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Chronic loss of noradrenergic tone produces β -arrestin2-mediated cocaine hypersensitivity and alters cellular D2 responses in the nucleus accumbens

Meriem Gaval-Cruz^{1,*}, Richard B. Goertz^{2,*}, Daniel J. Puttick¹, Dawn E. Bowles³, Rebecca C. Meyer⁴, Randy A. Hall⁴, Daijin Ko⁵, Carlos A. Paladini^{2,#}, and David Weinshenker^{1,#}

¹Department of Human Genetics, Emory University School of Medicine, Atlanta, GA 30322

²Department of Biology, Neurosciences Institute, University of Texas at San Antonio, San Antonio, TX 78249

³Department of Surgery, Duke University School of Medicine, Durham, NC 27710

⁴Department of Pharmacology, Emory University School of Medicine, Atlanta, GA 30322

⁵Department of Management Science and Statistics, University of Texas at San Antonio, San Antonio, TX 78249

Abstract

Cocaine blocks plasma membrane monoamine transporters and increases extracellular levels of dopamine (DA), norepinephrine (NE), and serotonin (5-HT). The addictive properties of cocaine are mediated primarily by DA, while NE and 5-HT play modulatory roles. Chronic inhibition of dopamine β -hydroxylase (DBH), which converts dopamine (DA) to norepinephrine (NE), increases the aversive effects of cocaine and reduces cocaine use in humans, and produces behavioral hypersensitivity to cocaine and D2 agonism in rodents, but the underlying mechanism is unknown. We found a decrease in β -arrestin2 (β Arr2) in the nucleus accumbens (NAc) following chronic genetic or pharmacological DBH inhibition, and overexpression of β Arr2 in the NAc normalized cocaine-induced locomotion in DBH knockout (*Dbh* $-/-$) mice. The D2/3 agonist quinpirole decreased excitability in NAc medium spiny neurons (MSNs) from control, but not *Dbh* $-/-$ animals, where instead there was a trend for an excitatory effect. The $G\alpha_i$ inhibitor NF023 abolished the quinpirole-induced decrease in excitability in control MSNs, but had no effect in *Dbh* $-/-$ MSNs, whereas the $G\alpha_s$ inhibitor NF449 restored the ability of quinpirole to decrease excitability in *Dbh* $-/-$ MSNs, but had no effect in control MSNs. These results suggest that chronic loss of noradrenergic tone alters behavioral responses to cocaine via decreases in

#Address correspondence to: David Weinshenker Ph.D., Department of Human Genetics, Emory University School of Medicine, Whitehead 301, 615 Michael St., Atlanta, GA 30322, Phone (404) 727-3106, Fax (404) 727-3949, dweinschenker@genetics.emory.edu; or Carlos A. Paladini, Ph.D., Department of Biology, University of Texas at San Antonio, 1 UTSA Circle, San Antonio, TX 78249-1644, Phone (210) 458-4458, Fax (210) 458-5658, Carlos.Paladini@utsa.edu.

*MGC and RBG contributed equally to the work.

Authorship Contributions. MGC, RBG, CAP, and DW participated in the research design, MGC, DJP, and RCM conducted the western blot experiments, MGC and DJP conducted the behavioral experiments, RBG conducted the electrophysiology experiments, DK conducted the statistical analysis of the electrophysiology experiments, RAH and DEB contributed analytic tools, and MGC, RBG, CAP, and DW wrote the manuscript.

β Arr2 and cellular responses to D2/D3 activation, potentially via changes in D2-like receptor G protein coupling in NAc MSNs.

Keywords

norepinephrine; dopamine; cocaine; dopamine β -hydroxylase; D2 receptor; mice

Introduction

Dopamine β -hydroxylase (DBH) is the enzyme that converts dopamine (DA) to norepinephrine (NE) in noradrenergic neurons, thereby controlling NE production and the DA/NE ratio (Weinshilboum, 1978). DBH is of clinical interest in cocaine dependence because (1) polymorphisms in the human DBH gene that are associated with reduced serum DBH enzymatic activity lead to greater cocaine-induced paranoia (Cubells et al., 2000; Kalayasiri et al., 2007), and (2) inhibition of DBH by the alcoholism medication, disulfiram, or the selective DBH inhibitor, nopicastat (Kapoor et al., 2011; Stanley et al., 1997), alters the subjective effects of cocaine and reduces cocaine use in humans (Gaval-Cruz and Weinshenker, 2009; Stanley et al., 1997) (K. Cunningham, personal communication). Genetic (DBH knockout; *Dbh* $-/-$) or pharmacological (disulfiram, nopicastat) DBH inhibition produces hypersensitivity to cocaine-induced locomotion, stereotypy, place preference, and place aversion in mice; it also enhances the discriminative stimulus effects of cocaine and attenuates cocaine-, cue-, and stress-induced reinstatement of cocaine seeking in rats (Gaval-Cruz et al., 2012; Manvich et al., 2013; Schank et al., 2006; Schroeder et al., 2013; Schroeder et al., 2010)

Because *Dbh* $-/-$ mice are hypersensitive to the D2/3 agonist, quinpirole, but not the D1 agonist, SKF81297, cocaine hypersensitivity would appear to be mediated by alterations in the D2 pathway (Schank et al., 2006; Weinshenker et al., 2002). These phenotypes are likely driven by compensatory responses in DA signaling following the chronic decrease in extracellular DA availability when noradrenergic excitatory drive on the mesocorticolimbic system is missing. We initially reported an increase in the abundance of high-affinity state D2 receptors in the striatum of *Dbh* $-/-$ mice, which could explain the cocaine and D2 hypersensitivity (Schank et al., 2006). However, subsequent work failed to confirm this finding (Skinbjerg et al., 2010), suggesting a contribution from downstream signaling molecules. Indeed, the behavioral alterations in *Dbh* $-/-$ mice were accompanied by a rise in striatal pERK and FosB protein levels (Rommelfanger et al., 2007).

The goals of the present study were to determine the molecular and cellular mechanisms behind the D2- and psychostimulant-induced hypersensitivity that follow chronic DBH inhibition. First, we found a decrease of β -arrestin2 (β Arr2), a protein involved in D2 desensitization and signaling (Beaulieu and Gainetdinov, 2011), in the NAc of *Dbh* $-/-$ mice and mice treated chronically with nopicastat. We next used viral-mediated overexpression to determine whether increasing β Arr2 levels in the NAc could normalize cocaine-induced behavior in *Dbh* $-/-$ mice. Finally, we assessed electrophysiological

responses to quinpirole in MSNs from the NAC of control and *Dbh* $-/-$ mice in the presence and absence of $G\alpha_i$ and $G\alpha_s$ inhibitors.

Materials and methods

Animals

Adult control (*Dbh* $+/-$) and *Dbh* $-/-$ mice were generated as previously described (Schank et al., 2006; Thomas et al., 1998). *Dbh* $-/-$ males were bred to *Dbh* $+/-$ females. Pregnant *Dbh* $+/-$ mice were given the AR agonists isoproterenol and phenylephrine (20 μ g/ml each) + vitamin C (2 mg/ml) from E9.5-E14.5, and L-3,4-dihydroxyphenylserine (DOPS; 2 mg/ml + vitamin C 2 mg/ml) from E14.5-birth in their drinking water to rescue the embryonic lethality associated with the homozygous *Dbh* $-/-$ mutation. Because of this treatment, NE and epinephrine were present in both *Dbh* $-/-$ animals before but not after birth. They were maintained on a mixed C57BL/6J and 129SvEv background and group-housed, and food and water were available *ad libitum* throughout the course of the study. Both sexes were used due to the extreme measures required to breed sufficient numbers of knockout mice for the experiments (Thomas et al., 1998; Thomas et al., 1995). Comparable numbers of male and female knockouts were used for each experiment, and sex-matched *Dbh* $+/-$ littermates were used as controls. Although the studies were not powered sufficiently to rigorously detect sex differences, no obvious ones were observed. The *Dbh* $+/-$ mice were used as controls because their brain catecholamine levels and behavior is indistinguishable from wild-type (*Dbh* $+/+$) mice (Bourdlat-Parks et al., 2005; Mitchell et al., 2006; Thomas et al., 1998). Some wild-type C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were also used as controls for the electrophysiology experiments.

All animals were treated in accordance with the National Institutes of Health (NIH) Intramural Animal Care and Use Program guidelines. The experiments described in this article followed the UTSA and Emory University Division of Animal Resources' Guide for the Care and Use of Laboratory Animals and were approved by the UTSA and Emory Institutional Animal Care and Use Committee.

Chronic nepicastat treatment

Nepicastat was administered to *Dbh* $+/-$ mice via daily i.p. injections (western blots) or osmotic minipumps (locomotor activity). For the i.p. administration, *Dbh* $+/-$ mice received vehicle or nepicastat (50 mg/kg, i.p. \times 3, each injection spaced 2 h apart) for 5 consecutive days. This dosing regimen reduces brain NE levels by \sim 75% and produces cocaine hypersensitivity (Gaval-Cruz et al., 2012). Mice were euthanized by CO_2 asphyxiation 11 days later, and their brains were removed, dissected on ice, and stored at $-80^\circ C$. For the minipump administration, nepicastat was dissolved in 50% saline and 50% DMSO and loaded into Alzet osmotic minipumps (Model #2004, 0.25 μ L/hour, 28 days; Durect, Cupertino, CA) to achieve a dose of 50 mg/kg/d. All pumps were placed in a sterile $37^\circ C$ saline bath for 1 d before implantation. Mice were anesthetized with isoflurane, and minipumps implanted in the intraperitoneal cavity. Buprenorphine (2.5mg/kg, s.c.) was given immediately after surgery. Cocaine-induced locomotion was recorded 21 d after pump implantation.

Locomotor recordings

Mice were placed in locomotion recording chambers (transparent Plexiglas cages placed into a rack with 7 infrared photobeams spaced 5 cm apart; San Diego Instruments Inc., La Jolla, CA) and allowed to habituate for 30 min before receiving a single injection of cocaine (10 or 15 mg/kg, i.p.). Novelty-induced locomotion was defined as ambulations during the first 10 min of the habituation period. Ambulations (consecutive beam breaks) were recorded for an additional 1–2 h following drug administration.

Western blotting

Mouse brain tissue was homogenized in 500 μ l harvest buffer (10 mM HEPES, 50 mM NaCl, 5 mM EDTA, pH 7.4, supplemented with protease inhibitors) using a sonicator. Laemmli sample buffer containing SDS, β -mercaptoethanol, glycerol, Tris-Cl, and bromophenol blue was added to samples after measuring protein concentrations with a BCA Assay (Thermo Fisher Scientific, Rockford, IL). Samples were resolved by SDS-PAGE on 4–20% Tris-Glycine precast gels followed by transfer to nitrocellulose membranes. Following transfer, membranes were incubated with Ponceau staining to assess even protein loading, then rinsed with distilled water. Membranes were then incubated in blocking buffer (10 mM HEPES, 50 mM NaCl, 1% Tween-20, 2% dry milk, pH 7.4, for most antibodies; (1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk, for pAKT, GSK3 β , and pGSK3 β) for 30 min, and then incubated with primary antibody overnight at 4°C. The primary incubation buffer was the same as blocking buffer for all antibodies except pAKT, GSK3 β , and pGSK3 β . For these, the primary incubation buffer was 1X TBS, 0.1% Tween-20 with 5% BSA. The membranes were washed 3 times in blocking buffer and incubated with either a fluorescent (1:10000) or HRP-conjugated secondary (1:4000) antibody (Invitrogen, Carlsbad, CA) for 30 min, washed more times, and then visualized using either the Odyssey imaging system (Li-Cor) or via ECL reagent (Thermo Fisher Scientific, Rockford, IL), followed by exposure to film. Membranes were stripped for 20 min at 37°C and 10 min at room temperature with stripping buffer and re-probed for α -actin to confirm equal loading of samples. Blots were analyzed by densitometry using Image J Software. A mean density value was calculated for the “control” group (i.e. *Dbh* +/- mice were the control for *Dbh* -/- mice, vehicle was the control for nepicastat, etc), and data were expressed as % control.

Antibodies

The antibodies used and their working dilutions were as follow: β Arr2 (anti-rabbit; 1:2500; Cell Signaling Technology, Danvers, MA, CS3857); α -actin (anti-mouse; 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, SC58671); pAkt-Ser473 (anti-rabbit; 1:1000; Cell Signaling CS9271); Akt (anti-mouse; 1:500; Santa Cruz Biotechnology; SC5298); pGSK3 β -Ser9 (anti-rabbit; 1:1000; Cell Signaling CS9322); pGSK3 β (anti-rabbit; 1:1000; Cell Signaling CS9315); fosB (anti-rabbit; 1:1000; Cell Signaling CS2551); FosB (anti-rabbit; 1:1000; Cell Signaling CS9890).

β -arrestin2 viral vectors

The original β Arr2 plasmid (rat sequence) was obtained from Sudha Shenoy in the lab of Dr. Robert Lefkowitz. The Duke Neurotransgenic Laboratory then removed the β Arr2 open

reading frame, and the insert was cloned into a pCMVShuttle plasmid (AdEasy System, Stratagene, Santa Clara, CA, USA). The AdEasy β Arr2 recombinant plasmid was generated per Stratagene instructions, and the β Arr2 adenoviral vector was expanded and purified. The viruses were harvested with a titer of $2 \times 10^{12}/\mu\text{L}$ (β Arr2) and $5 \times 10^9/\mu\text{L}$ (GFP control).

β -arrestin2 viral infusions

Mice (n = 16 for each treatment group: β Arr2 overexpression adenovirus and GFP adenovirus) were anesthetized using isoflurane and placed in a stereotaxic frame with a nose bar. The animal's scalp was opened and bregma and lambda aligned to flat-skull position. The stereotaxic arm was then lowered to the NAc core. The core subregion was chosen because it has been implicated in cocaine-induced locomotion and behavioral sensitization to cocaine. The anteroposterior (AP) and mediolateral (ML) coordinates of the NAc core in relation to bregma were AP = 1.4 mm, ML = \pm 1.0 mm, and a small hole was drilled in the skull at these coordinates. A 5- μL Hamilton microsyringe was lowered to target the NAc core (dorsoventral coordinate = -4.2 mm). The 26-gauge beveled tip of the Hamilton needle was precoated with 2% anti-bovine serum (BSA) prior to loading the virus to prevent molecular interactions between the syringe and the viral vectors. Animals received 1 μL of virus per side, injected at a rate of 0.2 $\mu\text{L}/\text{min}$, and the needle remained in place for 5 min after the injection and removed slowly. The skin was glued together using Vetbond tissue glue. All animals received meloxicam (0.5 mg/kg) for post-operative pain and water/liquid ibuprofen (0.1 mg/ml).

Ten days after the infusion of β Arr2 overexpression and GFP control vectors, all mice were placed in locomotor chambers, and their basal locomotion was recorded for 30 min before receiving an injection of cocaine (15 mg/kg, i.p.), and cocaine-induced locomotion was recorded for 2 h. Mice were anesthetized and transcardially perfused with saline and 4% paraformaldehyde 24–48 h later, their brains removed, stored in 4% paraformaldehyde for 4 days, and then transferred to 30% sucrose. Brains were sectioned and stained with antibodies against GFP or β Arr2, and expression in the NAc was assessed. Three mice that received the β Arr2 virus and 2 mice that received GFP virus were removed from the analysis due to incorrect placement of viral infusion.

Electrophysiological recordings of nucleus accumbens neurons

C57BL/6J, *Dbh* +/-, and *Dbh* -/- mice were used for electrophysiological recordings. C57BL/6J mice were used to (1) confirm that *Dbh* +/- mice NAc MSNs were similar to wild-type NAc MSNs, and (2) increase the number of cells in a few experiments when not enough appropriately sex- and age-matched *Dbh* +/- control animals were available. Mice were anesthetized with a lethal dose of isoflurane and decapitated. The brains were quickly removed and placed into an ice-cold, oxygenated cutting solution containing (in mM): 110 choline Cl, 2.5 KCl, 1.25 NaH_2PO_4 , 4 MgCl_2 , 2 CaCl_2 , 10 dextrose, 25 NaHCO_3 , 1.3 ascorbic acid, 2.4 sodium pyruvate, and 0.05 glutathione. Parasagittal brain slices containing the nucleus accumbens (250 μm) were cut using a vibrating tissue slicer (Microm HM 650V). The slices were then transferred to an incubation chamber containing warm (35°C) artificial cerebral spinal fluid (ACSF) for 1 h prior to recordings, and then stored at room temperature. The slices were transferred to a recording chamber for the experiments, where

they were submerged in oxygenated ACSF. The ACSF was equilibrated with 95% O₂–5% CO₂, had a pH of 7.2 and contained (in mM): 126 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 2 MgCl₂, 2 CaCl₂, 10 dextrose, 25 NaHCO₃, 1.3 ascorbic acid, and 2.4 sodium pyruvate. The slices were superfused with 34–36°C ACSF at a rate of 2 ml/min.

The cells were visualized using gradient contrast illumination through a 40X water-immersion lens attached to an Olympus BX51 (Olympus) upright microscope. Patch pipettes were pulled from borosilicate glass (o.d. – 1.5 mm, i.d. – 0.84 mm) using a P-97 Flaming/Brown electrode puller (Sutter Instruments). Pipettes were filled with a solution containing (in mM): 138 K-gluconate, 10 HEPES, 0.0001 CaCl₂, 0.2 EGTA, 4 NaATP, 0.4 NaGTP, 2 MgCl₂, with an osmolarity of 270–275 mOsm and adjusted to a pH of 7.3 with KOH. Recordings were made using a MultiClamp 700B amplifier (Molecular Devices). Signals were digitized at 15–30 kHz and saved to a hard drive for analysis using the software program, AxoGraph X (AxoGraph Scientific).

Spiny neurons in the NAc core were identified as having the following properties: a hyperpolarized membrane potential (< –70 mV), a low input resistance (< 350 MΩ), and delayed spiking upon current injection. Drugs were applied to the slice by superfusion at the indicated concentration. All experiments were performed in the presence of: 5 μM NBQX (AMPA antagonist), 25 μM D-APV (NMDA antagonist), 100 μM picrotoxin (GABA_A antagonist), and 10 μM SCH 23390 (D1 antagonist). The drug NF023 (10 μM) was applied internally. For NF 449 (1 μM) application, the slices were incubated in the Gα_s antagonist for 1 h prior to recording, and then continuously exposed to NF449 throughout the recording process. All drugs were obtained from Tocris Bioscience (Bristol, UK) or Sigma-Aldrich (St. Louis, MO). In current-clamp configuration, current was injected for 200 ms at 100-pA step intervals (100–500 pA) with 5 s between each pulse, until the cell was depolarized and spikes were evoked. An input/output curve was obtained under baseline conditions before and after superfusing 10 ml of a 5 μM solution of quinpirole for approximately 5 min. Action potentials were detected using an amplitude threshold, and spike frequency was calculated as the reciprocal of the inter-spike interval.

Statistical analysis

Western blot data were analyzed by t-test using GraphPad Prism 6.0 for Macintosh. Behavioral data were analyzed by two-way repeated measures ANOVA (RMANOVA), followed by Bonferroni posthoc tests, where appropriate, using Prism. Electrophysiological data were analyzed by RMANOVA with a generalized estimating equation (GEE). In our data, the number of observations differed between different current steps across cells. Due to the unbalanced design, the classic RMANOVA was therefore not an appropriate test. We used the RMANOVA with a GEE approach with an exchangeable correlation structure to take into account the unbalanced design, as well as correlated observations. For each test, GEE uses a robust test (Wald Chi-squared test based on robust variance estimators) for each effect. These analyses were performed using R (www.r-project.org).

Results

***Dbh* ^{-/-} mice have decreased β -arrestin2 in the nucleus accumbens**

We showed previously that FosB, which is induced in the NAc by chronic drug exposure and is known to promote psychostimulant-induced behaviors (Kelz et al., 1999), is elevated in the striatum of drug-naïve *Dbh* ^{-/-} mice (Rommelfanger et al., 2007). As part of a larger survey to identify potential upstream mediators of the cocaine hypersensitivity that follows chronic DBH inhibition, we found that *Dbh* ^{-/-} mice had significantly less β Arr2 in the NAc ($t_6 = 3.493$, $p < 0.05$) (Fig. 1a). Besides its role in G-protein-coupled receptor desensitization, β Arr2 can signal through a protein kinase B/glycogen synthase kinase-3 β (Akt/GSK3 β) pathway (Del'guidice et al., 2011); however, we detected no genotype differences in the proportion of phosphorylated Akt and GSK3 β proteins compared to the total protein levels when comparing *Dbh* ^{-/-} mice to control *Dbh* ^{+/-} mice (Fig. 1b, 1c).

Nepicastat-treated mice are hypersensitive to cocaine and have decreased β -arrestin2 and increased FosB in the nucleus accumbens

We next determined whether the cocaine hypersensitivity observed in *Dbh* ^{-/-} mice could be mimicked by chronic pharmacologic DBH inhibition in control mice. *Dbh* ^{+/-} mice with normal NE content that received chronic nepicastat (via osmotic minipump or daily i.p. injections) had no change in locomotion induced by a novel environment, but displayed increased cocaine-induced stereotypy (Gaval-Cruz et al., 2012) and/or locomotion (Fig. 1d), reminiscent of *Dbh* ^{-/-} mice (Gaval-Cruz et al., 2012; Schank et al., 2006; Weinschenker et al., 2002). Two-way ANOVA revealed a main effect of time ($F_{11,110} = 17.55$, $p < 0.0001$) and a treatment x time interaction ($F_{11,110} = 2.64$, $p < 0.01$). Posthoc tests showed that peak cocaine-induced locomotion was significantly enhanced by chronic nepicastat administration. Acute DBH inhibition, in contrast, does not augment cocaine responses and can even inhibit them (Haile et al., 2003; Maj et al., 1968; Schroeder et al., 2013). These results indicate that the hypersensitivity to psychostimulants seen in *Dbh* ^{-/-} mice cannot be attributed to developmental alterations produced solely by DBH knockout, but likely result from downstream changes in the signaling pathways that occur following prolonged deficits in NE.

Because *Dbh* ^{-/-} mice have decreased β Arr2 in the NAc and increased FosB in the striatum, we measured the relative levels of these proteins in the NAc of control mice following chronic treatment with nepicastat. We found that nepicastat-treated mice had decreased β Arr2 ($t_{14} = 3.49$, $p < 0.01$; Fig. 1e) and increased FosB ($t_{14} = 2.69$, $p < 0.05$; Fig. 1f), confirming that genetic and pharmacological inhibition of NE synthesis produce similar alterations in DA signaling proteins in the ventral striatum.

Overexpression of β -arrestin2 in the nucleus accumbens reverses cocaine hypersensitivity in *Dbh* ^{-/-} mice

While FosB is induced in the NAc by chronic drug exposure and is known to promote psychostimulant-induced behaviors (Kelz et al., 1999), the role of β Arr2 is less clear. To determine whether the decreased β Arr2 in the NAc of mice with chronic NE deficiency contributes to their behavioral hypersensitivity to cocaine, we overexpressed green

fluorescent protein (GFP) or β Arr2 in the NAc of *Dbh*^{-/-} mice using adenoviral vectors and assessed novelty- and cocaine-induced locomotor activity. High levels of β Arr2 immunoreactivity were evident along the needle track and in both the core and shell subregions (Fig. 2a) and β Arr2 protein levels were doubled in the *Dbh*^{-/-} NAc as assessed by western blot (Fig. 2b) 7–10 days following viral vector injection, indicating that β Arr2 overexpression was achieved and that the antibody we used was detecting β Arr2. β Arr2 overexpression had no effect on the reduced novelty-induced locomotor activity of *Dbh*^{-/-} mice (Fig. 2c). Two-way ANOVA revealed a main effect of time ($F_{2,66} = 99.44$, $p < 0.0001$) and genotype ($F_{2,33} = 4.46$, $p < 0.05$). Posthoc tests showed that *Dbh*^{-/-} mice overexpressing GFP or β Arr2 had decreased novelty-induced locomotion compared to *Dbh*^{+/-} controls with normal NE content at the 10-min time point. As expected, *Dbh*^{-/-} mice that were infused with the GFP virus were hypersensitive to cocaine-induced locomotion compared to mice with normal NE content. By contrast, overexpression of β Arr2 in the NAc core of *Dbh*^{-/-} mice completely normalized their cocaine response (Fig. 2d). Two-way ANOVA revealed a main effect of time ($F_{11,286} = 9.62$, $p < 0.0001$), genotype ($F_{2,26} = 5.20$, $p < 0.05$), and a time x genotype interaction ($F_{22,286} = 2.88$, $p < 0.0001$). Posthoc tests showed that *Dbh*^{-/-} mice overexpressing GFP displayed increased locomotion compared with *Dbh*^{+/-} mice and *Dbh*^{-/-} overexpressing β Arr2 at the 20-, 30-, and 40-min time points following cocaine administration, whereas there were no apparent differences between *Dbh*^{+/-} mice and *Dbh*^{-/-} mice overexpressing β Arr2 in the NAc at any time point. These results suggest that the cocaine hypersensitivity conferred by chronic DBH inhibition is mediated, at least in part, by reduced β Arr2 levels in the NAc.

***Dbh*^{-/-} nucleus accumbens medium spiny neurons have aberrant responses to quinpirole**

To uncover the cellular underpinnings of the D2 and cocaine hypersensitivity following chronic DBH inhibition, we measured the spike frequency of trains of action potentials elicited by the injection of current steps in NAc MSNs both at baseline and following bath application of quinpirole (5 μ M) from control and *Dbh*^{-/-} mice. The control group consisted of both *Dbh*^{+/-} and wild-type C57Bl/6J mice since results comparing baseline and quinpirole responses in these groups were not significantly different ($n = 22$ *Dbh*^{-/-}, 24 C57Bl/6J; Chi-square (df = 1) = 0.1, $p = 0.75$) (Fig. 3). We found no genotype differences between control and *Dbh*^{-/-} mice in baseline firing rate in untreated MSNs ($F_{4, 160} = 0.43$; $p = 0.79$; data not shown), and activation of D2 receptors by quinpirole did not significantly change the input resistance or the resting membrane potential in either control or *Dbh*^{-/-} mice (control $R_{in} = 5.84 \pm 4.28$ M Ω , *Dbh*^{-/-} $R_{in} = 6.57 \pm 8.55$ M Ω , $p = 0.54$; control $V_{rest} = -0.71 \pm 1.80$ mV, *Dbh*^{-/-} $V_{rest} = -1.36 \pm 1.04$ mV, $p = 0.43$). As reported previously and expected for a $G_{\alpha i/o}$ -coupled receptor (Perez et al., 2006; Surmeier and Kitai, 1993; Yasumoto et al., 2002; Zamponi and Snutch, 1998), activation of D2 receptors by quinpirole (5 μ M) reduced evoked mean spike frequency in MSNs from control animals (Chi-square (df = 1) = 5.2, $p = 0.02$) (Fig. 4a, 4c). However, the D2-mediated reduction in MSN excitability seen in control mice was absent in MSNs recorded from *Dbh*^{-/-} mice. Instead, quinpirole tended to have an excitatory effect in the knockout neurons, but it did not quite reach significance (Chi-square (df = 1) = 3.5, $p = 0.06$) (Fig. 4b, 4c). There was a highly significant difference between the spike frequency difference curves between MSNs from controls compared to MSNs from *Dbh*^{-/-} animals (Chi-square (df =

1) = 20.99, $p < 0.0001$) (Fig. 4c), indicating the excitability of MSNs is increased in *Dbh* $-/-$ animals compared to controls.

Because D2-like receptor abundance is normal in *Dbh* $-/-$ mice but the cellular response to quinpirole is altered, we suspected that D2 receptors might be aberrantly coupled in NAc MSNs of *Dbh* $-/-$ mice. To test this idea, we assessed the effects of quinpirole on MSN excitability in the presence of either a $G\alpha_i$ (NF023; 10 μ M), or $G\alpha_s$ (NF449; 1 μ M) inhibitor (Freissmuth et al., 1996; Hohenegger et al., 1998). As expected for a $G\alpha_i$ -coupled receptor like D2, application of NF023 to control MSNs abolished the quinpirole-induced decrease in excitability (Chi-square (df = 1) = 1.2, $p = 0.27$) and resulted in a significant difference in the spike frequency difference curve compared to the spike frequency difference curve for MSNs treated with quinpirole alone (Chi-square (df = 1) = 16.5; $p < 0.0001$) (Fig. 5a, 5c). By contrast, the spike frequency difference curve obtained from *Dbh* $-/-$ MSNs after application of NF023 was not significantly different from the spike frequency difference curve obtained from *Dbh* $-/-$ MSNs treated with quinpirole alone (Chi-square (df = 1) = 1.57, $p = 0.21$) (Fig. 5b, 5d).

Application of the $G\alpha_s$ inhibitor, NF449, did not occlude the inhibitory effects of D2 activation in MSNs from control mice; no changes in spike frequency were observed when compared to quinpirole alone (Chi-square (df = 1) = 0.14, $p = 0.71$) (Fig. 6a, 6c). However, application of NF449 in *Dbh* $-/-$ MSNs significantly reduced spike frequency compared to quinpirole alone (Chi-square (df = 1) = 14.99, $p = 0.0001$) (Fig. 6b, 6d). Combined, these results suggest that D2 receptors on NAc MSNs primarily couple to $G\alpha_i$ and suppress firing rate in control mice, but that D2-G protein coupling is altered and quinpirole-induced inhibition is lost in *Dbh* $-/-$ NAc MSNs.

Discussion

Pharmacological and genetic DBH inhibition lead to behavioral hypersensitivity to dopaminergic drugs, including psychostimulant drugs of abuse. (Gaval-Cruz et al., 2012; Haile et al., 2003; Schank et al., 2006; Weinshenker et al., 2008; Weinshenker et al., 2002). We identified a decrease in β Arr2 and an increase in FosB in the NAc following chronic genetic or pharmacological DBH inhibition, and overexpression of β Arr2 normalized cocaine responses in *Dbh* $-/-$ mice. Slice electrophysiology experiments revealed that MSNs from control mice are inhibited by quinpirole in a $G\alpha_i$ -dependent manner, while the effects of quinpirole are altered and become sensitive to $G\alpha_s$ blockade in *Dbh* $-/-$ NAc MSNs.

Chronic DBH inhibition alters the abundance of DA receptor signaling proteins in the nucleus accumbens

Because DBH catalyzes the conversion of NE to DA in noradrenergic neurons, DBH inhibition decreases NE production, with a concomitant increase in tissue DA levels (Bourdelat-Parks et al., 2005; Goldstein, 1966; Musacchio et al., 1966). However, because NE provides direct and indirect excitatory drive onto midbrain DA neurons, basal and stimulant-evoked DA overflow is actually reduced in *Dbh* $-/-$ mice (Schank et al., 2006; Weinshenker and Schroeder, 2007), producing a compensatory upregulation of D2 signaling

and hypersensitivity to psychostimulants and quinpirole (Schank et al., 2006; Weinshenker et al., 2008; Weinshenker et al., 2002). Indeed, neurotoxic ablation of brain NE neurons, which reduces NE without an increase in tissue DA, confers a similar pattern of drug responses (Harro et al., 2000; Nowak et al., 2009; Weinshenker et al., 2008).

We originally reported increased high affinity-state D2 receptors in the striatum of *Dbh* $-/-$ mice, which we speculated might underlie the behavioral hypersensitivity of the knockouts to psychostimulants (Schank et al., 2006). However, subsequent in vitro radioligand competition experiments failed to confirm these results (Skinbjerg et al., 2010) (our unpublished data). The discrepancy between studies measuring D2 affinity states may be due to some differences in the radioligands and approaches employed, but more concerning was our failure to observe a genotype difference in the abundance of high affinity-state D2 receptors in vivo using PET imaging (Skinbjerg et al., 2010).

Because of these issues and inconsistencies, we suspected that changes in downstream signaling molecules, rather than D2 receptor affinity state, were responsible for cocaine hypersensitivity following chronic NE deficiency. Both genetic and pharmacological DBH inhibition produced a decrease of β Arr2 and an increase in FosB in the NAc. FosB is a transcription factor that is induced by chronic exposure to drugs or other environmental stimuli and enhances behavioral responses to cocaine (Kelz et al., 1999). We chose to pursue the contribution of β Arr2 because it is upstream of FosB in the DA receptor signaling pathway and because it had not been implicated in cocaine-induced behaviors; locomotor activity and conditioned place preference following cocaine administration are unchanged in β Arr2 knockout mice (Bohn et al., 2003). We found that viral-mediated overexpression of β Arr2 in the NAc suppressed the cocaine hypersensitivity in *Dbh* $-/-$ mice. This effect was not due to a general suppression of motor activity because ambulatory behavior in a novel environment was unaffected by β Arr2 overexpression, and cocaine-induced locomotion was normalized to, but not below, control levels. Because we examined used a CMV promoter to drive overexpression of β Arr2, we cannot attribute its effects on cocaine-induced locomotion specifically to changes in D2 signaling. Future experiments using D1- and D2-specific promoters will be required to delineate the importance of β Arr2 in direct versus indirect pathway MSNs. The discrepancies between the β Arr2-mediated phenotypes in *Dbh* $-/-$ and β Arr2 knockout mice may be due to the complete lack of global β Arr2 in the β Arr2 knockout mice versus the partial reduction of β Arr2 specifically in the NAc of *Dbh* $-/-$ mice.

Aberrant cellular responses to quinpirole in nucleus accumbens medium spiny neurons of *Dbh* $-/-$ mice

Because normal D2 autoreceptor function is preserved in *Dbh* $-/-$ mice (Paladini et al., 2007), we focused our attention on potential changes in D2 receptor signaling in accumbal MSNs. Activation of D2 and other $G\alpha_{i/o}$ -coupled receptors typically inhibits evoked action potentials, reducing firing and spike frequency in MSNs. By contrast, $G\alpha_s$ -coupled receptors, such as D1, have a facilitatory effect on MSN responses (Hu and Wang, 1988; Surmeier et al., 2010; West and Grace, 2002). Our electrophysiological recordings from NAc neurons confirmed that the D2/3 agonist, quinpirole, suppressed evoked MSN firing in

slices from control mice. By contrast, the inhibitory effects of quinpirole were abolished in *Dbh*^{-/-} MSNs, and in fact there was a trend for quinpirole to be excitatory.

There are several potential explanations for this change in cellular response to D2/3 activation. For example, the involvement of β Arr2 in receptor desensitization and endocytosis suggests a possible contribution of altered D2 trafficking and localization. In addition, because β Arr2 recruits cAMP-degrading phosphodiesterase (PDE) to the membrane upon receptor binding (Kendall and Luttrell, 2009; Perry et al., 2002), reduction of β Arr2 could dampen inhibition and promote excitation by potentiating cAMP abundance. The reduction in β Arr2 could also affect neuronal firing by altering G protein-independent GSK3 β /Akt signaling, although we did not detect any differences in these proteins in the NAc of *Dbh*^{-/-} mice. It is also possible that the decrease in β Arr2 and the altered quinpirole response are unrelated. Future experiments to determine whether loss of β Arr2 directly *causes* aberrant D2/3 signaling will help identify the underlying mechanisms.

Given that NF023 abolished quinpirole-induced inhibition in MSNs from control mice, but had no effect on quinpirole response in *Dbh*^{-/-} MSNs, while NF449 had no effect on quinpirole-induced inhibition in control MSNs, but suppressed firing in the presence of quinpirole in *Dbh*^{-/-} mice, it is possible that at least some D2 receptors are coupled to $G\alpha_s$ instead of $G\alpha_i$ in *Dbh*^{-/-} MSNs. *In vivo* $G\alpha_i$ -to- $G\alpha_s$ switching has been reported for μ -opioid receptors and CB1 cannabinoid receptors following chronic agonist exposure (Paquette et al., 2007; Wang et al., 2005), and a reduction in β Arr2 promotes $G\alpha_s$ -to- $G\alpha_i$ switching of β -adrenergic receptors (Baillie et al., 2003). However, these data are associated with several limitations and must be interpreted with caution. Quinpirole is an agonist of both D2 and D3 DA receptors, and both subtypes are present in the NAc. D3 receptors and D2-D3 heterodimers receptors have been reported to couple to $G\alpha_q$, and thus promote neuronal excitation. However, increased D3- $G\alpha_q$ or D2/D3- $G\alpha_q$ heterodimer signaling is unlikely to underlie quinpirole-induced excitation in *Dbh*^{-/-} MSNs for two reasons. First, *Dbh*^{-/-} mice are hypersensitive to quinpirole but not the preferential D2/D3 heterodimer agonist SKF83959 (our unpublished data). thus the $G\alpha_s$ -to- $G\alpha_i$ switch could be solely or preferentially affecting one of these two subtypes. An increase in firing following quinpirole as that seen in *Dbh*^{-/-} mice could also be explained if these mice had a greater proportion of D2-D3 G_q -coupled heteromers. However, systemic administration of the preferential D2-D3 heteromer agonist SKF83959 does not result in differences in locomotion between *Dbh*^{-/-} and *Dbh*^{+/-} mice. NF023 and NF449 are not totally selective for $G\alpha_i$ and $G\alpha_s$; for example, both compounds are also X and P2Y receptor antagonists (Braun et al., 2001; Lambrecht, 1996). This is nevertheless unlikely given our results since P2X, as a ligand-gated ion channel, would have a direct effect on membrane potential and firing rate, which we did not observe. NF023 and NF449 also had different effects in our different groups of animals, which is inconsistent with these drugs acting as purinergic antagonists. Finally, our methods did not allow us to distinguish between D1 and D2 MSNs, which precludes assigning direct effects of quinpirole and could also account for the high variability in some of our experiments, particularly the ones involving *Dbh*^{-/-} recordings. In addition, it will be important to test alterations in D2-G protein associations using other techniques such as

co-immunoprecipitation. Unfortunately, D2 antibodies of sufficient quality and specificity for this approach are not currently available.

Clinical implications

The consequences of chronic reduction in DBH function may be relevant to drug addiction. Non-selective DBH inhibitors, like disulfiram, have shown promise in human laboratory studies and clinical trials for the treatment of stimulant dependence (Gaval-Cruz and Weinschenker, 2009), and a large phase II trial of nescicatat for cocaine addiction is ongoing (<http://clinicaltrials.gov/show/NCT01704196>). Genetic or chronic pharmacological reduction of DBH activity enhances some interoceptive properties of cocaine, particularly its aversive effects, such as paranoia and anxiety (Gaval-Cruz and Weinschenker, 2009; Hameedi et al., 1995; Kalayasiri et al., 2007; McCance-Katz et al., 1998a, b; Mutschler et al., 2009; Schank et al., 2006; Sofuoglu et al., 2008)_ENREF_46, suggesting that the clinical efficacy of DBH inhibitors is related to an increase in cocaine aversion. Interestingly, optogenetic inhibition of D2 MSNs drives, while excitation of D2 MSNs inhibits, compulsive cocaine seeking in mice (Bock et al., 2013). Thus, under normal conditions, cocaine may facilitate its own use by promoting DA signaling via the D2 receptor and suppressing D2 MSN activity; however, our data suggest that, under conditions of chronic DBH inhibition, cocaine may actually limit its own use because D2-mediated inhibition is absent, leading to increased cell excitability.

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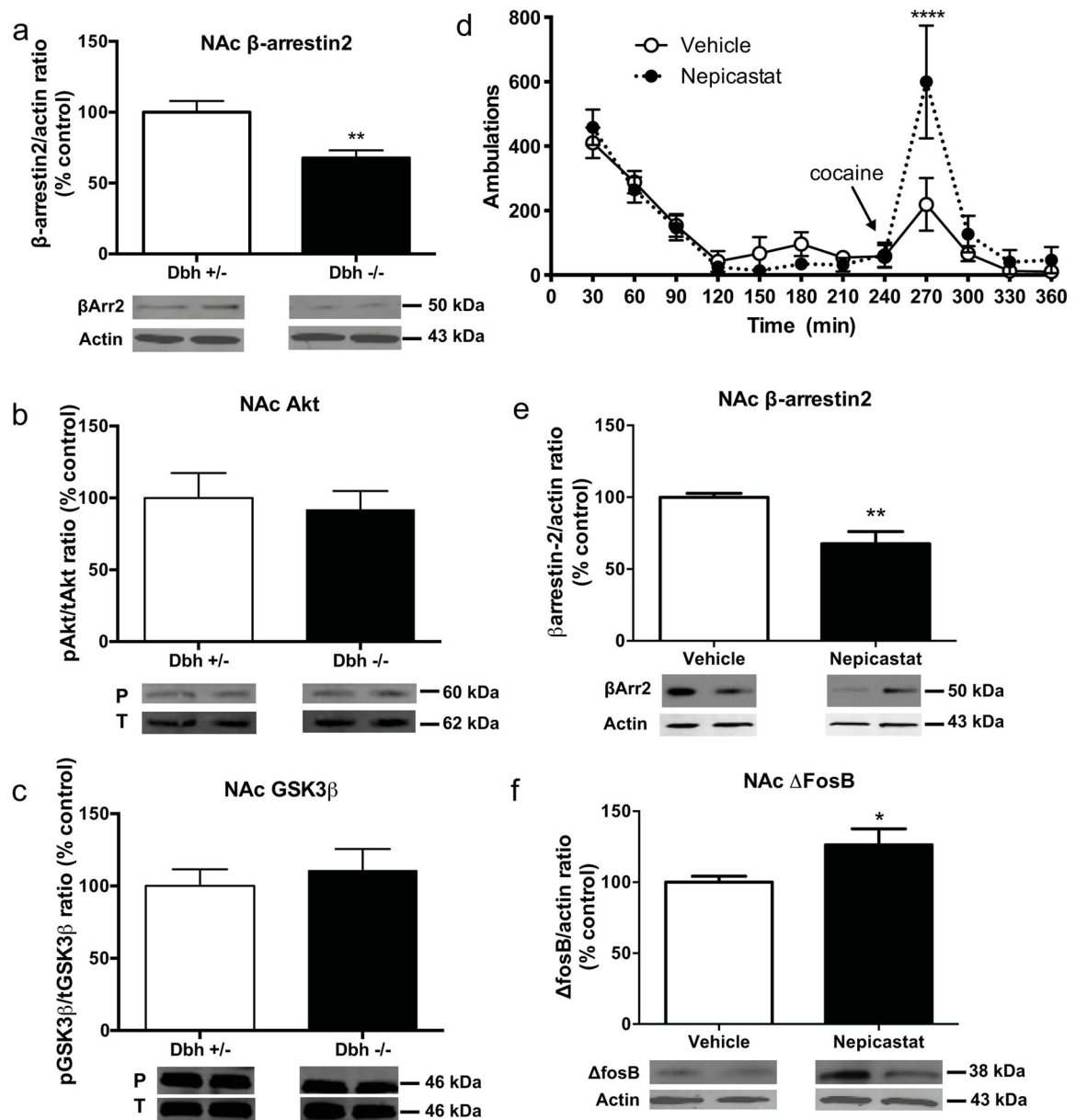


Figure 1. Behavioral and neurochemical phenotypes of mice with chronic DBH deficiency
 Western blot data (mean ± SEM above, representative blot below) for (a) βArr2:actin ratio, (b) protein kinase B (Akt; phospho:total ratio) and (c) glycogen synthase kinase-3β (GSK-3β; phosphor:total ratio) in the nucleus accumbens (NAc) of *Dbh* +/- and *Dbh* -/- mice (n = 8 per group). *p < 0.05 compared to *Dbh* +/- mice. (d) Cocaine-induced (10 mg/kg, i.p.) locomotion, (e) βArr2:actin ratio, and (f) FosB:actin ratio in the NAc of *Dbh* +/- control mice treated chronically with the selective DBH inhibitor nepicastat. *p < 0.05, **p < 0.01, ****p < 0.0001 compared to vehicle.

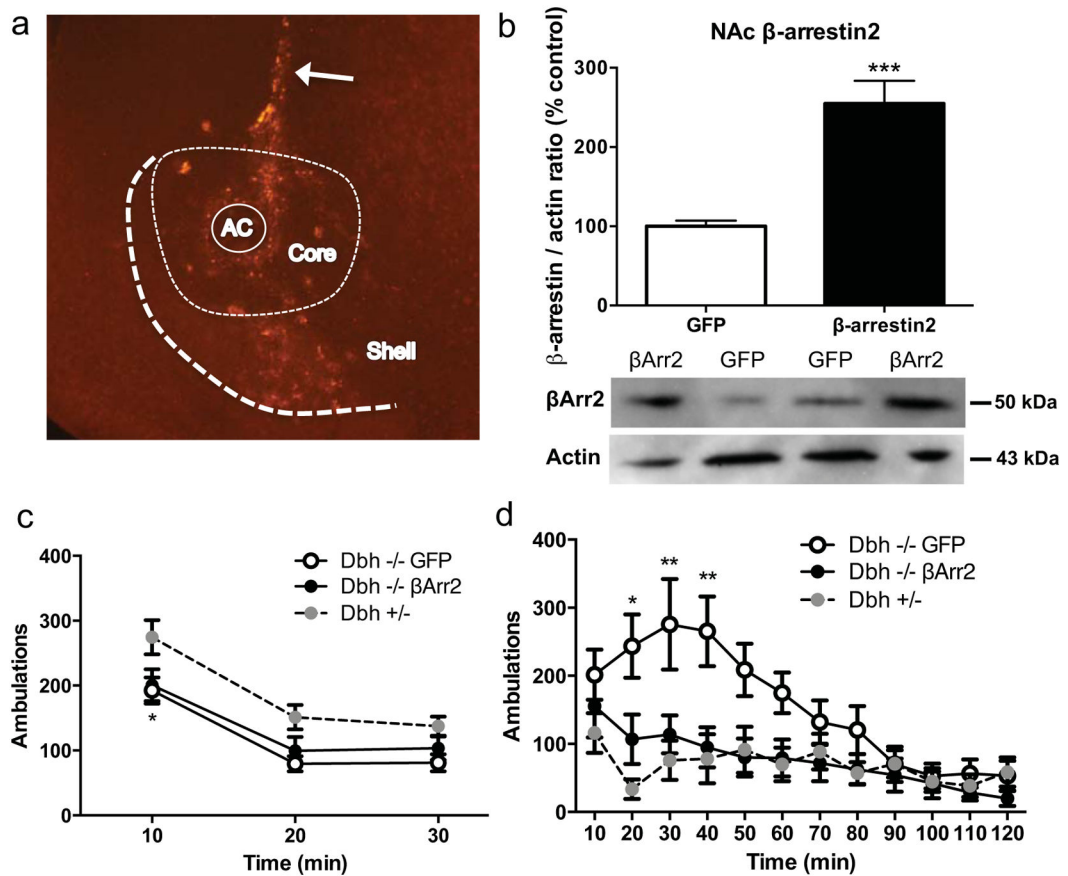


Figure 2. β -arrestin2 overexpression in the nucleus accumbens restores normal cocaine sensitivity to *Dbh*^{-/-} mice

(a) Representative picture of β Arr2 overexpression in the NAc of a *Dbh*^{-/-} mouse that received the β Arr2 virus. (b) Western blot data (mean \pm SEM above, representative blot below) for β Arr2:actin ratio in the NAc of *Dbh*^{-/-} mice that received GFP virus (n = 6) or β Arr2 virus (n = 7). ***p < 0.001 compared to *Dbh*^{-/-} with GFP virus. (c) Novelty-induced (drug-free state) and (d) cocaine-induced (15 mg/kg, i.p., administered after 30 min in chamber) locomotor activity in *Dbh*^{+/-} mice (n = 8), *Dbh*^{-/-} mice that received GFP virus (n = 10), and *Dbh*^{-/-} mice that received β Arr2 virus (n = 11). *p < 0.05, **p < 0.01 compared to *Dbh*^{+/-} controls. AC, anterior commissure; Core, NAc core; Shell, NAc shell; arrow, needle track.

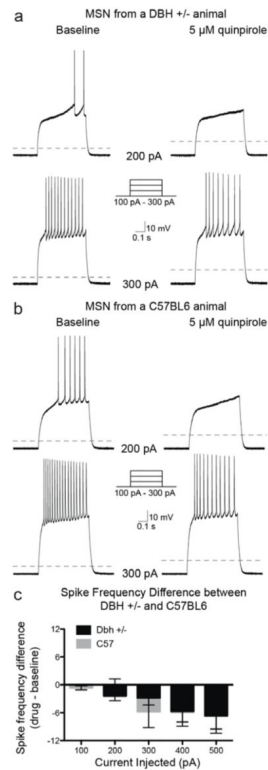


Figure 3. Activation of D2 receptors similarly inhibits evoked firing of NAc MSNs from *Dbh* +/- and C57BL/6J mice

Example traces of a MSN from a *Dbh* +/- (a) and a wild-type C57BL/6J (b) animal at baseline and following bath application of the D2/3 agonist, quinpirole (5 μM), while action potentials were evoked with a series of current steps. Dashed line indicates -70 mV. (c) Population data (mean ± SEM) showing the effects of D2 receptor activation on spike frequency differences (quinpirole – baseline) from cells recorded from *Dbh* +/- (n = 22) and C57BL/6J (n = 24) mice.

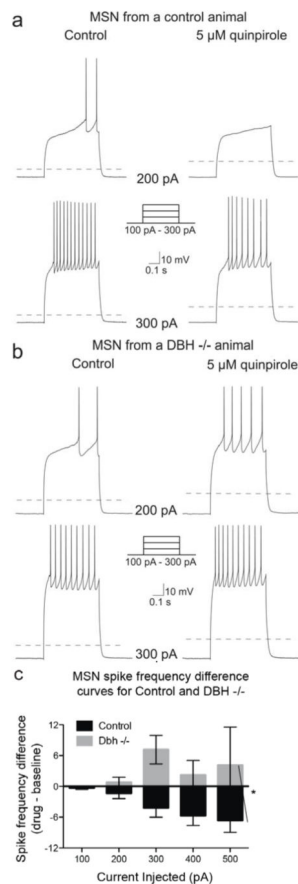


Figure 4. Quinpirole inhibits evoked firing of NAc MSNs from control, but not *Dbh*^{-/-} mice
 Example traces of a MSN from a control (a) and *Dbh*^{-/-} (b) animal at baseline, and following bath application of the D2/3 agonist, quinpirole (5 μ M), while action potentials were evoked with a series of current steps. Dashed line indicates -70 mV. (c) Population data (mean \pm SEM) showing the effects of D2-like receptor activation on evoked spike frequency differences (quinpirole - baseline) from cells recorded from control (n = 46) and *Dbh*^{-/-} (n = 15) mice. * indicates significant difference (p < 0.05) between control and *Dbh*^{-/-} population data.

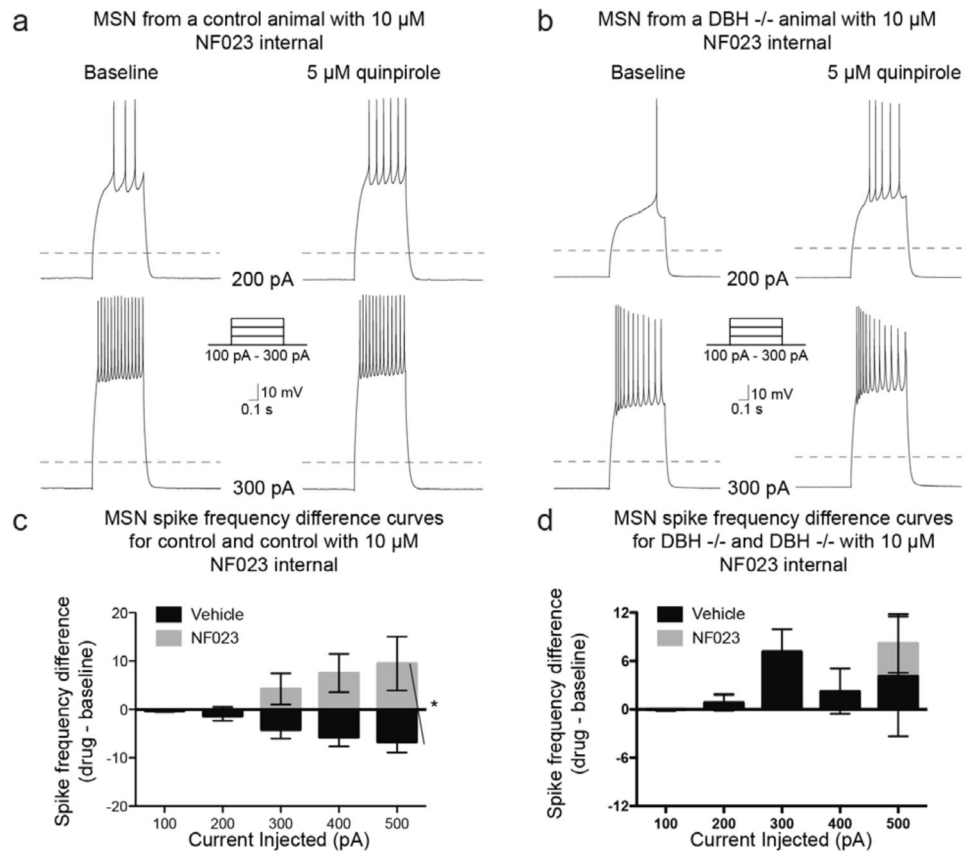


Figure 5. $G\alpha_i$ inhibition abolishes quinpirole-mediated inhibition of MSN spike excitability in control mice, but has no effect in *Dbh* $-/-$ mice

Example traces from MSNs recorded from a control (a) and a *Dbh* $-/-$ (b) animal at baseline or following bath application of quinpirole (5 μM) with the $G\alpha_i$ inhibitor NF023 (10 μM) applied internally, while action potentials were evoked with a series of current steps. Dashed line indicates -70 mV. (c) Population data (mean \pm SEM) showing the effects of D2-like receptor activation on evoked spike frequency differences (quinpirole – baseline) in the presence of vehicle (n = 46) or NF023 (n = 10) from cells recorded from control mice. (d) Population data (mean \pm SEM) showing the effects of D2-like receptor activation on evoked spike frequency differences (quinpirole – baseline) in the presence of vehicle (n = 15) or NF023 (n = 17) from cells recorded from *Dbh* $-/-$ mice. * indicates significant difference ($p < 0.05$) between vehicle and NF023 population data.

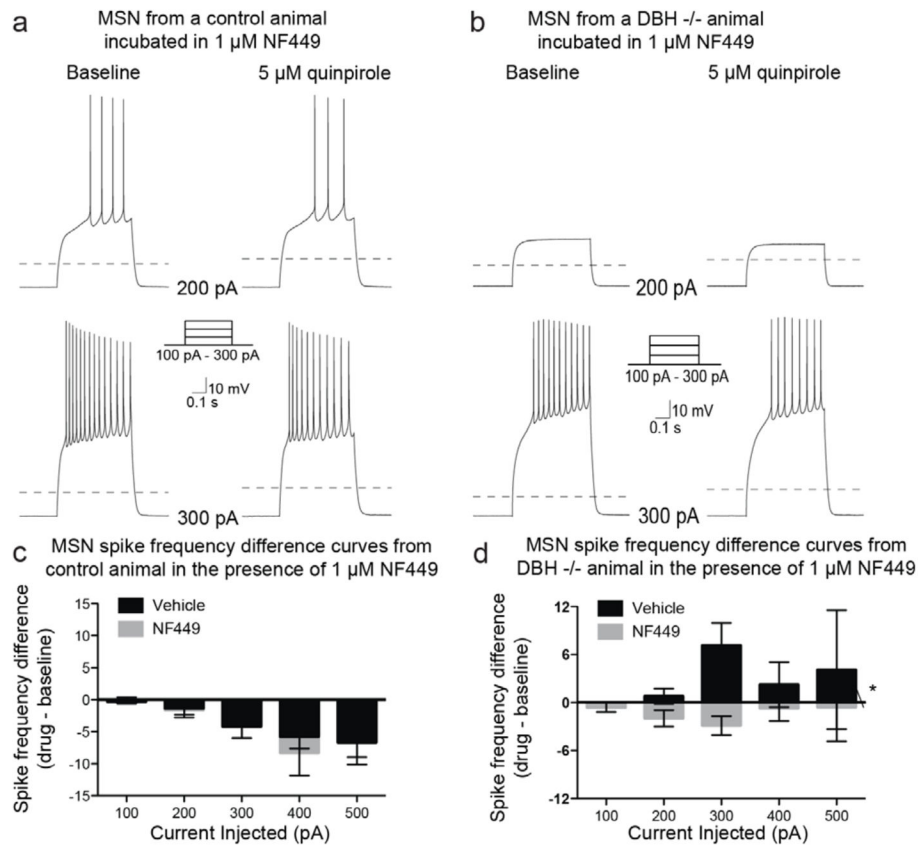


Figure 6. $G\alpha_s$ inhibition has no effect on quinpirole-mediated inhibition in control mice, but restores quinpirole-mediated inhibition of MSN spike frequency in *Dbh*^{-/-} mice

Example traces from MSNs recorded from a control (a) and a *Dbh*^{-/-} (b) animal at baseline or following bath application of quinpirole (5 μ M) with the $G\alpha_s$ inhibitor, NF449 (10 μ M; slices pre-incubated in the $G\alpha_s$ antagonist for 1 h prior to recording, and then continuously exposed to NF449 throughout the recording process), while action potentials were evoked with a series of current steps. Dashed line indicates -70 mV. (c) Population data (mean \pm SEM) showing the effects of D2-like receptor activation on evoked spike frequency differences (quinpirole - baseline) in the presence of vehicle (n = 46) or NF449 (n = 11) from cells recorded from control mice. (d) Population data (mean \pm SEM) showing the effects of D2-like receptor activation on evoked spike frequency differences (quinpirole - baseline) in the presence of vehicle (n = 15) or NF449 (n = 22) from cells recorded from *Dbh*^{-/-} mice. * indicates significant difference (p < 0.05) between vehicle and NF449 population data.