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

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Abstract

The dorsal striatum plays an important role in 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 a novel approach that relies on the viral-mediated expression of an engineered GPCR (hM_4D), we demonstrated 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 in rats, CNO did not change acute locomotor responses to amphetamine but altered 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 behaviors such as sensitization.

Despite the overwhelming negative consequences of drug addiction, psychostimulant usage and 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[1,2,3]. The striatum has been identified as a key site for many of the behavioral and neurobiological adaptations thought to form the core processes that mediate addiction[1,2,3]. The majority of neurons within the striatum (~95%) are GABAergic medium spiny projection neurons (MSNs) that differ in their neuropeptide expression and form two major efferent pathways[4]. Striatopallidal MSNs

Competing interests statement

The authors declare that they have no competing financial interests.

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[5],[6]. However, there is little empirical evidence to support their differential role in the control of behavior because 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 combined two novel strategies: viral vectors that use either the ENK or DYN gene promoters to target transgene expression to striatopallidal or striatonigral neurons, respectively, and an engineered GPCR ($G_{i/o}$-coupled human muscarinic M_4 DREADD; Designer Receptor Exclusively Activated by a Designer Drug; hM_4D)[7] that is activated by an otherwise pharmacologically inert ligand, clozapine-*N*-oxide[8],[9] (CNO; Fig. 1a, h). Following expression in cultured neurons, administration of CNO stimulates $G_{i/o}$-coupled hM_4D receptors thus activating inwardly rectifying potassium 3 (Kir3) channels, resulting in membrane hyperpolarization and transient neuronal silencing[9].

To test for 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-hM₄D*) or the DYN promoter (p*DYN-hM₄D*). We found that p*ENK-hM₄D* expression was primarily in ENK-containing MSNs (90% of hemagglutinin cells were ENK+, 85 out of 94; 6% of hemagglutinin cells were substance P +, 4 out of 70 cells; Fig. 1b) whereas p*DYN-hM₄D* expression was primarily in substance P-containing MSNs (95% of hemagglutinin cells were substance P+, 109 out of 115 cells; 5% of hemagglutinin cells were ENK+, 5 out of 97 cells; Fig. 1i). Similar results were obtained following infusion of promoter-specific viruses that express green fluorescent protein (p*ENK-GFP* and p*DYN-GFP*; SupplementaryFigs. 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 used injections of the retrograde tracer Fluoro-Gold into these brain regions followed by 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 co-localized with striatal Fluoro-Gold expression following infusions into the GPe but not the SNpr (Supplementary Fig. 2b) whereas p*DYN-GFP* cells co-localized with striatal Fluoro-Gold expression following infusions into the SNpr but not the GPe (Supplementary Fig. 3b). Expression of the viral vectors did not change the number of ENK+ or substance P+ neurons in the region of viral infection, suggesting that use of these promoters for viral-mediated gene transfer did not interfere with endogenous neuropeptide levels. All of these results demonstrate that the p*ENK* and p*DYN* viral vectors express genes in their appropriately segregated striatal cell populations.

Although hM_4D receptor-based techniques have been demonstrated to modulate activity of other neuronal types[9], their ability to affect striatal neurons has not been examined. Therefore, we infected striatal medium spiny neurons with hM_4D receptors under the control of a herpes simplex virus (HSV) promoter, prepared coronal slices of dorsal striatum two days later, and examined how hM_4D-expressing medium spiny striatal neurons respond to CNO. We observed that local application of CNO (10 μM) induced a hyperpolarization of the membrane potential (~7 mV, the baseline membrane potential was adjusted to −80 mV; Fig. 1c) and reduced the input resistance of the neurons after CNO application (Fig. 1d, e), suggesting that potassium conductance (i.e., Kir3-mediated current) is activated by CNO in hM_4D receptor-expressing neurons. Furthermore, perfusion of CNO substantially decreased the number of evoked action potentials in hM_4D-expressing neurons, but not in control cells, thereby effectively inhibiting the functional output of the virally-infected neurons Expression of hM_4D receptors alone did not alter input resistance ($P = 0.84$) or action potential firing ($P = 0.64$). (Fig. 1f, g). Taken together, these data suggest that, similar to hippocampal neurons[9], the hM_4D/CNO-based method can effectively decrease the excitability of rat striatal neurons.

As additional proof of concept, we tested whether hM_4D receptors would block neurotransmission in a well-established circuit where neural activity is predictably evoked by behaviorally-relevant stimuli. Accordingly, we infected ventral tegmental area (VTA) neurons with hM_4D receptors under control of a HSV promoter, which expresses strongly in dopamine neurons[10], and used fast-scan cyclic voltammetry to measure changes in dopamine release in the nucleus accumbens after unexpected delivery of a food reward[11]. Administration of CNO significantly attenuated food pellet-evoked dopamine release in the nucleus accumbens compared to vehicle (Supplementary Fig. 4). Finally, we tested whether decreasing activity of specific neuronal cell types in the striatum *in vivo* could alter the ability of amphetamine to stimulate Fos expression. Psychostimulants, such as amphetamine, are robust activators of c-*fos* in the striatum[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 play an important role in the initiation and maintenance of the neural adaptations associated with psychomotor sensitization[1],[14]. We found that CNO-mediated activation of p*ENK-hM_4D* receptors significantly reduced the total number of amphetamine-induced c-Fos cells in the striatum (Fig. 1k and Supplementary Fig. 5a). This reduction occurred in both hemagglutinin-positive neurons (i.e, those expressing hM_4D receptors) and hemagglutinin-negative neurons (i.e., those not expressing hM_4D receptors; Fig. 1l), suggesting a neuronal cross-talk effect between hM_4D-expressing neurons and uninfected neurons. Significant reductions in the total number of amphetamine-evoked c-Fos cells and in the number of hemagglutinin-positive c-Fos cells were also seen when hM_4D receptors were activated in direct pathway neurons (Fig. 1n, o and Supplementary Fig. 5b). Importantly, these effects are not simply caused by viral expression of a novel receptor since expression of either p*ENK-hM_4D* or p*DYN-hM_4D* receptors in the absence of CNO treatment had no effect on the number of amphetamine-evoked Fos cells (Supplementary Figs. 6 and 7). Thus, these findings demonstrate that the CNO-mediated activation of hM_4D 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, often referred to as behavioral sensitization. Importantly, sensitization involves some of the same neural circuits implicated in the development of human drug addiction[3]. Here we use our novel tools to investigate 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 where striatonigral neurons promote sensitization and striatopallidal neurons suppress sensitization, consistent with their conceptually-proposed roles in behavioral activation and inhibition respectively[5],[6]. Accordingly, we tested whether biochemically 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.

For the first study, we used a treatment regimen that induces a threshold level of locomotor sensitization in GFP controls (four drug exposures). Following a withdrawal period, a moderate challenge amphetamine dose (2 mg/kg) was administered in the absence of CNO-induced attenuation of neuronal activity to determine if sensitization was persistent. CNO-mediated activation of p*ENK-hM$_4$D* receptors during amphetamine treatment did not alter the acute locomotor response to amphetamine (Fig. 2a). However, CNO-mediated disruption of neuronal activity in indirect-pathway neurons facilitated the development of a significantly more robust sensitization compared to GFP controls (Fig. 2b). This enhancement of sensitization was maintained on the amphetamine challenge, which was conducted one-week later in the absence of CNO treatment (Fig. 2c, d). These effects can be attributed to a CNO-dependent reduction of activity of striatopallidal neurons by hM$_4$D receptors because hM$_4$D receptor expression without CNO treatment does not produce locomotor sensitization to this mild dosing regimen of amphetamine (Supplementary Fig. 6).

In order to determine whether striatonigral neurons can regulate drug responsiveness in an opposing fashion, we next tested the effect of biochemically diminishing excitability of striatonigral neurons in the dorsomedial striatum during a drug treatment regimen of amphetamine that produces robust sensitization in GFP controls (six drug exposures) as well as during a low challenge dose of amphetamine (0.5 mg/kg) in the absence of receptor-mediated disruption of neuronal activity. As with indirect pathway dampening, CNO-mediated decreases of excitability of direct pathway neurons during amphetamine treatment did not alter the acute locomotor response to amphetamine (Fig. 2e). Although the development of sensitization appeared similar to the GFP controls following CNO-induced activation of p*DYN-hM$_4$D* receptors during the treatment phase (Fig. 2f), 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 by hM$_4$D receptors because hM$_4$D receptor expression in the absence of CNO treatment did not block the development of locomotor sensitization as sensitization was observed during the treatment phase and on the drug challenge (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.

In conclusion, these data provide 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 novel and powerful approach for deconstructing the molecular basis of addiction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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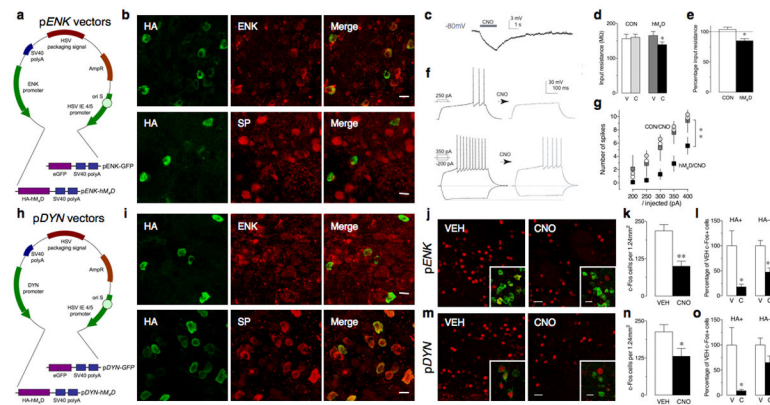14. Hyman SE, Malenka RC. Nat Rev Neurosci. 2001; 2:695–703. [PubMed: 11584307]

Figure 1.

Transient and targeted attenuation of striatal cell signaling. (**a**, **h**) Amplicon maps of p*ENK-hM$_4$D*/p*ENK-GFP* (**a**) and p*DYN-hM$_4$D*/p*DYN-GFP* (**h**) targeting vectors. (**b**, **i**) Confocal microscopy showed that p*ENK-hM$_4$D* receptors were selectively expressed in striatopallidal MSNs (**b**) whereas p*DYN-hM$_4$D* receptors were selectively expressed in striatonigral MSNs (**i**). Green, hemagglutinin (HA); Red, ENK (top) and substance P (SP, bottom); Yellow, co-localization of neurons. Scale bars, 10 μm. (**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 vs. hM$_4$D after CNO application, n=4–5. (**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 vs. hM$_4$D/CNO (**k**, **n**) CNO-mediated activation of p*ENK-hM$_4$D* (**k**) or p*DYN-hM$_4$D* (**n**) receptors decreased the number of amphetamine-induced Fos cells (CON: vehicle-treated p*ENK-hM$_4$D* and p*DYN-hM$_4$D*, respectively) (p*ENK*: $P = 0.002$, n=5–6/group; p*DYN*: $P < 0.05$, n=5–6/group). (**l**, **o**) Amphetamine-evoked c-Fos+ cells were reduced in both hemagglutinin-positive ($P < 0.05$) and hemagglutinin-negative ($P < 0.01$) neurons in the pENK-hM$_4$D experiment (**l**) and in hemagglutinin-positive neurons ($P < 0.05$) in the p*DYN-hM$_4$D* experiment (**o**). Representative Fos immunohistochemistry sections (red) from p*ENK-hM$_4$D* (**j**) and p*DYN-hM$_4$D* (**m**) infused striatum of vehicle (VEH) and CNO-treated rats. Scale bars, 50 μm. Insets depict single-labeled Fos cells (red), hemagglutinin cells (green) and dual-labeled cells (yellow). Scale bars, 10 μm. Data represent mean ± SEM. V = vehicle treatment, C = CNO treatment.
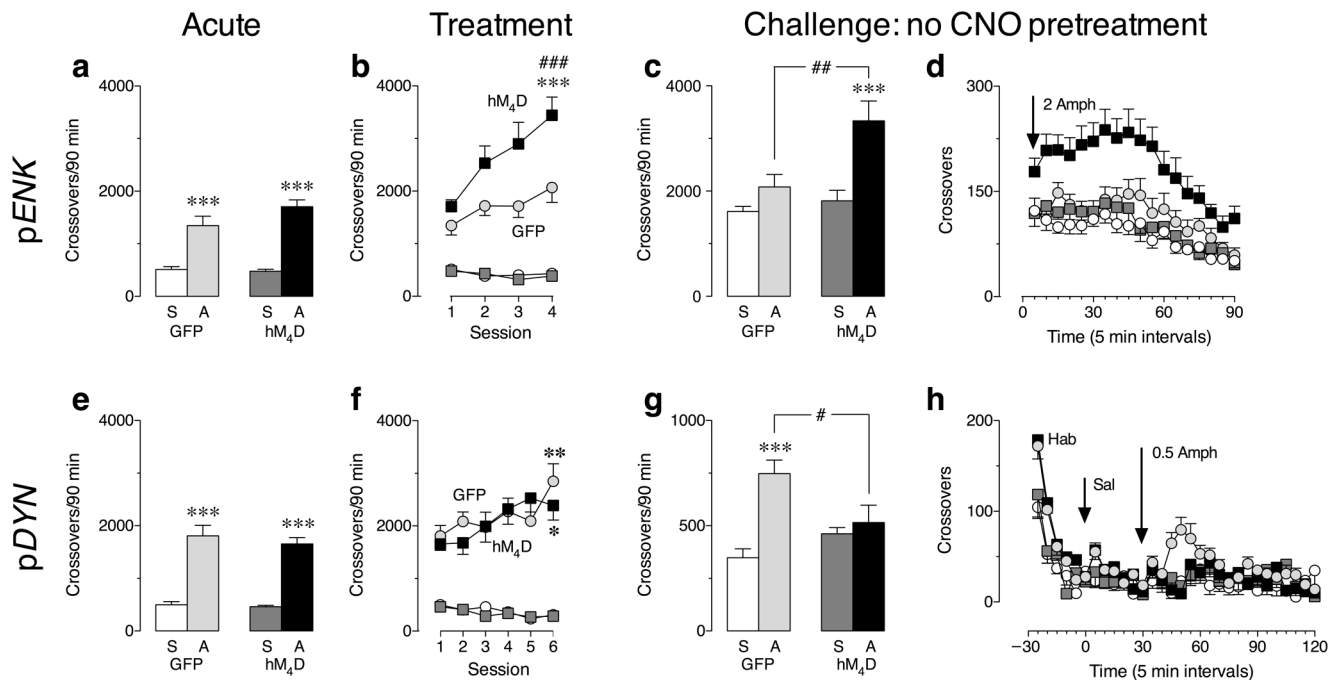
Figure 2.

Transiently reducing excitability of striatopallidal or striatonigral neurons had opposing effects on amphetamine sensitization. (**a**, **e**) Acute locomotor responses to amphetamine following CNO-induced activation of p*ENK-hM₄D* (**a**) and p*DYN-hM₄D* (**e**) receptors. p*ENK*, n = 9–10/group; p*DYN*, n = 8–10/group, ****P* < 0.001 versus saline-treated groups. (**b**) Activation of p*ENK-hM₄D* receptors during amphetamine treatmentenhanced the development of locomotor sensitization. ****P* < 0.001 versus Session 1, ###*P* < 0.001 versus amphetamine-treated GFP group). (**c**, **d**) Enhanced sensitization in the amphetamine-pretreated p*ENK-hM₄D* group was maintained during the challenge test. ****P* < 0.001 versus saline-pretreated group, ##*P* < 0.01 versus amphetamine-pretreated GFP group. (**f**) Activation of p*DYN-hM₄D* receptors during amphetamine treatment initially produced locomotor sensitization similar to p*DYN-GFP* controls. ***P* < 0.01 and **P* < 0.05 versus Session 1. (**g**, **h**) Sensitization in the amphetamine-pretreated p*DYN-hM₄D* 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 ± SEM. S = saline, A = amphetamine. Squares represent hM₄D 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. All experimental procedures were approved by the University of Washington Institutional Animal Care and Use Committee and were conducted in accordance with National Institutes of Health guidelines. See supplementary methods for additional statistics information.