



Published in final edited form as:

Mol Psychiatry. 2020 September ; 25(9): 2086–2100. doi:10.1038/s41380-018-0212-4.

Arrestin recruitment to dopamine D2 receptor mediates locomotion but not incentive motivation

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SUMMARY

The dopamine (DA) D2 receptor (D2R) is an important target for the treatment of neuropsychiatric disorders such as schizophrenia and Parkinson's disease. However, the development of improved therapeutic strategies has been hampered by our incomplete understanding of this receptor's downstream signaling processes *in vivo* and how these relate to the desired and undesired effects of drugs. D2R is a G protein-coupled receptor (GPCR) that activates G protein-independent as well

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Conflict of Interest

The authors declare no conflicts of interest.

Author contributions

P.D., E.G, D.C.B., E.S., Y.Z., and J.R.L. performed the experiments. L.B., K.A.N., C.K. and J.A.J. supervised the project. P.D., E.G., C.K. and J.A.J. wrote the manuscript, with input from J.R.L., L.B. and K.A.N. This work was supported by NIH grants DA044696 to P.D., MH093672 to C.K., DA009158 to L.M.B., MH54137 and DA022413 to J. A. J., MH107648 to E.G. by Merit Review Award BX003279 to K.A.N. from the US Department of Veterans Affairs, Veterans Health Administration, Office of Research and Development, Biomedical Laboratory Research, and Development, and by the Lieber Center for Schizophrenia Research.

Competing financial interests

The authors declare no competing financial interests.

Supplementary information is available at MP's website

as non-canonical arrestin-dependent signaling pathways. Whether these effector pathways act alone or in concert to facilitate specific D2R-dependent behaviors is unclear. Here, we report on the development of a D2R mutant that recruits arrestin but is devoid of G protein activity. When expressed virally in “indirect pathway” medium spiny neurons (iMSNs) in the ventral striatum of D2R knockout mice, this mutant restored basal locomotor activity and cocaine-induced locomotor activity in a manner indistinguishable from wildtype D2R, indicating that arrestin recruitment can drive locomotion in the absence of D2R-mediated G protein signaling. In contrast, incentive motivation was enhanced only by wildtype D2R, signifying a dissociation in the mechanisms that underlie distinct D2R-dependent behaviors, and opening the door to more targeted therapeutics.

Introduction

The neuromodulator dopamine (DA) plays major roles in motor function, motivation, reward learning, and cognition^{1–3}, and its dysregulation has been implicated in psychiatric and neurological disorders including schizophrenia⁴, Parkinson’s disease⁵, addiction⁶, ADHD⁷, obsessive compulsive disorder⁸, and Tourette’s syndrome⁹. DA confers its actions through five seven-transmembrane, G protein-coupled receptors (GPCRs) (DARs; D1-D5R) that are distributed heterogeneously throughout the central nervous system. D2R is notable for its clinical importance as a therapeutic target. D2R is blocked by all current antipsychotic medications^{10, 11}, is activated by anti-Parkinsonian medications¹², and has been implicated as a target for the treatment of addiction¹³. However, medications that target this receptor have numerous on- and off-target effects that impair quality of life and medication compliance. Furthermore, the precise D2R localization and downstream intracellular signal transduction mechanisms that must be targeted to bestow clinical efficacy remain unknown.

Elucidating the roles of D2R in living animals is challenging in part because it is expressed heterogeneously across diverse cell types and brain regions¹⁴. For example, D2R is highly enriched in the striatum¹⁵, where it is expressed in functionally distinct neurons including the “indirect pathway” medium spiny projection neurons (iMSNs) as well as cholinergic interneurons (ChIs)^{14, 16}. D2Rs also act as auto- and heteroreceptors at the terminals of dopaminergic midbrain¹⁷ and glutamatergic cortical neurons¹⁸, respectively, that innervate the striatum. Postand pre-synaptic striatal D2Rs are thought to play distinct roles, with the former regulating somato/dendritic excitability¹⁹ and the latter regulating transmitter synthesis and release²⁰.

D2R can engage a multitude of signaling pathways within the same cell. D2R couples to $G_{i/o/z}$ proteins¹⁵, which inhibit adenylyl cyclase to decrease cAMP levels, and also regulate signaling proteins such as DARPP-32²¹, ion channels^{22–26}, and transcription factors²⁷. D2R also recruits arrestins (arrestin-2 and -3)^{28, 29}, which terminate G protein signaling, facilitate receptor internalization, and engage non-canonical, G protein-independent signaling pathways³⁰. Whether G protein- and arrestin-dependent pathways act alone or in concert to facilitate specific D2R-dependent processes is not well understood.

Traditional pharmacological agents, i.e., agonists and antagonists of D2R, have two limitations. First, they target receptors in a cell type-independent manner and therefore it is difficult to identify their site of action, especially within the striatum. Second, they are

largely pathway-independent so it is unclear which signaling pathway is responsible for an observed behavioral effect. Recently, novel ligands have been developed that are functionally selective, or biased, in their ability to activate either G proteins or arrestins downstream of D2R^{31–34}. Though promising, these ligands are often partial agonists compared to DA and only have limited bias. Moreover, biased ligands cannot overcome the cell type specificity limitation, and like classical ligands, have off-target effects at other DARs and other targets. While the genetic knockout of specific signaling proteins can partially address these limitations^{35, 36}, this approach is not specific to D2R signaling and is complicated by potential compensation during development.

Limitations associated with existing pathway-selective approaches are highlighted by studies investigating the role of arrestin signaling on D2R-dependent locomotion. Biased ligands that preferentially engage arrestins downstream of D2R were shown to inhibit DA-dependent, psychostimulant-induced locomotion in mice, suggesting that arrestin signaling inhibits locomotion³¹. However, the global knockout of arrestin-3 attenuated psychostimulant-induced locomotion, suggesting that arrestins are necessary for locomotion³⁵. This discrepancy is likely due to the partial agonism of the biased compounds as well as the potential multitude of on- and off-target effects associated with these approaches.

We recently showed that upregulating D2R in iMSNs of the nucleus accumbens (NAc-iMSNs) of adult mice enhances both locomotion and motivation in mice^{37, 38}. These overexpressed receptors are activated by endogenous DA, thereby obviating the need for exogenous ligands and maintaining the spatio-temporal aspects of signaling. Here, we further refined this approach to study, with cell type specificity, the precise role of arrestin recruitment to D2R and its presumed downstream signaling effects. We developed an arrestin-specific biased mutant of D2R by disrupting the receptor's ability to activate G proteins. Virus-mediated expression of this mutant in NAc-iMSNs within a D2R-knockout background was sufficient to reverse blunted basal locomotor activity in these animals as well as to restore the locomotor response to cocaine to levels identical to that achieved by wildtype D2R. Remarkably, in contrast to wildtype, the expression of this mutant in NAc-iMSNs enhanced locomotion but not motivation, indicating that these behaviors are differentially regulated by D2R-mediated arrestin recruitment and that enhancing motivation requires G protein activation.

Materials and Methods

Modeling

The molecular representation of D2R co-crystallized with the antagonist risperidone (pdb: 6CM4)³⁹ was prepared using Chimera, which was developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311).

Molecular biology and heterologous expression

The short or long isoforms of human D2R were cloned into the expression vector pcDNA3.1 with a signal peptide to enhance receptor expression, a FLAG-tag at the receptor N-terminus, and with or without *Renilla* luciferase 8 (Rluc8) at the receptor C-terminus⁴⁰. Point mutations in D2R were introduced using the QuikChange site-directed mutagenesis method (Stratagene, La Jolla, CA) per the manufacturer's instructions. For the bioluminescence resonance energy transfer (BRET)-based assays used in this study, HEK293T cells were seeded onto 10 cm plates and transfected with a 1:1 ratio of DNA:polyethylenimine (linear, MW 25,000; PolySciences, Inc.). For the BRET-based D2R-mediated G₁₁ recruitment assay, we transfected plasmids encoding D2R-Rluc8 (0.2 µg), human G_{α11} with mVenus inserted at position 91 (G_{α11}-91-mVenus; 5 µg) and human G_{β1} and human G_{γ2} (5 µg each). For the BRET-based D2R-mediated G_γ-mVenus recruitment assay, HEK293T cells were seeded onto 6-well plates and transfected with a 1:6 ratio of DNA:polyethylenimine with plasmids encoding D2R-Rluc8 (50 ng), the pertussis toxin insensitive variants (cDNA resource center, Bloomsburg University, PA, 50–125 ng) of human G_{α11} C351I, G_{α12} C352I, G_{α13} C351I, G_{αoA} C351I, or WT human G_{αz}, human G_{β1} (125–250ng) and human G_{γ2}-mVenus (125–250 ng). For the D2R-mediated arrestin recruitment assay, we transfected D2RRluc8 (0.2 µg), bovine GRK2 (5 µg) and bovine arrestin-2 or human arrestin-3 fused to mVenus at their N-termini (8 µg)^{41, 42}. For the D2R-AP2 interaction assay, we transfected D2R-Rluc8 (0.2 µg), arrestin-3 (5 µg), GRK2 (5 µg) and β2-adaptin subunit of AP-2 fused to YFP⁴³ (10 µg). For the BRET-based D2R-mediated cAMP inhibition assay, we transfected plasmids encoding D2R (0.2 µg) and CAMYEL⁴⁴ (6 µg; ATCC). For cAMP inhibition experiments in which G₁₁ was overexpressed, HEK293T cells were seeded onto 6-well plates and transfected with CAMYEL (1 µg), D2R-WT or its mutants (0.06 µg), G_{α11} (0.165 µg), G_{β1} (0.5 µg) and G_{γ2} (0.5 µg). For measuring receptor surface expression as well as internalization, D2R-WT and its mutants were cloned into pcDNA5/FRT/TO with a signal peptide and FLAG-tag at the receptor N-terminus. These plasmids were stably transfected into Flp-in T-Rex HEK293 cells using the Flp-in system (Thermo Fisher). Stable cell lines were seeded onto 10 cm plates 48 hours prior to the assay. Receptor expression was induced with 1 µg/mL tetracycline 24 hours prior to the assay. For the internalization assay, cells were also transiently transfected with pcDNA3.1 plasmids encoding arrestin-3 and GRK2 using a 1:1 ratio of DNA:polyethylenimine 24 hours prior to the assay.

HEK293T cells used in this study tested negative for mycoplasma contamination.

Bioluminescence resonance energy transfer (BRET) assays

All BRET studies were performed in HEK293T cells that were maintained in DMEM (Invitrogen) with 10% fetal bovine serum at 37 °C under 5% CO₂. Cells were transiently transfected with the plasmids described above. Cells were prepared and assayed as described previously in detail⁴². Briefly, cells were washed, harvested and resuspended in DPBS containing 5 mM glucose at room temperature. Cells (~40 µg of protein per well according to a BCA protein assay kit, ThermoScientific) were distributed into a 96-well microplate (Wallac, PerkinElmer Life and Analytical Sciences). After incubation with coelenterazine H (5 µM) (Dalton Pharma Services) for 8 minutes, different ligands were injected and

incubated for 2 to 10 minutes. For the CAMYEL assay, 30 μM of forskolin was added 10 minutes prior to the injection of DA. For the G γ -mVenus recruitment assay, cells were harvested from 6-well plates 24 hours after transfection and plated into poly-D-lysine coated (Sigma-Aldrich) white-bottom 96-well optiplates (Wallac, PerkinElmer Life and Analytical Sciences) at a density of 50,000 cells per well. Cells were treated with 100 ng/mL pertussis toxin (Sigma-Aldrich) 16 hours prior to assay measurement. Twenty four hours after cells were transferred to plates, media was aspirated, cells washed once with DPBS and 80 μL DPBS containing 5 mM glucose was added to each well. Using a Pherastar FS plate reader (BMG Labtech), BRET¹ signal was determined by quantifying and calculating the ratio of the light emitted by mVenus (510–540 nm) over that emitted by Rluc8 (485 nm).

Receptor surface expression and internalization

To measure surface expression of D2R-WT or its mutants, live cells were washed, harvested and resuspended in DPBS. For the internalization assay, live cells were preincubated with or without 10 μM DA for one hour at 37 °C under 5% CO₂, and then washed, harvested and resuspended in DPBS. Cells were then incubated with mouse anti-FLAG M2 antibody (1:400 dilution; Sigma) for 30 minutes on ice, washed and resuspended in DPBS. Cells were then incubated with goat antimouse IgG(H+L)-Alexa 647 antibody (Thermo Fisher) for 30 minutes on ice, washed and resuspended in DPBS. Receptor expression was assessed using a BD Accuri C6 cytometer.

Animal

For D2R overexpression in wild-type mice we used adult hemizygous male and female D2-Cre (ER44; GENSAT, backcrossed for over 5 generations onto C57BL/6J background). For reexpression of D2Rs in indirect pathway of D2R KO mice, A2A-Cre(KG126;GENSAT) were crossed with *Drd2*^{-/-} mice (B.6129S2-*Drd2*^{tm1low} strain backcrossed for 20 generations onto C57BL/6J background⁴⁵). *Drd2*^{+/-}A2A-Cre (homozygous for A2A-Cre transgene) mice were intercrossed to obtain *Drd2*^{-/-}A2A-Cre (hemizygous for A2A-Cre transgene). Mice were housed 3–5 per cage for most experiments on a 12 hour light/dark cycle. All experiments were conducted in the light cycle. All experimental procedures were conducted following NIH guidelines and were approved by Institutional Animal Care and Use Committees of Columbia University, New York State Psychiatric Institute, and the VA Portland Health Care System.

D2-Cre^{+/-} or *Drd2*^{-/-}A2A-Cre^{+/-} mice (8 weeks old) were bilaterally injected with a Crependent double-inverted open reading frame (DIO) adeno-associated viruses (AAVs) encoding the long isoform of D2R-WT^{36, 37} or the arrestin-biased mutant (D2R-ARB) followed by IRESmVenus, or a DIO AAV encoding EGFP (UNC Vector Core, Chapel Hill, NC) into the nucleus accumbens (NAc) using stereotactic Bregma-based coordinates: AP, +1.70 mm; ML, \pm 1.20 mm; DV, -4.1 mm (from dura) for behavioral experiments. For the *ex vivo* GTP γ S incorporation assay, mice were injected bilaterally at two different coordinates in the dorsal striatum instead of the NAc to increase striatal tissue yield (AP, +1.3 mm; ML, \pm 1.4 mm; DV, -3.3 mm, and AP, +0.9; ML, \pm 2.0; DV, -3.4 mm from dura). Groups of mice used for experiments were first assigned their AAVgenotype in a counterbalanced fashion that accounted for sex, age and home cage origin.

Histology

Mice were transcardially perfused with ice-cold 4% paraformaldehyde (Sigma, St. Louis, MO) in PBS under deep anesthesia. Brains were harvested, post-fixed overnight and washed in PBS. Free-floating 30- μ m coronal sections were obtained using a Leica VT2000 vibratome (Richmond, VA). After incubation in blocking solution (10% fetal bovine serum, 0.5% bovine serum albumin in 0.5% TBS-Triton X-100) for 1 hour at room temperature, sections were labeled overnight at 4°C with primary antibodies against GFP/mVenus (chicken; 1:1000; AB13970 Abcam, Cambridge, MA) and D2R (rabbit; 1:500; in-house). Sections were incubated with fluorescent secondary antibodies (donkey anti-chicken FITC, 1:400; 703-096-155; Jackson ImmunoResearch, West Grove, PA and donkey anti-rabbit Alexa 546; 1:400; A10040; Invitrogen, Carlsbad, CA) for 2 hours at room temperature. Sections were then mounted on slides and coverslipped with Vectashield containing DAPI (Vector, Burlingame, CA). Digital images were acquired using a Nikon epifluorescence microscope, and processed with NIH Image J software. For D2R fluorescence intensity analysis, NIH Image J software was used to draw an ROI outlining the regions of the NAC with virus-induced D2R expression in each section, and mean intensity values were calculated. These values were normalized to background fluorescence in an adjacent unlabeled region (medial septum) for *Drd2*^{-/-}A2A-Cre or to endogenous D2R immunofluorescence in striatum for D2-Cre mice. Values were obtained from individual hemispheres and used as individual data points for analysis.

Ex vivo GTP γ S incorporation assay

Four weeks after surgery, brains were harvested into ice-cold PBS promptly following decapitation. Striatal tissue was dissected from each hemisphere on ice and then snap frozen in liquid nitrogen and stored at -80°C and shipped overnight on dry ice to Jupiter, FL where experimenters were blinded as to treatment groups. G protein-coupling in mouse brain was measured using a previously published protocol^{46,47}. Membranes were prepared following glasson-glass dounce homogenization in homogenization buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM DTT). The homogenate was passed through a 26-gauge needle, centrifuged twice at 20,000 \times g for 30 min at 4 °C, and resuspended in ice-cold assay buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM DTT and 2 μ M GDP for CB1 activation and 100 μ M GDP for D2 activation). In a 96-well plate format, 2.5 μ g of membrane protein was incubated in assay buffer containing ~0.1 nM [³⁵S]GTP γ S and increasing concentrations of test compound in a total volume of 200 μ L for 1 h at room temperature. Test compounds (quinpirole or CP-55940) added to the assays at final DMSO concentration of 1%. Reactions were terminated by filtering through GF/B filters using a plate harvester (Brandel Inc., Gaithersburg, MD) and rinsing with cold dH₂O. Filters were dried overnight, and radioactivity was determined with a microplate scintillation counter. The average of n=3 individual mouse striatum preparations were performed in duplicate presented as mean \pm S. E. M.

Open field locomotor activity

Basal locomotion was assessed in open field boxes equipped with infrared photobeams to measure locomotor activity (Med Associates, St. Albans, VT) over 90 minute sessions. Data,

expressed as distance traveled, were acquired using Kinder Scientific Motor Monitor software (Poway, CA). Cocaine-stimulated activity was measured in activity monitors (40 cm²; Accuscan Instruments, Inc., Columbus, OH), with 15 mg/kg of the drug injected i.p. after 90 minutes of acclimation to the chambers.

Operant apparatus

Eight operant chambers (model Env-307w; Med-Associates, St. Albans, VT) equipped with liquid dippers were used. Each chamber was located in a light- and sound-attenuating cabinet. The dimensions of the experimental chamber interior were 22 × 18 × 13 cm, with flooring consisting of metal rods placed 0.87 cm apart. A feeder trough was centered on one wall of the chamber. An infrared photocell detector was used to record head entries into the trough. Raising of the dipper inside the trough delivered a drop of evaporated milk. A retractable lever was mounted on the same wall as the feeder trough. A house light located on wall opposite to trough illuminated the chamber throughout all sessions.

Dipper and lever press training

Four weeks after AAV surgery, mice underwent operant training. Mice were weighed daily and food-restricted to 85–90% of baseline weight; water was available *ad libitum*. Mice were trained as previously described⁴⁸. In the first training session, 20 dipper presentations were separated by a variable inter-trial interval (ITI) and ended after 20 rewards were earned or after 30 minutes, whichever occurred first. Mice reached criterion when head entries were made during 20 dipper presentations in one session. In the second training session, criterion was achieved when mice made head entries during 30 of 30 dipper presentations. Mice were trained to lever press on a continuous reinforcement (CRF) schedule. Levers were retracted after each reinforcer and were presented again after a variable ITI (average 30 seconds). The reward consisted of raising the dipper for 5 seconds, and the session ended when the mouse earned 60 reinforcements, or one hour elapsed, whichever occurred first. Sessions were repeated daily until mice achieved 60 reinforcements. Next, mice underwent fixed interval (FI) training wherein lever presses were reinforced until after a fixed interval (timed relative to the lever extension) had elapsed. Each reinforcement was followed by a variable inter-trial interval (average 30 seconds) during which the lever remained retracted, and then a new trial started, signaled by lever extension. Mice began with FI-4 s session and proceeded successively to longer interval sessions after earning 30 rewards in each session. The FI durations were 4, 8, 12, 16, and 24 seconds.

Progressive ratio (PR) task

Following FI schedules, mice were tested in a PR task, as previously described^{38, 49}. Briefly, a reward was obtained after the mice made the required number of lever presses. The criterion was set at two lever presses for the first trial and the requirement doubled with each successive trial. The session ended after 2 hours or after 3 minutes had elapsed without a lever press. Breakpoint was defined as the last criterion successfully completed. Mean values from 4 daily PR sessions were analyzed. Investigators were blind to genotype of mice during the experiment and during data analysis. Mice were matched for age and sex.

Statistics and data analysis

For dose-response curves, nonlinear regression analysis was performed using the sigmoidal dose-response function in GraphPad Prism. All values reported are mean \pm standard error mean (S.E.M.). Statistical analyses were performed using Graphpad Prism.

Sample sizes for behavioral studies were determined by performing statistical power analyses based on effect sizes observed in preliminary data or on similar work in the literature. Histological verification of viral expression was used to exclude mice with misplaced injections from behavioral data analysis in a manner blinded to genotype. Statistical analyses were performed using Graphpad Prism. Data are expressed as mean \pm S.E.M.. Paired and unpaired two-tailed Student's t-tests were used to compare 2-group data, as appropriate. Multiple comparisons were evaluated by one-way or two-way ANOVA and Bonferroni's *post hoc* test, when appropriate. Log-rank tests were used to analyze survival curves. All statistical tests met their respective assumptions. A pvalue of < 0.05 was considered statistically significant. Behavioral findings were successfully replicated with mice from different litters, ages, or sexes, and in several instances, across independent cohorts or related mouse strains.

Results

Development of an arrestin-biased D2R

Biased D2R mutants have been reported previously but their utility has been constrained by their incomplete bias towards either G proteins or arrestins^{41, 50} or diminished expression^{41, 51}. The only reported arrestin-biased D2R mutant to date, D2R(A135R:M140D), enhanced locomotion when overexpressed in striatal D2R-expressing neurons of mice^{50, 52}. However, this mutant was shown to be only partially biased toward arrestin recruitment⁵⁰, which we confirmed using bioluminescence resonance energy transfer (BRET)-based G protein activation and arrestin recruitment assays in HEK293T cells. Under our assay conditions, maximal DA-induced G protein-dependent cAMP inhibition was impaired but still substantial ($28 \pm 2\%$ of wildtype D2R (D2R-WT), $n=3$; **Supplementary Figures 1A & C**). The potency of DA was only slightly reduced compared to D2R-WT (~ 3 -fold, **Supplementary Figures 1A & D**). Maximal DA-induced arrestin-3 recruitment to D2R(A135R:M140D) was relatively less impaired, but still considerably lower than D2R-WT ($55 \pm 3\%$ of D2R-WT, $n=3$; **Supplementary Figures 1B & C**). Thus, although D2R(A135R:M140D) is partially arrestin-biased, it is unclear whether arrestin or residual G protein signaling (or both) downstream of D2R is responsible for enhanced locomotion *in vivo*.

To develop a more arrestin-biased D2R mutant with levels of arrestin recruitment more comparable to D2R-WT, we took advantage of a previously reported arrestin-biased, muscarinic acetylcholine receptor (mAChR)-based Designer Receptor Exclusively Activated by Designer Drugs (DREADD)⁵³. A single point mutation of an arginine (R^{3.50} according to the Ballesteros-Weinstein GPCR numbering system⁵⁴) to leucine in this receptor's "DRY motif" resulted in strong arrestin bias⁵³. The DRY motif is critically involved in receptor activation⁵⁵ and is conserved across Family A GPCRs, including mAChRs and DARs.

Therefore, we mutated the DRY motif in D2R to DLY (D2R(R132L); **Figures 1A & 1B**), which greatly impaired DA-induced recruitment of the G_{α} subunit of G_{i1} to the receptor ($9 \pm 5\%$ of D2R-WT, $n=5$; **Figure 1C**)⁵⁶. In contrast, R132L's effect on DA-induced arrestin-3 recruitment was less severe, though it still significantly reduced maximal arrestin-3 recruitment ($47 \pm 8\%$ of D2R-WT, $n=6$; two-tailed t-test, $p<0.001$) and potency (~ 16 -fold lower compared to D2R-WT, $n=6$; two-tailed t-test, $p<0.0001$) (**Figure 1D**). Similar results were observed for arrestin-2 recruitment to D2R(R132L) (**Supplementary Figure 2**).

Even if D2R (R132L) preferentially recruits arrestin, its attenuated arrestin activity could still mask a role for arrestin-dependent processes *in vivo*. We hypothesized that combining R132L with mutations that increase receptor activity would enhance arrestin recruitment. The mutation of L^{3,41} to W (L123W in D2R) was shown previously to thermostabilize Family A GPCRs and facilitate their crystallization⁵⁷, e.g., this mutation is present in the crystal structure of a close homolog of D2R, DA D3R⁵⁸. Interestingly, DA was significantly more potent at D2R(L123W) (**Figures 1A & 1B**) compared to D2R-WT in assays measuring the recruitment of $G_{\alpha i1}$ (~ 4 -fold; two-tailed t-test; $p<0.01$; **Figure 1C**), arrestin-2 (~ 14 -fold; two-tailed t-test; $p<0.01$; **Supplementary Figure 2**) and arrestin-3 (~ 14 -fold; two-tailed t-test; $p<0.0001$; **Figure 1D**), suggesting that L123W enhances the affinity of the receptor for DA and/or the ability of the receptor to populate an active conformational state when bound to agonist.

As hypothesized, combining R132L and L123W created a mutant, D2R(L123W:R132L) (**Figures 1A & B**), that was capable of robustly recruiting arrestin-3 ($100 \pm 7\%$ of D2R-WT, $n=3$; **Figure 1D**) and arrestin-2 ($132 \pm 9\%$ of D2R-WT, $n=3$; **Supplementary Figure 2**). Consistent with its effect on D2R-WT, the L123W mutation combined with R132L enhanced the potency of DA by ~ 4 -fold relative to that in R132L in both the arrestin-2 and -3 recruitment assays. When compared to D2R WT, D2R(L123W:R132L) was still ~ 2 - to ~ 4 -fold less sensitive to DA for the recruitment of arrestin-2 and -3, respectively. Remarkably, D2R(L123W:R132L) was devoid of detectable $G_{\alpha i1}$ activity ($n=3$; **Figure 1C**). D2R(L123W:R132L) expressed at the plasma membrane of HEK293T cells at $75 \pm 8\%$ ($n=6$) of D2R-WT (**Supplementary Figure S3 & Figure 1E**). Furthermore, L123W:R132L conferred arrestin-bias to both the short (**Figure 1**) and long isoforms of D2R (**Supplementary Figure 4**).

Of note, D2R has been reported to couple more potently to G_o than G_i isoforms^{59, 60}, and it has been inferred based on knockout data that G_o is a critical mediator of D2R activity *in vivo*^{61, 62}. Thus, we carried out a BRET-based assay comparing the ability of each $G_{\alpha_{i/o/z}}$ isoform to support DA-induced recruitment of the G protein subunit G_{γ} to D2R-WT or D2R(L123W:R132L). Consistent with our results for $G_{\alpha i1}$ recruitment (**Figure 1C**), D2R(L123W:R132L) failed to recruit the G_{γ} subunit of G_{i1} , G_{i2} , G_{i3} , G_{oa} , or G_z (**Figure 1F**).

We next tested whether G protein or arrestin recruitment to D2R-WT and its mutants correlated with the engagement of processes downstream of these signaling proteins. We first measured inhibition of cyclic AMP (cAMP) accumulation by endogenous $G_{i/o}$ in HEK293T cells. Whereas DA robustly inhibited cAMP accumulation in cells expressing

D2R(L123W) ($107 \pm 3\%$ of D2R-WT, $n=3$), it had essentially no effect in those expressing D2R(R132L) and D2R(L123W:R132L) ($1 \pm 1\%$ and $3 \pm 3\%$ of D2R-WT, respectively, $n=3$; **Figure 1G**). Similar effects were observed for D2R(L123W:R132L) even in cells overexpressing *gii* ($3 \pm 5\%$ of D2R-WT; $n=4$; **Supplementary Figure 5**). Overall, these results are consistent with the impaired G protein recruitment to D2R(R132L) and D2R(L123W:R132L) (**Figure 1C**).

We showed previously that arrestin-mediated internalization of D2R requires an interaction between the receptor and the $\beta 2$ -adaplin subunit of clathrin-associated adaptor protein-2 (AP-2)⁴¹. In a BRET-based assay that we developed previously⁴¹, DA dose-dependently enhanced the interaction between AP-2 and D2R-WT (**Supplementary Figure 6**)⁴¹. There was no significant difference in the DA-induced interaction between AP-2 and D2R-WT or D2R(L123W:R132L) (two-tailed t-test, $p=0.98$). Consistent with this observation, there was no significant difference in arrestin-dependent, DA-induced internalization of D2R-WT and D2R(L123W:R132L) (two-tailed t-test, $p=0.49$; **Figure 1H**). These results indicate that D2R(L123W:R132L) recruits arrestin that can then bind accessory proteins and facilitate processes downstream of receptor activation.

It is conceivable that D2R(L123W:R132L) only appears biased towards arrestins because its basal (constitutive) activation of G proteins is extremely high, and thus the receptor is incapable of further activating these signaling proteins in response to DA. We used the D2R inverse agonist sulpiride⁶³ to assess the constitutive G protein activity of D2R-WT, D2R(L123W:R132L) or D2R(E339A:T343R), a reported constitutively active mutant (CAM) of D2R⁶⁴, in the cAMP assay. Sulpiride robustly reversed constitutive inhibition of cAMP accumulation by the CAM, and to a lesser degree that by D2R-WT (**Supplementary Figure 7**). However, it had no effect on cells expressing D2R(L123W:R132L) or mock transfected cells not expressing receptor (**Supplementary Figure 7**), indicating that this mutant does not constitutively activate G proteins. Overall, these results indicate that G protein activation by D2R(L123W:R132L) is disrupted whereas arrestin recruitment is intact.

Bias is maintained in the brain

To express the arrestin-biased D2R(L123W:R132L) mutant (henceforth called **D2R-ARB**) *in vivo*, we generated a Cre recombinase (Cre)-dependent AAV encoding a double-inverted open reading frame (DIO) containing this mutant followed by an internal ribosome entry site (IRES) and the YFP variant mVenus under the control of the synapsin promoter I (SynI; **Figure 2A**). We selectively expressed this receptor in iMSNs by injecting AAV in the NAc of adult *Drd2*^{-/-}A2A-Cre mice (**Figure 2B**). We also injected AAVs containing EGFP or D2R-WT. Four weeks following injection of the AAVs, we observed EGFP or mVenus expression in the NAc (**Figure 2C**). The presence of D2R-WT or D2R-ARB was also verified by D2R immunofluorescence (**Figure 2C**). Comparable D2R dendritic-like immunoreactivity was observed in mice infected with D2R-WT and D2R-ARB AAVs (**Figure 2C**), consistent with their similar levels of expression in HEK293T cells (**Figure 1E**).

By expressing D2R-ARB in a D2R-KO background, we could assess its signaling properties independently of native D2R-mediated G protein signaling and thus determine whether the bias observed in heterologous cells is maintained *ex vivo* in the iMSNs. We measured G protein activity using a GTP γ S incorporation assay in dorsal striatal homogenates⁴⁶ (see Methods). Experiments were performed with the selective D2-like receptor agonist quinpirole to avoid activation of D1-like receptors and because quinpirole has similar efficacy as DA at both G protein activation (**Supplementary Figure 8**) and arrestin recruitment⁶⁵. Not surprisingly, quinpirole induced robust G protein activation in heterozygotic D2R-KO mice (*Drd2*^{+/-}A2A-Cre), but had no effect in homozygous D2R-KO (*Drd2*^{-/-}A2A-Cre) mice infected with EGFP (**Figure 2D**). Whereas the selective restoration of D2R-WT in NAc-iMSNs rescued robust G protein activation in the D2RKO background, D2R-ARB had no effect (**Figure 2D**), consistent with its inability to activate G proteins in HEK293T cells (**Figures 1C & F**). To verify that AAV-mediated D2R overexpression did not affect the function of other G_{i/o/z}-coupled receptors, we activated cannabinoid receptors (CBRs) with the agonist CP55,940⁶⁶. There was no significant difference in the ability of this ligand to activate G proteins downstream of CBRs in any of the groups, indicating that G_{i/o/z} activation was not generally disrupted (**Figure 2E**). Furthermore, baseline GTP γ S was not significantly different between any of the groups (**Figure 2F**), suggesting that overexpression of EGFP, D2RWT, or D2R-ARB did not result in compensatory changes in basal G protein activity.

Selective restoration of D2R-mediated arrestin recruitment in accumbens-iMSNs reverses blunted locomotion in D2R-deficient mice

To address whether D2R-mediated G protein signaling is essential for D2R-mediated locomotion, we directed the expression of EGFP, D2R-WT or D2R-ARB to NAc-iMSNs of mice that lack native D2R (*Drd2*^{-/-}A2A-Cre mice) using Cre-dependent AAVs, as described above (**Figures 2A-C**).

A number of studies have shown that ablation of the D2R gene in mice results in hypolocomotion as well as a blunted locomotor response to an acute administration of cocaine^{67, 68}, which enhances DAR signaling by preventing the uptake of DA by its transporter (DAT)⁶⁸. We tested whether D2R-WT or D2R-ARB is sufficient to reverse these phenotypes when expressed selectively in NAc-iMSNs of *Drd2*^{-/-}A2A-Cre mice. Locomotor activity was assessed across three consecutive days for 90 minutes after intraperitoneal injections of either saline on days one and two or cocaine (15 mg/kg) on day three (**Figure 3**).

There was no significant difference in locomotor activity between EGFP, D2R-WT and D2R-ARB-virus-injected mice on day one (one-way ANOVA, $F_{(2,31)}=2.11$, $p=0.14$, $n=11-12$ mice in each group; **Figure 3A**). However, on day two, locomotor activity was significantly higher in D2R-WT and D2R-ARB virus-injected mice compared to EGFP mice (one-way ANOVA, $F_{(2,31)}=5.23$, $p<0.05$, Bonferroni, $t=2.97$ and 2.61 , respectively, $p<0.05$, $n=11-12$ mice in each group; **Figure 3B**). Moreover, there was no significant difference in locomotor activity between D2R-WT and D2R-ARB virus-injected mice (one-way ANOVA, Bonferroni, $t=0.43$, $p>0.05$; **Figure 3B**).

Both D2R-WT and D2R-ARB mice exhibited a robust increase in locomotor activity in response to administration of cocaine that was significantly different from that for EGFP mice (one-way ANOVA, $F_{(2,31)}=7.85$, $p=0.002$, Bonferroni, $t=3.54$ and 3.34 , respectively, $p<0.05$, $n=11-12$ mice in each group; **Figures 3C**). Moreover, there was no difference in the total distance traveled between D2R-WT and D2R-ARB mice in response to cocaine (one-way ANOVA, Bonferroni, $t=0.28$; **Figure 3C**). These results indicate the restoration of D2R-mediated arrestin recruitment in NAc-iMSNs, in the apparent absence of D2R-mediated G protein signaling, is sufficient to reverse basal hypolocomotion in D2R-KO mice. Moreover, with respect to the role of D2R, arrestin recruitment in iMSNs is sufficient to mediate the full psycholocomotor actions of cocaine.

Upregulated D2R-mediated arrestin recruitment enhances locomotion but not motivation

We recently upregulated D2R-WT in D2R-expressing NAc-iMSNs of D2-Cre mice that express native D2R-WT and observed a robust increase in basal locomotion in an open field assay as well as increased motivation in a progressive ratio task measuring motivation^{38, 48}. Our recent mechanistic analysis suggests that D2R in iMSNs promotes motivation mainly by weakening canonical iMSN output to the ventral pallidum (VP) through their actions at the iMSN terminals⁴⁸. This effect, by analogy with presynaptic D2-autoreceptor function in dopamine neurons, is most likely to be G protein-mediated⁶⁹⁻⁷¹. Therefore, we hypothesized that the D2R-mediated enhancement of motivation would be dependent on G protein signaling at iMSN terminals, and thus compared the ability of the D2R-ARB to regulate these D2R-mediated behaviors with that of D2R-WT and the EGFP control when the receptors were upregulated in D2R wild-type mice.

We selectively overexpressed D2R-ARB as well as EGFP or D2R-WT in NAc-iMSNs of D2-Cre mice using the AAVs described above (Figure 4A). Four weeks after injection of the AAVs, we observed EGFP or mVenus expression in the NAc, as well as comparable levels of overexpressed D2R-WT or D2R-ARB (Figure 4B).

In agreement with our previous work³⁸, basal locomotion was significantly higher in mice overexpressing D2R-WT than in those expressing EGFP (one-way ANOVA, $F_{(2,24)} = 9.06$, $p<0.05$, Bonferroni, $t=3.47$, $p<0.05$, $n=8-10$ mice in each group; **Figure 4C, 4D & Supplementary Figure 9A**). Interestingly, overexpression of D2R-ARB significantly enhanced locomotion relative to the EGFP control (Bonferroni $t=3.78$, $p<0.05$) to the same level as D2R-WT overexpression (**Figure 4D**). Thus, the overexpression of a receptor capable of recruiting arrestins but not G proteins is sufficient to enhance basal locomotion, just as it can rescue basal locomotion in the D2R-KO background (**Figure 3**).

Using the same mice, we next investigated whether the D2R-ARB acts similarly to D2R-WT in the regulation of motivation. We showed previously that upregulation of D2R-WT in NAc-iMSNs increases motivation but does not alter consummatory behavior^{48, 49}. Using a progressive ratio (PR) lever pressing task, we measured the willingness to expend effort to obtain a food reward by D2-Cre mice expressing EGFP, D2R-WT or D2R-ARB. Consistent with our previous findings, lever pressing (**Figure 5A**) and breakpoint (**Figure 5B**) were significantly higher in mice expressing D2R-WT than those expressing the EGFP control (one-way ANOVA, lever pressing: $F_{(2,23)}=11.66$, $p<0.05$, Bonferroni, $t=4.57$, $p<0.05$;

breakpoint: $F(2,23)=10.39$, $p<0.05$, Bonferroni, $t=4.14$, $p<0.05$). Remarkably, lever pressing and breakpoint were not significantly different between mice expressing EGFP and D2R-ARB (lever pressing: Bonferroni, $t=0.63$, $p>0.05$; breakpoint: Bonferroni, $t=0.14$, $p>0.05$, **Figure 5A & 5B**). A survival curve analysis of the percentage of mice that continued to respond as a function of time detected an overall group effect (Log-rank test; $\chi^2=13.33$, $p<0.005$), and pairwise log-rank tests comparing groups indicated that a greater number of D2R-ARB mice lasted significantly less time in the PR session than D2RWT mice but more time than EGFP mice (EGFP vs D2R-WT, $\chi^2=12.54$, $p<0.0005$; D2R-WT vs. D2R-ARB, $\chi^2=7.407$, $p=0.0065$; EGFP vs. D2R-ARB, $\chi^2=2.222$, $p=0.1361$; **Figure 5C**). Since a single press of the lever during a three-minute time period will extend the PR session, a very small increase in lever presses can extend session time without a significant increase in total lever presses or rewards received. Press rate, was unaltered by expression of either D2R-WT or D2RARB (one-way ANOVA, $F(2, 23)=1.42$, $p=0.26$; **Figure 5D**). Thus, these data indicate that overexpression of a D2R capable of selectively recruiting arrestin enhances locomotor activity, but is not sufficient to increase responding in a task measuring incentive motivation.

Discussion

Our understanding of the mechanisms by which D2R regulates diverse physiological processes has been limited by the nature of available genetic^{35, 50} and pharmacological tools³¹. While optogenetic and chemogenetic approaches are uncovering details regarding the circuitry underlying distinct behaviors⁷²⁻⁷⁵, very little is known about the roles in DA-mediated behaviors of distinct D2R-mediated signaling pathways in specific cell types. Using a novel arrestin-biased D2R mutant (L123W:R132L), termed D2R-ARB, we have shown in global D2R-KO mice that D2R-mediated arrestin recruitment in NAc-iMSNs enhances basal locomotion and supports cocaine-induced hyperlocomotion to the same extent as D2R-WT. These results indicate that arrestin recruitment to D2R in iMSNs can facilitate locomotor activity in the apparent absence of D2R-mediated G protein signaling. The D2R has also been shown to interact with a number of other proteins⁷⁶, and it is conceivable that the mutations in the D2R-ARB also disrupt other as yet unidentified functions.

The effects of D2R-ARB on locomotion are consistent with previous work showing that a global loss of arrestin-3 attenuates psychostimulant-induced locomotion³⁵. These results, however, appear inconsistent with results showing that biased-D2R ligands, which selectively promote arrestin signaling, inhibit psychostimulant-induced locomotion³¹. This inconsistency may be due to the partial agonism of these first-generation biased ligands, which can confound the impact of bias by acting as partial antagonists to limit the ability of endogenously released DA to fully activate the arrestin pathway. Recent work with an engineered, partially arrestin-biased D2R showed that arrestin recruitment enhanced basal locomotion to only about half the level produced by D2R-WT⁵². This stands in contrast to our results and reflects the complexity of engineering biased receptors; indeed, consistent with the published behavioral results, we find that this receptor can only recruit arrestin to about 50% of the level of D2R-WT (**Supplementary Figures 1B & C**). In contrast, our arrestin-biased mutant, D2R-ARB, mediates full arrestin recruitment (**Figure 1D**), normal internalization (**Figure 1G**) and fully phenocopies the basal hyperlocomotor effects of D2R-

WT (**Figures 4C & D**), even in a D2R-KO background (**Figure 3**). Furthermore, in the global D2R-KO background, cocaine enhanced locomotion to the same extent in mice expressing D2R-WT or D2R-ARB (**Figures 3B & D**), suggesting that acute D2R-mediated arrestin recruitment is sufficient to facilitate locomotion, whereas D2R-mediated G protein activation does not appear to be necessary. While we cannot rule out a role for some residual G protein activity in our D2R-ARB, any such activity is not detectable in the GTP γ S assay (**Figure 2D**) or the G protein recruitment assays (**Figure 1 and Supplementary Figure 4**).

Our findings are consistent with an important role for arrestin-mediated signaling downstream of D2R. Most likely this signaling is mediated by arrestin scaffolding of Akt and PP2A, which decreases Akt activity, thereby reducing GSK3 β phosphorylation and enhancing GSK3 β activity^{35, 77}. Indeed, an increasing body of work implicates GSK3 β activation in DA-mediated locomotion⁷⁷. Moreover, cocaine⁷⁸- and amphetamine⁷⁹-induced locomotion is inhibited by the specific GSK3 β inhibitor SB 216763 as well as by heterologous knockout of GSK3 β ⁷⁹. The target or targets of GSK3 β phosphorylation that lead to enhanced locomotion are unknown, but some evidence points to GSK3 β -mediated regulation of NMDA and AMPA receptors^{80, 81}, which merits further study as a potential mechanism for how arrestin recruitment can affect locomotor activity.

In contrast to locomotion, incentive motivation was enhanced by the upregulation of D2R-WT but not by D2R-ARB. To our knowledge, this is the first study to dissociate between the mechanisms that underlie D2R-dependent locomotion and incentive motivation. Enhanced PR performance can result from enhanced arousal and an attendant enhancement in the activational component of motivation. In addition, enhanced PR performance can also be due to an enhancement in the directional component of motivation driven by the reward value. Our results suggest different mechanisms for each of these contributions. While the D2R-ARB did not produce a significant increase in lever presses or rewards, consistent with its inability to enhance incentive motivation, D2R-ARB did significantly extend survival in the PR protocol to a level intermediate between control and D2R-WT (**Figure 5C**). The survival analysis is a measure that is highly sensitive to the activational component of motivation, since a single press of the lever during a three-minute time period extends the PR session. Thus, behavioral activation, through a very small increase in lever presses, can extend session time without a significant increase in total lever presses or rewards received. This demonstrates that the PR protocol captures both the activational and goal-directed aspects of incentive motivation, which we have now disentangled with our pathway-selective receptor construct. Importantly, our results offer the possibility that therapeutic approaches that target distinct D2R-mediated signaling pathways in a cell typespecific manner may avoid adverse effects associated with conventional D2R-targeting medications by selectively engaging only a subset of D2R-mediated behavioral processes.

It is indeed remarkable that the activation of different signaling pathways in the same neurons can result in enhancement of distinct behaviors. Even though the receptors are expressed in the same population of iMSNs, we do not yet know whether a distinct subpopulation of neurons within the iMSN population mediates the different behaviors through different effector mechanisms, or whether activation of the different pathways in the same neurons can produce these divergent behaviors. Using slice electrophysiology paired

with optogenetics, we have recently shown that D2R upregulation attenuates inhibitory transmission of iMSN output to the ventral pallidum (VP)⁴⁸. *In vivo* recordings confirmed the reduction in indirect pathway inhibitory transmission to the VP, and localized pharmacogenetic inhibition of iMSN terminals within the VP was sufficient to enhance motivation. These results suggest that D2R in iMSNs promotes incentive motivation mainly by weakening their canonical output to the VP through their actions at the iMSN terminals⁴⁸.

In dopaminergic neurons, presynaptic D2-autoreceptors are thought to inhibit dopamine release from axon terminals via G $\beta\gamma$ -mediated inhibition of voltage-gated calcium channels and activation of Kv1.2 potassium channels^{69–71}. Likewise, slice physiology studies have shown that pharmacological activation of D2R reduces inhibitory transmission at striatopallidal synapses^{82–85}. These data suggest that D2R-mediated G protein activation is essential to inhibit iMSN terminals in the VP to enhance incentive motivation, in accordance with our findings that D2R-ARB does not enhance motivation. Analogous studies with a G protein-biased D2R mutant will be critical for providing insight into the roles of G protein signaling on incentive motivation and other D2R-dependent behaviors.

Of note, our studies have focused on the role of D2R-mediated signaling in the NAc. We previously have shown that inhibition of indirect pathway function produced by activating the Gi/arrestin coupled DREADD receptor hM4D enhances locomotion both in the dorsal and ventral striatum⁷⁴. Thus, D2R upregulation in the dorsal striatum is also likely to enhance activity. It is conceivable, however, that the underlying signaling mechanisms may differ due to differences between the role of G protein-dependent and -independent signaling in iMSNs of the dorsal versus the ventral striatum^{41, 50, 51}, and this will require further investigation. The expression of biased D2Rs in D2R in neuronal populations other than the NAc may provide further insight into the role of D2R, not only for the regulation of locomotion and motivation, but for other DA-dependent behaviors as well. Such studies may ultimately shed light on the D2R populations and signaling pathways that must be targeted to maximize clinical efficacy and reduce adverse side effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Eric Teboul and Jeremy Sherman for technical assistance, and the Rodent Neurobehavioral Analysis Core at the New York Psychiatric Institute.

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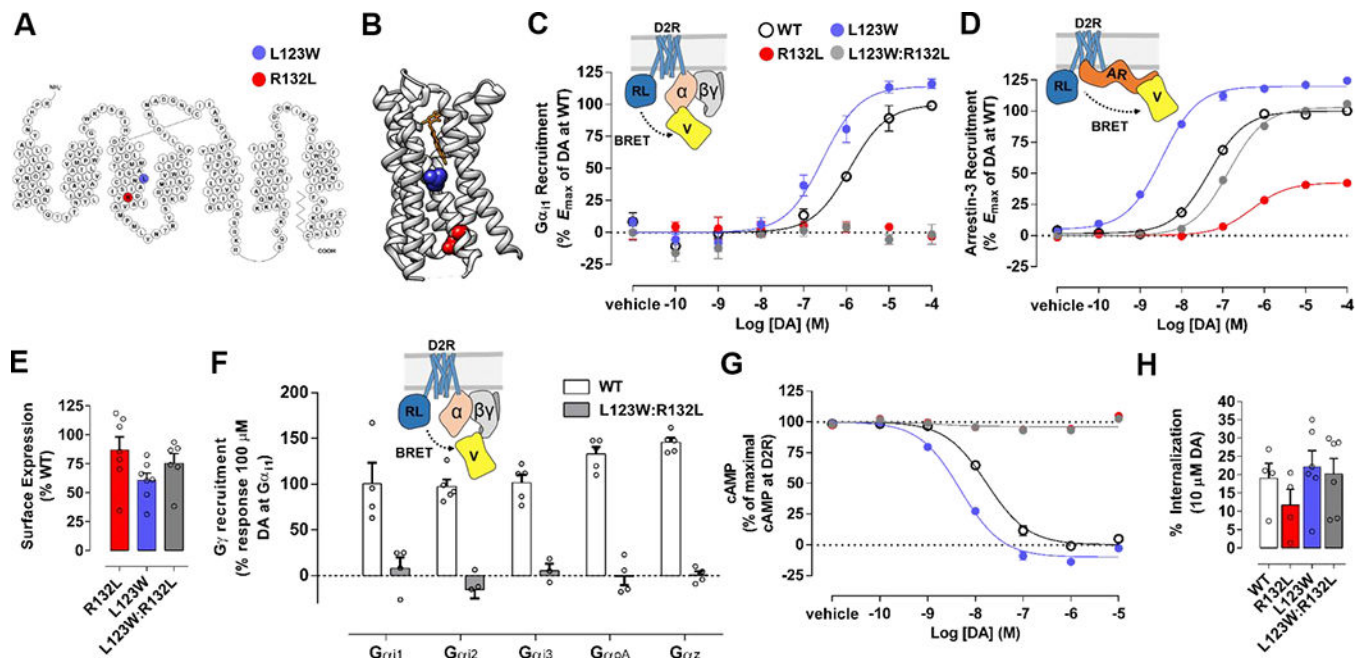


Figure 1. An extremely arrestin-biased D2R mutant receptor, D2R(L123W:R132L).

A) Snake-plot representation of D2R highlighting mutated residues R132 (red) to L and L123 (blue) to W in transmembrane segment 3 (TM3).

B) Ribbon representation of D2R co-crystallized with the D2-like receptor antagonist risperidone. Risperidone (orange) is shown bound to the DA-binding orthosteric binding site (OBS) of the receptor, which is formed by the TM bundle and lies above the mutated residues R132 (red) and L123 (blue).

C) Schematic representation of a bioluminescence resonance energy transfer (BRET)-based assay that measures agonist-induced recruitment of $G_{\alpha i1}$ (α) fused to mVenus (V) in its α -helical domain to D2R fused to *Renilla* luciferase 8 (RL) at its C-terminus (inset). $G_{\alpha i1}$ -recruitment to D2R-WT, D2R(R132L), D2R(L123W), or D2R(L123W:R132L) in response to increasing concentrations of DA. Dose-response curves are representative of independent experiments ($n=6, 6, 3,$ and $3,$ respectively) performed in triplicate.

D) Schematic representation of a BRET-based assay that measures agonist-induced recruitment of arrestin-3 (AR) fused at its N-terminus to mVenus (V) to D2R-Rluc8 (inset). Arrestin-3-recruitment to D2R-WT, D2R(R132L), D2R(L123W), or D2R(L123W:R132L) in response to increasing concentrations of DA (refer to Figure 1C for figure legend). Dose-response curves are representative of independent experiments ($n=6, 6, 3,$ and $3,$ respectively) performed in triplicate.

E) Surface expression of D2R(R132L), D2R(L123W) and D2R(L123W:R132L) in HEK293T cells as a percentage of D2R-WT. Bars represent the average of six to seven independent experiments.

F) $G\gamma$ -mVenus recruitment to D2R-WT and D2R(L123W:R132L) with co-expression of pertussis toxin insensitive variants of human $G_{\alpha i1-3}$ or $G_{\alpha oA}$ or wildtype human $G_{\alpha z}$ in response to $100 \mu\text{M}$ DA. Cells were treated with 100 ng/mL pertussis toxin prior to measurement of response. Responses represent a change in BRET normalized to that of 100

μM DA at the D2R-WT with coexpression of $\text{G}\alpha\text{i}1$. Bars represent the average of four to five independent experiments.

G) $\text{G}_{i/o/z}$ -mediated inhibition of forskolin ($30 \mu\text{M}$)-induced cyclic AMP accumulation (cAMPI) for D2R-WT, D2R(R132L), D2R(L123W), and D2R(L123W:R132L) in response to increasing concentrations of DA (refer to Figure 1C for figure legend). Dose-response curves are representative of three independent experiments performed in triplicate.

H) The percentage of D2R-WT, D2R(R132L), D2R(L123W), and D2R(L123W:R132L) that internalizes in response to DA ($10 \mu\text{M}$) after one hour. Bars represent the average of four to six independent experiments.

The short isoform of D2R (D2Rs) was used for all experiments with the exception of the $\text{G}\gamma$ -mVenus recruitment assay. Error bars represent S.E.M.

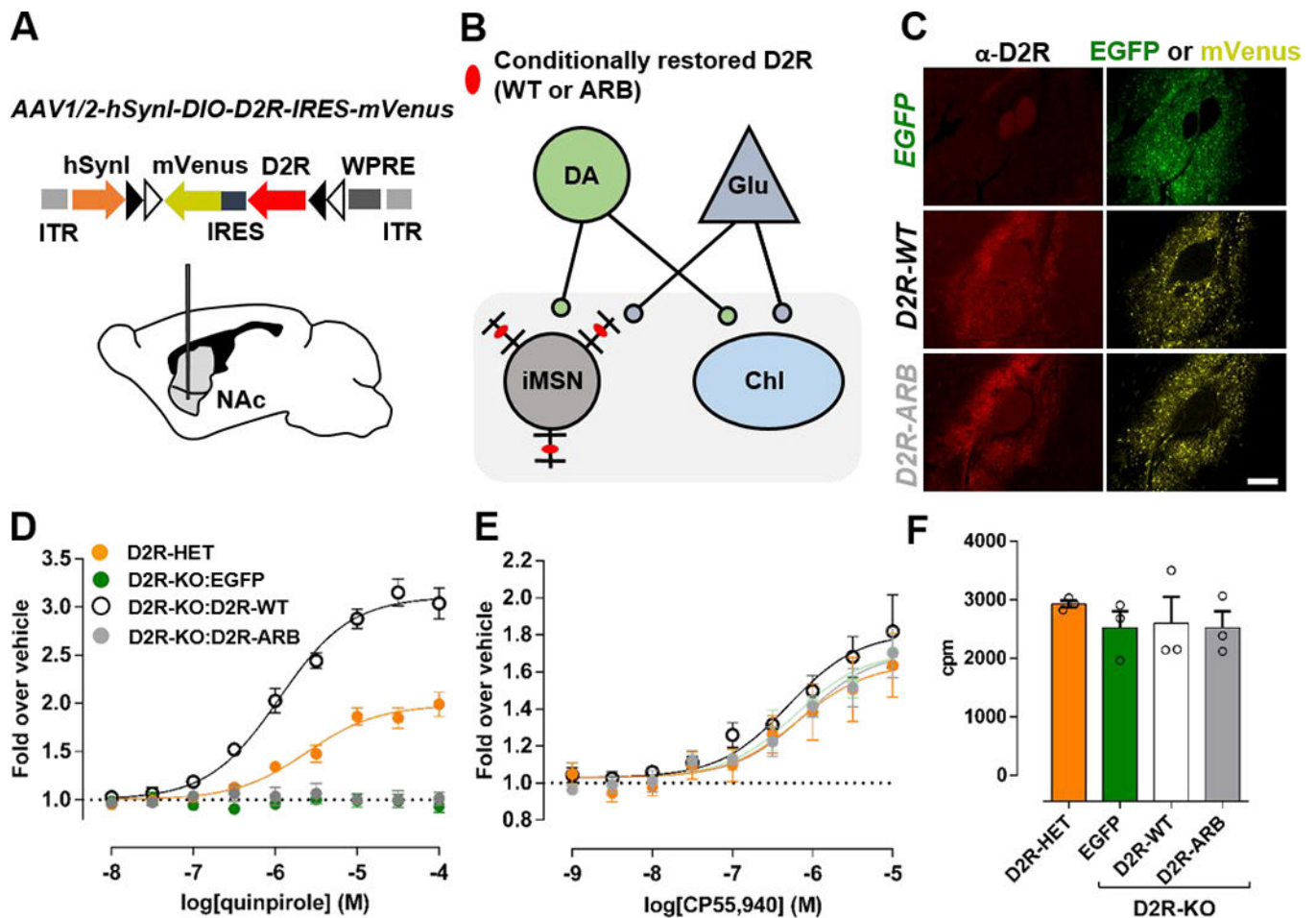


Figure 2. Arrestin bias downstream of D2R in iMSNs of the NAc.

A) Schematic representation of AAV1/2-hSyn1-DIO-SF-D2R-IRES-mVenus. The expression cassette is oriented in the forward direction in the presence of Cre-recombinase, which recognizes flox sites (black and white arrows) (*top*). The AAV was injected into the NAc of *Drd2*^{-/-}A2A-Cre mice (*bottom*).

B) Schematic of striatal neurons from mice lacking native D2Rs. D2R-WT or D2R-ARB (red ovals) was selectively expressed in NAc-iMSNs by injecting Cre-dependent AAVs into the ventral striatum of *Drd2*^{-/-}A2A-Cre mice.

C) AAV-induced expression of EGFP, D2R-WT, or D2R-ARB in NAc four weeks after injection. Shown are D2R immunoreactivity (*left*) and EGFP (*top right*) or mVenus (*middle, bottom right*) fluorescence. Mean D2R immunofluorescence intensity analysis showed similar D2R levels in both D2R groups (D2R-WT: 1.70 ± 0.07 A.U., n=4 mice; D2R-ARB: 1.83 ± 0.07 A.U., n=5 mice; p = 0.21). Scale bar equals 100 μ m.

(D) Quinpirole- or (E) CP55,940-induced ³⁵S-GTP γ S binding in dorsal striatal membranes prepared from *Drd2*^{+/-}A2A-Cre (D2R-HET) or *Drd2*^{-/-}mice. *Drd2*^{-/-}A2A-Cre mice were injected with Cre-dependent AAVs encoding EGFP, D2R-WT, or D2R-ARB. Dose-response curves were fit globally from three independent experiments performed in duplicate. Dose-response curves were normalized to a vehicle control.

F) Baseline ^{35}S -GTP γ S incorporation did not differ between the samples in assays performed in parallel.

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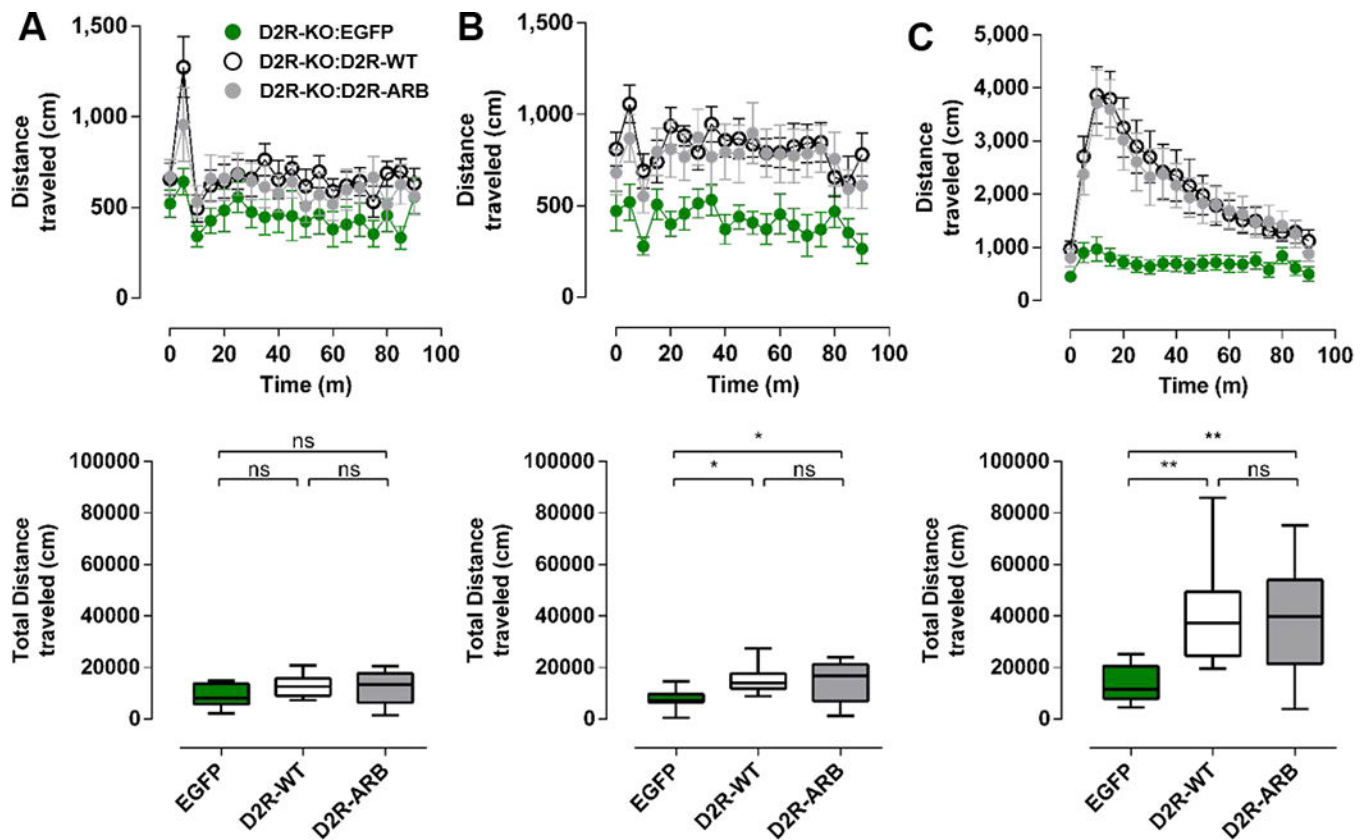


Figure 3. Selective expression of D2R-ARB in iMSNs of the NAc reverses blunted locomotor responses in D2R-knockout mice.

Time course of distance traveled (cm) by *Drd2*^{-/-}A2A-Cre mice expressing EGFP, D2R-WT, or D2R-ARB 90 minutes following the injection of saline on day 1 (A, top), saline on day 2 (B, top), and cocaine (15 mg/kg) on day 3 (C, top). Total distance traveled by EGFP, D2R-WT, or D2R-ARB mice in an open field over 90 minutes after the injection of saline on day 1 (A, bottom), saline on day 2 (B, bottom), and cocaine on day 3 (C, bottom).

One-way ANOVA, Bonferroni. * $p < 0.05$, ** $p < 0.01$, EGFP (n=11), D2R-WT (n=11), and D2R-ARB (n=12), respectively. Error bars represent S.E.M.

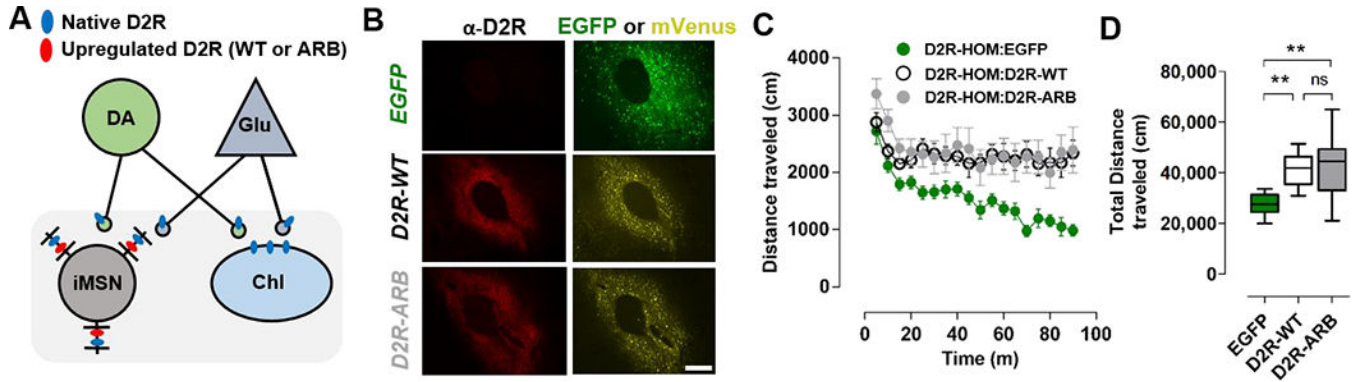


Figure 4. Overexpression of an arrestin-biased D2R in the NAc of mice enhances locomotion.
 A) Schematic depicting neurons in the striatum that express native D2R (blue ovals). These include iMSNs, cholinergic interneurons (ChIs) as well as dopaminergic (DA) and glutamatergic (Glu) neurons, which express D2Rs at their terminals. D2R-WT or D2R-ARB (red ovals) were upregulated selectively in postsynaptic D2R-expressing iMSNs of D2-Cre mice.
 B) AAV-induced expression of EGFP, D2R-WT, or D2-ARB in NAc four weeks following injection. Shown are D2R immunoreactivity (*left*), EGFP (*top right*), or mVenus (*middle, bottom right*) fluorescence. Mean D2R immunofluorescence intensity analysis showed similar D2R upregulation in both D2R groups (D2R-WT: 5.70 ± 0.30 A.U., $n=3$ mice; D2R-ARB: 5.95 ± 0.77 A.U., $n=3$ mice; $p = 0.77$). Scale bar equals 100 μ m.
 C, D) Distance traveled (cm) over 90 minutes in an open field by D2-Cre mice expressing EGFP, D2R-WT, or D2R-ARB.
 One-way ANOVA, Bonferroni. $**p < 0.01$, EGFP ($n=10$), D2R-WT ($n=9$), and D2R-ARB ($n=8$), respectively. Error bars represent S.E.M.

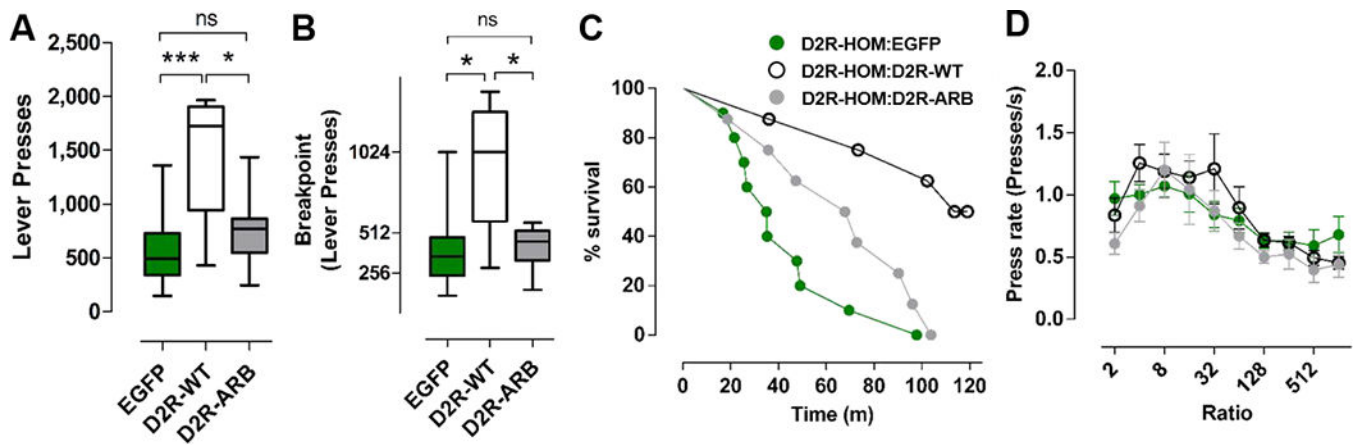


Figure 5. Overexpression of an arrestin-biased D2R in the NAc of mice does not enhance motivation.

A) Average lever presses in a progressive ratio (PR)-task by D2-Cre mice expressing EGFP, D2R-WT, or D2R-ARB and (B) breakpoint were significantly increased in D2R-WT relative to D2R-ARB or EGFP. One-way ANOVA, Bonferroni. * $p < 0.05$ and *** $p < 0.001$, EGFP (n=10), D2RWT (n=9), and D2R-ARB (n=9), respectively.

C) Effect of overexpressing D2R-WT or D2R-ARB on PR session duration. Kaplan-Meier survival function showing the percentage of mice that continue to respond on the PR schedule as a function of session time. Average data of four PR sessions are shown. Log-rank test showed overall group effect ($\chi^2=13.33$, $p < 0.005$), and pairwise log-rank tests comparing between groups indicated that D2R-ARB (n=8) and EGFP (n=10) mice lasted significantly less time in the PR session than D2R-WT (n = 8) mice (EGFP vs D2R-WT, $\chi^2=12.54$, $p < 0.0005$; D2R-WT vs. D2RARB, $\chi^2=7.407$, $p=0.0065$; EGFP vs. D2R-ARB, $\chi^2=2.222$, $p=0.1361$).

D) Press rate, plotted here as a function of the ratio requirement, was not altered. One-way ANOVA, $p=0.26$, EGFP (n=10), D2R-WT (n=9), and D2R-ARB (n=9), respectively. Error bars represent S.E.M.