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Normalizing Dopamine D2 Receptor-Mediated Responses in D2 Null Mutant Mice by Virus-Mediated Receptor Restoration: Comparing D2_L and D2_S

Kim A. Neve^{a,*}, Christopher P. Ford^{b,1}, David C. Buck^a, David K. Grandy^c, Rachael L. Neve^d, and Tamara J. Phillips^a

^aResearch Service, Portland VA Medical Center, and Department of Behavioral Neuroscience and Methamphetamine Abuse Research Center, Oregon Health & Science University, Portland, OR 97239, USA

^bVollum Institute, Oregon Health & Science University, Portland, OR 97239, USA

^cDepartment of Physiology & Pharmacology, Oregon Health & Science University, Portland, OR 97239, USA

^dDepartment of Brain and Cognitive Sciences, MIT, Cambridge, MA 02139, USA

Abstract

D2 receptor null mutant (*Drd2*^{-/-}) mice have altered responses to the rewarding and locomotor effects of psychostimulant drugs, which is evidence of a necessary role for D2 receptors in these behaviors. Furthermore, work with mice that constitutively express only the D2 receptor short form (D2_S), as a result of genetic deletion of the long form (D2_L), provides the basis for a current model in which D2_L is thought to be the postsynaptic D2 receptor on medium spiny neurons in the basal forebrain, and D2_S the autoreceptor that regulates the activity of dopamine neurons and dopamine synthesis and release. Because constitutive genetic deletion of the D2 or D2_L receptor may cause compensatory changes that influence functional outcomes, our approach is to identify aspects of the abnormal phenotype of a *Drd2*^{-/-} mouse that can be normalized by virus-mediated D2 receptor expression. *Drd2*^{-/-} mice are deficient in basal and methamphetamine-induced locomotor activation and lack D2 receptor agonist-induced activation of G protein-regulated inward rectifying potassium channels (GIRKs) in dopaminergic neurons. Virus-mediated expression of D2_L in the nucleus accumbens significantly restored methamphetamine-induced locomotor activation, but not basal locomotor activity, compared to mice receiving control virus. It also restored the effect of methamphetamine to decrease time spent in the center of the activity chamber in female but not male *Drd2*^{-/-} mice. Furthermore, the effect of expression of D2_S was

*Corresponding author. Tel: +1-503-721-7911; fax: +1-503-721-7839 nevek@ohsu.edu Research Service (R&D-30) VA Medical Center 3710 SW US Veterans Hospital Rd Portland, OR 97239, USA.

¹Present address: Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, OH 44106, USA
Email addresses, in order: nevek@ohsu.edu, cpf21@case.edu, buckd@ohsu.edu, grandyd@ohsu.edu, meve@MIT.EDU, phillipt@ohsu.edu

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indistinguishable from D2_L. Similarly, virus-mediated expression of either D2_S or D2_L in substantia nigra neurons restored D2 agonist-induced activation of GIRKs. In this acute expression system, the alternatively spliced forms of the D2 receptor appear to be equally capable of acting as postsynaptic receptors and autoreceptors.

Keywords

dopamine D2 receptor; methamphetamine; D2 receptor null mutant mouse; potassium channel

INTRODUCTION

The actions of dopamine are mediated by D1-like (D1 and D5) and D2-like (D2, D3, and D4) receptors. The dopamine D2 receptor belongs to a subfamily of 7-transmembrane domain G protein-coupled receptors that interact with the G proteins Gα_i and Gα_o to modulate several effectors, including adenylate cyclase, potassium channels, and mitogen-activated protein kinases (Neve et al., 2004). Splice variants of the D2 receptor, D2_L and D2_S, differ in the presence or absence of an alternatively spliced exon that encodes a 29-amino acid insert in the third intracellular loop (Dal Toso et al., 1989; Giros et al., 1989; Grandy et al., 1989; Monsma et al., 1989). Although pharmacological and cell biological studies have not consistently observed functional differences between D2_L and D2_S in heterologous expression systems, selective sparing of some D2 receptor-mediated responses in mice that constitutively express only the D2_S as a result of genetic deletion of D2_L (*Drd2*^{ex6-/ex6-} mice) suggests that the splice variants differ in subcellular localization or function. Thus, *Drd2*^{ex6-/ex6-} mice are deficient in several responses that are thought to reflect activation of postsynaptic D2 receptors in the basal forebrain, such as spontaneous and agonist-stimulated locomotor activity and inhibition by D2 receptors of protein kinase A-catalyzed phosphorylation of DARPP-32 (Lindgren et al., 2003; Usiello et al., 2000; Wang et al., 2000). This deficiency occurs even though, in *Drd2*^{ex6-/ex6-} mice, D2_L expression is replaced by D2_S, instead of simply being deleted, and suggests that only D2_L, not D2_S, is capable of mediating responses to dopamine in the medium spiny neurons in the neostriatum and nucleus accumbens that receive dopaminergic input. In contrast, D2 autoreceptor responses such as inhibition of locomotor activity and inhibition of the firing rate of and dopamine synthesis in mid-brain dopaminergic neurons are spared in *Drd2*^{ex6-/ex6-} mice (Lindgren et al., 2003; Wang et al., 2000). Although those data show only that D2_S can be an autoreceptor and do not address the question of whether D2_L can also be an autoreceptor, studies such as these have been interpreted in terms of a model in which D2_L is thought to be the postsynaptic D2 receptor on medium spiny neurons in the basal forebrain, and D2_S the autoreceptor that regulates the activity of dopamine neurons and dopamine synthesis/release.

Constitutive genetic deletion of the D2 or D2_L receptor may cause compensatory changes that influence functional outcomes; to address this, our approach is to identify aspects of the abnormal phenotype of a *Drd2*^{-/-} mouse that can be normalized by virus-mediated D2 receptor restoration. Because *Drd2*^{-/-} mice are deficient in methamphetamine-induced locomotor activation (Kelly et al., 2008) and lack D2 receptor agonist-induced activation of

potassium channels (GIRKs) in dopaminergic neurons (Beckstead et al., 2004; Mercuri et al., 1997) we decided to investigate whether these deficits could be restored by virus-mediated expression of different isoforms of the D2 receptor directly injected in the nucleus accumbens or substantia nigra, respectively. Virus-mediated expression of D2 receptors in the nucleus accumbens significantly restored methamphetamine-induced locomotor stimulation compared to mice receiving control virus, and the effect of expression of D2_S was indistinguishable from D2_L. Similarly, virus-mediated expression of either D2_S or D2_L in substantia nigra neurons restored D2 agonist-induced activation of GIRKs. In this acute expression system, the alternatively spliced forms of the D2 receptor appear to be equally capable of acting as postsynaptic receptors and autoreceptors

EXPERIMENTAL PROCEDURES

Generation and packaging of HSV and AAV vectors

cDNAs encoding rat D2_S and D2_L were subcloned into the Herpes simplex virus 1 (HSV-1) amplicons pHSVPrPUC or p1005, and replication-defective HSV vectors expressing the receptors were prepared as described (Neve et al., 1997). HSV-*LacZ* and HSV-GFP were prepared simultaneously with receptor-encoding vectors with a titer of $3\text{--}5 \times 10^8$ infectious units/ml ($\sim 1 \times 10^{11}$ viral genomes per ml). cDNAs encoding rat D2_S and D2_L were also subcloned into the AAV vector pFB-AAV-CMV-SV40pA and were packaged by Virovek, Inc. (Hayward, CA). Titers exceeded 1×10^{13} viral genomes per ml.

Virus injection

Infectious viral particles in which D2_L, D2_S, or control protein (either green fluorescent protein (GFP) or β -galactosidase) was expressed by either herpes simplex virus (HSV) or adeno-associated virus (AAV) were used to restore D2 receptor expression in the nucleus accumbens or in the ventral mesencephalon of *Drd2*^{-/-} mice. The generation and basic phenotypic analysis of the B6.129S2-*Drd2*^{tm1low} strain of D2R-deficient mice used in this study have been described previously (Kelly et al., 1997; Kelly et al., 1998). Mice used here were the offspring of congenic mice that had been backcrossed for 20 generations to the C57BL/6J strain. Littermates of both sexes (60–100 d old), born to heterozygous breeder pairs in the Veterinary Medical Unit of the Portland VA Medical Center, were used. Mice under anesthesia were placed in a stereotaxic instrument (Cartesian Research, Sandy, OR), and standard procedures were used to expose the skull and drill holes above the nucleus accumbens or the substantia nigra, pars compacta (SNc). Mice received bilateral infusions of infectious viral particles, 0.5 μ l/side, delivered over 7.5 min using a Hamilton 0.5 μ l syringe, 7000SER, 32 ga. Cole-Parmer dual infusion pump. Coordinates for the nucleus accumbens were 1.18 rostral, 1.2 lateral, and 4.8 ventral, whereas coordinates for the SNc were 3.16 caudal, 1.2 lateral, and 4.3 ventral.

Immunohistochemistry

Mouse brains were soaked in 4% paraformaldehyde in PBS at 4°C overnight, then transferred to 20% sucrose/PBS at 4°C for 1 week before being stored in 30% sucrose/PBS at 4°C until sectioning. The tissue was sliced (30 μ m coronal sections) on a Leica CM1850 cryostat and stored in PBS with 0.1% sodium azide at 4°C in 24-well plates. Staining was

done using the VECTASTAIN® ABC immunoperoxidase system PK6100 (Vector Labs, Burlingame CA). All incubations were performed at room temperature. Prior to staining the sections were washed in PBS for 5 min. They were then incubated in 0.3% H₂O₂ in PBS for 15 minutes, washed 3 times in PBS (5 min each), then blocked in a solution of 5% goat serum and 0.3% Triton X-100 in PBS for 4 hr. The sections were incubated overnight in rabbit anti-D2 receptor primary antibody (1:5000 in PBS/0.3% Triton X-100/0.1% BSA; AB5084P from Chemicon International, Temecula, CA), then washed 3 times for 5 minutes each. They were then incubated for 2 hr in 1:200 biotinylated goat anti rabbit IgG (Vectastain) diluted in PBS/0.3 % Triton X-100/0.1% BSA. The sections were washed 3 times for 5 min each. After incubation in the ABC solution (Vectastain) for 1 hr, the sections were washed 3 times for 5 min each, immersed in DAB solution (Thermo Scientific, Rockford IL) for 3 min, washed once in water, and mounted on glass slides prior to imaging on a Leica SP5 confocal microscope.

Locomotor activity measurements

Locomotor activity was measured in automated Accuscan activity monitors (40 cm²; AccuScan Instruments, Inc., Columbus, OH, USA), with tests performed between 1000 and 1400 h, during the light phase of the 12:12 h L:D cycle. Monitors were encased by sound attenuating external chambers that were illuminated (3.3 W incandescent light bulb) and ventilated. Mice were tested on 3 consecutive days, beginning 2 days after nucleus accumbens infusion of HSV. On days 1 and 2, each animal received an i.p. injection of saline, and activity was recorded for 60 min; day 1 provided level of activity in the novel environment and day 2 provided baseline activity data in a now familiar environment. On day 3, each animal received an injection of 2 mg/kg methamphetamine, and the magnitude of methamphetamine-stimulated activity was determined by subtracting baseline activity data on day 2 from activity after methamphetamine treatment on day 3 for each animal. This procedure controls for differences among animals in baseline activity level and is our standard method for obtaining a “drug response” score for methamphetamine (Kamens et al., 2005) and other drugs (Gubner et al., 2013). Accuscan software translated patterns of photocell beam breaks for beams and sensors located 2 cm above the chamber floor into distance traveled in centimeters, which we used as a measure of locomotor activity in each 5-min sample period. Distance traveled and time spent in the center of the chamber was also examined, which may indicate level of anxiety, as mice typically display thigmotactic (wall hugging) behavior. All mice were euthanized 1 hr following the day 3 activity testing to verify the placement of the intracerebral nucleus accumbens HSV injections.

Electrophysiological recording

Recordings from dopamine neurons in the SNc were performed as previously reported (Ford et al., 2006; Ford et al., 2009). Midbrain slices (220 µm) prepared from mice euthanized 2–3 weeks after injection of AAV into the SNC were horizontally cut in ice-cold physiological saline solution containing (in mM) 126 NaCl, 2.5 KCl, 1.2 MgCl₂, 2.4 CaCl₂, 1.4 NaH₂PO₄, 25 NaHCO₃, 11 D-glucose, and 0.005 MK-801 using a vibratome (Leica, Nussloch, Germany). Slices were incubated in warm (35°C) 95% O₂/5% CO₂ oxygenated saline containing MK-801 (10 µM) prior to being transferred to a recording chamber. All recordings were done at 35°C. Whole cell recordings were made with an Axopatch 200A

amplifier (Axon Instruments, Foster City, CA) using 1.5–2.5 M Ω pipettes. Pipette internal solution contained (in mM) 115 K-methylsulphate, 20 NaCl, 1.5 MgCl₂, 5 HEPES, 10 BAPTA, 2 ATP, 0.3 GTP; pH 7.3, 270 mOsm. Cells were voltage clamped at -60 mV. Initially, infected cells were identified under fluorescence. Dopamine neurons in the SNc were identified by pacemaker (1–5 Hz) firing with spikes exhibiting AP widths of 1.2 ms (measured in cell-attached voltage clamp mode from the initial inward current to the peak of the outward current) and the presence of a large hyperpolarization-activated current (I_h). Dopamine hydrochloride was ejected as a cation with a single pulse (3 sec, 100 nA) from thin walled iontophoretic electrodes with an Axoclamp-2 amplifier (Axon Instruments, Foster City, CA). Electrophysiological data were acquired using Axograph X (Axograph Scientific). For recordings of dopamine synaptic currents, dopamine release was evoked with a train of 5 stimuli (0.5ms, 40 Hz) using an extracellular mono-polar saline-filled glass electrode. The D2 receptor inhibitory postsynaptic current (IPSC) was isolated pharmacologically using an external solution containing picrotoxin (100 μ M), DNQX (10 μ M), CGP 55845 (200 nM) and MK-801 (10 μ M).

Statistical Analyses

Behavioral data were analyzed with STATISTICA (StatSoft, Tulsa, OK, USA) software. Drug response scores were analyzed by repeated measures ANOVA, with genotype, infusion type (active or control), and sex as possible grouping factors and time as the repeated measure. Simple main effects analysis was used to identify the source of significant two-way interactions, and post-hoc mean comparisons were performed using the more conservative Tukey HSD test to account for multiple comparisons. Differences were considered to be significant at $P < 0.05$.

RESULTS

D2 receptor expression

Transgene expression driven by the native HSV IE 4/5 promoter is typically rapid and transient, peaking within 2–5 days after infection (Neve et al., 2005). To confirm rapid expression of both D2_L and D2_S in the nucleus accumbens, *Drd2*^{-/-} mice were euthanized 2, 4, or 6 days after receiving intracerebral injections of HSV-D2_S or HSV-D2_L, and D2 receptor expression was evaluated by immunohistochemistry. Both receptor isoforms were expressed at modest levels by day 2, with more robust expression by day 4 (Fig. 1). Expression was typically centered in the nucleus accumbens core, extending also into the shell. Expression appeared to wane by 6 days, particularly within the nucleus accumbens, although some immunoreactivity could still be detected above the nucleus accumbens. We chose the nucleus accumbens as the site of injection to assess postsynaptic receptor function because of the importance of this brain region for the effects of psychostimulant drugs (Di Chiara and Imperato, 1988).

Locomotor activity

In this initial study, sex was not included as a factor in the analysis because the number per sex was too small for reliable analysis (< 4 per sex, genotype, and treatment group); final group size was 8–10 per genotype and treatment. Both baseline activity and

methamphetamine-induced locomotor activation were greatly reduced in *Drd2*^{-/-} mice compared to wild type littermates (Fig. 2). For baseline activity (Fig. 2A), there was a significant genotype (wild type vs. *Drd2*^{-/-}) × treatment (± D2_L infusion) × time interaction ($F[11,341]=2.0$, $P < 0.05$) that appeared to be associated with a somewhat larger difference between *Drd2*^{-/-} and wild type mice (3-fold) that received the control virus (LacZ) expressing β-galactosidase than mice that were HSV-D2_L treated (1.8-fold). However, further analysis indicated that locomotor responses across time were similar for all groups and that the activity difference between *Drd2*^{-/-} and wild type mice persisted for both HSV-LacZ ($P < 0.001$) and HSV-D2_L treated ($P < 0.001$) groups. Therefore, the reduced baseline activity in *Drd2*^{-/-} mice was not restored by bilateral infusion of HSV-D2_L into the nucleus accumbens.

Bilateral infusion of HSV-D2_L into the nucleus accumbens of *Drd2*^{-/-} mice, however, significantly restored methamphetamine-induced activation, compared to mice that received the control virus (Fig. 2B). Data for one *Drd2*^{-/-} control mouse were removed based on an activation score that was > 2 SD higher than the mean of that treatment group. The genotype × treatment × time interaction was significant ($F[11,330]=1.9$; $P < 0.05$). Simple main effect analysis identified a significant time × treatment interaction for the control virus and HSV-D2_L treated KO mice ($P < 0.01$). Post-hoc tests confirmed significantly greater activity levels in D2_L-treated KO mice compared to LacZ controls at 20 min and all subsequent time points. There was no significant time × treatment interaction for the control virus and HSV-D2_L treated wild type mice; importantly, infusion of D2_L into wild type mice had little effect on baseline or methamphetamine-induced locomotor activation compared to mice receiving control virus.

Additional mice received intra-accumbal infusions of HSV-D2_L or HSV-D2_S. This experiment used a version of the HSV vector in which GFP is co-expressed with the D2 receptor; the control virus expresses only GFP. Group size per genotype, treatment and sex was 7–9; therefore, sex was included in the analysis. There were no significant effects of sex. Again we observed that *Drd2*^{-/-} mice receiving HSV-GFP had reduced baseline and methamphetamine-induced activity compared to wild type littermates that received the control virus (Fig. 3). D2_S and D2_L were equally capable of restoring sensitivity to methamphetamine-induced activation (Fig. 3B). Baseline (saline-induced) activity, on the other hand, was considerably less in *Drd2*^{-/-} mice than in wild type mice ($F[3,61]=35.3$, $P < 0.001$ for the main effect of group), regardless of whether the mice received HSV-D2 or HSV-GFP (Fig. 3A). Post-hoc comparisons indicated that wild type mice were more active than all groups of KO mice, but the KO groups were not different from each other, indicating that D2 receptor restoration in the nucleus accumbens had little effect on the reduced spontaneous activity of *Drd2*^{-/-} mice, at least during the light part of the diurnal cycle. Level of activity changed similarly across time ($F[11,671]=59.0$, $P < 0.001$ for the main effect of time); there was no interaction of time with group. For methamphetamine-induced activation, there was a significant group × time interaction ($F[33,660]=9.7$, $P < 0.001$). Simple main effect analyses identified significant group differences at all time points except the first 5 minutes. Post-hoc tests confirmed significantly greater activity levels in D2_L- and D2_S-treated KO mice, compared to GFP control KO mice, at most subsequent

time points (Fig. 3B). The tendency for *Drd2*^{-/-} mice receiving HSV-D2_L or HSV-D2_S to show higher methamphetamine-induced stimulation than wild type mice at early time points (Fig. 3B) may have been due to the difference in baseline activity level in *Drd2*^{-/-} vs. wild type mice.

Distance and time spent in center

We separately analyzed the data for time spent and distance traveled in the center and at the margins of the activity chambers. The margin distance measurements looked very much like the overall distance measurements described above. We confirmed that methamphetamine increased distance traveled at the margins with a corresponding decrease in distance traveled in the center in wild type mice, consistent with anxiogenic effects of amphetamines (e.g., Pogorelov et al., 2007). We also observed that methamphetamine had little effect on distance in *Drd2*^{-/-} mice, that basal distance traveled at the margins was greater for wild type mice than for *Drd2*^{-/-} mice, and that intra-accumbal infusion of HSV-D2_L or HSV-D2_S restored methamphetamine-increased margin distance in *Drd2*^{-/-} mice, but did not restore their reduced basal margin distance (data not shown).

Analysis of methamphetamine effects on time spent in the center, on the other hand, identified a group $\times$ sex interaction ($F[3,57]=5.3$, $P < 0.005$). Separate analyses identified group differences for each sex ($F[3,27]=6.1$, $P < 0.01$ and $F[3,30]=4.3$, $P < 0.02$ for males and females, respectively), and showed that a deficit in sensitivity to methamphetamine-induced decreased time spent in the center in *Drd2*^{-/-} mice was restored by intra-accumbal infusion of HSV-D2_L or HSV-D2_S in female (Fig. 4A), but not, male (Fig 4B), mice.

D2 autoreceptor activity

To determine if both D2_S and D2_L could function as autoreceptors, virus-mediated expression was evaluated in dopaminergic neurons of the SNc. We chose SNc neurons for this investigation because they have an established role in locomotion, and are more homogeneous than ventral tegmental neurons in their synaptic inputs and firing properties (Beckstead and Phillips, 2009). Although HSV produced GFP expression in dopaminergic neurons of wild type mice, we were unable to identify GFP-labeled dopaminergic neurons in the SNc or ventral tegmental area of *Drd2*^{-/-} mice that received midbrain injections of HSV-D2_L or HSV-D2_S (results not shown). For this reason an adeno-associated virus (AAV) vector was used as an alternative approach to express D2_S and D2_L in dopaminergic neurons of the SNc of *Drd2*^{-/-} mice.

Infected neurons were identified in mid-brain slices by co-expression of GFP. SNc dopamine neurons were confirmed by the presence of a large hyperpolarization-activated current (I_h) and by pacemaker firing (1–5 Hz). Iontophoretic application of a saturating concentration of dopamine (3 sec, 100 nA) evoked large, rapidly activating, outward currents in AAV-infected SNc dopamine neurons from *Drd2*^{-/-} mice receiving either AAV-D2_S or AAV-D2_L (Fig. 5), indicating that both forms of the receptor functionally couple to GIRK channels. D2 receptor function was further assessed by the bath application of a sub-saturating concentration of the D2 agonist quinpirole (100 nM). Current amplitudes for quinpirole were normalized to the amplitude of GABA_B receptor-mediated GIRK currents

induced by baclofen (10 μ M). Currents induced by quinpirole and baclofen were reversed by the D2 antagonist sulpiride (200 nM) and by the GABA_B antagonist CGP55845 (200 nM), respectively. Current amplitudes mediated by D2_S and D2_L were similar in terms of absolute value and as a percentage of the current induced by baclofen (Fig. 4; Table 1). Application of dopamine or quinpirole failed to evoke any current in recordings from fluorescent cells in *Drd2*^{-/-} animals that received AAV expressing only GFP, although the dopamine neurons continued to exhibit baclofen-activated currents.

In the midbrain, the dendritic release of dopamine causes a D2-receptor mediated IPSC on adjacent dopamine neurons (Beckstead et al., 2004; Ford et al., 2006; Ford et al., 2009). To determine whether virus-mediated expression of D2_L and D2_S resulted in delivery of functional D2 receptors to synaptic sites, IPSCs were examined in *Drd2*^{-/-} mice receiving either AAV-D2_S or AAV-D2_L. In both sets of animals a train of stimuli evoked D2 receptor-mediated IPSCs. The amplitude and time to peak of the IPSCs from both groups were similar and were similar to previous recordings made from wild type animals (Table 2).

DISCUSSION

Studies comparing the function of recombinant D2_S and D2_L expressed in mammalian cell lines have reported qualitative and quantitative differences in signaling and coupling to G proteins, but with little consistency in the results among different cell lines or laboratories (Neve et al., 2004). In contrast, two independently derived lines of mice in which D2_L was selectively ablated have been used in studies that, with remarkable consistency, demonstrate that responses thought to be mediated by postsynaptic D2 receptors on striatal medium spiny neurons are lost in the *Drd2*^{ex6-/ex6-} mice, whereas D2 autoreceptor-mediated responses are spared (Usiello et al., 2000; Wang et al., 2000). Behaviorally, *Drd2*^{ex6-/ex6-} mice are deficient in haloperidol-induced catalepsy, locomotor activation induced by mixed D1/D2 agonists, and spontaneous locomotion and rearing. *Drd2*^{ex6-/ex6-} mice are also deficient in inhibition of the phosphorylation of DARPP-32 (Lindgren et al., 2003) and the protein kinase Akt (Beaulieu et al., 2007) in the neostriatum and the ability of haloperidol to promote corticostriatal long-term potentiation (Centonze et al., 2004). Importantly, these deficits are observed even though the mice express D2_S at levels normally observed for combined expression of D2_S and D2_L, so the deficits cannot be attributed simply to a lower level of D2 receptor expression (Usiello et al., 2000; Wang et al., 2000). D2 receptor-mediated responses that are spared in *Drd2*^{ex6-/ex6-} mice include inhibition of the cyclic AMP-dependent phosphorylation and activation of tyrosine hydroxylase, quinpirole-induced inhibition of locomotor activity, inhibition of the firing rate of SNc neurons, and inhibition of neostriatal dopamine release (Lindgren et al., 2003; Usiello et al., 2000; Wang et al., 2000), all of which are autoreceptor-mediated responses. Both D2_S and D2_L are capable of regulating GABAergic inhibition of striatal interneurons (Centonze et al., 2003).

These data indicate that D2_L on striatal medium spiny neurons serves as the postsynaptic receptor mediating the functions described above, and that D2_S, even when overexpressed in those neurons, does not. These results also demonstrate that D2_S can function as a dopamine autoreceptor but do not address the possibility that D2_L may also be an autoreceptor; nevertheless, it has become common to refer to D2_S as the dopamine autoreceptor. The only

data to suggest that D2_S actually is the autoreceptor are from studies reporting that, in the rhesus monkey brain, dopamine neurons express mainly D2_S immunoreactivity (Khan et al., 1998) and that D2_S mRNA is more abundant than D2_L mRNA in the murine brainstem (Montmayeur et al., 1991). On the other hand, Jang et al. (2011) used single-cell reverse transcription-PCR to determine that D2_L is expressed in more dopamine neurons of the rat substantia nigra than D2_S, and that either receptor variant can mediate inhibition of cell firing by quinpirole, and our analysis of mRNA expression in the rat midbrain found that D2_L was modestly more abundant than D2_S (Neve et al., 1991).

In the present studies, we confirmed that *Drd2*^{-/-} mice show reduced basal activity (Baik et al., 1995; Kelly et al., 1998) and are deficient in methamphetamine-induced locomotion (Kelly et al., 2008). HSV-mediated expression of the D2 receptor in the nucleus accumbens of *Drd2*^{-/-} mice restored methamphetamine-induced locomotion, but not basal locomotor activity in the activity chamber. The lack of effect on baseline (saline-induced) activity might indicate that this behavior requires D2 receptor expression elsewhere in the brain, such as in the neostriatum, or requires a different level of receptor expression. D2_S and D2_L were indistinguishable with regard to restoring methamphetamine-induced locomotion. Methamphetamine also enhanced thigmotaxis in wild type mice, as indicated by decreased time in the center of the activity chambers, but not in *Drd2*^{-/-} mice; methamphetamine-enhanced thigmotaxis was restored by D2_S or D2_L expression in the nucleus accumbens of female, but not male, *Drd2*^{-/-} mice. Male mice have been reported to be more sensitive than female mice to the anxiogenic effect of amphetamine (Pogorelov et al., 2007), a difference that seems to be reflected in the results observed for methamphetamine in the present study. It may be that receptor expression is more effective at normalizing the response in the female mice where the genotypic difference was less extreme than in the male mice where there was a greater difference between wild type and *Drd2*^{-/-} mice.

Indirect dopamine receptor agonists such as methamphetamine activate all subtypes of dopamine receptors as a result of enhanced release of dopamine, with activation of both D1 and D2 receptors required for full stimulation of locomotor activity (Marshall et al., 1997). These data demonstrating that either subtype of the D2 receptor can meet the requirement for D2 receptor activation appear to contradict the finding that expression of the D2_S subtype alone in *Drd2*^{ex6-/ex6-} mice is not sufficient for locomotor activation by D1/D2 agonists (Usiello et al., 2000; Wang et al., 2000). There are many respects in which the present model differs from the *Drd2*^{ex6-/ex6-} model, but it is not obvious how any of the differences would explain the discrepant results.

Prior studies addressed the relative ability of the D2 receptor splice variants to serve as autoreceptors by expressing recombinant D2_S and D2_L in cultures of rat mesencephalic neurons (Jomphe et al., 2006) and by functional analysis of D2 receptors in acutely isolated dopamine neurons that had been characterized for expression of D2_S and D2_L (Jang et al., 2011). Results from both studies suggest that either variant, if expressed in dopamine neurons, can inhibit cell firing and dopamine release. We addressed this question by AAV-mediated expression of D2_S and D2_L in the ventral midbrain of *Drd2*^{-/-} mice, followed by electrophysiological analysis of D2 receptor-activated GIRK currents in identified dopamine neurons in midbrain slices prepared from the mice. Whereas dopamine neurons from

Drd2^{-/-} mice infected with AAV-GFP had no response to iontophoretically applied dopamine or bath-applied quinpirole, GFP-expressing dopamine neurons from mice infected with either AAV-D2_S or AAV-D2_L exhibited robust D2 receptor-mediated activation of GIRK currents. Furthermore, inhibitory postsynaptic currents elicited by a brief train of stimuli, reflecting the release of dopamine from terminals in the midbrain, were observed in mice expressing either D2_S or D2_L, demonstrating that both D2 receptor variants traffic to postsynaptic sites in dopamine neurons. Although our approach characterized autoreceptors in only the somatodendritic compartment, and autoreceptors located on dopamine neuron axons in the forebrain might have very different trafficking or signaling properties (Fulton et al., 2011), the preponderance of evidence supports the conclusion that both D2_S and D2_L are expressed in dopamine neurons in the rodent substantia nigra, and that both receptor variants can function as autoreceptors.

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Abbreviations

AAV	adeno-associated virus
DARPP-32	Dopamine- and cyclic AMP-regulated phosphoprotein of about 32 kD
<i>Drd2</i> ^{-/-}	D2 receptor null mutant <i>Drd2</i> ^{tm1Low}
<i>Drd2</i> ^{ex6/ex6-}	D2 _L receptor null mutant mouse
GFP	green fluorescent protein
GIRK	G protein-regulated inward rectifying potassium channel
HSV	herpes simplex virus
IPSC	inhibitory postsynaptic current, SNc, substantia nigra, pars compacta

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Highlights

- We compare the function of splice variants of the dopamine D2 receptor D2s and D2L.
- We used virus-mediated expression of the receptors in D2 receptor null mutant mice.
- Restoring D2 receptors (D2R) in the accumbens restored response to methamphetamine.
- Restoring D2R in the substantia nigra restored autoreceptor regulation of GIRK channels.
- D2 receptor splice variants were indistinguishable in their ability to restore function.

Fig. 1.

D2 receptor immunoreactivity. Representative images are shown of D2 receptor immunoreactivity in the nucleus accumbens of *Drd2*^{-/-} mice that received intra-accumbal injections of HSV-D2_S or HSV-D2_L and were euthanized 2, 4, or 6 days later. AC, anterior part of the anterior commissure.

Fig. 2.

Locomotor activity, experiment 1. *Drd2*^{-/-} (KO) mice or wild type littermates (WT) received bilateral injections of HSV-D2_L (D2L) or control virus expressing β -galactosidase (LacZ) into the nucleus accumbens. Beginning two days later, all mice were placed in the activity chambers after saline injections on days 1 and 2, followed by methamphetamine (2 mg/kg, i.p.) on day 3. The graphs depict mean $\pm$ SEM distance traveled (cm) vs. time after injection of saline (day 2) or methamphetamine corrected for saline baseline (day 3 minus day 2). (A) Activity levels for mice placed in the activity chambers for the second time after receiving a saline injection (day 2). *Drd2*^{-/-} mice had significantly less baseline activity than wild type (C57BL/6) mice, whether receiving control virus or HSV-D2_L. Specific mean differences are not indicated because there was no significant genotype $\times$ time or treatment $\times$ time interaction. (B) Methamphetamine-stimulated activity. Expression of D2_L in the nucleus accumbens of *Drd2*^{-/-} mice increased methamphetamine-stimulated locomotor activity compared to *Drd2*^{-/-} mice that received the negative control HSV-*LacZ*. In wild type littermates, HSV-D2_L had no effect compared to *LacZ*. * $P < 0.05$, ** $P < 0.01$ compared to *Drd2*^{-/-} mice receiving HSV-*LacZ*, $n = 7-10$ per group.

Fig. 3.

Locomotor activity, experiment 2. *Drd2*^{-/-} (KO) mice received bilateral infusions of HSV-D2_L (D2L), HSV-D2_S (D2S) or control virus expressing GFP into the nucleus accumbens; wild type littermates (WT) received control virus. Beginning two days later, all mice were placed in the activity chambers after saline injections on days 1 and 2, followed by methamphetamine (2 mg/kg, i.p.) on day 3. The figures depict mean ± SEM distance traveled (cm) vs. time after injection of saline (day 2) or methamphetamine corrected for saline baseline (day 3 minus day 2). (A) Activity levels for mice placed in the activity chambers for the second time after receiving a saline injection (day 2). *Drd2*^{-/-} mice had significantly less baseline activity than wild type (C57BL/6) mice, whether receiving control virus, HSV-D2L, or HSV-D2S. Specific mean differences are not indicated because there was no significant group × time interaction. (B) Methamphetamine-stimulated activity. Expression of D2_L or D2_S in the nucleus accumbens of *Drd2*^{-/-} mice increased methamphetamine-stimulated locomotor activity compared to *Drd2*^{-/-} mice that received the negative control HSV-GFP to a level statistically indistinguishable from wild type mice. The upper row of asterisks denotes the level of significance for *Drd2*^{-/-} mice receiving HSV-D2_S compared *Drd2*^{-/-} mice receiving control virus, and the lower level of asterisks denotes the same for mice receiving HSV-D2_L. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *n* = 15–17 per group.

Fig. 4.

Time in center of activity chamber. Locomotor activity was measured as described in figures 2 and 3, and data are shown for the methamphetamine-induced change in time spent in the center of the activity chamber, calculated as the amount of time in center after methamphetamine (day 3) minus the amount of time in center after saline (day 2). A) Methamphetamine caused female wild type (WT) mice to spend less time in the center, an effect absent in *Drd2*^{-/-} (KO) mice that was restored by expression of either D2_S or D2_L in the nucleus accumbens. B) Methamphetamine caused male wild type (WT) mice to spend less time in the center, an effect absent in *Drd2*^{-/-} (KO) mice that was not restored by expression of either D2_S or D2_L in the nucleus accumbens.

Fig. 5.

D2 receptor regulation of GIRK currents. Representative traces are shown for D2 receptor-regulated GIRK currents examined in the substantia nigra pars compacta 2–3 weeks after injection of AAV-D2_S (panel A), AAV-D2_L (panel B), or control virus (GFP; panel C) into the ventral midbrain of *Drd2*^{-/-} mice. AAV-infected neurons were identified in mid-brain slices by expression of GFP. In all panels, DA indicates iontophoretic application of dopamine (3 sec, 100 nA), whereas the GABA_B agonist baclofen (10 μM) and antagonist CGP55845 (200 nM) and the D2 agonist quinpirole (Quin, 100 nM) and antagonist sulpiride (100 nM) were all applied in the bath. Results from all cells are compiled in Table 1.

Table 1

Iontophoretic application of dopamine (DA; 3 sec, 100 nA) or bath application of quinpirole (quin, 100 nM) evoked large outward currents in AAV-infected SNc dopamine neurons from *Drd2*^{-/-} mice receiving either AAV-D2S or AAV-D2L, but not from cells expressing only GFP. Current amplitudes for quinpirole were also normalized to the amplitude of GABA_B receptor-mediated GIRK currents induced by baclofen (10 μ M; % baclofen).

Transgene	Cells/Mice	Current Amplitude (DA, pA)	Current Amplitude (Quin, pA)	Quin Amplitude (% baclofen)	Current Amplitude (Ih, pA)
D2 _S	9–15/5	315 $\pm$ 27	188 $\pm$ 18	110 $\pm$ 15	434 $\pm$ 49
D2 _L	10–14/4–5	307 $\pm$ 33	220 $\pm$ 19	104 $\pm$ 10	583 $\pm$ 49
GFP only	5–8/2–3	–6 $\pm$ 2	0 $\pm$ 1	N/A	673 $\pm$ 50

Table 2

Characteristics of D2 receptor-mediated IPSCs in dopamine neurons after viral expression of D2_S or D2_L in the substantia nigra of *Drd2*^{-/-} mice. IPSCs were elicited with a train of 5 stimuli (0.5ms, 40 Hz), and were confirmed to be mediated by D2-like receptors using 200 nM sulpiride (data not shown).

Transgene	Cells/Mice	Current Amplitude (pA)	Time to peak (ms)	Half-width time (ms)
D2 _S	10/4	103 ± 15	409 ± 23	595 ± 59
D2 _L	12/5	99 ± 14	370 ± 10	594 ± 53