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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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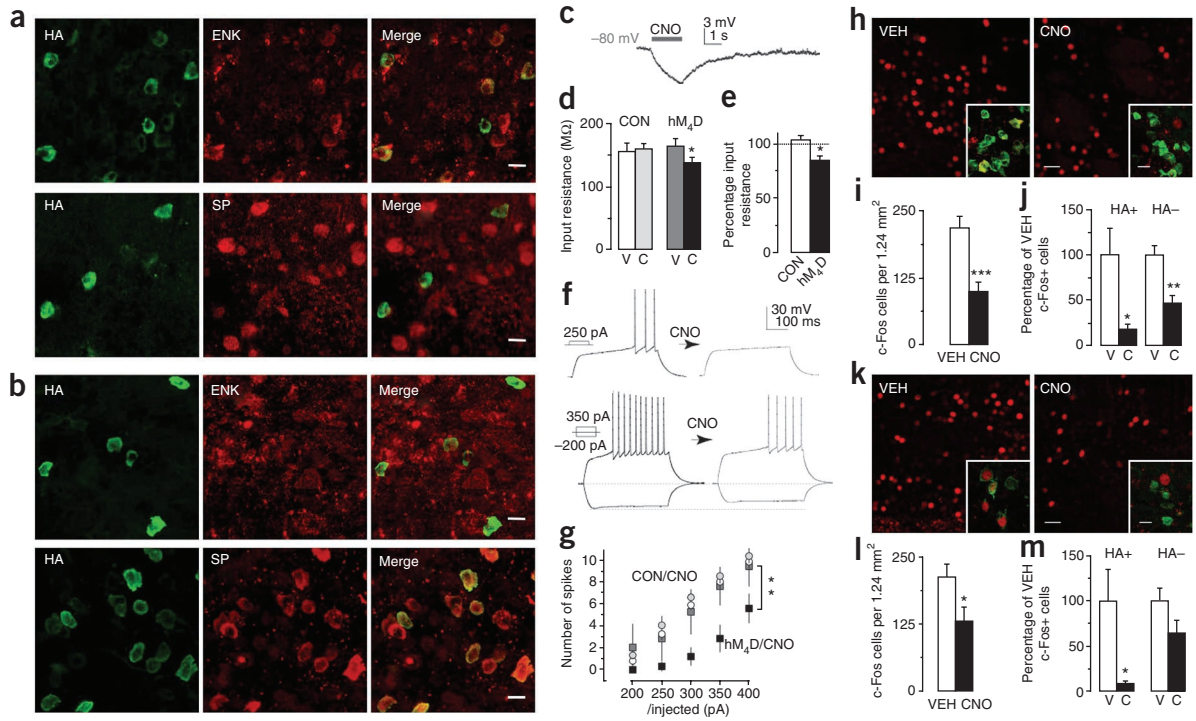

Figure 1 Transient and targeted attenuation of striatal cell signaling. (**a**) p*Enk-hM*$_4$*D* receptors were selectively expressed in striatopallidal MSNs. Green, hemagglutinin (HA); red, ENK (top) and substance P (SP, bottom); yellow, colocalization of neurons. Scale bars represent 10 µm. (**b**) 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

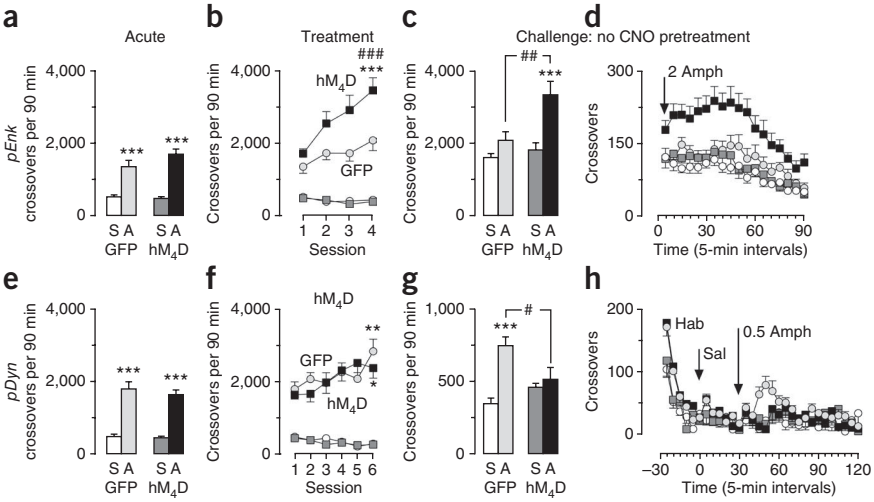

Figure 2 Transiently reducing excitability of striatopallidal or striatonigral neurons had opposing effects on amphetamine sensitization. (**a**) Acute locomotor responses to amphetamine following activation of p*Enk-hM*$_4$*D* receptors ($n = 9$–10 per group). $^{***}P < 0.001$ versus saline-treated 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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