent 10 µm. (**b**) p*Dyn-hM*$_4$*D* receptors were selectively expressed in striatonigral MSNs. Data are presented as in **a**. (**c**) Representative voltage trace of CNO-induced hyperpolarization of an hM$_4$D-expressing striatal neuron. (**d**,**e**) CNO decreased input resistance in hM$_4$D-expressing neurons. $^*P < 0.05$ hM$_4$D before versus hM$_4$D after CNO application ($n = 4$–5). C, CNO treatment; V, vehicle treatment. (**f**,**g**) Representative traces (**f**) and summarized data (**g**) showed that CNO decreased the number of evoked action potentials in hM$_4$D-expressing neurons. $^{**}P < 0.01$ hM$_4$D versus hM$_4$D/CNO. (**h**) Representative Fos immunohistochemistry sections (red) from p*Enk-hM*$_4$*D* infused striatum of vehicle (VEH) and CNO-treated rats. Insets depict single-labeled Fos cells (red), hemagglutinin cells (green) and dual-labeled cells (yellow). Scale bars represent 50 µm and 10 µm (insets). (**i**) Activation of p*Enk-hM*$_4$*D* receptors decreased the number of amphetamine-induced Fos cells ($^{***}P = 0.002$, $n = 5$–6 per group). (**j**) Amphetamine-evoked c-Fos–positive cells were reduced in both hemagglutinin-positive ($^*P < 0.05$) and hemagglutinin-negative ($^{**}P < 0.01$) neurons in the pENK-hM$_4$D experiment. (**k**) Representative Fos immunohistochemistry sections (red) from p*Dyn-hM*$_4$*D* infused striatum of vehicle (VEH) and CNO-treated rats. Data are presented as in **h**. (**l**) Activation of p*Dyn-hM*$_4$*D* receptors decreased the number of amphetamine-induced Fos cells ($^*P < 0.05$, $n = 5$–6 per group). (**m**) Amphetamine-evoked c-Fos–positive cells were reduced in hemagglutinin-positive neurons ($^*P < 0.05$) in the p*Dyn-hM*$_4$*D* experiment. All data represent mean ± s.e.m.

($P = 0.64$) (**Fig. 1f**,**g**). These data suggest that the hM$_4$D/CNO-based method can effectively decrease the excitability of rat striatal neurons.

We also tested whether hM$_4$D receptors would block neurotransmission in a circuit in which neural activity is predictably evoked by behaviorally relevant stimuli. Accordingly, we infected ventral tegmental area (VTA) neurons with hM$_4$D receptors under the control of a herpes simplex virus promoter and used fast-scan cyclic voltammetry to measure changes in dopamine release in the nucleus accumbens after unexpected delivery of a food reward[11]. CNO significantly attenuated ($P < 0.05$) food pellet–evoked dopamine release in the nucleus accumbens (**Supplementary Fig. 4**). Finally, we tested whether decreasing activity of specific striatal cell types could alter the ability of amphetamine to stimulate Fos expression. Psychostimulants are robust activators of *c-fos*[12] and will increase *c-fos* in both striatonigral and striatopallidal neurons under our experimental conditions[13]. In addition to its use as a marker of neuronal activity, psychostimulant induction of *c-fos* is thought to be important in the initiation and maintenance of the neural adaptations associated with psychomotor sensitization[1,14]. We found that activation of p*Enk-hM*$_4$*D* receptors significantly reduced ($P = 0.002$) the number of amphetamine-induced c-Fos cells (**Fig. 1h**,**i** and **Supplementary Fig. 5a**). This reduction occurred in both hemagglutinin-positive neurons (that is, those expressing hM$_4$D receptors) and hemagglutinin-negative neurons (that is, those not expressing hM$_4$D receptors; **Fig. 1j**), suggesting a neuronal cross-talk effect between hM$_4$D-expressing neurons and uninfected neurons. Significant reductions ($P < 0.05$) in amphetamine-evoked c-Fos cells and in hemagglutinin-positive c-Fos cells were also seen when hM$_4$D receptors were activated in direct pathway neurons (**Fig. 1k**,**l**,**m** and **Supplementary Fig. 5b**). These effects were not simply caused by viral expression of a receptor, as expression of either p*Enk-hM*$_4$*D* or p*Dyn-hM*$_4$*D* receptors in the absence of CNO had no effect on the number of amphetamine-evoked Fos cells (**Supplementary Figs. 6** and **7**). These findings indicate that activation of hM$_4$D receptors can also lead to decreases in neuronal activity by reducing neurotransmitter release and attenuating intracellular signaling.

Repeated exposure to addictive drugs can lead to a progressive and persistent increase in behavioral responsiveness, known as behavioral sensitization. Notably, sensitization involves some of the same neural circuits that have been implicated in the development of human drug addiction[3]. We investigated the effect of circuit-specific dampening of striatal neuron activity on the development of amphetamine sensitization. We hypothesized that direct and indirect pathway neurons have opposing roles in which striatonigral neurons promote sensitization and striatopallidal neurons suppress sensitization, consistent with their conceptually proposed roles in behavioral activation and inhibition, respectively[6,7]. Accordingly, we tested whether biochemically

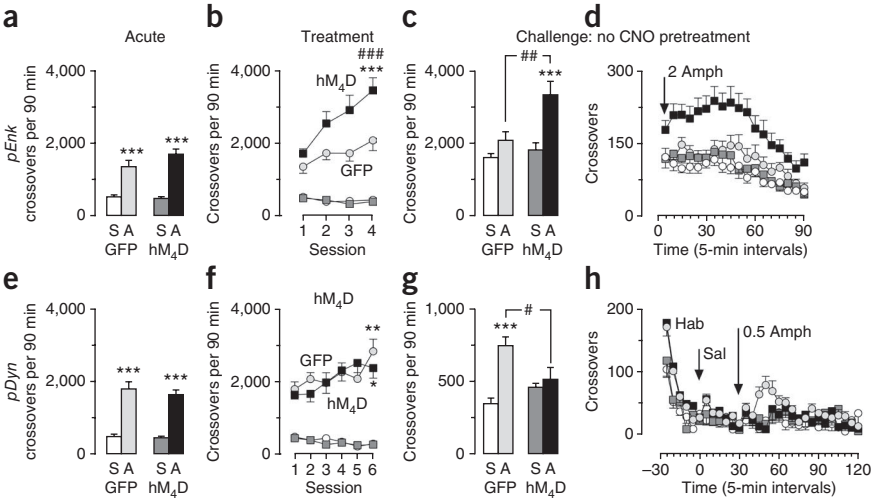

Figure 2 Transiently reducing excitability of striatopallidal or striatonigral neurons had opposing effects on amphetamine sensitization. (**a**) Acute locomotor responses to amphetamine following activation of p*Enk-hM*$_4$*D* receptors ($n = 9$–10 per group). $^{***}P < 0.001$ versus saline-treated groups. (**b**) Activation of p*Enk-hM*$_4$*D* receptors during amphetamine treatment enhanced the development of locomotor sensitization. $^{***}P < 0.001$ versus session 1 of amphetamine-treated hM$_4$D group, $^{###}P < 0.001$ versus amphetamine-treated GFP group. (**c**,**d**) Enhanced sensitization in the amphetamine-pretreated p*Enk-hM*$_4$*D* group was maintained during the challenge test. $^{***}P < 0.001$ versus saline-pretreated group, $^{##}P < 0.01$ versus amphetamine-pretreated GFP group. (**e**) Acute locomotor responses to amphetamine following activation of p*Dyn-hM*$_4$*D* receptors ($n = 8$–10 per group). (**f**) Activation of p*Dyn-hM*$_4$*D* receptors during amphetamine treatment initially produced locomotor sensitization similar to that of p*Dyn-GFP* controls. $^{**}P < 0.01$ and $^{*}P < 0.05$ versus session 1. (**g**,**h**) Sensitization in the amphetamine-pretreated p*Dyn-hM*$_4$*D* group was no longer evident on the challenge test. $^{***}P < 0.001$ versus saline-pretreated groups, $^{#}P < 0.05$ versus amphetamine-pretreated GFP group. Data represent mean ± s.e.m. A, amphetamine; S, saline. Squares represent hM$_4$D groups, circles represent GFP groups. Light gray and black symbols represent rats that received amphetamine during the treatment phase, and white and dark gray symbols represent rats that received saline during the treatment phase. All experimental procedures were approved by the University of Washington Institutional Animal Care and Use Committee and were conducted in accordance with US National Institutes of Health guidelines. See **Supplementary Methods** for additional statistical information.

decreasing neuronal excitability of striatopallidal neurons would induce sensitization to an amphetamine-dosing regimen that elicits a threshold level of sensitization and whether decreasing neuronal excitability of striatonigral neurons would prevent sensitization in a protocol that normally produces robust sensitization.

We found that activation of p*Enk-hM*$_4$*D* receptors during amphetamine treatment did not alter the acute locomotor response to amphetamine (**Fig. 2a**). However, disruption of neuronal activity in indirect-pathway neurons facilitated the development of a significantly more robust sensitization ($P < 0.001$) than in GFP controls (**Fig. 2b**). This enhancement of sensitization was maintained during the amphetamine challenge, which was conducted 1 week later in the absence of CNO (**Fig. 2c**,**d**). These effects can be attributed to a CNO-dependent reduction of activity of striatopallidal neurons because hM$_4$D receptor expression without CNO treatment did not produce locomotor sensitization (**Supplementary Fig. 6**).

As with indirect pathway dampening, decreased excitability of direct pathway neurons during amphetamine treatment did not alter the acute locomotor response to amphetamine (**Fig. 2e**). Although activation of p*Dyn*-hM$_4$D receptors during amphetamine treatment did not appear to effect the development of sensitization (**Fig. 2f**), the amphetamine challenge revealed that sensitization did not persist in the p*Dyn-hM*$_4$*D* group, but was still robustly maintained in the GFP controls (**Fig. 2g**,**h**). These effects can also be attributed to a CNO-dependent decrease of activity of striatonigral neurons because hM$_4$D receptor expression in the absence of CNO treatment did not block the development of locomotor sensitization (**Supplementary Fig. 7**). These data suggest that striatonigral neurons may be particularly important for regulating the long-term behavioral adaptations that are a consequence of repeated drug use.

These data provide, to the best of our knowledge, the first evidence for the critical and opposing roles of striatopallidal and striatonigral neurons in the regulation of drug experience–dependent behavior plasticity. In addition, the lack of effect of neuronal inhibition on the acute locomotor response to amphetamine provides further evidence that the mechanisms that regulate acute responses to drugs are distinct from those that modulate the enduring adaptations that occur with repeated drug exposure. Finally, pairing phenotypic-specific viral vectors with designer receptors capable of altering neuronal activity without permanently disrupting cell function provides a new and powerful approach for deconstructing the molecular basis of addiction.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

S.M.F. and D.E. generated the viral vector constructs. S.M.F. did the behavioral and immunohistochemical experiments. M.I. and Y.D. did the electrophysiology experiments. M.J.W. and P.E.M.P. did the voltammetry experiments. B.L.R. provided the hM$_4$D plasmids and assisted with experimental design. S.M.F. and J.F.N. designed the overall study and wrote the manuscript. All of the authors contributed to data interpretation and manuscript editing.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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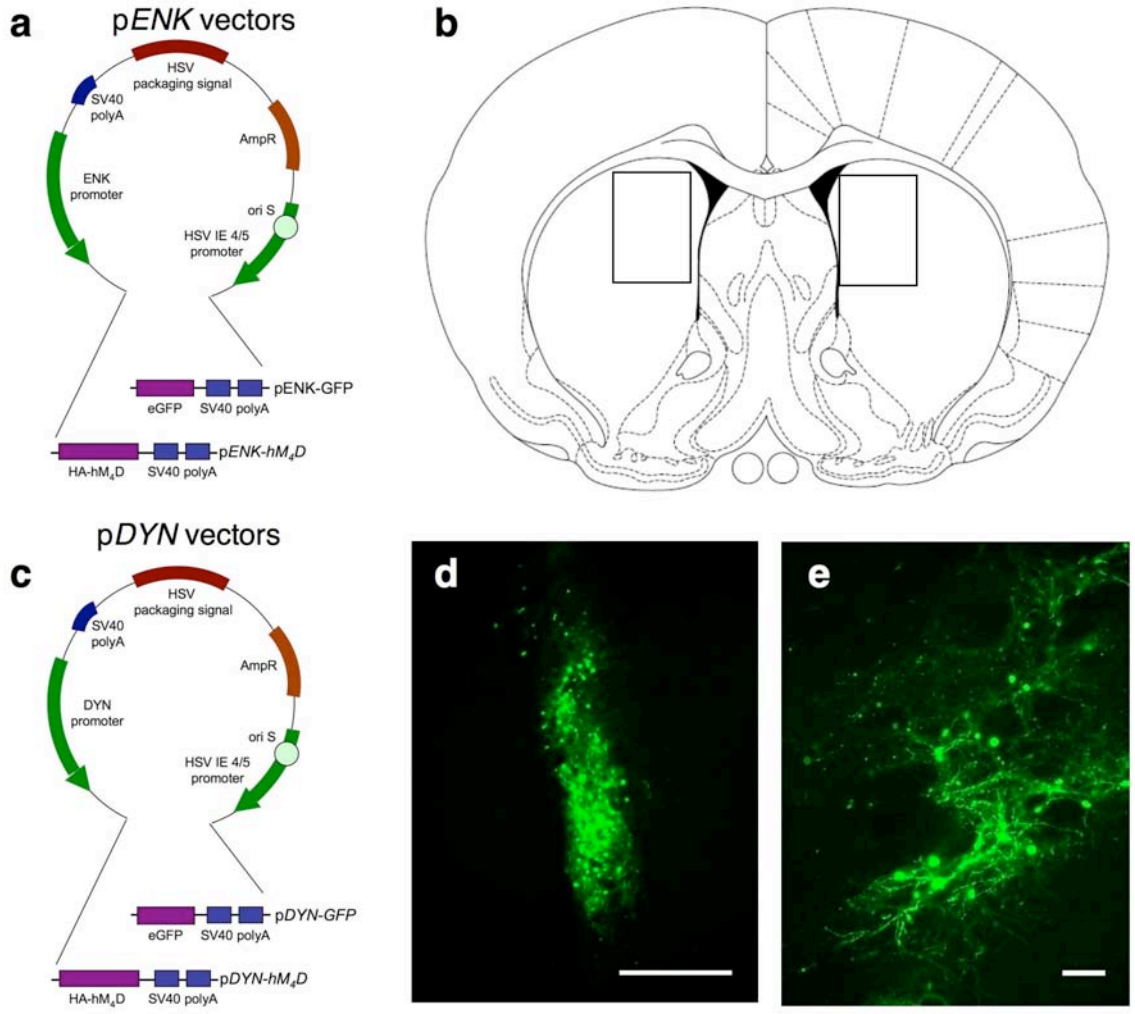

Supplementary Figure 1 Viral vector maps and representative depiction of viral spread. (**a**) Amplicon map of the p*ENK-hM4D*/p*ENK-GFP* targeting vectors. (**b**) Drawing

adapted from plate 17 (0.2 mm from bregma) of the Paxinos and Watson rat atlas illustrating the region of viral spread on one coronal brain section. (**c**) Amplicon map of the p*DYN-hM*$_4$*D*/p*DYN-GFP* targeting vectors. (**d,e**) Representative histological photomicrographs demonstrating GFP expression from a coronal section of the dorsomedial striatum following viral infusion. Scale bars, 1 mm (**d**) and 100 μm (**e**).

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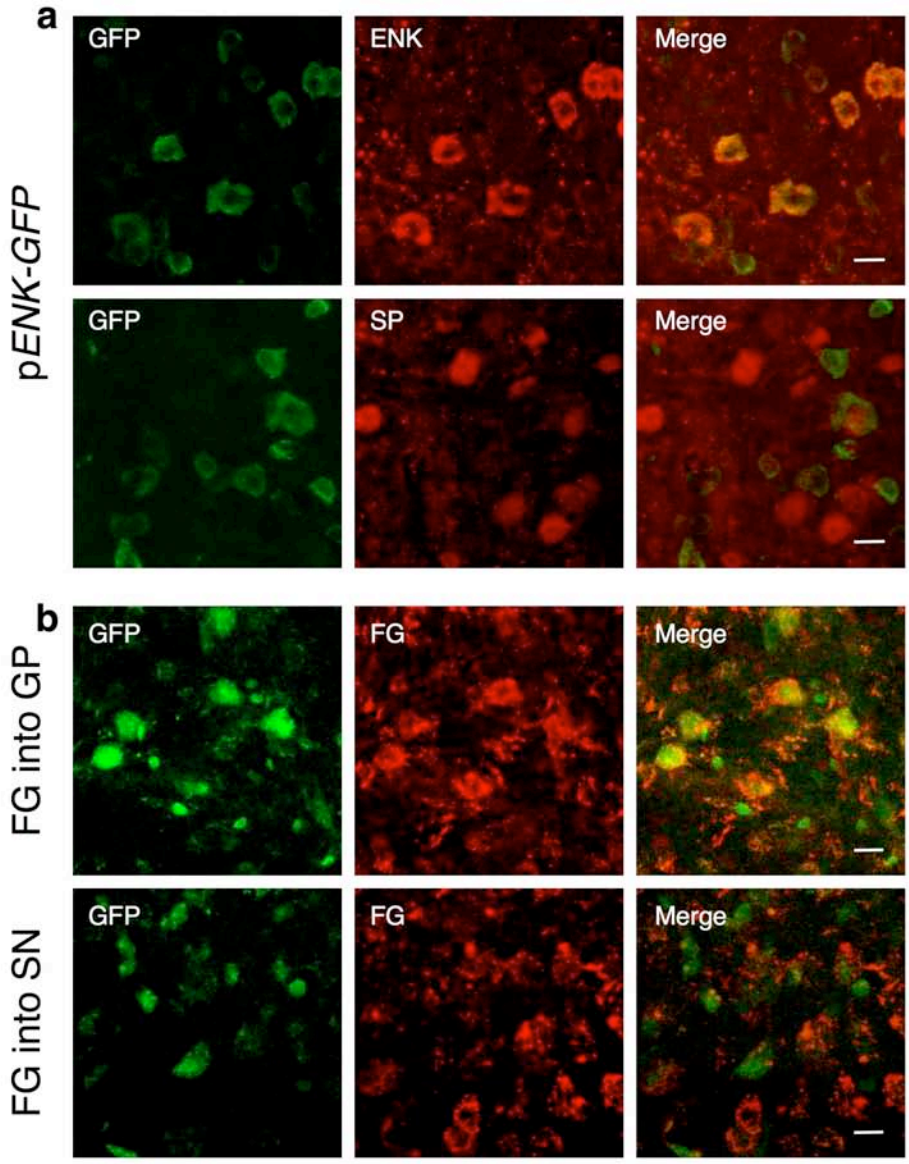

Supplementary Figure 2 Expression of p*ENK* viral vectors was restricted to striatopallidal neurons. (**a**) p*ENK-GFP* was selectively expressed in striatopallidal MSNs (87% of GFP cells were ENK+, 150 out of 179; 4% of GFP cells were substance P+, 8 out of 195 cells). This is shown by co-localization (right, yellow) of GFP (left, green) and ENK+ striatopallidal MSNs (top middle, red) and absence of co-localization of GFP and substance P+ striatonigral MSNs (bottom middle, red). Scale bars, 10 μm. (**b**) p*ENK-GFP* was selectively expressed in indirect pathway neurons (90% of GFP cells were

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Fluoro-Gold (FG)+ after GPe infusions, 111 out of 124 cells; 5% of GFP cells were FG+ after SNpr infusions, 3 out of 71 cells). This is shown by co-localization (right, yellow) of GFP (left, green) and striatal FG immunoreactivity (top middle, red) following infusions of FG into the GPe and absence of co-localization of GFP and striatal FG immunoreactivity (bottom middle, red) following infusions of FG into the SNpr. Expression of the viral vectors did not change the number of ENK positive or substance P positive neurons in the region of viral infection, suggesting that these promoters for viral-mediated gene transfer did not interfere with endogenous neuropeptide levels. Scale bars, 10 μm.

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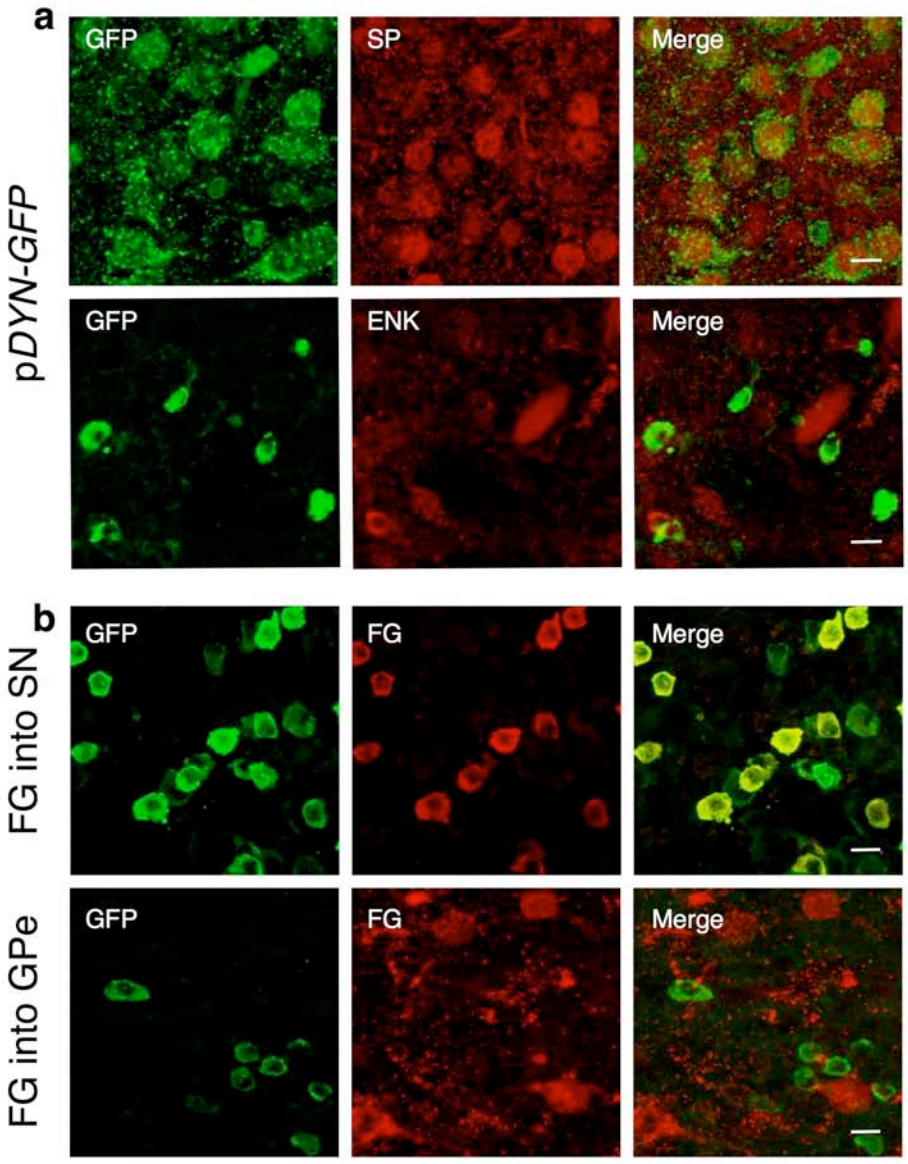

Supplementary Figure 3 Expression of p*DYN* viral vectors was restricted to striatonigral neurons. (**a**) p*DYN-GFP* was selectively expressed in striatonigral MSNs (92% of GFP cells were substance P+, 232 out of 251 cells; 8% of GFP cells were ENK+, 10 out of 129 cells). This is shown by co-localization (right, yellow) of GFP (left, green) and substance P+ striatonigral MSNs (top middle, red) and absence of co-localization of GFP and ENK+ striatopallidal MSNs (bottom middle, red). Scale bars, 10 μm. (**b**) p*DYN-GFP* was selectively expressed in direct pathway neurons (89% of GFP cells were FG+ after

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SNpr infusions, 157 out of 177 cells; 3% of GFP cells were FG+ after GPe infusions, 3 out of 90 cells). This is shown by co-localization (right, yellow) of GFP (left, green) and striatal FG immunoreactivity (top middle, red) following infusions of FG into the SNpr and absence of co-localization of GFP and striatal FG-immunoreactivity (bottom middle, red) following infusions of FG into the GPe. Similar to the p*ENK* viral vectors, expression of these viral vectors did not alter the number of ENK positive or substance P positive neurons in the region of viral infection. Scale bars, 10 μm.

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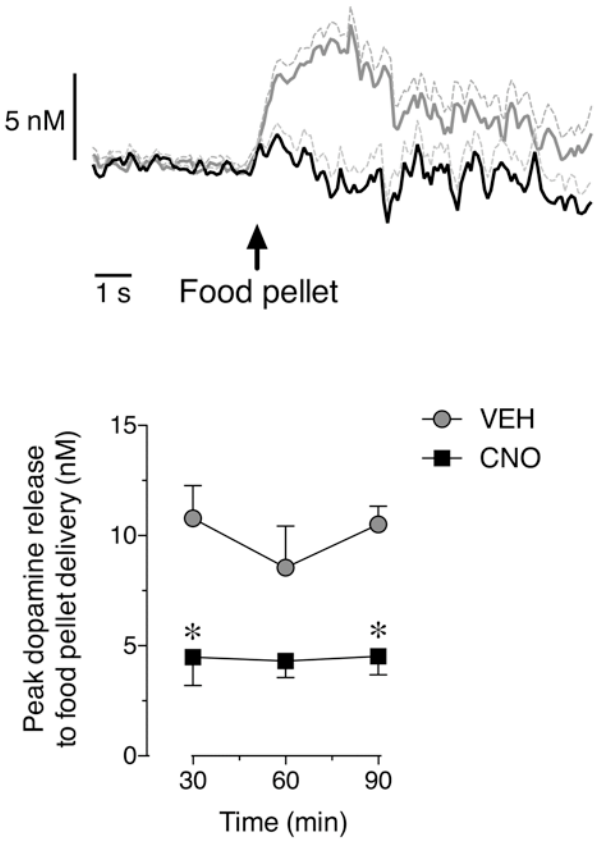

Supplementary Figure 4 Activation of hM_4D receptors in the VTA altered dopamine neurotransmission. Activation of hM_4D receptors in the VTA during food pellet delivery significantly attenuated dopamine release at 0-30 min and 60-90 min post-CNO administration (Main effect of Treatment: $F_{1,12} = 16.3$, $P < 0.01$, $^*P < 0.05$ paired t-test versus VEH-treated group, bottom). Data is averaged across 90 min following administration of vehicle (VEH) or CNO (n=4, top).

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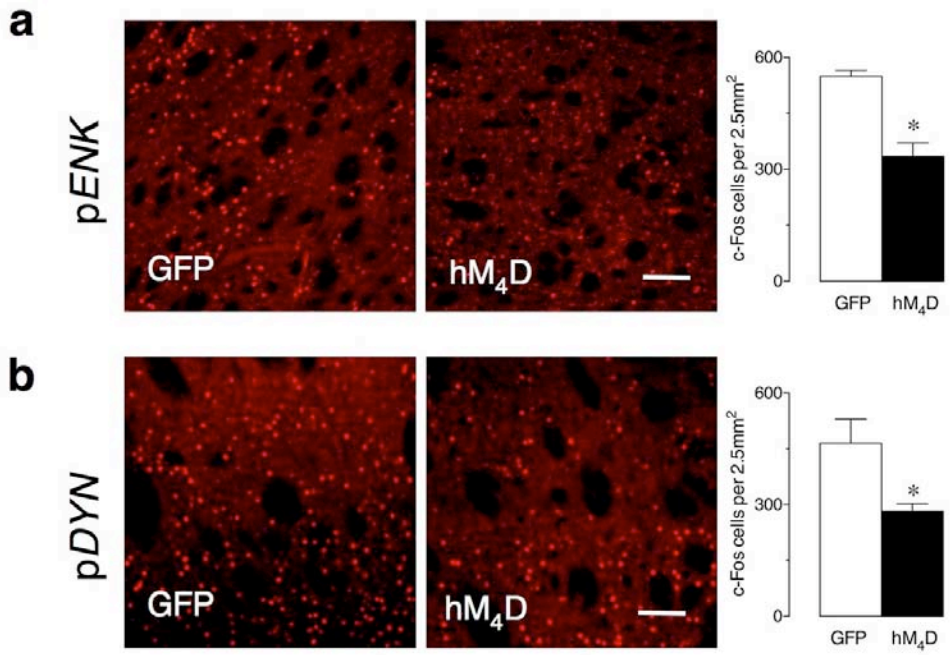

Supplementary Figure 5 Activation of hM_4D receptors in specific striatal cell populations reduced amphetamine-evoked c-Fos expression. CNO-mediated activation of p*ENK-hM4D* (**a**) or p*DYN-hM4D* (**b**) receptors significantly decreased the number of amphetamine-induced Fos cells in dorsomedial striatum compared to control (GFP: p*ENK-GFP* and p*DYN-GFP*, respectively) (p*ENK*: $t_6 = 5.41$, $P = 0.002$, n=4/group; p*DYN*: $t_9 = 2.48$, $P = 0.04$, n=5-6/group). Data represent mean ± SEM. Representative sections of Fos immunohistochemistry (red) are shown from GFP and p*ENK-hM4D* (**a**) and p*DYN-hM4D* (**b**) infused striatum. Scale bars, 100 μm.

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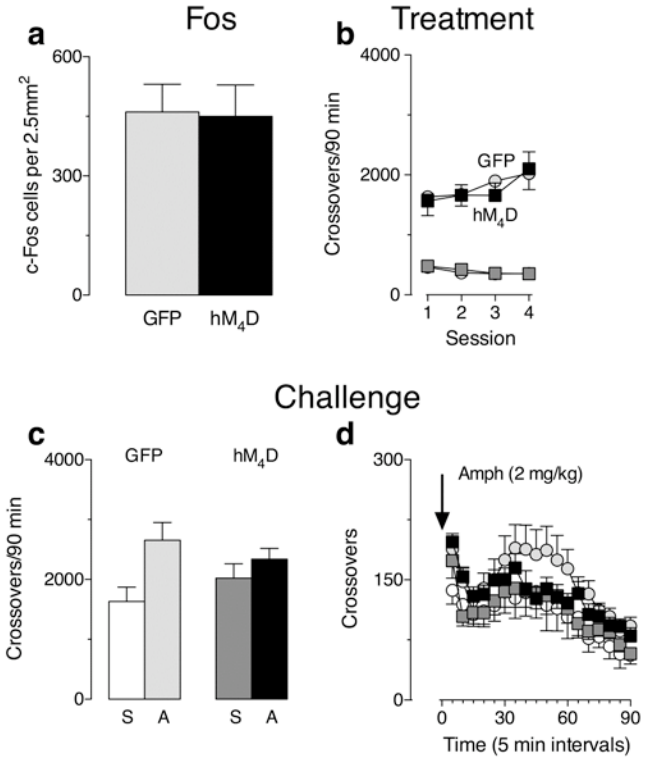

Supplementary Figure 6 Expression of p*ENK-hM4D* receptors in the absence of CNO-mediated reduction of neuronal activity had no effect on biological responses to amphetamine. (**a**) Mere expression of p*ENK-hM4D* receptors in the dorsomedial striatum had no effect on the number of amphetamine-evoked c-Fos cells ($t_{10} = 0.10$, $P = 0.92$, n=6/group). (**b**,**c**,**d**) In the absence of activation, expression of p*ENK-hM4D* receptors in the striatum had no effect on the development of amphetamine sensitization (n=8-10/group). Treatment phase (**b**): main effect of Session (S1 vs. S4: $F_{1,17} = 5.19$, $P = 0.04$; main effect of Virus: $F_{1,17} = 0.0002$, $P = 0.99$ and interaction between Virus and Session factors: $F_{1,17} = 0.13$, $P = 0.73$ not significant. Challenge phase (**c**): main effect of Pretreatment: $F_{1,32} = 4.23$, $P = 0.048$; main effect of Virus: $F_{1,32} = 0.13$, $P = 0.72$ and interaction between Pretreatment and Virus factors: $F_{1,32} = 0.70$, $P = 0.41$ not significant.

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Time course of the challenge response is shown in (**d**). Thus, in the presence of CNO, p*ENK-hM$_4$D* receptor expression enhanced amphetamine sensitization compared to p*ENK-GFP* (Figure 2), whereas, in the absence of CNO, p*ENK-hM$_4$D* receptor expression had no effect on sensitization to this threshold sensitization procedure. Data represent mean ± SEM. S = saline, A = amphetamine. Squares represent hM$_4$D groups, circles represent GFP groups. Light grey and black symbols represent rats that received amphetamine during the treatment phase, white and dark grey symbols represent rats that received saline during the treatment phase.

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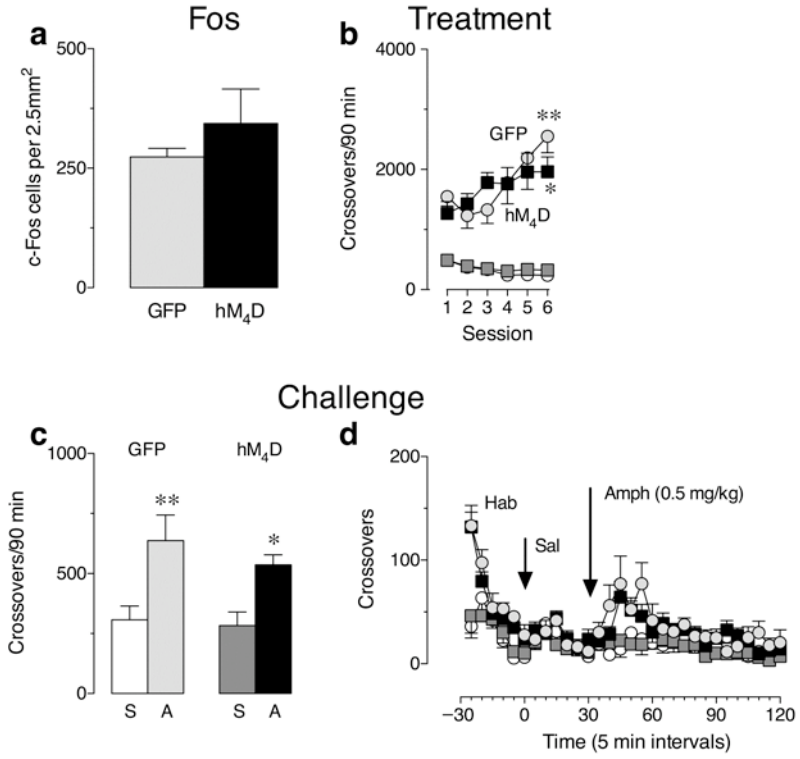

Supplementary Figure 7 Expression of p*DYN-hM4D* receptors in the absence of CNO-mediated reduction of neuronal activity had no effect on biological responses to amphetamine. (**a**) Mere expression of p*DYN-hM4D* receptors in the dorsomedial striatum had no effect on the number of amphetamine-evoked c-Fos cells (t_9 = 1.03, P = 0.33, n=5-6/group). (**b**,**c**,**d**) Expression without activation of p*DYN-hM4D* receptors in the striatum had no effect on the development of amphetamine sensitization (n=6/group). Treatment phase (**b**): main effect of Session (S1 vs. S6): $F_{1,10}$ = 22.95, P = 0.0007; main effect of Virus: $F_{1,1}$ = 2.75, P = 0.13 and interaction between Virus and Session factors: $F_{1,10}$ = 0.75, P = 0.41 not significant, **P < 0.01 and *P < 0.05 versus Session 1. Challenge phase (**c**): main effect of Pretreatment: $F_{1,20}$ = 17.35, P = 0.0005; main effect of Virus: $F_{1,20}$ = 0.80, P = 0.38 and interaction between Pretreatment and Virus factors: $F_{1,20}$ = 0.30, P = 0.59 not significant, **P < 0.01 and *P < 0.05 versus saline-pretreated

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group. Time course of the challenge response is shown in (**d**). Thus, p*DYN-hM$_4$D* receptor activation prevented the persistence of amphetamine sensitization (Figure 2), whereas in the absence of CNO, p*DYN-hM$_4$D* receptor expression had no effect on the development of sensitization. Data represent mean ± SEM. S = saline, A = amphetamine. Squares represent hM$_4$D groups, circles represent GFP groups. Light grey and black symbols represent rats that received amphetamine during the treatment phase, white and dark grey symbols represent rats that received saline during the treatment phase.

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Supplemental Methods

Subjects. Male Sprague-Dawley rats (Harlan, Hollister, CA) weighing 250-274 grams upon arrival were housed two per cage and given a one-week acclimation period prior to any experimental manipulation. The housing room was temperature- and humidity-controlled and maintained on a 12:12 h light:dark cycle, with food and water available ad libitum.

Drugs. Amphetamine (Sigma, St. Louis, MO) was dissolved in sterile 0.9% saline and clozapine-*N*-oxide (BIOMOL Int., Plymouth Meeting, PA) was dissolved in sterile water. Drugs were administered by intraperitoneal (ip) injection in a volume of 1-2 ml/kg.

Viral Vector Construction. *pHSV-hM$_4$D plasmid*. In order to construct a herpes simplex virus (HSV) vector that expresses a triple hemagglutinin epitope-tagged hM$_4$D gene (1567 Kb), the hM$_4$D gene was excised from a pc*DNA3.1* plasmid and inserted into a modified version of p*HSV-PrPUC* (kindly provided by Dr. Rachael Neve, McLean Hospital, Boston, MA). *pENK plasmids*. In order to construct an HSV vector that expresses green fluorescent protein (GFP) under the control of the enkephalin promoter, a ~2.7 Kb fragment (-2609 to +52) upstream of the enkephalin gene was excised from a p*REJCAT* plasmid (kindly provided by Dr. Sabol, NIH), subcloned into an intermediary p*GL3-basic* plasmid and inserted into a modified version of p*HSV-PrPUC*. In order to make an HSV vector that expresses the hM$_4$D gene under the control of the enkephalin promoter, the hemagglutinin-tagged hM$_4$D gene was excised from a pc*DNA3.1* plasmid and blunt-cloned into the p*ENK-GFP* plasmid after removal of the GFP gene. *pDYN*

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plasmids. To construct an HSV vector that expresses GFP under the control of the dynorphin promoter, a ~2.0 Kb fragment (-1858 to +135) upstream of the dynorphin gene was PCR cloned from rat genomic DNA using an upstream primer (5'-AAAGCTTAGGATAGAGATGAGAGAGGGCAGG-3') and a downstream primer (5'-GCTCTAGGTACCGATACTTACCTGCGTGCTGCTTTGTC-3') that also introduced a multiple-cloning site. The PCR product was sub-cloned into a TOPO plasmid using the Zero Blunt TOPO PCR cloning Kit (Invitrogen, Carlsbad, CA) and then inserted into the pHSV-PrPUC plasmid. To produce a version of this plasmid that expresses the hM_4D gene, the hemagglutinin-tagged hM_4D gene was excised from a pc*DNA3.1* plasmid and blunt-cloned into the p*DYN-GFP* plasmid after removal of the GFP gene. For all plasmids, restriction mapping was used to identify successfully ligated clones and their entire sequences were confirmed by PCR. In order to prevent HSV promoter-driven "leakage" expression in non-targeted neurons, the promoter fragments and the GFP (or hM_4D) genes were inserted in a reverse orientation with respect to the endogenous HSV promoter/origin of replication sequence, and two SV40 polyadenylation sequences were positioned between the end of the HSV promoter and the end of the GFP (or hM_4D) genes. The amplicons were packaged into viral vectors using replication-deficient helper virus as described previously[1].

Surgery and viral gene transfer. Rats were anesthetized with 2-4% isoflurane (Webster Veterinary Supply, Sterling, MA). Using standard stereotaxic procedures, 27-gauge stainless steel injectors were placed above targeted brain regions. Coordinates from bregma (mm) for dorsomedial striatum: A/P 0.2; M/L ±2.3; D/V -5.1 from skull surface,

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for substantia nigra pars reticulata: A/P -5.3; M/L ±2.4; D/V -7.7 and for globus pallidus external: A/P -0.9; M/L ±2.7; D/V -6.2. Then, 3 μl of either GFP (p*ENK* or p*DYN*, control) or hM_4D (p*ENK* or p*DYN*) viral vector (~200,000 infectious units in 10% sucrose) was infused (unilaterally or bilaterally, depending on experiment) over a 15 min period at a flow rate of 0.2 μl/min. The injector was left in place an additional 5 min to minimize diffusion up the injector tract. For tract tracing experiments, 2 μl of either p*ENK-GFP* or p*DYN-GFP* viral vector was infused into the dorsomedial striatum and 1 μl of a 2% Fluoro-Gold solution (Fluorochrome, Denver, CO) was infused into the SNpr or GPe. Experiments were carried out at 7-10 d post-infusion, based on pilot studies that examined the onset of gene expression. For electrophysiology experiments, 1 μl of either p*HSV-GFP* or p*HSV-hM$_4$D* was infused into the dorsal striatum. For voltammetry experiments, in-house constructed carbon-fiber electrodes were chronically implanted into the nucleus accumbens core (coordinates relative to bregma (mm): A/P 1.3; M/L ±1.3; D/V -7.0) for unilateral or bilateral voltammetric recordings and bilateral guide cannula were implanted above the ventral tegmental area (coordinates relative to bregma (mm): A/P -5.6; M/L ±0.5; D/V -7.0) for viral infusions. Starting 3 weeks post-surgery, rats were food restricted to ~ 90% of the free-feeding weight with an increase of 1.5% per week. To minimize neophobia, rats were pre-exposed to food pellets (45-mg food pellets, BioServ, NJ) in the home cage prior to magazine training in an operant chamber (Med Associates, VT). Once delivery of an unexpected food pellet delivery elicited a signal with a cyclic voltammagram that correlated ($r^2 > 0.75$) with the template voltammagram of dopamine (see Fast-scan cyclic voltammetry section), rats received 2 μl infusions of the neuron-specific p*HSV-hM$_4$D* viral vector, which is strongly expressed

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in dopamine neurons[2], into the ventral tegmental area through an infusion cannula that extended 1 mm past the guide cannula at a rate of 0.2 μl/min. Experimental treatments were performed on days 3 and 4 post-virus infusion, corresponding to the time of maximal gene expression with this viral vector[3]. For all experiments, accuracy of injection coordinates was confirmed by visualization of GFP or hemagglutinin immunofluorescence or by cresyl violet staining of the injection needle tracts in 40 mm tissue sections. Rats with injection sites outside of the targeted brain region were excluded from the experiments.

Immunohistochemistry/Photomicrograph preparation. Floating sections (40 μm) were washed in 0.5% Triton-X/PBS for 10 min, then blocked in 5% normal goat serum (NGS)-0.25%Triton-X/PBS for 1 h. Sections were then incubated in 2.5% NGS-0.25%Triton-X/PBS containing antibodies to substance P (1:400, Chemicon/Millipore), GFP (1:400, Chemicon/Millipore), hemagglutinin (1:200, Chemicon/Millipore), Fluoro-Gold (1:8,000, Fluorochrome), and/or c-Fos (1:400, Santa Cruz) and/or in PBS containing methionine enkephalin (1:100, Immunostar) with gentle agitation at 4ºC for 24 to 72 h. Next, sections were rinsed 4 times in PBS and incubated in species-appropriate Alexa 488 (green) and/or Alexa 568 (red)-conjugated goat secondary antibodies (1:500, Invitrogen, Carlesbad, CA) for 1 h. Sections were washed 2 times in PBS, mounted on slides and cover-slipped with Vectashield mounting medium with DAPI (Vectorlabs, Burlingame, CA). Images were captured with a Bio-Rad Radiance 2000 confocal system and an associated Nikon fluorescence microscope using an argon/krypton laser and red laser diode. For photomicrographs without immunohistochemistry, tissue sections were

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mounted on slides and cover-slipped with Vectashield mounting medium. Slides were visualized with a Nikon Eclipse E600 with HyQ FITC, HyQ TRITC, and DAPI epi-fluorescence filters.

Electrophysiology. Two days following viral infusions of HSV viral vectors into the dorsal striatum, rats were decapitated following isoflurane anesthesia. Brains were removed, glued to a block and sliced with a vibratome in 4°C modified artificial cerebrospinal fluid (aCSF). Similar to previously described[4], coronal striatal slices (300 μm) were cut such that the preparation contained the signature anatomical landmarks (e.g. the anterior commissure) that delineated striatal subregions. After a 1-2 h recovery period, slices were transferred to a holding chamber to a submerged recording chamber where they were continuously perfused with oxygenated aCSF mainained at 33°C. Standard whole-cell recordings were made from the infected cells (identified by their GFP signals) and the uninfected cells (controls) using a MultiClamp 700B amplifier (Molecular Device) through an electrode (2-6 MΩ) in all electrophysiological experiments[4,5,6,7]. The slices were continuously perfused with regular oxygenated aCSF (in mM: 119 NaCl, 2.5 KCl, 1.0 NaH_2PO_4, 1.3 $MgCl_2$, 2.5 $CaCl_2$, 26.2 $NaHCO_3$, and 11 glucose, 290–295 mOsm, equilibrated at 31–34°C with 95% O_2/5% CO_2). Current-clamp recordings were used to measure evoked action potential firing, in which the resting membrane potential was adjusted to -80 mV. Input resistance was measured as the potential changes upon injected currents between – 150 pA and 150 pA). For these experiments, a K^+-based internal solution was used (in mM: 130 K-methansulfate, 10 KCl, 10 HEPES, 0.4 EGTA, 2.0 $MgCl_2$, 3.0 MgATP, 0. 5 Na_3GTP, pH 7.2–7.4; 290–300

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mOsm). Electrophysiological recordings were made from MSNs located in the striatum. The MSNs, which comprise >90% of all neuronal types in the striatum, could be readily identified in the experimental condition by their mid-sized somas as well as their electrophysiological characteristics, such as hyperpolarized resting membrane potentials, long latency before the first action potential, lack of the I_h component, and rectification of the I-V curve at hyperpolarized voltages[8].

Fast-scan cyclic voltammetry. During all experimental sessions, the chronically implanted microelectrodes were connected to a head-mounted voltammetric amplifier, which interfaces with a PC-driven data acquisition system (National Instruments, TX) through an electrical swivel (Med Associates, VT) mounted above the operant chamber. The electrodes were held at -0.4 V against an Ag/AgCl reference. Scans every 100 ms consist of ramping up to +1.3 V and back to -0.4 V at 400 V/s in a triangular fashion. Waveform generation for voltage ramps, data acquisition and analysis was carried out on a PC-based system using software written in LabVIEW (National Instruments, TX). Data were five-point smoothed and the concentration of dopamine was calculated through chemometric analysis[9]. Rats received CNO (3 mg/kg) or vehicle (counterbalanced within subject design on days 3 and 4 post-virus infusion) 10 minutes prior to initiating data collection. A single food pellet was delivered on a variable interval schedule of 5 minutes over the next 90 minutes (18 pellets given).

Locomotor sensitization. For experiments using the p*ENK* viral vectors, the locomotor activating effects of amphetamine were measured using locomotor activity boxes (22 x

45 x 23 cm; San Diego Instruments, San Diego, CA). Briefly, 7 days following viral infusions rats received four injections of amphetamine (2 mg/kg) or vehicle over an 8-day treatment period (one injection ~ every other day). This protocol was designed to induce threshold amphetamine sensitization in GFP control rats. Thirty minutes prior to each drug treatment, rats received an injection of CNO (1 mg/kg) or vehicle and returned to their home cage. Rats then received injections of amphetamine or vehicle and were placed into the locomotor activity boxes. Following a one-week withdrawal period all rats received a 2 mg/kg amphetamine challenge in the absence of CNO pre-treatment. Behavior was recorded for 90 min during each test session. As an additional control, a similar experiment was performed in animals that received p*ENK* viral infusions, except all animals received a vehicle pre-treatment during the treatment sessions (i.e., no animals received CNO injections during the experiment). The number of cage crossovers, defined as two consecutive beam breaks - photobeams spaced 2" apart, was used as an index of locomotor activity. For experiments using the p*DYN* viral vectors, rats received six injections of amphetamine (2 mg/kg) or vehicle over an 8-day treatment period (one injection ~ every day). This protocol produces consistent amphetamine sensitization in GFP control rats. Thirty minutes prior to each drug treatment, rats received an injection of CNO (1 mg/kg) or vehicle and returned to their home cage. Following a one-week withdrawal period all rats received a 30-min habituation period to the locomotor activity boxes, followed by an injection of saline (behavior recorded for 30 min) and a 0.5 mg/kg amphetamine challenge in the absence of CNO pre-treatment (behavior recorded for 90 min).

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Fos expression. Ten days following viral infusions, rats were transported to a novel test environment and given an injection of vehicle or CNO (3 mg/kg) followed 30 minutes later by an injection of amphetamine (5 mg/kg). Two hours later, rats were perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. Brains were post-fixed for 4 h in paraformaldehyde and transferred to PBS until processed for immunohistochemistry.

Statistics. Group differences in locomotor activity, electrophysiology, fast-scan cyclic voltammetry and the number of c-Fos+ cells were tested using two-way analyses of variance (ANOVAs; with or without repeated measures as warranted) followed by Bonferroni's post-hoc tests with corrections or unpaired t-tests. For all comparisons, α = 0.05. Statistical values for the Supplementary Figures are included in the figure legends. Statistical values from experiments in Figures 1 and 2 are included below.

Electrophysiology: Figure 1d,e; Data from d is expressed as a percent change from baseline in e. Paired t-test, * $P < 0.05$ hM_4D before vs. hM_4D after CNO application; P = 0.46 control before vs. control after CNO application, n=4-5. Figure 1g; $F_{3,85} = 11.08$, two-factor ANOVA; ** $P < 0.01$ hM_4D vs. hM_4D/CNO; $P = 1.0$, control vs. control/CNO). Expression of hM_4D receptors alone did not alter input resistance (P = 0.84) or action potential firing ($P = 0.64$).

c-Fos: Figure 1k; t-test, $t_9 = 4.197$, $P = 0.002$, n=5-6/group; Figure 1n: t-test, $t_9 = 2.29$, $P < 0.05$, n=5-6/group). Figure 1l: t-test, hemagglutinin-positive, $t_9 = 2.46$, $P < 0.05$; hemagglutinin-negative, $t_9 = 3.75$, $P < 0.01$; Figure 1o: t-test, hemagglutinin-positive, t_9 = 2.36, $P < 0.05$; hemagglutinin-negative, $t_9 = 1.82$, $P = 0.1$.

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Acute amphetamine response: Figure 2a: 2 way ANOVA, main effect of Treatment: $F_{1,34}$ = 71.67, $P < 0.0001$, n = 9-10/group, ***$P < 0.001$ versus saline-treated groups; main effect of Virus: $F_{1,34}$ = 1.82, P = 0.19 and interaction between Treatment and Virus factors: $F_{1,34}$ = 2.58, P = 0.12 not significant. Figure 2e: 2 way ANOVA, main effect of Treatment: $F_{1,32}$ = 85.62, $P < 0.0001$, n = 8-10, ***$P < 0.001$ versus saline-treated groups; main effect of Virus: $F_{1,32}$ = 0.49, P = 0.49 and interaction between Treatment and Virus factors: $F_{1,32}$ = 0.19, P = 0.67 not significant.

Sensitization: Figure 2b; 2 way RM ANOVA, main effect of Virus: $F_{1,18}$ = 10.61, P = 0.004; main effect of Session (S1 vs. S4): $F_{1,18}$ = 27.68, $P < 0.0001$ and interaction between Virus and Session factors: $F_{1,18}$ = 4.71, P = 0.04, ***$P < 0.001$ versus Session 1, ###$P < 0.001$ versus amphetamine-treated GFP group). Figure 2c,d; 2 way ANVOA, main effect of Virus: $F_{1,34}$ = 8.09, P = 0.008; main effect of Pretreatment: $F_{1,34}$ = 14.96, P = 0.0005 and interaction between Virus and Pretreatment factors: $F_{1,34}$ = 4.22, P = 0.047, ***$P < 0.001$ versus saline-pretreated group, ##$P < 0.01$ versus amphetamine-pretreated GFP group). Figure 2f; 2 way RM ANOVA, main effect of Session (S1 vs. S6): $F_{1,18}$ = 22.81, P = 0.0002; main effect of Virus: $F_{1,18}$ = 1.11, P = 0.31 and interaction between Virus and Session factors: $F_{1,18}$ = 0.68, P = 0.42 not significant, **$P < 0.01$ and *$P < 0.05$ versus Session 1. Figure 2g,h; 2 way ANOVA, main effect of Pretreatment: $F_{1,32}$ = 12.97, P = 0.001 and interaction between Virus and Pretreatment factors: $F_{1,32}$ = 7.56, P = 0.01; main effect of Virus: $F_{1,32}$ = 0.87, P = 0.36 not significant; ***$P < 0.001$ versus saline-pretreated groups, #$P < 0.05$ versus amphetamine-pretreated GFP group.

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