Supersensitive presynaptic dopamine D2 receptor inhibition of the striatopallidal projection in nigrostriatal dopamine-deficient mice

Wei Wei¹,², Li Li³, Guoliang Yu¹, Shengyuan Ding¹, Chengyao Li²*, Fu-Ming Zhou¹

¹Department of Pharmacology, College of Medicine, University of Tennessee Health Science Center, Memphis, Tennessee 38163 USA; ²School of Biotechnology, Southern Medical University, Guangzhou, China

Running Head: Supersensitive D2 receptors on striatopallidal terminals

Corresponding author: Fu-Ming Zhou, Department of Pharmacology, University of Tennessee College of Medicine, Memphis, TN 38163
Email: fzhou3@uthsc.edu
Phone: 901-448-1779
*, equal corresponding author

Abstract 190 words
Introduction 591 words
Discussion 2091 words
Figures 9
Table 1

Keywords: basal ganglia, dopamine receptor, globus pallidus, presynaptic inhibition, Parkinson’s disease

Conflict of interest: The authors declare no conflict of interest.

Acknowledgements
This work was supported by NIH grants R01NS058850 and R03NS085380 to FMZ. The authors thank Drs. Robert Foehring and Doug Guan for help with measuring liquid junction potentials.

Copyright © 2013 by the American Physiological Society.
Abstract
The dopamine (DA) D2 receptor (D2R)-expressing medium spiny neurons (D2-MSN) in the striatum project to and inhibit the GABAergic neurons in the globus pallidus (GP), forming an important link in the indirect pathway of the basal ganglia movement control circuit. These striatopallidal axon terminals express presynaptic D2Rs that inhibit GABA release and thus regulate basal ganglia function. Here we show that in transcription factor Pitx3 gene mutant mice with a severe DA loss in the dorsal striatum mimicking the DA denervation in Parkinson’s disease (PD), the striatopallidal GABAergic synaptic transmission displayed a heightened sensitivity to presynaptic D2R-mediated inhibition with the dose-response curve shifted to the left, although the maximal inhibition was not changed. Functionally, low concentrations of DA were able to more efficaciously reduce the striatopallidal inhibition-induced pauses of GP neuron activity in DA-deficient Pitx3 mutant mice than in WT mice. These results demonstrate that presynaptic D2R inhibition of the striatopallidal synapse become supersensitized following DA loss. These supersensitive D2Rs may compensate for the lost DA in PD and also induce a strong disinhibition of GP neuron activity that may contribute to the motor-stimulating effects of dopaminergic treatments in PD.
Introduction

The striatopallidal projection is being increasingly appreciated as an important component of the basal ganglia motor control circuit in health and disease (Kravitz et al. 2010; Kita and Kita 2011a; Nambu et al. 2011). Studies have established that the GABAergic, phasically active, medium spiny neurons (MSNs) expressing a high level of D2Rs project to and inhibit the globus pallidus (GP) in rodents or the external segment of the globus pallidus (GPe) in primates (Gerfen and Bolam 2010; Sano et al. 2012). Most GP neurons are GABAergic projection neurons and fire spikes spontaneously (Soares et al. 2004; Starr et al. 2005; Kita 2010; Benhamou et al. 2012). Classic and recent studies indicate that the activity of pallidal neurons is critical to the function and dysfunction of the basal ganglia (DeLong 1990; Raz et al. 2000; Bateup et al. 2010; Kravitz et al. 2010). In PD, GPe neuron activity may be decreased with increased burstiness (Kita and Kita 2011a,b; Soares et al. 2004; Wichmann and Dostrovsky 2011). Anatomical studies have established that D2R-MSNs innervate GP neurons (Bolam et al. 2000; Levesque and Parent 2005; Gerfen and Bolam 2010; Kita 2010; Fujiyama et al. 2011). Ultrastructural studies have demonstrated that striatopallidal axon terminals express D2Rs (Levey et al. 1993; Yung et al. 1995; Smith and Villalba 2008). Functionally, these presynaptic D2Rs inhibit GABA release from striatopallidal axon terminals (Cooper and Stanford 2001; Watanabe et al. 2009; Chuhma et al. 2011).

Matching the strong D2R expression, the striatum receives an intense DA innervation originating in the DA neurons in the substantia nigra (Björklund and Lindvall 1984; Matsuda et al. 2009). The GPe (in primates) or GP (in rodents) also receives a DA innervation from axon collaterals of the nigrostriatal DA projection and also dedicated nigropallidal DA neurons, although the pallidal DA innervation is much smaller than that in the striatum (Hedreen 1999; Jan et al. 2000; Smith and Kieval 2000). In postmortem late stage PD brains, DA loss was up to 80% in GPe (Hornykiewicz 2001; Rajput et al. 2008). In comparison, the DA loss was 98% in the putamen. Severe DA loss can induce upregulated or supersensitive molecular and behavioral responses upon reintroduction of DA agonists (Ungerstedt 1971; Creese et al. 1977; Staunton et al. 1982; Schwarting and Huston 1996). A prominent receptor that is upregulated by DA loss in PD is the DA D2R (Hornykiewicz 2001). Studies indicate that the upregulation may result from an increased D2R expression and also an increased functionality such as higher D2R binding affinity and more efficient receptor-G protein coupling and downstream signaling (Qin et al. 1994; Kostrzewa 1995; Geurts et al. 1999; Cai et al. 2002; Betarbet and Greenamyre 2004; Seeman et al. 2005a). Supersensitive DA receptors may compensate for the lost DA (Bezard et al. 2010) and may be even the primary mediators of the therapeutic effects of
dopaminergic treatments for PD (Seeman et al. 2005b; Seeman 2007). Despite the clear importance, the potential D2R supersensitivity at the striatopallidal axon terminals has not been studied and the functional consequences also remain unknown. We hypothesize that presynaptic D2Rs at the striatopallidal axon terminals are supersensitive following DA depletion, and during dopaminergic treatment, these supersensitive presynaptic D2Rs may disinhibit GP neurons more potently in DA-deficient mice than in normal mice. We have tested these ideas in transcription factor Pitx3 mutant mice that have a severe and consistent nigral DA neuron loss (Nunes et al. 2003; van den Munckhof et al. 2003; Li et al. 2013; Li and Zhou 2013), a feature that is helpful to our experimental questions.

Materials and Methods

Animals. Two breeding pairs of heterozygous Pitx3+/- mice were purchased from the Jackson Laboratory (Bar Harbor, Maine), resulting in a small colony of homozygous Pitx3-/- (Pitx3Null), heterozygous Pitx3+/-, and wild-type Pitx3+/+ (Pitx3WT) mice (Li et al. 2013; Li and Zhou 2013). Pitx3Null mice are aphakic and thus clearly identified (Fig. 1). The genotypes were further determined by PCR-based genotyping to identify WT, homozygotes, and heterozygotes (Li et al. 2013). The genotyping results are identical to our published results (Li et al. 2013). Since Pitx3 gene is recessive, the nigrostriatal dopamine system is normal in heterozygous Pitx3+/- mice (Hwang et al., 2003; Nunes et al. 2003; van den Munckhof et al. 2003). To avoid any potential ambiguity on data interpretation and also to avoid genotyping, heterozygous Pitx3+/- mice were not used; only Pitx3WT mice (produced by mating Pitx3WT mice) and Pitx3Null mice (produced by mating Pitx3Null mice) were used in our experiments. Mice had free access to food and water. All procedures were approved by The Institutional Animal Care and Use Committee of The University of Tennessee Health Science Center in Memphis, Tennessee.

Reserpine treatment. Pitx3WT mice of postnatal 18 days of age (PN18) received injections of reserpine, following published protocols (Trugman and James 1992; Taverna et al. 2008; Day et al. 2006; Ding et al. 2006; Kreitzer and Malenka 2007). Reserpine (5 mg) was first dissolved in glacial acetic acid (0.2 mL) and then mixed with a 1.8 mL mix of 2 parts (in volume) of polyethylene glycol (PEG)-400 and 1 part of Tween-20 (i.e. 2 mL vehicle). The reserpine dose was 2.5 mg/Kg injected in 0.01 ml once per day in the morning about 10 AM. The mice started to display severe akinesia after the third reserpine injection. Two hours after the 4th reserpine injection, the mice were sacrificed for in vitro electrophysiological experiments.
**Brain slice preparation.** Parasagittal slices were prepared following the methods described in the literature (Beurrier et al. 2006; Ding et al. 2013). Mice of postnatal days 20–24 were used in our experiments because brain slices from these mice at this age were highly viable and no age-dependent changes in cellular properties during these days were detected. These mice were killed by decapitation, and brains were dissected out quickly and immediately immersed in an oxygenated ice-cold cutting solution (in mM: 220 glycerol, 2.5 KCl, 1.25 NaH$_2$PO$_4$, 25 NaHCO$_3$, 0.5 CaCl$_2$, 7 MgCl$_2$, 20 D-glucose) for 2 min. Three hundred (300) μm-thick, 15° angular parasagittal slices were cut using a Leica Zero Z VT1200S vibratome (Leica Microsystems, Wetzlar, Germany). The parasagittal slices were transferred to a standard extracellular bathing solution (in mM: 125 NaCl, 2.5 KCl, 25 NaHCO$_3$, 1.25 NaH$_2$PO$_4$, 2.5 CaCl$_2$, 1.3 MgCl$_2$, and 10 D-glucose) that was continuously bubbled with 95% O$_2$ and 5% CO$_2$ for 30 min at 34 °C in a holding chamber. After that, the holding chamber with the brain slices were kept at room temperature (25 °C) for at least 30 min. Brain slices were used 1-6 hours after being prepared. Ascorbic acid (vitamin C, 0.4 mM) is added to all solutions including the cutting solution to protect the tissue and DA and be consistent (Rice 1999). Fresh DA was added to the perfusing solution at desired concentrations immediately before use, and this DA-containing solution was used only for one DA application that lasted for 10 min.

**Synaptic stimulation.** Conventional electrical stimulation was used to evoke IPSCs or IPSPs. A bipolar tungsten stimulating electrode (World Precision Instruments) was placed in the dorsal striatum. Stimulating pulses were controlled by a pulse generator (Master-8, AMPI) and delivered via a stimulus isolator (A365, World Precision Instruments). A single stimulating pulse or a pair of pulses was delivered every 20 seconds to evoke IPSCs or IPSPs. The stimulation intensity ranged 20 to 100 μA with a constant duration of 0.2 ms, and was adjusted to evoke roughly 70% of the maximal response.

**Electrophysiology.** Slices were placed in a recording chamber and continuously perfused at 2 ml/min with the normal extracellular bathing solution saturated with 95% O$_2$ and 5% CO$_2$. Recordings were made under visual guidance of a video microscope (Olympus BX51WI and Zeiss Axiocam MRm digital camera) equipped with Nomarski optics and a 60X water immersion lens. A Multiclamp 700B amplifier, pClamp 9.2 software and Digidata 1322A interface (Molecular Devices, Sunnyvale, CA) were used to acquire data. Patch pipettes were pulled from borosilicate glass capillary tubing (KG-33, 1.1 mm i.d., 1.65 mm o.d., King Precision Glass, Claremont, CA) using a PC-10 puller (Narishige, Tokyo, Japan) and had resistances of about 2
MΩ when filled with one of the following intracellular solutions. A low Cl⁻ intracellular solution (in mM: 135 KSO₃CH₄, 5 KCl, 0.5 EGTA, 10 HEPES, 2 Mg-ATP, 0.2 Na-GTP, and 4 Na₂-phosphocreatine, pH 7.25, 280-290 mOsm) was used for recording hyperpolarizing GABA_A inhibitory postsynaptic potentials (IPSPs) and spiking activity. A high Cl⁻ intracellular solution (in mM: 135 KCl, 0.5 EGTA, 10 HEPES, 2 Mg-ATP, 0.2 Na-GTP, and 4 Na₂-phosphocreatine, pH 7.25, 280-290 mOsm) was used for recording inward GABA_A inhibitory postsynaptic currents (IPSCs) at –70 mV. Series resistance was monitored by 10 mV, 50 ms pulses. The series resistance was 4-8 MΩ among different cells and the cells were discarded when series resistance increase was >15%. The 8 mV liquid junction potential detected for the KSO₃CH₄-based intracellular solution used in the current clamp experiments was subtracted from the membrane potential values presented in this report, whereas the 4 mV liquid junction potential for the KCl-based intracellular solution used in the voltage clamp experiments was not corrected because its impact on the IPSCs at –70 mV was minimal. Signals were filtered at 10 kHz using the built-in 4-pole low-pass Bessel filter in the patch clamp amplifier and digitized at 20 KHz. All recordings were made at 30-32 °C. At least 5 mice were used to obtain an averaged electrophysiological data point with each mouse yielding 1 or 2 useful cells, depending on how difficulty the experiment was.

Immunohistochemistry

Conventional immunofluorescence methods were used to detect DA neurons and their axons according to our published procedures (Zhou et al. 2009; Li et al. 2013; Li and Zhou 2013). The brains were fixed in 4% paraformaldehyde dissolved in a phosphate buffer at 4°C overnight and then sectioned on a vibratome. The free-floating sections (50 μm in thickness) were incubated with 2% fat-free milk, 1% bovine serum albumin, and 0.4% Triton X-100 in a phosphate buffered saline (PBS) for 1 hours at room temperature to block nonspecific binding and permeabilize the cell membrane, respectively. After thorough rinsing, the free-floating sections were incubated for 48 hours at 4°C with the primary antibody, a polyclonal tyrosine hydroxylase (TH) antibody raised in rabbit (diluted at 1:1000; Novus Biologicals, Littleton, CO), and then rinsed in the PBS, followed by incubating with a donkey anti-rabbit secondary antibody conjugated with Alexa Fluor 488 (diluted at 1:200; Invitrogen) for 3 hours at room temperature. Fluorescence images were acquired on a Zeiss 710 confocal laser scanning microscope.
High-pressure liquid chromatography (HPLC) quantification of tissue DA contents

Brain slices (0.5 mm in thickness) containing the striatum and GP were cut in the same manner as the brain slices for electrophysiology. Immediately after cutting, an individual brain slice was placed on a block of agar gel partially submerged in the iced-cold cutting solution. Using a glass pipet (outer diameter: 1.0 mm, inner diameter: 0.8 mm), tissue punches were obtained in the dorsal, middle and ventral striatum and the dorsal GP. Tissue punches were collected into 1:50-diluted 70% perchloric acid (final concentration: 0.33 M) and fully homogenized with a tissue tearor. After 3 min centrifugation at 12000g at 4 °C, the supernatant was aspirated and stored at –80 °C. For HPLC analysis, chromatographic separation of DA was achieved by using a 150 × 2 mm ODS C18 column (ESA, Inc., Chelmsford, MA) connected to an ESA 582 HPLC pump. The mobile phase, was perfused at 0.2 ml/m, contained 80 mM, NaH2PO4•H2O, 2.0 mM 1-octanesulfonic acid sodium salt, 100 μl/l triethylamine, 5 nM EDTA and 10% acetonitrile (pH 3.0). The samples (20 μl) were automatically injected onto column by an ESA 542 autosampler, and were analyzed by an ESA Coulochem II 5200A electrochemical detector with an ESA 5041 high-sensitivity analytical cell. Electrochemical detection was performed at a potential of 220 mV with the current gain at 1.0 nA. Serially diluted DA standards containing known quantities were included in each assay. Under these conditions, the limit of detection for DA was 0.5 pg/injection.

Chemicals and drugs

Routine chemicals were purchased from Sigma-Aldrich (St Louis, MO). 6,7-dinitroquinoxaline-2,3-dione (DNQX), 2-amino-5-phosphonopentanoic acid (AP5), picrotoxin, dopamine, reserpine, quinpirole, SKF81297 hydrobromide and sulpiride were purchased from Sigma-Aldrich or Tocris. SKF81297 hydrobromide was also supplied by the Drug Supply Program of the National Institute of Mental Health of the National Institutes of Health.

Statistics

Data were expressed as mean ± SEM. The paired Student’s t test was used to compare changes before and after drug administration within the same group. The unpaired t test, one-way ANOVA with post-hoc Tukey test and two-way ANOVA with post-hoc least significant difference (LSD) test were used to compare the measurements among different groups. p<0.05 was considered statistically significant. Calculations were performed using the IBM SPSS Statistics 21 program.
Results

Characterization of the intrinsic membrane properties of the GP neurons in the DA-deficient Pitx3Null mice

Anatomical studies have established that D2-MSNs in the dorsal striatum project to the dorsal GP (Selemon and Goldman-Rakic 1990; Hedreen and DeLong 1991; Levesque and Parent 2005; Fujiiyama et al. 2011; Nambu 2011). Since our main goal was to determine if the presynaptic D2Rs at the striatopallidal synapse is supersensitive, we chose to use the Pitx3Null mice based on the fact that these mice have a consistent and severe loss in their SNc DA neurons that normally innervate the GP and the dorsal striatum (Fig. 2A-C). Since the DA loss is the severest in the dorsal striatum with about 98% DA lost (Fig. 2C), as reported in published studies (Nunes et al. 2003; van den Munckhof et al. 2003), and DA receptor supersensitization is positively correlated to the severity of DA loss (Schwarting and Huston 1996), we focused on the striatopallidal projection from the dorsal striatum to the dorsal GP. The anatomical location of the dorsal striatum and dorsal GP were reliably identified in our angular sagittal brain slices (Fig. 3A).

We first characterized the intrinsic membrane and action potential properties of dorsal GP neurons (Fig. 3A, B). As illustrated in Fig. 3C-G and Table 1, injection of current pulses revealed that GP neurons in both Pitx3WT and Pitx3Null mice had widely distributed intrinsic and action potential properties, consistent with literature reports on the variation of GP neuron membrane properties (e.g. Günay et al. 2008 and Deister et al. 2013). However, scatter plots of spike duration and input resistance placed our recorded GP neurons into two clusters or groups (Fig. 3C, Table 1). Type A GP neurons had a higher input resistance, a longer membrane time constant and a longer spike duration than type B GP neurons (Fig. 3C, Table 1), consistent with the original characterization of Cooper and Stanford (2000). Equally important, for both cell types, these parameters of intrinsic membrane and spike properties were similar in Pitx3WT mice and Pitx3Null mice (Table 1). Additionally, we also examined the I_h-mediated, hyperpolarization-induced depolarization sag in GP neurons. As illustrated in Fig. 3D-G, the sag was detected in both type A and type B GP neurons in Pitx3WT mice and Pitx3Null mice. At a trough membrane potential about −100 mV, the sag amplitude was larger in type A cells than in type B cells (9 mV vs. 5 mV, p<0.01, unpaired t test; Table 1). Similar values for the sag were obtained in type A cells and type B cells in Pitx3Null mice (Fig. 3D-G, Table 1). These results indicate that loss of the nigral DA system does not alter the intrinsic membrane properties of neurons in the dorsal GP. These results also pave the way for us to study the potential abnormalities in striatopallidal synaptic transmission.
Because our main goal was to determine whether the presynaptic D2Rs are supersensitive and because DA loss is severest in the dorsal striatum in Pitx3Null mice, we next focused on the dorsal striatum-evoked striatopallidal IPSCs in dorsal GP neurons. As indicated in Fig. 3A and B, we placed the stimulating electrode in the dorsal striatum and recorded striatopallidal IPSCs in dorsal GP neurons in the presence of 10 μM DNQX and 20 μM D-AP5 to block ionotropic glutamate receptors. These IPSCs in Pitx3WT and Pitx3Null mice were also blocked by 100 μM picrotoxin, confirming they were GABA<sub>A</sub> receptor-mediated IPSCs. Additionally, under our experimental conditions, the dorsal striatum-evoked IPSCs were facilitating (described below) in the majority of dorsal GP neurons and were thus predominantly striatopallidal IPSCs with minimal contamination from antidromically activated intra-GP recurrent IPSCs (Voorn 2010; Mallet et al. 2012; Miguelez et al. 2012). In about 20% of GP neurons, the striatum-evoked IPSCs were depressing or flat when 2 20-Hz stimuli were used, indicating a significant contamination by antidromically activated intra-GP recurrent IPSCs (Miguelez et al. 2012). These depressing IPSCs were excluded from this report. Under our experimental conditions, the striatopallidal IPSCs were similar in type A and type B GP neurons and thus data were pooled. Under these conditions, we found that the striatopallidal IPSCs had a similar 10-90% rise time (1.2±0.1 ms vs. 1.2±0.1 ms) and decay time (τ = 5.5±0.2 ms vs. τ = 5.4±0.2 ms, p>0.05, unpaired t test) in 20 GP neurons in Pitx3WT mice and in 20 GP neurons in Pitx3Null mice, consistent with the kinetic parameters of the striatopallidal IPSCs in the literature (Sims et al. 2008).

We next examined the release properties of striatopallidal axon terminals by comparing the paired pulse ratios (PPR) of the striatopallidal IPSCs in dorsal GP neurons in Pitx3WT and Pitx3Null mice (Fig. 4A-C). Since MSNs in freely moving WT and Pitx3Null mice may either be silent or fire at low frequencies (~ 1 Hz) or at bursty high frequencies (5-40 Hz) (Miller et al. 2008; Hernandez et al. 2013; unpublished data of Ben Sagot and Fu-Ming Zhou), we used paired pulses of 1, 2, 5, 10, 20 and 40 Hz. We found that the PPR was about 1 for paired pulses separated by 1 or 0.5 s, indicating that low frequency firing of MSNs at 1-2 Hz induces non-facilitating, independent IPSCs in GP neurons in Pitx3WT and Pitx3Null mice. When the interval between the 2 pulses shortened to 200 ms, the paired IPSCs became facilitating with the PPR increasing to about 1.4 in Pitx3WT and Pitx3Null mice (Fig. 4C). The maximal PPR was about 1.7, achieved with paired pulses separated by 50 ms, in Pitx3WT and Pitx3Null mice (Fig. 4A-C). The PPR was about 1.5 for paired pulses separated by 25 ms in Pitx3WT and Pitx3Null mice (Fig. 4A-C). These results indicate that MSN firing at 5-40 Hz, the possible phasic firing...
frequencies for MSNs, may cause intensifying inhibition of GP neuron activity. There was no
difference in the PPRs between Pitx3WT and Pitx3Null mice (Fig. 4C). These results show that
the baseline properties of these striatopallidal axon terminals are similar in Pitx3WT and
Pitx3Null mice, setting the stage for our main question: is the presynaptic DA inhibition of
striatopallidal IPSCs supersensitive?

Low doses of DA induce stronger presynaptic inhibition of striatopallidal IPSCs in DA-
deficient Pitx3Null mice than in Pitx3WT mice

First, we tested the effects of low doses of DA to determine if DA is more efficacious in inhibiting
striatopallidal IPSCs in DA-deficient mice than in Pitx3WT mice. At 0.1 and 0.5 μM, bath applied
DA had no significant effect on striatopallidal IPSCs in 7 GP cells in Pitx3WT mice, but
significantly reduced the amplitude of striatopallidal IPSCs in Pitx3Null mice by 13.4±2.9% (n=7
cells) and 40.9±4.8% (n=7 cells), respectively. At 1 μM, DA reduced the peak amplitude of the
striatopallidal IPSC by 15.2±1.7% (n=7 cells) in Pitx3WT mice and by 63.8±6.8% (n=7 cells) in
Pitx3Null mice (Fig. 5A,D). At 3 μM, DA reduced the striatopallidal IPSC by 33.2±3.4% (n=7
cells) in Pitx3WT mice and 68.3±7.2% (n=7 cells) in Pitx3Null mice. At 5 μM, DA reduced the
striatopallidal IPSC by 48.4±2.3% (n=8 cells) in Pitx3WT mice and 72.2±1.5% (n=7 cells) in
Pitx3Null mice (Fig. 5B,E). However, at 10, 20 and 50 μM, DA had a similar effect, reducing the
peak amplitude of the striatopallidal IPSCs by about 72% in both Pitx3WT and Pitx3Null mice
and indicating that DA had reached a saturating dose at 10 μM (Fig. 5C,F,G). The DA-induced
inhibition was recovered upon washout. Two-way ANOVA indicates a significant difference in
the inhibitory DA effect on the striatopallidal IPSC amplitude in Pitx3Null mice and Pitx3WT mice
(F(1,91)=78.3, p<0.001). Post hoc LSD tests confirm that the DA effect was significantly stronger
in Pitx3Null mice than in Pitx3WT mice (p<0.001 for 1 and 3 μM DA and p<0.05 for 5 μM DA),
but the effect was not significantly different for DA concentrations ≥ 10 μM in the two types of
mice (p>0.01). As illustrated in Fig. 5G, fitting the data points to the Hill equation showed that
while the maximal effect was similar, the IC₅₀ was 3.22 μM for Pitx3WT mice and 0.36 μM for
Pitx3Null mice, indicating a substantial increase in the efficacy of DA or supersensitivity in
mediating inhibition of striatopallidal IPSCs in Pitx3Null mice.

Pitx3Null mice offer a convenient mouse model to study the consequences of DA loss.
However, its early loss of DA is different from the DA denervation in PD. To support our idea
that DA loss is sufficient to induce supersensitive DA inhibition of the striatopallidal IPSCs, we
treated Pitx3WT mice with a reserpine regimen (2.5 mg/Kg, 1 injection/day) that has been
widely used to deplete DA and induce akinesia (Trugman and James 1992; Taverna et al. 2008;
Day et al. 2006; Ding et al. 2006; Kreitzer and Malenka 2007). The mice started to display severe akinesia after 3 days of reserpine treatment. Two hours after the 4th reserpine injection, the mice were sacrificed for in vitro electrophysiological experiments. As shown in Fig. 6, at 1 μM, bath applied DA reduced the peak amplitude of the striatopallidal IPSC by 62.2±5.1% (n=7 cells paired t-test, p<0.01) in reserpine-treated Pitx3WT mice and by only 15.6±1.9% (n=7 cells paired t-test, p<0.01) in vehicle-treated Pitx3WT mice. Unpaired t test indicated that the DA effect was significantly stronger in reserpine-treated Pitx3WT mice than in vehicle-treated Pitx3WT mice (p<0.001). These results indicate that DA depletion is sufficient to induce enhanced or supersensitive DA-mediated inhibition of the striatopallidal IPSCs.

**DA increases paired-pulse ratios of striatopallidal IPSCs in Pitx3WT mice and Pitx3Null mice**

Striatopallidal axon terminals express D2Rs (Levey et al. 1993; Yung et al. 1995; Smith and Villalba 2008), providing a firm anatomical foundation for presynaptic D2R-mediated inhibition. It was also reported, however, that GP neurons may express D2-like receptors such as D4Rs that may reduce IPSCs postsynaptically (Shin et al. 2003), although this postsynaptic D2-like effect was not observed in other studies (Cooper and Stanford 2001; Miguelez et al. 2012). To determine if the DA inhibition of striatopallidal IPSCs described above was presynaptic or postsynaptic in origin, we performed paired-pulse experiments. It is established that presynaptic inhibition of neurotransmitter release may increase the paired pulse ratio (PPR) whereas postsynaptic inhibition or enhancement of GABA_A receptor function generally does not alter the PPR (Thomson 2000, 2003; Zucker and Regehr 2002; Fioravante and Regehr 2011). As shown in Fig. 6, during saturating DA 50 μM application, the PPR of the striatopallidal IPSCs, evoked by a pair of stimuli spaced at 50 ms or 20 Hz, increased from 1.65±0.25 to 3.30±0.39 in 7 GP neurons in Pitx3WT mice and from 1.56±0.15 to 3.03±0.36 in 6 GP neurons in Pitx3Null mice (paired t-test, p<0.05 for both genotypes). These results indicate a presynaptic D2R-mediated reduction in GABA release. To further support this conclusion, we calculated the coefficient of variation (CV) of the evoked striatopallidal IPSCs by dividing the standard deviation by the mean of the 10 or more individual IPSCs, as described in Michaeli and Yaka (2010). Under control condition, the baseline CV was 0.47±0.07 in Pitx3WT mice (n=12) and 0.43±0.05 in Pitx3Null mice (n=12). During 50 μM DA, the CV was 0.89±0.11 in Pitx3WT and 0.90±0.08 in Pitx3Null mice, respectively. The increase in CV was significant for each genotype with p<0.05 (paired t-test), indicating a decreased vesicular release probability during 50 μM DA.
D2R agonist quinpirole mimics DA’s inhibitory effects on the striatopallidal IPSCs in Pitx3WT mice and Pitx3Null mice

Since it is firmly established that striatopallidal MSNs express only or predominantly D2Rs (Gerfen and Surmeier 2011), we did the following 3 experiments to confirm that it was the D2Rs that were mediating the heightened presynaptic DA inhibition of striatopallidal IPSCs in the DA-deficient Pitx3Null mice. First, as shown in Fig. 8A,B, bath application of D2-like receptor agonist quinpirole mimicked the effect of DA. At 1 μM, quinpirole reduced the peak amplitude of the striatopallidal IPSCs by 39.67±3.1% in Pitx3Null mice (n= 9 cells, paired t-test, p<0.01) and by 11.6±1.6% in Pitx3WT mice (n=7 cells, paired t-test, p<0.05). At 10 μM, quinpirole reduced the peak amplitude of the striatopallidal IPSCs by 63.0±3.8% (n=6 cells, paired t-test, p<0.01) in Pitx3Null mice and 56.3±2.2% (n=6 cells, paired t-test, p<0.01) in Pitx3WT mice. The effects of quinpirole were recovered upon washing. Two-way ANOVA indicates a significant difference in the inhibitory quinpirole effect on the striatopallidal IPSC amplitude in Pitx3Null mice and Pitx3WT mice (F (1,24)=36.118, p<0.001). Post hoc LSD tests confirm that the effect of 1 μM quinpirole effect was stronger in Pitx3Null mice than in Pitx3WT mice (p<0.05), but the effect of 10 μM quinpirole was not significantly different in the two types of mice. Second, bath application of 10 μM D1-like agonist SFK81297 had no detectable effect on striatopallidal IPSCs in Pitx3WT mice (6 neurons) or Pitx3Null mice (6 neurons) (Fig. 8D). Third, in the presence of 10 μM sulpiride, a D2-like antagonist, 10 μM DA did not inhibit the striatopallidal IPSCs in 5 cells in WT mice and 5 cells in Pitx3Null mice (Fig. 8D). (Sulpiride did not cause a detectable effect on the baseline striatopallidal IPSCs. This is likely due to the lack of sufficient ambient extracellular DA in the GPe in a 300 μm-thick brain slice preparation where the modest amount of DA may dissipate easily.) These results together with the paired pulse data indicate that DA inhibited the peak amplitudes of the striatopallidal IPSCs in both Pitx3WT mice and Pitx3Null mice through the D2Rs at the striatopallidal axon terminals.

DA disinhibits GP neuron firing more efficaciously in DA-deficient Pitx3Null mice than in Pitx3WT mice

Striatopallidal GABAergic inputs induce pauses in the spontaneous firing in GP neurons (Elias et al. 2007; Sani et al. 2009; Kita and Kita 2011a,b). We reasoned that the supersensitive presynaptic D2R inhibition of the striatopallidal terminals in Pitx3Null mice may enable low concentrations of DA to more effectively reduce the pausing effect on GP neuron firing. To test this possibility, we evoked striatopallidal inhibitory postsynaptic potentials (IPSPs) that caused pauses in the spontaneous firing in GP neurons both in Pitx3WT mice and Pitx3Null mice (Fig.
A KSO$_3$CH$_3$–based pipet solution was used such that the GABA$_A$ IPSPs were hyperpolarizing. To quantify the DA effect, we measured the duration of the firing pause induced by the striatopallidal IPSPs. Here the pause duration was defined as the time window between the stimulus artifact and the first spike in each sweep (Fig. 9A1, B1). We found that bath application of 1 μM DA reduced the pause duration by 10.7±0.8% (n=6 GP neurons, p<0.05, paired t test) [from the control duration of 250.8±41.7 ms (range: 394~140 ms)] in Pitx3WT mice, but by 32.7±3.3% (n=6 GP neurons, p<0.05, paired t test) [from the control duration of 244.5±34.3 ms (range: 371~132 ms)] in Pitx3Null mice (Fig. 9C). Increasing DA to 3 μM reduced the pause duration by 25.4±2.9% (n=9 GP cells, paired t-test, p<0.05) [from the control duration of 250.8±41.7 ms (range: 394~140 ms)] in Pitx3WT mice, and 54.7±4.8% (n=6 GP cells, paired t-test, p<0.01) [from the control duration of 273.4±38.0 ms (range: 372~150 ms)] in Pitx3Null mice (Fig. 9A-C). When a saturating 50 μM DA was bath-applied, the pause duration was reduced by 57.1±5.1% (n=10 GP cells, paired t-test, p<0.01) [from the control duration of 250.8±41.7 ms (range: 394~140 ms)] in Pitx3WT mice, and 56.6±3.8% (n=10 GP cells, paired t-test, p<0.01) [from the control duration of 250.8±41.7 ms (range: 394~140 ms)] in Pitx3Null mice (Fig. 9C). Two-way ANOVA indicates a significant difference in the DA effect on striatopallidal IPSP-induced pause duration in GP neuron firing in Pitx3WT mice and Pitx3Null mice ($F(1,41)=28.067$, p<0.001). Post hoc LSD tests confirm that at 1 and 3 μM, the DA effect was significantly stronger in Pitx3Null mice than in Pitx3WT mice (p<0.001), whereas there was no significant difference at 50 μM (p>0.05). These results indicate that low concentrations of DA were more efficacious in reducing the pausing effect of the striatopallidal IPSP in the DA-deficient Pitx3Null mice, although the maximal inhibition under saturating doses of DA was similar, consistent with our data in the preceding sections showing that the D2Rs on the striatopallidal axon terminals were supersensitive to DA with a left-ward shift in dose-response curve in Pitx3Null mice (Fig. 5).

Discussion

The main finding of this study is that in mice with severe nigral DA neuron loss resulting from transcription factor Pitx3 gene null mutation, the D2Rs at the striatopallidal axon terminals are supersensitive, leading to reduced inhibitory output from indirect pathway MSNs and consequently reduced pausing in GP neuron activity upon introduction of D2R agonism. In the following sections, we will first discuss the suitability of Pitx3 mutant mice as a model to study DA receptor supersensitivity and then our main findings.
Pitx3Null mice are a suitable model to study DA receptor supersensitivity

In this study, we used the Pitx3Null mice that have a severe and consistent loss of the nigral DA neurons and hence the nigrostriatal DA projection and other nigra-originated DA innervation (Hwang et al. 2003; Li et al. 2013; Li and Zhou 2013). The DA neuron loss occurs early in Pitx3Null mice such that the majority of the nigral DA neurons is lost when the animal is born, even though a substantial number of DA neurons in the ventral tegmental area survive into adulthood (Nunes et al. 2003; van den Munckhof et al. 2003; Smidt et al. 2004). It is known that the expression levels of D1Rs and D2Rs in MSNs start to increase rapidly in the first two postnatal weeks (Rao et al. 1991; Schambra et al. 1994; Jung and Bennett 1996). Consequently, most of the D1Rs and D2Rs in the dorsal striatum in Pitx3Null mice have little or no exposure to DA. This raises a concern on the suitability of Pitx3Null mice as a model to study DA receptor supersensitivity. We argue that a lack of prior exposure to DA does not affect the supersensitization of DA receptors based on the following reasons. First, DA depletion by reserpine treatment in normal mice led to a similar supersensitive DA inhibition of the striatopallidal IPSCs. Second, literature evidence and our prior work have shown that Pitx3Null mice have the behavioral and molecular hallmarks of typical DA denervation supersensitivity (Hwang et al. 2005; van den Munckhof et al. 2006; Ding et al. 2007; Li et al. 2013; Li and Zhou 2013). Third, studies have indicated that prior DA innervation is not required for DA receptor supersensitivity to develop. For example, fetal MSNs that had no or very little DA innervation developed DA receptor supersensitivity when these fetal MSNs were transplanted into the cortex or thalamus (Guerra et al. 1996). DA receptor supersensitivity also occurred in animals whose DA system was lesioned during the neonatal period before the expression of the majority of DA receptors (Kostrzewa 1995). Fourth, studies using mice with null tyrosine hydroxylase (TH) gene in their DA neurons suggested that D1R and D2R supersensitivity at behavioral and molecular levels can be reversed or resensitized, depending on DA availability, regardless of prior history of DA innervation or exposure (Kim et al. 2000, Kim et al. 2002). These studies (Kim et al. 2000, Kim et al. 2002) even suggested that DA receptors expressed during the embryonic and first few postnatal days in normal animals were supersensitive, due to a lack of sufficient DA innervation, until the arrival of the massive nigrostriatal DA projection. Fifth, the induction of normal movements and dyskinesias by the first dose of L-dopa in young children with TH deficiency (Pons et al. 2013) also indicates that the intensity of DA loss, not the age nor repeated use of L-dopa, is critical to the supersensitive DA response. Taken together, it is reasonable to conclude that the DA receptor supersensitivity caused by an early loss of DA in Pitx3Null mice is similar or identical to the DA supersensitivity induced by a DA loss at a later
point of the animal’s life. Therefore, Pitx3Null mice are suitable for studying the cellular
neurophysiological aspects of DA denervation supersensitivity. In fact, the pre-made and
consistent DA loss in Pitx3Null mice that are viable without the need of any special care is a
highly convenient feature to cellular experimental questions that usually require a large number
of animals (Ding et al. 2011; present study).

Nigral DA neuron loss does not alter GP neuron intrinsic membrane properties in
Pitx3Null mice
We detected the I_h-mediated depolarizing sag in both type A and type B GP neurons, although
the amplitude of the sag was larger in type A neurons than in type B neurons. This is in partial
agreement with Cooper and Stanford (2000) who reported that type A GP neurons had a strong
I_h sag whereas type B neurons had no I_h, although the figure 5A of that paper shows a clear I_h
sag in a type B GP neuron. We also found that the severe loss of nigral DA neurons in Pitx3Null
mice did not induce any detectable change in the I_h sag or other intrinsic properties such as
input resistance in GP neurons, indicating that I_h amplitude was not affected in GP neurons in
the DA-deficient Pitx3Null mice. This is in contrast with Chan et al. (2011) who reported that DA
depletion with 6-OHDA or reserpine reduced I_h. Despite this discrepancy, our data are solid and
also entirely possible for the following two reasons. First, even though the GP receives a
dopaminergic input from SNc DA neurons (Hedreen 1999; Smith and Kieval 2000; Prensa and
Parent 2001), this DA innervation is very small compared with the massive DA innervation in the
striatum in both rodents and primates including humans (Hornykiewicz 2001; Rajput et al. 2008).
Furthermore, a significant portion of the modest DA content in the GP clearly belongs to the DA
fibers of passage en route to the striatum (Jan et al. 2000), leaving the DA fibers truly
innervating GP even more sparse. Second, the expression of DA receptors in GP neurons is
very low compared to the intense DA receptor expression in the striatum (Mansour et al. 1990;
Levey et al. 1993; Yung et al. 1995). These two anatomical facts suggest that it is entirely
possible that DA loss in the GP may not induce any detectable effect in the intrinsic properties
of GP neurons simply because the DA innervation and DA receptor expression are too low. In
contrast, the drastic loss of the intense DA innervation in the striatum and strong D2R
expression in the striatopallidal neurons provide a rich substrate for DA loss to induce robust
homeostatic responses. Indeed, increased D2R functionality (due to increased receptor
expression and signaling efficiency) at the somatodendritic area, following severe DA loss, is an
established observation (Graham et al. 1990a,b; Mandel et al. 1993; Qin et al. 1994; Kostrzewa
1995; Geurts et al. 1999; Newman-Tancredi et al. 2001; Cai et al. 2002; Betarbet and
Greenamyre 2004). These changes may spread to the axon terminals of D2-MSNs, leading to the increased functionality of D2Rs at the striatopallidal axon terminals.

Presynaptic D2R inhibition of the striatopallidal synapse is supersensitive in DA-deficient mice

DA is known to reduce the striatopallidal synaptic transmission through the inhibitory presynaptic D2Rs (Cooper and Stanford 2001; Watanabe et al. 2009; Chuhma et al. 2011; Kim and Kita 2013). As the D2R-expressing striatopallidal MSNs need to be inhibited for the basal ganglia motor circuit to function properly (Sano et al. 2012), these presynaptic D2Rs serve as the final layer of negative control on these indirect pathway MSNs. Despite the apparent importance, the effect of DA loss on these D2Rs at the striatopallidal axon terminals was not known. A recent study indicated that 6-OHDA-induced DA loss did not alter the baseline striatopallidal synaptic transmission but did not examine if DA sensitivity was altered (Miguelez et al. 2012). In our present study, we found that in the DA-deficient Pitx3Null mice, the baseline striatopallidal synaptic transmission was identical to that in Pitx3WT mice, consistent with (Miguelez et al. 2012); however, the presynaptic DA inhibition of the striatopallidal synapse was much more efficacious in Pitx3Null mice (IC$_{50}$ ~ 0.35 μM) than in Pitx3WT mice (IC$_{50}$ ~ 3 μM).

Thus, to our knowledge, these data provide the first evidence that the presynaptic D2Rs at the striatopallidal axon terminals become supersensitive after a severe loss of nigral DA neurons. This upregulation of D2R functionality at the striatopallidal axon terminal may result from the loss of DA innervation in both the striatum and GP. First, a loss of the massive DA innervation in the striatum, coupled with the strong expression of D2Rs in D2-MSNs, may induce substantial homeostatic responses in D2-MSN somata that are likely to spread to axon terminals. Second, the loss of DA in GP may further increase the presynaptic D2R supersensitivity at the striatopallidal axon terminals. The normal DA innervation in GP is low (see the discussion in the preceding section). Therefore, even a moderate DA loss in the GP may reduce the synaptic DA level to a DA deficient state, contributing to the supersensitization of D2Rs at the striatopallidal axon terminals. Presynaptic D2Rs inhibit GABA release from D2-MSNs in normal animals (Delgado et al. 2000; Guzmán et al. 2003; Salgado et al. 2005; Geldwert et al. 2006; Tecuapetla et al. 2007; Tecuapetla et al. 2009; Kohnomi et al. 2012). Ca$^{2+}$ imaging studies in medium spiny neurons in the striatum indicate that D2Rs inhibited the action potential-induced Ca$^{2+}$ signal in the axon terminals (Wu et al. 2006; Mizuno et al. 2007), Ca$^{2+}$ currents in the somatodendritic area and the Ca$^{2+}$ signal in the dendritic spines (Hernandez-Lopez et al. 2000; Rakhilin et al. 2004; Olson et al. 2005; Salgado et al. 2005; Higley and Sabatini 2010). Furthermore, it has
been documented that toxin-induced DA denervation causes a supersensitive D2R inhibition of voltage-activated Ca\(^{2+}\) currents in the somatodendritic area of the medium spiny neurons (Prieto et al. 2009). DA denervation also led to supersensitive DA presynaptic inhibition of GABA and glutamate release in the subthalamic nucleus (Shen et al. 2003). Therefore, the supersensitive D2R is fully capable of inhibiting GABA release from striatopallidal axon terminals in a heightened manner.

Although not the subject of our present study, several mechanisms have been proposed to underlie D2R supersensitivity at D2-MSN somata, including an increased D2R expression at the cell surface and an increased functionality such as higher D2R binding affinity and more efficient receptor-G protein coupling and downstream signaling (Qin et al. 1994; Kostrzewa 1995; Geurts et al. 1999; Cai et al. 2002; Betarbet and Greenamyre 2004; Seeman et al. 2005a). How DA loss in Pitx3Null mice leads to a supersensitive D2Rs on the striatopallidal axon terminals remains to be determined. However, the fact that the IC\(_{50}\) of DA inhibition of the striatopallidal IPSCs was decreased while the maximal inhibition remained unchanged indicates that DA receptor affinity probably was increased following DA loss, as a homeostatic response to the decreased DA availability (Seeman et al. 2005a).

**Supersensitive DA inhibition of the inhibitory striatopallidal synapse in DA-deficient Pitx3Null mice: functional implications**

Pre-clinical and clinical studies indicate an importance of GP neuronal activity and its temporary inhibition (pausing) in the physiology and pathophysiology of the basal ganglia and hence movement control (Obeso et al. 2008; Kita and Kita 2011a,b; Soares et al. 2004; Nambu et al. 2011; Wichmann and Dostrovsky 2011; Sano et al. 2013). DA, via presynaptic D2Rs, inhibits this inhibitory effect of striatopallidal IPSPs, leading to a disinhibition of, or a smaller pausing in, GP neuron firing in normal mice. This result indicates that in normal animals, the presynaptic D2Rs on striatopallidal axon terminals work, in concert with the somatic inhibitory D2Rs (West and Grace 2002), to reduce the output from D2-MSNs. Such a coordinated D2R-mediated inhibition of D2-MSNs likely facilitates movements since inhibition or ablation of D2-MSNs is known to increase motor activity (Sano et al. 2003; Durieux et al. 2009; Bateup et al. 2010). In DA-deficient animals, owing to the supersensitive presynaptic D2Rs, low concentrations of DA may be able to disinhibit GP neurons from the striatopallidal inhibition more efficaciously than in normal animals. This supersensitive presynaptic D2R-mediated disinhibition of GP neurons has important functional implications for PD pathophysiology and dopaminergic treatments. First, it may compensate for the lower DA level in GP in PD, thus minimizing a possible decrease in GP
neuron activity under a DA deficient or PD condition and minimizing motor deficits. Second, during the treatment of L-dopa [converted to DA by residual DA axon terminals and the rich 5-HT axon terminals in the GP (Parent et al. 2011)] or a D2-like agonist, the striatopallidal synapse may be inhibited more strongly because of the supersensitive presynaptic D2Rs. Consequently, GP neurons may also be more strongly disinhibited, potentially contributing to the motor-stimulating effects of these dopaminergic treatments.
References


Geurts M, Hermans E, Cumps J, Maloteaux JM. Dopamine receptor-modulated 

Graham WC, Clarke CE, Boyce S, Sambrook MA, Crossman AR, Woodruff GN. 
Autoradiographic studies in animal models of hemi-parkinsonism reveal dopamine D2 but not 
D1 receptor supersensitivity. II. Unilateral intra-carotid infusion of MPTP in the monkey (Macaca 

Graham WC, Crossman AR, Woodruff GN. Autoradiographic studies in animal models of hemi-
parkinsonism reveal dopamine D2 but not D1 receptor supersensitivity. I. 6-OHDA lesions of 

Guerra MJ, Liste I, Rozas G, Tobio J, Labandeira-Garcia JL. Previous dopaminergic innervation 
is not necessary for the development of dopamine supersensitivity in rat striatal neurons. 

Günay C, Edgerton JR, Jaeger D. Channel density distributions explain spiking variability in the 
globus pallidus: a combined physiology and computer simulation database approach. *J 

Dopaminergic modulation of axon collaterals interconnecting spiny neurons of the rat striatum. *J 

Hedreen JC. Tyrosine hydroxylase-immunoreactive elements in the human globus pallidus and 

Hedreen JC, DeLong MR. Organization of striatopallidal, striatonigral, and nigrostriatal 

Hernandez LF, Kubota Y, Hu D, Howe MW, Lemaire N, Graybiel AM. Selective effects of 
dopamine depletion and L-DOPA therapy on learning-related firing dynamics of striatal neurons. 

D2 dopamine receptors in striatal medium spiny neurons reduce L-type Ca2+ currents and 
excitability via a novel PLC[beta]1-IP3-calcineurin-signaling cascade. *J Neurosci* 20: 8987-8995, 
2000.

Higley MJ, Sabatini BL. Competitive regulation of synaptic Ca2+ influx by D2 dopamine and 

Hornykiewicz O. Chemical neuroanatomy of the basal ganglia--normal and in Parkinson's 

Hwang DY, Ardayfio P, Kang UJ, Semina EV, Kim KS. Selective loss of dopaminergic neurons 
in the substantia nigra of Pitx3-deficient aphakia mice. *Brain Res Mol Brain Res* 114: 123-131, 
2003.


Jung AB, Bennett JP Jr. Development of striatal dopaminergic function. I. Pre- and postnatal development of mRNAs and binding sites for striatal D1 (D1a) and D2 (D2a) receptors. *Brain Res Dev Brain Res* 94: 109-120, 1996.


**Figure legends**

**Figure 1.** Visual identification of Pitx3+/+ (Pitx3WT) mice and Pitx3-/- mutant (Pitx3Null) mice. Pitx3WT mice (A) and Pitx3Null mice (B) look identical except that Pitx3Null mice have malformed eyes that clearly identified Pitx3Null mice on or after postnatal day 14 when Pitx3WT and Pitx3-/+ mice open their eyes. These 2 example mice were of 35 days old. A’ and B’ show that compared with the Pitx3WT mouse, the eye of the Pitx3Null mouse (both mice were 30 days old) is smaller with no clearly identifiable lens or cornea. The scale in B’ applies to A’.

However, Pitx3Null mice eat well, are fertile, and do not need any special care.

**Figure 2.** The DA loss pattern in Pitx3Null mice. A, B. TH immunofluorescent stain reveals the intense DA innervation in the dorsal striatum (A1), the modest DA innervation in the dorsal GP (A2) and the densely packed DA neurons in the substantia nigra pars compacta (SNC) and in the ventral tegmental area (VTA) (A3) in WT mice. A1-A3 show the DA innervation in the dorsal striatum and the dorsal GP and DA neurons in the SNC and VTA DA neurons. Their counterparts in Pitx3Null mice are shown in B1-B3. These pictures are raw, unprocessed confocal images obtained at identical acquisition parameters. Note that the dorsal striatum is largely devoid of DA fibers (B1). The scale bar in A1 applies to A2, B1 and B2. The scale bar in A3 applies to A3. C. HPLC quantification of tissue DA content in the dorsal (1.01±0.08 µmole/g wet tissue in Pitx3WT vs. 0.02±0.00 µmole/g in Pitx3Null), middle (0.93±0.10 µmole/g in Pitx3WT vs. 0.09±0.02 µmole/g in Pitx3Null) and ventral striatum (0.84±0.11 µmole/g in Pitx3WT vs. 0.34±0.07 µmole/g in Pitx3Null) and dorsal GP (0.07±0.01 µmole/g in Pitx3WT vs. 0.03±0.01 µmole/g in Pitx3Null) (all p<0.01, 2-way ANOVA and LSD posthoc test). Five mice for each genotype. Note that in Pitx3WT mice, the DA content in the dorsal GP is only 6.5% of that in the dorsal striatum, and the DA content in the dorsal GP in Pitx3Null mice is reduced to 45% of that in Pitx3WT mice. The DA loss is smaller in GP compared to the dorsal striatum at least partially due to the residual DA fibers of passage en route to the middle striatum. The inset (C1) illustrates the locations for the dorsal striatum punch, the middle striatum punch and the ventral striatum punch. *, the anterior commissure.

**Figure 3.** Similar intrinsic membrane properties of GP neurons in Pitx3WT and DA-deficient Pitx3Null mice. A: A live sagittal brain slice showing the stimulating site in the dorsal striatum and the recording site in the dorsal GP, photographed with a 4X objective. *, the anterior commissure. IC, internal capsule. B: Patch clamping of a typical GP neuron, photographed with
a 60X objective. C: A scatter plot of input resistance and action potential base duration of type A and type B GP neurons in Pitx3WT and Pitx3Null mice. The inset shows the shorter spike duration in type B neurons. D-G: Example recordings show intrinsic membrane and action potential properties of a representative type A and type B GP neuron from a PN20 Pitx3WT mouse (D, E) and a representative type A and type B GP neuron from a PN20 Pitx3Null mouse (F, G). The Ih-mediated sag is larger in these 2 type A neurons than in the 2 type B GP neurons, but no apparent difference between the genotypes. See Table for statistical comparisons.

Figure 4. Similar baseline properties of striatopallidal IPSCs in GP neurons in Pitx3WT and DA-deficient Pitx3Null mice. A and B: Example striatopallidal IPSCs evoked by a pair of stimuli spaced at 50 ms apart in a GP neuron in a Pitx3WT mouse (A) and a Pitx3Null mouse (B). Each trace is an average of 10 sweeps. C: Summary of paired pulse experiments at 1 Hz, 5 Hz, 10 Hz, 20 Hz and 40 Hz in GP neurons in Pitx3WT and Pitx3Null mice. Holding potential was –70 mV. There was no difference in the PPR at each of these frequencies between Pitx3WT and Pitx3Null mice (p>0.05, unpaired t test).

Figure 5. Low concentrations of DA reduce striatopallidal IPSCs more efficaciously in DA-deficient Pitx3Null mice than in WT mice. A, B and C: Examples of the inhibitory effect of 1, 5 and 50 μM DA on striatopallidal IPSCs in Pitx3WT mice. D, E and F: Examples of the inhibitory effect of 1, 5 and 50 μM DA on striatopallidal IPSCs in Pitx3Null mice. G: Pooled data showing different dose-response relationships of DA inhibition of striatopallidal IPSCs in Pitx3WT mice and Pitx3Null mice. n = 7-8 cells for each data point. Holding potential was –70 mV. The 2 continuous lines are the fits to the Hill equation: Y = A·X^n/[1/(K^n+X^n)], where A is the maximal inhibition, X is the DA concentration, K is the IC_{50}, and n is the Hill number.

Figure 6. DA reduces striatopallidal IPSCs more efficaciously in reserpine-treated WT mice. A: Averaged traces of striatopallidal IPSCs before, during and after bath application of 1 μM DA in vehicle-treated Pitx3WT mice. B: Averaged traces of striatopallidal IPSCs before, during and after bath application of 1 μM DA in reserpine-treated Pitx3WT mice. C: Pooled data showing 1 μM DA effects on striatopallidal IPSCs in vehicle- and reserpine-treated Pitx3WT mice, respectively.

Figure 7. DA increases the paired pulse ratio of striatopallidal IPSCs in Pitx3WT and Pitx3Null mice. A: Averaged traces of striatopallidal paired IPSCs (evoked by 2 stimuli 50 ms apart)
before and during bath application of saturating 50 μM DA in Pitx3WT mice. These two traces were then scaled to the peak of their 1\textsuperscript{st} IPSC to show the relative increase of the 2\textsuperscript{nd} IPSC peak.

**B:** Summary showing a clear DA-induced increase in PPR in Pitx3WT mice. **C:** Averaged traces of striatopallidal paired IPSCs (evoked by 2 stimuli 50 ms apart) before and during bath application of saturating 50 μM DA in Pitx3Null mice. **D:** Summary showing a clear DA-induced increase in PPR in Pitx3Null mice. These two traces were then scaled to the peak of their 1\textsuperscript{st} IPSC to show the relative increase of the 2\textsuperscript{nd} IPSC peak. Holding potential was –70 mV for all cells. *, p<0.05, paired t test.

**Figure 8.** D2-like agonist quinpirole mimics the DA effects on striatopallidal IPSCs in Pitx3Null mice. **A:** Averaged traces of striatopallidal IPSCs before, during and after bath application of 1 and 10 μM quinpirole in Pitx3WT mice. **B:** Averaged traces of striatopallidal IPSCs before, during and after bath application of 1 and 10 μM quinpirole in Pitx3Null mice. **C:** Pooled data showing 1 μM quinpirole reduced the striatopallidal IPSCs more strongly in Pitx3Null mice than in Pitx3WT mice, *, p<0.01, post-hoc LSD test following 2-way ANOVA, but the effects of 10 μM quinpirole were not significantly different in the two genotypes. **D:** Pooled data showing 10 μM SKF81297 had no effect on the striatopallidal IPSCs in Pitx3WT or Pitx3Null mice. Holding potential was –70 mV for all cells.

**Figure 9.** Low concentrations of DA reduce the pausing effect of striatopallidal IPSPs on GP neuron spike firing more efficaciously in DA-deficient Pitx3Null mice than in WT mice. **A:** In normal Pitx3WT mice, striatopallidal IPSPs induced a pause in the spontaneous firing in GP neurons (A1) that was reduced by bath application of 3 μM DA (A2). **B:** In DA-deficient Pitx3Null mice, striatopallidal IPSPs induced a pause in the spontaneous firing in GP neurons (B1) that was strongly reduced by bath application of 3 μM DA (B2). **C:** Summary showing the effects of 1, 3 and 50 μM on the IPSP-induced pause duration in GP neuron firing in Pitx3WT mice and Pitx3Null mice. ***, p<0.001, post-hoc LSD test following 2-way ANOVA.

**Table 1.** Table title: Intrinsic membrane properties of GP neurons in Pitx3WT and DA-deficient Pitx3Null mice.

Table footnote: The number of cells included for analysis for each parameter is indicated by n=.

Values are mean ± SEM. The values in the parentheses below the mean values indicate the ranges of individual values. p values are from unpaired t-tests.
Fig. 9. B/W

A Pitx3WT
  A1 control
  A2 3 μM DA

B Pitx3Null
  B1 control
  B2 3 μM DA

C

% decrease of pause duration

- Pitx3WT
- Pitx3Null

1 μM DA  3 μM DA  50 μM DA
<table>
<thead>
<tr>
<th>Mouse type</th>
<th>Cell type</th>
<th>Input resistance (MΩ)</th>
<th>Membrane time constant (ms)</th>
<th>AP base duration (ms)</th>
<th>AP amplitude (mV)</th>
<th>AP threshold (mV)</th>
<th>RMP amplitude (mV)</th>
<th>L-sag (mV)</th>
<th>L-sag (mV) through</th>
<th>potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pitx3WT</td>
<td>Type A</td>
<td>373.8±28.7</td>
<td>37.4±2.3</td>
<td>1.34±0.06</td>
<td>70.1±1.8</td>
<td>-41.1±0.7</td>
<td>-17.4±0.7</td>
<td>8.9±0.7</td>
<td>8.9±0.7 (n=25)</td>
<td>(6.1–14.9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(100.3–705.6)</td>
<td>(14.2–66.9)</td>
<td>(0.92–1.06)</td>
<td>(54.0–61.0)</td>
<td>(-34.2–50.0)</td>
<td>(-11.0–24.6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>n=37</td>
<td>n=37</td>
<td>n=37</td>
<td>n=37</td>
<td>n=37</td>
<td>n=37</td>
<td>n=37</td>
<td>n=37</td>
<td></td>
</tr>
<tr>
<td>Pitx3WT</td>
<td>Type B</td>
<td>167.2±10.5</td>
<td>20.2±1.8</td>
<td>0.73±0.01</td>
<td>71.4±1.8</td>
<td>-43.3±1.1</td>
<td>-21.5±0.8</td>
<td>4.8±0.6</td>
<td>4.8±0.6 (n=11)</td>
<td>(2.7–7.7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(81.0–253.6)</td>
<td>(14.6–33.8)</td>
<td>(0.61–0.89)</td>
<td>(53.3–89.5)</td>
<td>(-31.6–58.4)</td>
<td>(-16.4–30.5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>n=26</td>
<td>n=26</td>
<td>n=26</td>
<td>n=26</td>
<td>n=26</td>
<td>n=26</td>
<td>n=26</td>
<td>n=26</td>
<td></td>
</tr>
<tr>
<td>Pitx3Null</td>
<td>Type A</td>
<td>377.6±24.7</td>
<td>37.2±2.5</td>
<td>1.38±0.04</td>
<td>70.8±1.4</td>
<td>-40.2±0.7</td>
<td>-17.6±0.6</td>
<td>8.8±0.6</td>
<td>8.8±0.6 (n=31)</td>
<td>(6.4–12.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(170.4–220.9)</td>
<td>(15.2–73.8)</td>
<td>(0.95–1.33)</td>
<td>(58.6–90.9)</td>
<td>(-35.2–51.4)</td>
<td>(-10.9–24.5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>n=35</td>
<td>n=35</td>
<td>n=35</td>
<td>n=35</td>
<td>n=35</td>
<td>n=35</td>
<td>n=35</td>
<td>n=35</td>
<td></td>
</tr>
<tr>
<td>Pitx3Null</td>
<td>Type B</td>
<td>171.7±10.4</td>
<td>21.1±1.3</td>
<td>0.72±0.02</td>
<td>71.2±1.2</td>
<td>-43.4±0.8</td>
<td>-21.4±0.7</td>
<td>4.9±0.5</td>
<td>4.9±0.5 (n=14)</td>
<td>(0.0–6.9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(96.8–286.5)</td>
<td>(12.0–33.3)</td>
<td>(0.51–0.88)</td>
<td>(53.3–90.7)</td>
<td>(-38.1–56.1)</td>
<td>(-16.5–34.8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>n=39</td>
<td>n=30</td>
<td>n=30</td>
<td>n=30</td>
<td>n=30</td>
<td>n=30</td>
<td>n=30</td>
<td>n=30</td>
<td></td>
</tr>
<tr>
<td>Pitx3WT vs.</td>
<td>Type A</td>
<td>p=0.05</td>
<td>p=0.05</td>
<td>p=0.05</td>
<td>p=0.05</td>
<td>p=0.05</td>
<td>p=0.05</td>
<td>p=0.05</td>
<td>p=0.05</td>
<td>p=0.05</td>
</tr>
<tr>
<td>Pitx3Null</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type A vs.</td>
<td>WT</td>
<td>p=0.001</td>
<td>p=0.001</td>
<td>p=0.001</td>
<td>p=0.001</td>
<td>p=0.001</td>
<td>p=0.001</td>
<td>p=0.001</td>
<td>p=0.001</td>
<td>p=0.001</td>
</tr>
<tr>
<td>Type B</td>
<td>Homo</td>
<td>p=0.001</td>
<td>p=0.001</td>
<td>p=0.001</td>
<td>p=0.001</td>
<td>p=0.001</td>
<td>p=0.001</td>
<td>p=0.001</td>
<td>p=0.001</td>
<td>p=0.001</td>
</tr>
</tbody>
</table>