

A $G\alpha_s$ DREADD Mouse for Selective Modulation of cAMP Production in Striatopallidal Neurons

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Here, we describe a newly generated transgenic mouse in which the Gs DREADD (rM3Ds), an engineered G protein-coupled receptor, is selectively expressed in striatopallidal medium spiny neurons (MSNs). We first show that *in vitro*, rM3Ds can couple to $G\alpha_{\text{OIR}}$ and induce cAMP accumulation in cultured neurons and HEK-T cells. The rM3Ds was then selectively and stably expressed in striatopallidal neurons by creating a transgenic mouse in which an adenosine2A (adora2a) receptor-containing bacterial artificial chromosome was employed to drive rM3Ds expression. In the adora2A-rM3Ds mouse, activation of rM3Ds by clozapine-N-oxide (CNO) induces DARPP-32 phosphorylation, consistent with the known consequence of activation of endogenous striatal $G\alpha_s$ -coupled GPCRs. We then tested whether CNO administration would produce behavioral responses associated with striatopallidal G_s signaling and in this regard CNO dose-dependently decreases spontaneous locomotor activity and inhibits novelty induced locomotor activity. Last, we show that CNO prevented behavioral sensitization to amphetamine and increased AMPAR/NMDAR ratios in transgene-expressing neurons of the nucleus accumbens shell. These studies demonstrate the utility of adora2A-rM3Ds transgenic mice for the selective and noninvasive modulation of $G\alpha_s$ signaling in specific neuronal populations *in vivo*. This unique tool provides a new resource for elucidating the roles of striatopallidal MSN $G\alpha_s$ signaling in other neurobehavioral contexts.

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INTRODUCTION

Cell type-specific control of neuronal signaling can provide valuable insight into the neuronal correlates of behavior, disease, and mechanisms of therapeutic efficacy. Medium spiny neurons (MSNs) are the projection neurons of the striatum and are segregated into two populations defined by their efferent projections and their neuropeptide- and receptor-expression profiles. Striatonigral MSNs project to the substantia nigra and express dynorphin and substance P

neuropeptides, in addition to being enriched in D1-dopamine receptors. Striatopallidal MSNs project to the globus pallidus, express enkephalin, and are enriched in D2-dopamine and A2A-adenosine receptors (Ferre *et al*, 1992; Gerfen *et al*, 1990; Svenningsson *et al*, 1998). Activity of striatopallidal neurons is thought to produce an inhibitory effect on motor behavior (DeLong, 1990; Kravitz *et al*, 2010), and these D2-dopamine receptor-containing neurons have been implicated in both the etiology and potential therapy of many neuropsychiatric diseases (Beaulieu and Gainetdinov, 2011; Emilien *et al*, 1999).

The $G\alpha_s$ - and $G\alpha_i$ -G protein signaling cascades, modulated by D1 receptors and D2 receptors, respectively, are implicated in both the short-term excitability and the long-term plasticity of MSNs (Centonze *et al*, 2001; Surmeier *et al*, 2007). Striatum G protein-signaling cascades have primarily been studied as a consequence of activating

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dopamine receptors, but activation of G α_s -signaling downstream of other GPCRs also has significant effects. For example, striatopallidal G α_s signaling modulated by the A2A-adenosine receptor has been proposed to regulate psychostimulant activity (Brown and Short, 2008). To create a mouse model in which the cellular and behavioral consequences of striatopallidal-specific G α_s signaling (G protein signaling that increases cAMP production) can be studied, we took advantage of technology we developed, whereby evolved GPCRs (DREADDs, or Designer Receptor Exclusively Activated by Designer Drug) are expressed in a cell type-specific fashion to remotely control cellular signaling (Alexander *et al*, 2009; Armbruster *et al*, 2007).

In prior work, the ability of the rM3Ds DREADD to remotely control G α_s signaling in an inducible and cell type-specific fashion was demonstrated by transgenic expression of rM3Ds in pancreatic β -cells (Guettier *et al*, 2009). Here, we validate the rM3Ds in a neuronal context *in vivo* by creating a bacterial artificial chromosome (BAC) transgenic mouse line carrying the G α_s -DREADD (rM3Ds) downstream of the *adora2A* (adenosine A2A receptor) promoter. Adora2A-rM3Ds transgenic mice (JAX Stock No. 017863) afford a unique pharmacogenetic means to specifically modulate G $\alpha_{s/olf}$ signaling *in vivo* in a spatially and temporally controlled fashion using the pharmacologically inert, designer drug clozapine-N-oxide (CNO; see (Armbruster *et al*, 2007)). Herein, we use this novel transgenic line to validate the rM3Ds by measuring the biochemical, electrophysiological, and behavioral consequences of CNO administration to *adora2A*-rM3Ds transgenic mice.

MATERIALS AND METHODS

Plasmids

Plasmid maps are available at the DREADD site: www.dreadd.org

Drugs

Clozapine-N-oxide (CNO) was obtained from the NIH as part of a Rapid Access to Investigative Drug Program funded by the National Institute of Neurological Disorders and Stroke (NINDS). D-amphetamine (AMPH) and isoproterenol were purchased from Sigma (St Louis, MO). For experiments in mice, CNO was first dissolved in DMSO, then brought to final concentration with 0.9% saline and a final concentration of DMSO of 0.5%. Amphetamine was dissolved directly into 0.9% saline. For all experiments, the appropriate (eg, 0.9% saline for amphetamine experiments and 0.5% DMSO in saline for CNO experiments) vehicle controls were utilized. Unless otherwise noted, the dose of CNO was 1.0 mg/kg and the dose of amphetamine was 2.0 mg/kg. Drugs were injected *i.p.* at a volume 100 μ l/10 g body weight. For *in vitro* studies, drugs were dissolved in DMSO at 10 mM as stocks and then diluted into sample buffer.

In vitro Studies

Agonist-induced cAMP production was measured in living cells, as described previously (Abbas *et al*, 2009; Kimple

et al, 2009). Lentiviral studies expressing rM3Ds in cultured neurons were performed, as previously described with modification (Abbas *et al*, 2009; Alexander *et al*, 2009). For details, see Supplementary material and methods.

Plasmid maps and sequences are available on-line at: <http://pdsplit3.mml.unc.edu/projects/dreadd/wiki/WikiStart>.

Animal Subjects

Behavioral, biochemical, and electrophysiology experiments were performed at the University of North Carolina and Duke University in accordance with the National Institutes of Health's guidelines for the care and use of animals and with approved animal protocols from the Institutional Animal Care and Use Committees of the aforementioned institutions.

Generation of adora2A-rM3Ds mice. Transgenic mice were created by the Duke Neurotransgenic Core (Durham, NC) using the standard techniques as previously described (Gong *et al*, 2003). The rM3Ds-IRES-mCherry construct was recombinered into the adenosine2A bacterial artificial chromosome (BAC; GENSAT1-BX868, BAC address: RP24-238K3) downstream of the endogenous ATG codon, and this *adora2A*-rM3Ds-IRES-mCherry BAC was then injected into the pronucleus of B6SJLF1/J mouse oocytes. Genotyping was performed by PCR of genomic DNA extracted from tail clips in 20- μ l reaction volume using 2 \times Choice Taq Blue Mastermix (Denville Scientific, South Plainfield, NJ) and 0.4 nM each of the following primers for mCherry: FW 5'-GTGAGCAAGGGCGAGGAGG-3' REV: 5'-GTCGGCGGGG TGCTTAC-3' using the following cycle: initial denaturation: 94–4 min, followed by 30 cycles of 94 $^{\circ}$ C 30 s/65 $^{\circ}$ C 30 s/72 $^{\circ}$ C 30 s, followed by 72 $^{\circ}$ C 4 min final extension. PCR products were analyzed using gel electrophoresis (1%, Aqua Por LE, National Diagnostic, Atlanta, GA), and rM3Ds-positive mouse samples present a clear band at 200 bp (Supplementary Figure S2). From this screen, nine genotype-positive mice were found. These mice were bred to wild-type (C57BL/6J) mice, and their offspring were screened for mCherry expression via immunohistochemistry for mCherry following the methods detailed below. Three mCherry-positive founder lines were identified and named AD6, AD8, and AD10. The AD6 line was used in the present study. Following initial screening, AD6 mice were bred onto the C57BL/6J background. The breeding strategy had consistent pairing with an AD6 mouse always paired with a C57BL/6J mouse; thus, all mice used in these studies were hemizygous for the *adora2A*-rM3Ds gene. The AD6 line of mice is referred to as *adora2A*-rM3Ds mice in this manuscript. Mice used for behavioral studies were bred in large cohorts using a harem-breeding strategy, and littermate pairings between conditions were used. All behavioral studies were performed on mice of the F3 generation or later. The amphetamine behavioral sensitization studies and behavior core screen were performed on the F3 generation. Electrophysiology studies were performed on the F6 generation or later.

Other lines of mice used. Drd1a-EGFP (000297-MU) and Drd2-EGFP (000230-UNC) reporter mice were obtained

from the Mutant Mouse Regional Resource Centers and crossed with adora2A-rM3Ds mice for quantification of expression of rM3Ds in D1- and D2-expressing neurons. C57BL/6J mice were obtained from Jackson laboratories (Bar Harbor, ME).

Immunohistochemistry and Image Analysis

Immunohistochemistry was performed as described previously (Abbas *et al*, 2009). For complete information, see Supplementary materials and methods. Primary antibodies used: anti-RFP, ab65856, (1:1000, AbCam, Cambridge, MA); Anti-GFP, A11122, (1:1000, Invitrogen, Carlsbad, CA); anti-parvalbumin, P3088 (1:500, Sigma-Aldrich, St Louis, MO). Secondary antibodies used: goat anti-rabbit AlexaFluor-488 and goat anti-mouse AlexaFluor-594 antisera (1:250, Invitrogen). For colocalization studies, images of coronal slices were analyzed using ImageJ software (NIH). A region of interest in the body of the striatum was selected from one slice per mouse for $N=3$ mice, and the number of EGFP-positive and mCherry-positive cell bodies was quantified.

DARPP-32 Study

Mice were injected i.p. with CNO (5.0 mg/kg), cocaine (20.0 mg/kg), or vehicle and then sacrificed 15 min later by cervical dislocation. The ventral striatum was isolated using a rapid head-freeze-dissection technique as described previously (Beaulieu *et al*, 2004). Frozen-tissue samples were probe sonicated in 95 °C 1% SDS buffer containing 1 \times Halt phosphatase inhibitor (Halt, Pierce, 87786) and 1 \times protease inhibitor (Roche Diagnostics, Complete, no. 11697498001). Protein concentration of sample was determined using the BCA method (Pierce). Samples were boiled in Laemmli buffer, and 25 μ g of protein was loaded into 10% SDS-polyacrylamide gel electrophoresis gels, transferred onto nitrocellulose membranes, and incubated overnight at 4 °C with antibodies to pT34 DARPP-32 (phosphosolutions, Aurora, CO, p1025-34, 1:300, TBST/3% BSA), pT75 DARPP-32 (Cell Signaling, Danvers, MA, 9101, 1:500, TBST/5% BSA), total DARPP-32 (BD Transduction, San Diego, CA, 611520, 1:1500, TBST/5% BSA), pERK1/2 (Cell Signaling, 9101, 1:500, TBST/5% milk), total ERK1/2 (9107, 1:500, TBST/5% milk), pAKT308 (2965, 1:100, TBST/5% BSA), or total AKT (2920, 1:1500, TBST/5% BSA). Blots were imaged on the LI-COR Odyssey instrument (LI-COR Biosciences, Lincoln, NE). The phospho-specific probe band intensity was measured using NIH ImageJ software and was normalized to total probe band intensity. The fold stimulation was determined by normalizing these values to the average of the vehicle treatment group.

Locomotor Behavior Studies

Locomotor activity was assessed in photocell-based activity chambers under standardized environmental conditions using an AccuScan activity monitor (AccuScan Instruments, Columbus, OH) with a 41 \times 41 \times 30 cm Plexiglas chamber and a beam spacing of 1.52 cm as described (Abbas *et al*, 2009). Horizontal activity was measured as the total

distance covered in centimeters, as the total of all vectored X-Y coordinate changes and recorded in 5-min bins.

Spontaneous locomotor activity. Effect of CNO on spontaneous locomotion was measured during the dark phase of the light cycle to monitor behavior during a period of relatively high basal activity. Mice were placed in dark locomotor activity boxes at 2000 hours. At 2100 hours, mice were injected with a dose of CNO and returned to the locomotor chamber for 2 hours. *Novelty-Induced Locomotor Activity:* a separate cohort of adora2A-rM3Ds mice and wild-type mice were injected with CNO (1.0 mg/kg) or vehicle or CGS 21680 (0.1, 0.5 mg/kg) (Supplementary Figure S9) and placed in activity boxes 20 min later. Locomotor activity was recorded for 1 h.

Amphetamine Sensitization Study

Development phase. Mice were placed in locomotor activity boxes for 1 h to acclimate. Mice were then injected (i.p.) with drug(s) and/or vehicle and then returned to the activity chamber for 2 h. This was repeated once daily for 5 days. Drug doses were 2.0 mg/kg amphetamine and 1.0 mg/kg CNO. The following conditions were tested in separate cohorts: Cohort 1 (all adora2A-rM3Ds mice): amphetamine + CNO vs amphetamine + vehicle; Cohort 2 (all wild-type mice): amphetamine + CNO vs amphetamine + vehicle. *Incubation phase:* On days 6–14, mice were left in their home cages with no drug treatment. *Expression phase:* On day 15, mice were placed in locomotor chambers. One hour later, mice were injected with amphetamine (2.0 mg/kg) and returned to the activity chamber for 2 h. Locomotor activity chambers were located in a room separate from the mouse colony. All behavioral sensitization sessions were conducted from 1400–1700 hours. *Data analysis:* For each individual mouse, total distance traveled during the hour post injection was summed. Day-1 distance of a cohort was averaged, and this value was used to calculate each mouse's percentage of Day-1 distance traveled. Data are presented as the average of these percentages. Significance was determined using a Student's *t*-test on Day-15 data between the two groups for each cohort.

Electrophysiology. P23 mice were subject to the amphetamine behavioral sensitization procedure as detailed above. On day 15, mice were killed (without drug challenge) and tissue preparation and recording were performed as previously described (Thomas *et al*, 2001). Sagittal striatal slices (240 μ m in thickness) were cut containing the nucleus accumbens shell. After at least 1-hour recovery, slices were transferred to a recording chamber, where they were continuously perfused with oxygenated artificial cerebrospinal fluid containing 124 NaCl, 2.5 KCl, 1.2 NaH₂PO₄, 1 MgCl₂, 2.0 CaCl₂, 26 NaHCO₃, and 10 mM glucose. Whole-cell recordings were made from rM3Ds-containing cells (identified by their mCherry-fluorescent signals) at room temperature (24–25 °C) in the presence of 50 μ M picrotoxin and 1 μ M glycine. Recording pipette resistances were 2.5–3.5 M Ω with internal solution containing 103 mM cesium gluconate, 2.8 NaCl, 5 TEA-Cl, 20 HEPES, 0.2 EGTA,

5 lidocaine *N*-ethyl chloride, 4 Mg-ATP, 0.3 Na-GTP, 10 Na-phosphocreatine and pH 7.2–7.3. Experiments were discarded if series resistance (typically 15–20 M Ω) changed by >20%. Signals were low-pass filtered at 2 kHz and sampled at 10–20 kHz with an Axopatch 200B amplifier and a Digidata 1440A (Axon Instruments) for subsequent off-line analysis. To evoke excitatory postsynaptic currents, tungsten bipolar electrodes were placed at the prelimbic cortex-NAc border to stimulate afferents preferentially from prelimbic cortex. Stimuli with 150- μ s duration were delivered at 0.05 Hz. AMPAR/NMDAR ratio is the ratio of the peak of the excitatory postsynaptic current at -70 mV to the magnitude of the excitatory postsynaptic current at $+40$ mV at 60 ms following the stimulation.

Distribution of Resources

The *adora2A*-rM3Ds transgenic mice reported here are available from the Jackson Laboratory (JAX Stock No. 017863).

RESULTS

Generation and Characterization of *Adora2A*-rM3Ds Mice

The rM3Ds is an engineered and evolved muscarinic receptor that selectively couples to G_s G proteins (2), is activated by the inert designer drug CNO, and is insensitive to the native ligand acetylcholine (Guettier *et al*, 2009). To determine whether the rM3Ds can activate canonical G_s -type signaling in a neuronal environment, cAMP accumulation in response to increasing concentration of CNO was measured in cultured striatal (Figure 1b) and pyramidal (Supplementary Figure S1b) neurons infected with a lentivirus-expressing rM3Ds. CNO did not cause cAMP accumulation in uninfected, wild-type striatal neurons (Supplementary Figure S1a). As striatal neurons express $G\alpha_{olf}$, a G_s G protein enriched in striatum (Corvol *et al*, 2001; Drinnan *et al*, 1991; Zhuang *et al*, 2000), HEK-T cells were transfected with a 1:0, 1:1, and 1:3 ratio of rM3Ds to $G\alpha_{olf}$ and their cAMP accumulation in response to CNO measured. In Figure 1c, it can be seen that rM3Ds induces cAMP accumulation through the endogenous $G\alpha_s$ present in HEK-T cells in the 1:0 condition, and this accumulation is increased when $G\alpha_{olf}$ is co-transfected at ratios of 1:1 and 1:3. These experiments verify the functionality of rM3Ds in neurons and demonstrate that it can couple to both $G\alpha_s$ and $G\alpha_{olf}$.

To create striatopallidal-targeted rM3Ds transgenic mice, the *adora2A* BAC (GENSAT1-BX868)—a gene preferentially expressed in striatopallidal MSNs (Brown and Short, 2008; Chen *et al*, 2001)—was used to create a transgene carrying the rM3Ds construct. The *adora2A* BAC was recombined to include an rM3Ds—IRES—mCherry construct (Figure 1a) downstream of the *adora2A* start site, and mouse oocyte pronuclei were injected with the recombined and purified BAC to create mice expressing rM3Ds under control of the *adora2A* BAC. Three founder lines had detectable and essentially identical patterns of mCherry fluorescence, and the line denoted that 'AD6' was used for subsequent studies, referred to as '*adora2A*-rM3Ds mice'.

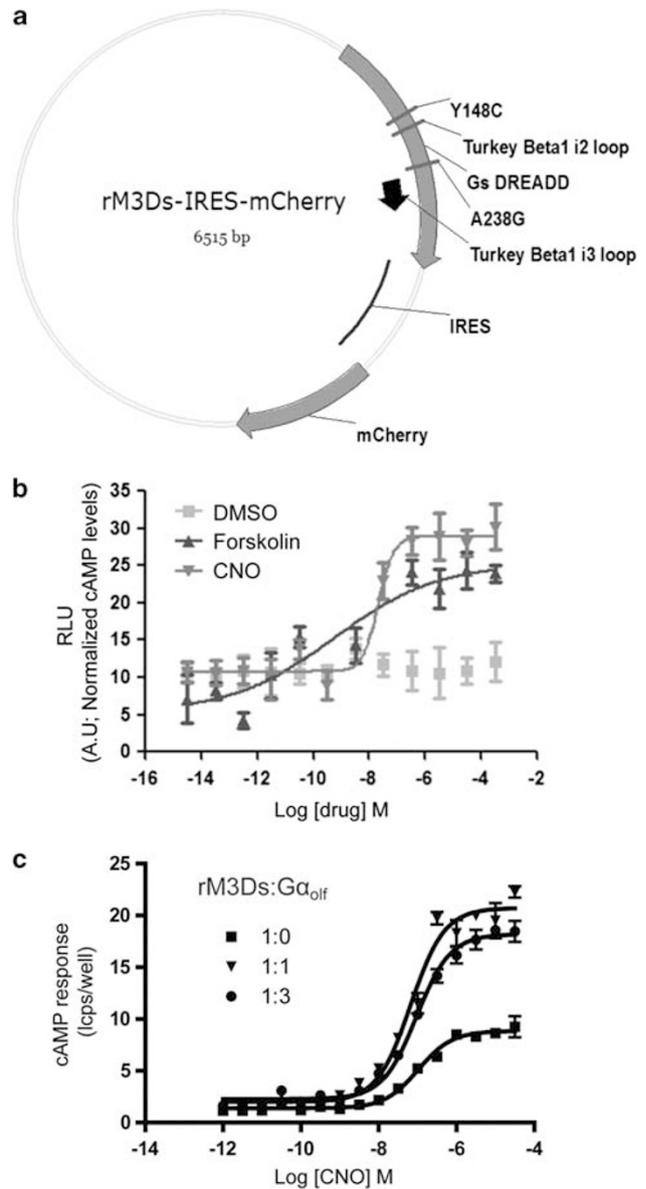


Figure 1 Creation and validation of *adora2A*-rM3Ds mice. (a) Schematic of rM3Ds-IRES-mCherry construct that was recombined into the *adora2A* BAC. (b) Activation of rM3Ds by clozapine-*N*-oxide (CNO) in mouse striatal neurons infected with pLenti-rM3Ds-mCherry causes a concentration-dependent increase in cAMP accumulation ($n=2$). (c) Increasing concentrations of $G\alpha_{olf}$ plasmid relative to rM3Ds-IRES-mCherry plasmid increase cAMP accumulation in response to CNO application in HEK293T cells ($n=3$).

Immunofluorescence microscopy revealed an expression pattern limited to the dorsal and ventral striata and consistent with striatopallidal projection (Figure 2a and Supplementary Figure S14). Cell type specificity was subsequently determined by crossing the *adora2A*-rM3Ds mice with the GENSAT *Drd1a*-EGFP and *Drd2*-EGFP reporter mice. *Drd2*-EGFP/*adora2A*-rM3Ds double-transgenic mice displayed 82.15% (± 5.14 , $n=695$ D2 cells in three mice) colocalization between *adora2A*-rM3Ds-mCherry cells and *Drd2*-EGFP cells (Figure 2b), whereas *Drd1a*-EGFP/*adora2A*-rM3Ds double-transgenic mice show a 2.51% (± 1.262 , $n=671$ D1 cells in 3 mice) colocalization between *adora2A*-rM3Ds-mCherry cells and *Drd1a*-EGFP

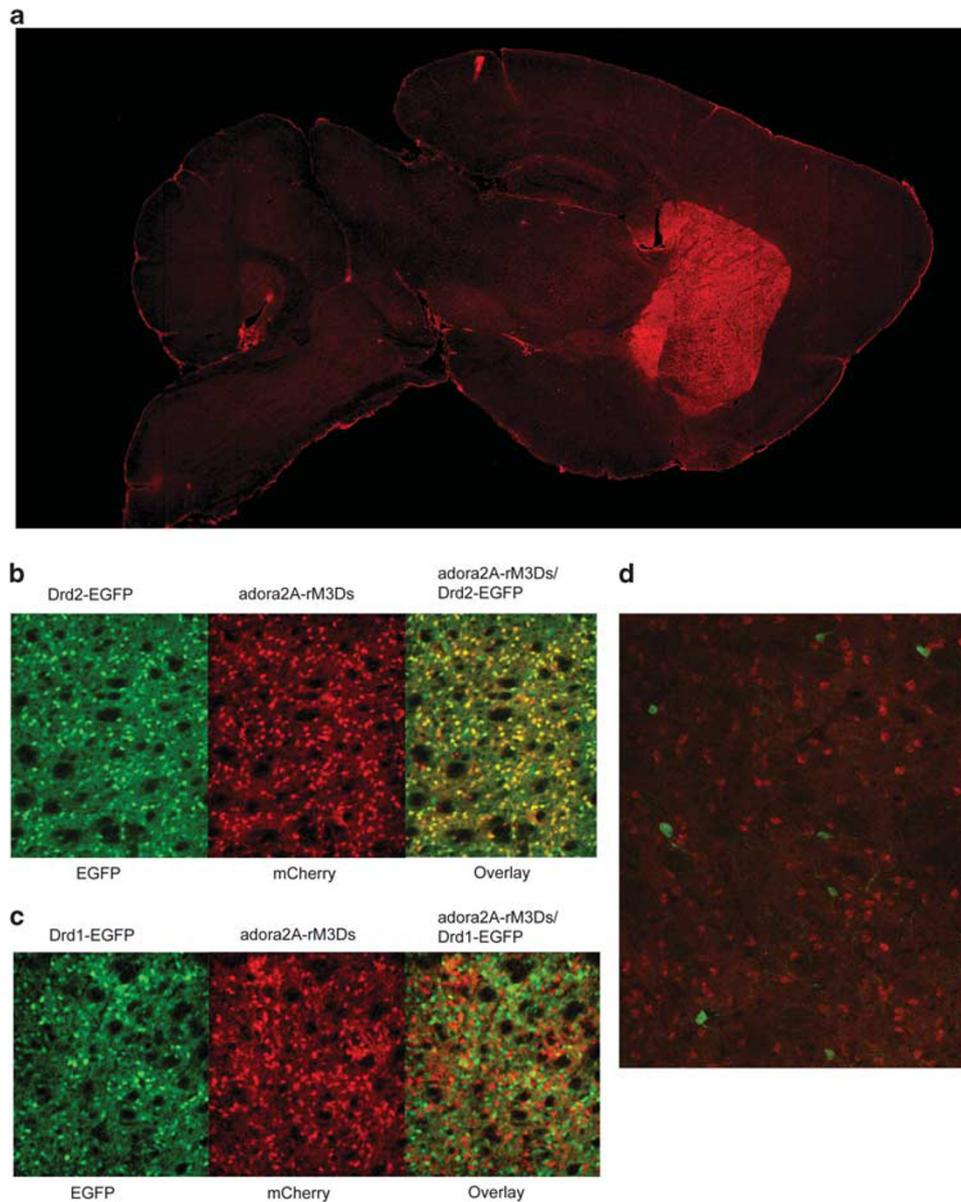


Figure 2 rM3Ds is expressed in striatopallidal neurons in adora2A-rM3Ds mice. (a) Sagittal whole-brain expression pattern of adora2A-rM3Ds mice. (b) Representative immunohistochemistry for mCherry (red) and EGFP (green) in adora2A-rM3Ds/Drd2-EGFP double-transgenic mice. (c) Representative immunohistochemistry for mCherry (red) and EGFP (green) in adora2A-rM3Ds/Drd1a-EGFP double-transgenic mice. (d) Immunohistochemistry for mCherry (red) and parvalbumin interneurons (green).

cells (Figure 2c). As expected, there was no colocalization between parvalbumin-containing interneurons and mCherry (Figure 2d). These data demonstrate that the adora2A-rM3Ds mice express rM3Ds selectively in striatopallidal D2-dopamine receptor-expressing MSNs.

A key signaling protein in MSNs is DARPP-32 (dopamine- and cyclic AMP-regulated phosphoprotein of 32 kD) (Svenningsson *et al*, 2004). DARPP-32 is directly phosphorylated at threonine 34 by protein kinase A, the canonical downstream effector of the $G_{\alpha_s/olf}$ signaling cascade. To determine whether the rM3Ds activates canonical $G_{\alpha_s/olf}$ signaling pathways in MSNs, we tested whether CNO caused phosphorylation of DARPP-32 at threonine 34. Mice were treated with CNO (5.0 mg/kg) or vehicle and striatal tissue was analyzed for pT34 DARPP-32 levels by western blot analysis. We found that CNO

increased pT34 DARPP-32 levels in adora2A-rM3Ds mice, but not wild-type mice (Figure 3). In addition, we found that CNO decreased pErk1/2 levels in adora2A-rM3Ds mice, but not wild-type mice and that CNO did not cause a change in pAKT308 levels (Supplementary Figure S11). To determine whether the rM3Ds was interfering with canonical G_{α_s} -type signaling in these neurons, we also measured the biochemical response to cocaine administration in adora2A-rM3Ds and wild-type mice. We observed no difference in cocaine-induced DARPP-32 Thr34 levels between wild-type and adora2A-rM3Ds mice (Supplementary Figure S12). CNO had no effect on pT75 DARPP-32 levels (Supplementary Figure S13). These findings demonstrate that rM3Ds activates canonical G_{α_s} -type signaling pathways *in vivo* and indicate that the endogenous signaling mechanisms are not disturbed.

To determine whether the adora2A-rM3Ds transgene had any CNO-independent activity that significantly altered behavior, transgenic mice and littermate controls were tested in a battery of behavioral tests in their naive state without CNO treatment. In comparison to wild-type littermates, adora2A-rM3Ds mice did not have significant differences in weight, locomotion, rotarod performance, prepulse inhibition, Morris water maze performance, sociability, elevated plus maze performance, olfaction, or

thermal sensation (Supplementary Figures S3–S8 and Supplementary Tables S1 and S2). To determine whether the presence of the striatopallidal rM3Ds influenced endogenous $G\alpha_s$ -type signaling, adora2A-rM3Ds and wild-type mice were injected with vehicle, 0.1, or 0.5 mg/kg CGS21680, a selective agonist for the adenosine A2A receptor, and placed in a locomotor chamber 20 min later to record novelty-induced locomotor activity (Supplementary Figure S9). A two-way ANOVA indicated a significant effect of dose ($p < 0.05$) but not genotype or interaction ($p = 0.66$ and $p = 0.51$, respectively). These data indicate that in the absence of designer-drug activation (CNO), the presence of the adora2A-rM3Ds transgene does not significantly alter mouse behavior. Combined, these control studies establish the suitability of adora2A-rM3Ds mice to activate $G\alpha_s$ -type signaling in striatopallidal neurons in a spatiotemporal manner.

CNO-Induced Modulation of Locomotion in Adora2A-rM3Ds Mice

Striatopallidal medium spiny neurons of the indirect pathway are thought to exert an inhibitory effect on locomotor behavior when activated (Albin *et al*, 1989; Alexander and Crutcher, 1990; DeLong, 1990; Kravitz *et al*, 2010). To determine whether rM3Ds activation in striatopallidal MSNs inhibits locomotion, adora2A-rM3Ds mice were injected with CNO (1.0 mg/kg) and placed into a novel open-field testing chamber 20 min later. CNO treatment of transgenic, but not wild-type, mice robustly decreased locomotion (Figure 4a). Similar effects and a dose dependency were demonstrated by testing spontaneous locomotion during the active period (dark phase) of the diurnal cycle (Figures 4b and c). These two data sets indicate that $G\alpha_{s/olf}$ -activation in striatopallidal neurons of adora2A-rM3Ds mice is sufficient to inhibit locomotion.

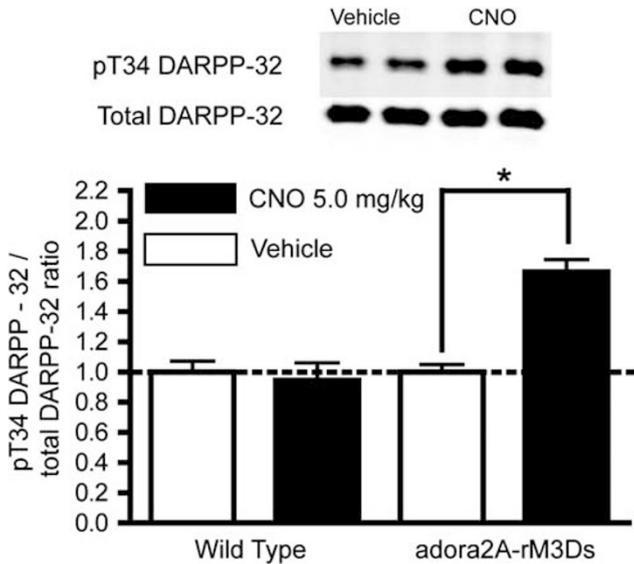


Figure 3 Clozapine-N-oxide (CNO) activates canonical $G\alpha_s$ -type signaling in adora2A-rM3Ds mice. CNO (5.0 mg/kg) increases pT34 DARPP-32 levels in the ventral striatum of adora2A-rM3Ds mice but not wild type (WT) mice. Representative western blots of pT34 DARPP-32 levels in adora2A-rM3Ds transgenic mice administered CNO (5.0 mg/kg) or vehicle are shown. * $p < 0.01$, two-tailed t -test, $n = 4$ –5.

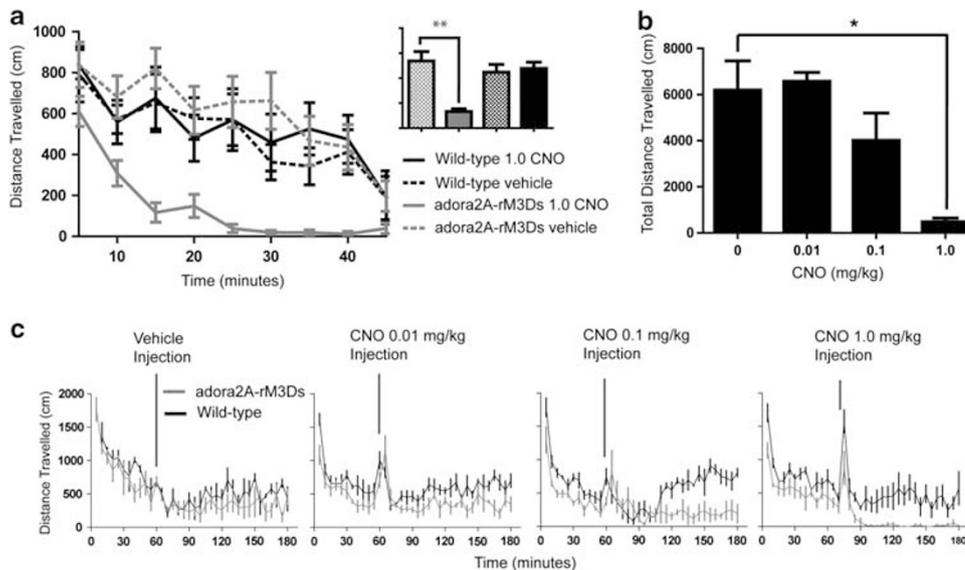


Figure 4 Clozapine-N-oxide (CNO) administration inhibits locomotor activity in adora2A-rM3Ds mice. (a) CNO blocks novelty-induced locomotor activity. Inset—histogram of data summed over time. Data are presented as distance traveled (cm) in 5 min bins \pm SEM starting 5 min after placement into chamber. Histogram is the total distance traveled as summed from minute 5 to minute 45 \pm SEM (b) Histogram of dark-phase locomotor activity summed for 100 min post-injection. Data are presented as total distance traveled as summed for 100 min starting 10 min after injection \pm SEM (c) CNO inhibits dark-phase spontaneous locomotor activity, time course. Data are presented as distance traveled (cm) in 5 min bins \pm SEM * $p < 0.05$; two-tailed t test, $n = 4$. ** $p < 0.005$; two-tailed t test, $n = 8$.

CNO-Induced Modulation of Amphetamine Sensitization

In rodent models, repeated exposure to psychostimulants produces an enhanced behavioral responsiveness, known as behavioral sensitization (Steketee and Kalivas, 2011). Amphetamine causes an increase in synaptic dopamine release, thereby inducing excessive stimulation of the D1- and D2-dopamine receptors (McKenzie and Szerb, 1968; Sulzer, 2011). The increased locomotor effects produced in behavioral sensitization may thus arise as a result of $G_{\alpha_s/olf}$ signaling in D1R-containing striatonigral MSNs, G_{α_i} signaling in D2R-containing striatopallidal MSNs, or a combination of these two effects. A potential role for G_{α_s} -type signaling in striatopallidal neurons in amphetamine-induced behavioral sensitization is suggested by pharmacological studies in rodents in which an A2AR agonist during the development phase inhibits sensitization (Shimazoe *et al*, 2000). Given these observations, we tested whether rM3Ds activation during development of amphetamine sensitization significantly altered behavioral sensitization. Mice were administered amphetamine (2.0 mg/kg) with or without CNO (1.0 mg/kg) for 5 days and their locomotor activity recorded. Mice were then given a 10-day 'break' period and the expression of sensitization was tested on day 15 by the administration of amphetamine (2.0 mg/kg) and vehicle. When CNO was co-administered with amphetamine in *adora2A*-rM3Ds mice during development, behavioral sensitization was inhibited (Figure 5a, red line and box). In contrast, wild-type mice treated with amphetamine, wild-type mice treated with amphetamine and CNO, and *adora2A*-rM3Ds mice treated with amphetamine alone all had normal sensitization (Figure 5a). These data indicate that (1) rM3Ds activation blocks development of amphetamine sensitization, (2) in the absence of CNO *adora2A*-rM3Ds mice sensitize normally to amphetamine, and (3) that CNO does not have off-target effects. Last, these findings were replicated in an independent founder line of *adora2A*-rM3Ds mice (Supplementary Figure S10).

In the nucleus accumbens, behavioral sensitization to psychostimulants can be accompanied by long-lasting changes in fast glutamatergic synaptic transmission (Bowers *et al*, 2010; Thomas *et al*, 2001; Wolf and Ferrario, 2010). Thus, we investigated whether repeated rM3Ds activation during the amphetamine sensitization protocol induced long-lasting changes in glutamatergic synaptic transmission in *adora2A* MSNs of *adora2A*-rM3Ds mice. Three-week-old mice (to facilitate whole cell recordings) were treated with amphetamine daily for 5 days along with CNO (1 mg/kg) or vehicle, as done in the behavioral sensitization paradigm described above. On day 15, mice were killed (without amphetamine challenge) and excitatory postsynaptic currents of mCherry-expressing (transgene marker) MSNs in the nucleus accumbens shell of acute brain slices were measured. Under recording conditions in which responses of AMPA- (2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid) and NMDA- (N-Methyl-D-aspartic acid) type glutamate receptors (AMPA and NMDAR, respectively) could be compared, we found that CNO treatment during the induction of behavioral sensitization caused a significant and long-lasting increase in the AMPAR/NMDAR ratio compared with amphetamine treatment alone

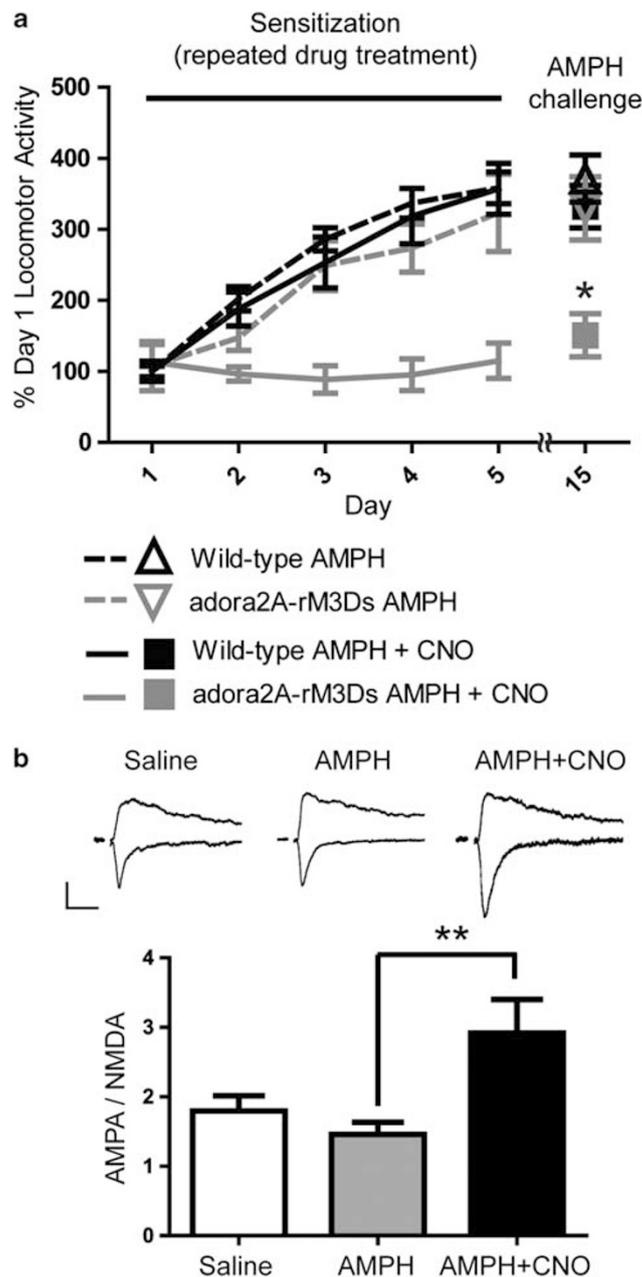


Figure 5 Clozapine-N-oxide (CNO) administration modulates amphetamine-induced physiological changes. (a) Co-administration of CNO (1.0 mg/kg) blocks the development of behavioral sensitization caused by amphetamine (2.0 mg/kg) in *adora2A*-rM3Ds mice. Data shown are mean percentage of day 1 total horizontal distance traveled over 60 min (\pm SEM). (b) CNO + AMPH increases the AMPAR/NMDAR receptor ratio in *adora2A* medium spiny neurons (MSNs) of the NAC shell relative to AMPH alone treated mice on day 15. Stimulus intensity was set to obtain an evoked AMPAR excitatory postsynaptic currents (EPSC) of about 70 pA at -70 mV for all three conditions. Scale bar: 25 pA and 20 ms. Sample traces shown on top. All data are presented as means \pm SEM. * $p < 0.05$; two-tailed t test, $n = 7$. ** $p < 0.01$; two-tailed t test, CNO/AMPH $n = 12$, AMPH $n = 14$, saline $n = 12$.

($p = 0.006$, t -test; $p = 0.016$, rank test) (Figure 5b). Of note, relative to nonsensitized (saline) controls, the effects of amphetamine on the AMPAR/NMDAR ratio trended in opposite directions depending on the presence or absence of CNO. Treatment with amphetamine alone trended to

decrease the AMPAR/NMDAR ratio compared to saline ($p = 0.108$, two-tailed t -test; $p = 0.100$, rank test), whereas treatment with amphetamine and rM3Ds activation by CNO trended to increase the ratio compared with saline ($p = 0.108$, two-tailed t -test; $p = 0.184$, rank test). These data demonstrate that concurrent activation of rM3Ds during amphetamine sensitization is accompanied by long-lasting changes in synaptic strength of adora2A MSNs that oppose the effects of amphetamine alone.

DISCUSSION

Although striatal GPCR signaling has been studied for decades, the role of whole-striatum, cell type-specific, GPCR-mediated signaling on behavior remains unknown and has been heretofore unknowable. Using a newly developed mouse model in which we selectively and stably expressed a G_s -DREADD (rM3Ds) in striatopallidal neurons, we have validated the use of the DREADD technology to noninvasively control G_s -type signaling in a neuronal context *in vivo*. Because of the noninvasive spatio-temporal control afforded, DREADD technology has far-reaching applicability to test the role of GPCR activity in a broad array of neuronal and non-neuronal contexts. In this report, we further demonstrate that G_s -DREADD is well tolerated when expressed long term in a transgenic context and this construct can be used in future studies to reveal new insights on the cellular and behavioral significance of G_{α_s} signaling in defined cellular populations.

Since the introduction of the DREADD technology, several independent groups have reported success activating and silencing neurons with hM3Dq and hM4Di, respectively (Alexander *et al*, 2009; Ferguson *et al*, 2011; Garner *et al*, 2012; Krashes *et al*, 2011; Ray *et al*, 2011; Sasaki *et al*, 2011), but have not explored the slow neurotransmission-type $G_{\alpha_{s/olf}}$ -mediated signaling. In this report, we demonstrate the suitability of DREADD technology to manipulate the $G_{\alpha_{s/olf}}$ -mediated signaling pathway in neurons and thereby modulate behavior. Our evidence for this is as follows: adora2A-rM3Ds mice, when administered CNO, (1) show increased DARPP-32 phosphorylation indicative of MSN $G_{\alpha_{s/olf}}$ activity; (2) show a dose-dependent decrease in locomotor activity; and (3) show a blunted behavioral sensitization response to amphetamine indicative of long-term G_{α_s} -induced modulatory effects in striatopallidal MSNs. In addition, our findings complement recent evidence that selective perturbation of D1 MSN activity influences behavioral sensitization (Pascoli *et al*, 2012). In this study, we find that manipulations specifically targeting A2AR MSNs are also sufficient. Moreover, the findings of the two studies are parsimonious with the idea of opposing effects of D1R and A2AR MSNs on motor activity. D1 MSN synaptic weakening inhibited expression of behavioral sensitization (Pascoli *et al*, 2012), and we find that A2AR MSN synaptic strengthening is sufficient to block behavioral sensitization.

It is important to note the differences between the pharmacogenetic and the optogenetic approaches to remotely control neuronal activity. Differences in the technical/methodological considerations have been previously

discussed, as have differences in the type of signaling afforded by these technologies (Alexander *et al*, 2009; Rogan and Roth, 2011). A key difference not fully appreciated is the nature of the neuronal modulation provided by these two technologies. Pharmacogenetics provides a system that more closely resembles the hormonal signaling mechanisms found in the brain, whereas optogenetics more closely resembles electrochemical signaling phenomena. These differences should fundamentally affect the type of experimentation performed with the respective technologies. To date, the implementation of pharmacogenetics as ‘synthetic pharmacology’ has yet to occur, in that the modulation afforded by peripheral CNO administration—bathing the brain with modulator—and the corresponding widespread DREADD expression afforded by genomic transgene expression closely resembles pharmacotherapeutic drug action. Potential applications of this aspect can be further appreciated by the fact that DREADDs are engineered GPCRs, and GPCRs are the target for >50% of currently prescribed psychiatric therapeutics (Roth *et al*, 2004). In this regard, the differences between the two technologies can be observed as differences in scientific objective: pharmacogenetics is best suited for the study of how drugs modulate the function of the brain (neuropharmacology), whereas optogenetics is best suited for the study of brain function itself (neurophysiology).

In conclusion, we here provide the first evidence that the G_s -DREADD technology can afford modulation of *in vivo* neuronal populations in a reproducible and noninvasive manner, and thereby providing the neuroscience community with a new tool to selectively and noninvasively modulate G_{α_s} signaling in a neuronal cell type-specific manner.

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