Upregulation of D₂-class signaling in dopamine-denervated striatum is in part mediated by D₃ receptors acting on Caᵥ2.1 channels via PIP₂ depletion

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PARKINSON’S DISEASE IS ACCOMPANYED by the loss of dopaminergic neurons that innervate the neostriatum (Albin et al. 1989; Hornykiewicz 1974). An accepted experimental model of striatal dopamine (DA) depletion is the unilateral ablation of dopaminergic neurons in the substantia nigra compacta (SNc) by 6-hydroxydopamine (6-OHDA) (Simola et al. 2007). Striatal dopaminergic receptor supersensitivity follows DA depleti

METHODS
6-OHDA lesion of the nigrostriatal pathway. Studies were approved by the Universidad Nacional Autónoma de México Committee of Bioethics and followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH approval no. A5281-01). DA depletion after 6-OHDA has been previously described (Ungerstedt and Arbuthnott 1970). Briefly, anesthetized (ketamine-xylazine, 87 and 13 mg/kg ip, respectively) male Wistar rats (180–200 g) were injected stereotaxically with 4 μg of 6-OHDA (Sigma, St. Louis, MO) into the left substantia nigra (SN; 4.80 mm caudal, 1.6
mm lateral to bregma, 8.2 mm ventral to the skull surface). Similarly, D1R-enhanced green fluorescent protein (eGFP) transgenic mice (30 days old) on an FVB background developed by the GENSAT project (Heintz 2004) were injected stereotaxically with 1 mg of 6-OHDA (SN: 3.40 mm caudal, 1.1 mm lateral to bregma, 4.4 mm ventral to the skull surface). Coordinates for rats and mice were set according to the atlases of Paxinos and Watson (1996) and Paxinos and Franklin (2004), respectively. 6-OHDA solution (4 µg/µl saline with 0.2 mg/ml ascorbic acid) was ejected at a rate of 0.1 µl/min. It was confirmed that this procedure efficiently reduces (>95%) the dopaminergic innervation in the striatum, as measured by tyrosine hydroxylase (TH) staining (Supplemental Fig. S1). (Supplemental Material for this article is available online at the Journal of Neurophysiology website.) In addition, lesion of the SN did not induce an apparent nonspecific damage in other neighboring nuclei. After 14–17 days, animals were selected on the basis of their rotational behavior in response to amphetamine (4 mg/kg ip; Sigma) (Ungerstedt and Arbuthnott 1970). Animals showing >500 stereotyped turns ipsilateral to the lesioned side after a single dose of amphetamine were considered as having >95% of DA depletion in the neostriatum (Inaji et al. 2005). Experiments were carried out after 2–3 wk of the behavioral test.

Preparation of dissociated cells. Neurons were obtained as previously described (Bargas et al. 1994; Perez-Burgos et al. 2008). Briefly, 6-OHDA-lesioned animals were deeply anesthetized and their brains quickly removed into ice-cold saline (in mM: 126 NaCl, 3 KCl, 26 NaHCO3, 2 CaCl2, 1 MgCl2, 11 glucose, 0.2 thiosurea, and 0.2 ascorbic acid; pH 7.4) and then incubated with 1 mg/ml pronase E type XIV (Sigma). After about 20 min of digestion, cells were dissociated by using a graded series of fire-polished Pasteur pipettes. Cell suspension was plated into a petri dish containing saline [in mM: 0.001 tetrodotoxin (TTX), 130 NaCl, 3 KCl, 5 CaCl2, 2 MgCl2, 10 HEPES, and 10 glucose; pH 7.4 adjusted with NaOH; 300 ± 5 mosM with glucose)]. eGFP-positive neurons were visualized using an UV lamp (X-Cite; EXFO, Ontario, Canada).

Voltage-clamp recordings of Ca2+ currents. Recordings were performed on medium-sized neostriatal neurons (main diameter 10–12 µm, whole cell capacitance 6–7 pF) (Bargas et al. 1994). The patch pipettes were made from borosilicate glass (WPI, Sarasota, FL), pulled in a Flaming-Brown puller (Sutter Instrument, Novato, CA), and fire-polished before use. Whole cell recordings were obtained with an Axopatch-200B patch-clamp amplifier (Axon Instruments, Foster City, CA) and controlled and monitored with pClamp software (version 8) with a 125-kHz direct memory access interface (Axon Instruments). Resistance of electrodes was 3–6 MΩ when filled with pipette solution (in mM: 180 N-methyl-D-glucamine, 40 HEPES, 10 EGTA, 4 MgCl2, 2 ATP, 0.4 GTP, and 0.1 leupeptin; pH 7.2 adjusted with H3SO4; 280 ± 5 mosM).

Currents were recorded (carried by Ba2+, a potent K+ channel blocker) while Na+ channels were blocked (1 µM TTX). Current was carried by Ba2+; however, because Ba2+ flows through Ca2+ channels, it was referred to as Ca2+ current. Intracellular K+ was replaced by 180 mM N-methyl-D-glucamine. Current-voltage (I–V) relationships were built, before and after drug applications, both with 20-ms voltage commands from −80 to 50 mV in 10-mV steps and with current responses to voltage-ramp commands (0.7 mV/ms) from −80 to 50 mV. Drugs were applied with a gravity-fed system that positioned a glass capillary tube 100 µm from the recording cell in the direction of superfusion flow. Solution changes were performed with a direct current-controlled microvalve system (Lee, Essex, CT). This method allowed reversible drug applications (Perez-Burgos et al. 2008).

Drugs. Chemicals used were TTX (Alomone Labs, Jerusalem, Israel), α-agatoxin TK (α-agato-TX; PKD Peptides International, Louisville, KY), nitrendipine, quinolacine, L-741,626, U-99914/A, L-750,667 (Sigma), FKS06 (A.G. Scientific, San Diego, CA), SL327, m-3M3-FBS, α-3M3-FBS (Tocris Cookson,Ellisville, MO), U-73122, U-73343, and sn-1-stearoyl-2-arachidonoyl phosphatidylcholine-4,5-bisphosphate (PIP2; Calbiochem, La Jolla, CA). Most active substances were dissolved in water to get stock solutions and, after thawing, were added to the superfuse to give the final concentration. Nitrendipine, FKS06, SL327, and U-73122 were prepared in 1% DMSO, m-3M3-FBS in 0.1% DMSO, and PIP2 in 0.1% chloroform. In each case, control saline also contained DMSO or chloroform at the same final concentration.

Data analysis. Means ± SE of peak (maximum value) Ca2+ currents are reported. Distribution-free statistical tests were used to assess statistical significance: Mann-Whitney’s U-test or Wilcoxon’s t-test (depending on paired or nonpaired samples) and one-way analysis of variance (ANOVA) with post hoc Tukey’s test were used to assess significance between multiple samples in which agonist modulation was tested in the presence or absence of different drugs.

Western blotting. Rats were deeply anesthetized and their brains quickly removed into ice-cold saline (4°C). Dorsal striata were dissected from sagittal brain slices (300–400 µm thick) and homogenized in 320 mM sucrose containing a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) by using a tissue grinder with Teflon pestle. Homogenate was centrifuged at 2,000 g for 5 min; supernatant was recovered and centrifuged at 10,000 g for 1 h. Pellet was dissolved in the same solution and stored at −70°C. The protein content in the brain membrane preparation was determined using the Bradford assay with BSA as standard. Protein extracts (30–50 µg) were submitted to 10% SDS-PAGE. Resolved proteins were transferred onto nitrocellulose or polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA) and immunoblotted with the following antibodies: anti-D1R (1:500 dilution; Calbiochem), anti-D2R (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-Na+/K+ ATPase α-1 (1:250; Affinity Bioreagents, Rockford, IL). Specificity of anti-D1R and anti-D2R was demonstrated using lysates from rat eyes (Supplemental Fig. S2), tissue that does not express D1R (Fujieda et al. 2003). Immune complexes were revealed by using appropriate peroxidase-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA) along with a chemiluminescent reagent (Amersham Biosciences, Piscataway, NJ). Densitometric analysis was carried out by using Quantity One software (Bio-Rad).

Tyrosine hydroxylase immunoreactivity. Rats were anesthetized and perfused transcardially with a solution of 0.9% NaCl for 15 min and then perfused with 4% paraformaldehyde in 0.1 M PBS, pH 7.4, for 25 min. Brains were then cut on a vibratome in 30-µm-thick sections, which were incubated for 24 h at 4°C with a rabbit anti-TH antibody (dilution 1:1,000; Chemicon, Temecula, CA) in PBS containing 2.5% BSA and 0.1% Triton X-100. After being washed in PBS, the sections were incubated for 2 h at room temperature with biotinylated donkey anti-rabbit IgG (Chemicon) diluted 1:250 in 0.1% Triton X-100/PBS containing 2.5% BSA. The sections were then washed and reacted with streptavidin-biotin peroxidase complex at room temperature for 2 h. The immunolabeling was revealed using diaminobenzidine as chromogen for peroxidase reaction. Images were captured with a digital camera mounted on a microscope. Optical density values were corrected for background staining, and TH staining in the ipsilateral striatum was expressed as a percentage of TH staining in the contralateral striatum (Supplemental Fig. S1).

RESULTS

Enhanced Ca2+ current modulation during D1R-class supersensitivity is in part due to the enhanced activity of D1R type. Neurons were obtained from the noninjured striatum of hemiparkinsonian animals (control side) or from the ipsilateral DA-depleted striatum of the 6-OHDA-lesioned animals (DA-depleted side) (Prieto et al. 2009). Ba2+ currents were evoked

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with depolarizing voltage commands using steps and ramp protocols (Supplemental Fig. S3, A and B). The match between both protocols after the I-V plots were constructed suggests acceptable space-clamp and voltage control (Supplemental Fig. S3C). For clarity, only I-V plots obtained with ramp commands are shown (Perez-Rosello et al. 2005). Average Ca²⁺ current amplitude was 375 ± 22 pA (n = 70) in neurons from control striatum and 360 ± 17 pA (n = 126) in neurons from the DA-depleted side [no significant difference (NS)]. In agreement with previous data (Prieto et al. 2009), there were no significant differences in current density (current/whole cell capacitance) when the results from control and DA-depleted striata (60 ± 4 and 62 ± 3 pA/pF, respectively) were compared.

In a previous work, D₃R-class supersensitivity was detected when 0.1 nM–100 μM quinelorane was used (Prieto et al. 2009). Under these conditions, D₂R, D₃R, and D₄R can be activated (Coldwell et al. 1999; Gackenheimer et al. 1995; Newman-Tancredi et al. 1997). Therefore, to dissect the role of D₂R, D₃R, and D₄R on Ca²⁺ current modulation, we exposed neurons to the D₃R-class agonist quinelorane in the presence of antagonists for the different receptor types. Quinelorane was used at 100 μM, given that when Ca²⁺ channel modulation is measured, this concentration reaches the maximal effect (see Prieto et al. 2009). All the antagonists used have been previously described in the literature (Kulagowski et al. 1996; Patel et al. 1996; Waters et al. 1993). They exhibit preferential selectivity for a receptor type belonging to the D₃R class (>20–1,000 times compared with the other D₃R class). On the basis of this information, the antagonist concentration used for blocking a particular receptor was at least 20 times larger than its dissociation constant. By adding low but effective concentrations of antagonists, we ensured blocking most of their respective receptor populations without interfering significantly with other dopamine receptor types. To ensure that high agonist concentrations are not enough to displace the binding of antagonists to their receptors, we used three different independent protocols with different drug combinations and animal species (see below). Quinelorane-responsive (D₃R-class responsive) neurons from both control and DA-depleted sides were 71% of tested neurons (n = 107/151).

Afterward, quinelorane was administered together with 500 nM U-99194A, a selective D₄R-type antagonist at this concentration (Waters et al. 1993), plus 50 nM L-750,667, a selective D₂R-type antagonist at this concentration (Patel et al. 1996), so that responses induced by quinelorane were putative-D₃R type only (Fig. 1, A and B). Because percentages of responsive neurons in samples from both sides were comparable (see Fig. 1C), measurements were pooled. It was found that 68% of rat MSNs (n = 19/28) responded to quinelorane during D₂R- and D₄R-type blockade; that is, they were D₃R-type responsive, coincident with the reported number of neurons belonging to the indirect pathway plus neurons that colocalize D₁R and D₃R types (Surmeier et al. 1996).

In these conditions, and once the remaining Ca²⁺ current was stable, D₃R-type antagonist was washed out to disclose D₂R-type modulation of remaining Ca²⁺ current (Fig. 1, A and B). D₃R-type responsiveness was comparable when samples were compared from both sides, and results were then pooled. D₃R-type responsiveness was attained in n = 17/28 of tested neurons (61%, Fig. 1C). Finally, D₃R-type antagonist was also washed out to finally disclose D₃R-type action on the remaining Ca²⁺ current. D₃R-type responsiveness was also comparable when samples from both sides were compared; it was obtained in n = 15/28 of tested neurons (54%, Fig. 1C). Antagonists used during these experiments, 500 nM U-99194A and 50 nM L-750,667, did not significantly modify Ca²⁺ current by themselves (data not shown). Notice that not all neurons responding to the D₂R-class agonist (quinelorane) responded to all and each receptor type (D₂, D₃, D₄; see below). Importantly, the percentages of responsive neurons for each receptor type were similar when control and DA-depleted sides were compared (Fig. 1C). Percentages of neurons responding to each receptor type agree with single-cell RT-PCR studies (Surmeier et al. 1996).

In particular, D₃R-responsive neurons were obtained in n = 7/11 (64%) and n = 10/17 (59%) control and DA-depleted striata, respectively (Fig. 1C; NS). However, Ca²⁺ current inhibition by the putative D₃R type was significantly larger in neurons from DA-depleted striatum compared with the controls (Fig. 1D). Hence, the percentage of cells exhibiting D₃R response did not change significantly after DA depletion. What changed significantly was the magnitude of the D₃R response: it was nearly doubled in neurons from denervated striata. No changes were observed in the responses associated with the other receptor types belonging to D₂R class (D₂, D₄). Therefore, their supersensitivity could have other manifestations (Prieto et al. 2009), and the results obtained were specific for D₄R.

A representative time course of one experiment is shown in Fig. 1E. D₃R-type action was clear, reversible, and reproducible in responsive neurons (dark shaded background). It was also clearly independent from D₂R-type reversible action (light shaded background, on and off arrows; n = 10). Although agonist-induced D₂R desensitization has been described in dopaminergic neurons of the ventral tegmental area (Bartlett et al. 2005), in our Ca²⁺ current recordings D₂R action occurred without apparent changes in sensitivity; perhaps other ionic currents have to be investigated to observe its electrophysiological manifestation. It is known that the antagonist concentration needed to block a given receptor depends on the concentration and nature of the agonist used (i.e., when high concentrations of agonist are used, the antagonism could be surmountable) (Wyllie and Chen 2007). Therefore, considering that quinelorane exhibits its high affinity for all D₃R-class receptors (<100 nM) (Coldwell et al. 1999; Aceves et al. 2011; Gackenheimer et al. 1995; Newman-Tancredi et al. 1997), it was plausible that when 100 μM quinelorane was used, D₃R and D₄R were only partially blocked by 500 nM U-99194A and 50 nM L-750,667, respectively. However, a clear disclosure of the action of these receptor types was obvious in each experiment after D₂R had been occupied by high agonist concentrations. Moreover, even if D₂R and D₄R actions are underestimated, the interest presently lies in the relative change in the action of one of them.

Nonetheless, to corroborate the pharmacological separation of receptor types and quantify the possible underestimation of receptor actions, we used a different and independent protocol to stimulate D₃R (Fig. 1, F and G). First, the cells were exposed to both D₃R- and D₄R-type antagonists (used at concentrations that block their respective receptors: 50 nM
L-741,626 + 50 nM L-750,667) (Kulagowski et al. 1996; Patel et al. 1996), and then quinelorane (100 μM) was introduced to act only on D3R. Note that in these experiments, underestimated actions would rely on D2R and D4R, because the high agonist concentration would first act on D3R. This alternative isolation procedure, performed as described above, suggests that the separation of receptor types can be achieved with these procedures with little estimation error. Furthermore, this alternative isolation procedure confirmed that the D3R-mediated inhibition of Ca2+ current was significantly stronger in neurons obtained from DA-depleted striatum compared with the controls (Fig. 1H) and in the same proportion.

Histograms in Fig. 1, D and H, summarize the above results. With the first protocol (Fig. 1D), percentages of D2R, D3R, and D4R Ca2+ current inhibition distributed among the three receptor types were as follows: D2R: 33 ± 4% in the control side (n = 8) and 35 ± 3% in the DA-depleted side (n = 11; NS); D3R: 17 ± 2% in the control side (n = 7) and 33 ± 4% in the DA-depleted side (n = 10; P < 0.05); and D4R: 13 ± 2% in the control side (n = 8) and 19 ± 2% in the DA-depleted side (n = 7; NS). Noticeably, the only significant enhancement occurred for D3R-type modulation. With the second protocol (Fig. 1H), putative D3R-type-mediated modulation increased from 17 ± 2% in neurons from the control side to 33 ± 6% in neurons from the DA-depleted side (n = 10; P < 0.05). Noticeably, this D3R-type enhancement is consistent with the whole Ca2+ current (16%) matches the Emax.
increment (13%) associated with the D2R-class supersensitivity described in a previous work (Prieto et al. 2009). This percentage may seem small compared with the whole cell current; however, it doubled the amount of D3R modulation, not at all negligible when seen as the action of a single receptor type. Therefore, we hypothesized that this selective doubling of the modulation should target a specific channel class. Moreover, given that both independent protocols are in full agreement, the results suggest that the pharmacological procedure to dissect receptor type actions is correct.

However, the D1R and D2R types are for the most part segregated in MSNs of the “direct” and “indirect” basal ganglia pathways, respectively: the “two pathways model” of the basal ganglia (Albin et al. 1989; Gerfen et al. 1990). As stated above, in our experiments not all MSNs responded to all receptor types. Interestingly, some rat neurons that responded to D1R activation did not respond to D2R activation (n = 2/6), suggesting that some direct pathway neurons may be D1R responsive (Hopf et al. 2003; Mizuno et al. 2007; Surmeier et al. 1996). Because bacterial artificial chromosome transgenic mice expressing eGFP under the control of the promoter for D1R (BAC D1R eGFP; Supplemental Fig. S4A) can be used to identify direct pathway neurons, we used these mice to confirm that a D1R response can be obtained independently or in the absence of a D2R response. Notably, a D1R response was not only obtained in isolation, but it was similarly enhanced by DA depletion in rat D2R-nonresponsive and mouse BAC D1R eGFP neurons (n = 4/15; Supplemental Fig. S4, B and C). BAC D1R eGFP mice were only used in these experiments. It was found that about 30% of BAC D1R MSNs exhibited a D1R response (Hopf et al. 2003; Mizuno et al. 2007; Surmeier et al. 1996). This result further supports the pharmacological separation of receptor types, and moreover, our measurements of D1R action were in complete agreement after the use of three different independent protocols that employed different drugs and animal species. The next step was to study signaling mechanisms and functions of D1R after DA depletion.

**Molecular mechanisms underlying Ca2+ current modulation by D1R.** 
Ca2+ channels can be modulated by G protein-coupled receptors (GPCRs) through two different mechanisms. One is a voltage-dependent mechanism (also referred to as the “fast” pathway), which involves direct binding of G protein subunits to cytoplasmic regions of the channel. This mechanism is sensitive to membrane depolarization and pertussis toxin, thus involving Gαi proteins, but insensitive to calcium chelators. By contrast, other mechanisms (also referred to as the “slow” pathway) are voltage independent, sometimes insensitive to pertussis toxin but sensitive to Ca2+ chelators and mediated by second messengers (Tedford and Zamponi 2006). In MSNs, modulation of Ca2+ channels by D1R-class agonists, such as quinpirole and quinelorane, is mediated by a voltage-independent (slow) mechanism: it is affected by Ca2+ chelators and second messengers but not by strong depolarizing prepulses (Hernandez-Lopez et al. 2000; Perez-Burgos et al. 2008). It has been described that D2R-class activation in MSNs inhibits Ca2+ current by a PLCβ-dependent signaling pathway that activates protein phosphatase 2B downstream (PP-2B or calcineurin) (Hernandez-Lopez et al. 2000). It is known that most channels targeted by this pathway are Cα1v channels (Hernandez-Lopez et al. 2000; Nishi et al. 1997; Oliveria et al. 2007; Olson et al. 2005; Perez-Burgos et al. 2008). First, we confirmed that this signaling pathway modulates a significant portion of the whole Ca2+ current in neurons obtained from the control side of hemiparkinsonian rats: inhibition of PP-2B with FK506 (1 μM) significantly reduced Ca2+ current modulation mediated by both D1R and D2R (Supplemental Fig. S5; n = 6; P < 0.05). Moreover, we next demonstrated that this slow pathway is also activated in neurons from DA-depleted striata and, moreover, that D3R also activates it. Thus the involvement of PLC signaling was tested, because it has been demonstrated to be the first step of the signaling cascade of the slow pathway in MSNs from naive rats (Hernandez-Lopez et al. 2000): The PLC inhibitor U-73122 (10 μM; n = 5), but not the inactive

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**Fig. 2.** D1R-type modulation is not voltage dependent. A: neurons dissociated from ipsilateral striata of 6-hydroxydopamine (6-OHDA)-lesioned rats were subjected to a standard double-pulse protocol (inset). Current amplitude evoked by depolarizing commands to 0 mV before and after a 80-mV prepulse was the same (control; solid trace). After exposure to 100 μM quinelorane in the presence of D3R-ant (50 nM L-741,626) and D4R-ant (50 nM L-750,667), current reduction was the same before and after the prepulse (+quinelorane; shaded trace). B: histogram summarizes the results of experimental sample (n = 6).
Following the previously described steps of this slow pathway, we next investigated whether D3R-type action was affected by PP-2B inactivation (Chen et al. 2009) in neurons obtained from the DA-depleted side, because this is the end of the cascade described in neurons from naive animals as it is in neurons from the control side (Supplemental Fig. S5). In this study we demonstrated that both D2R and D3R PP-2B modulations were inhibited by 1 μM of FK506 (Hernandez-Lopez et al. 2000; Nishi et al. 1997; Oliveria et al. 2007; Perez-Burgos et al. 2008) in neurons from the lesioned side (Fig. 4; A; *P < 0.05). Nonetheless, the action of D2R was reduced to 16 ± 4% (Fig. 4, A and C; n = 6; *P < 0.05), so the remaining FK506-insensitive D3R-type modulation is substantial in de-

![Diagram](https://via.placeholder.com/150)

Fig. 3. D3R-type modulation depends on PLC activation. All experiments were carried out in neurons from DA-depleted striata exposed to 100 μM quinolone in the presence of D3R-ant (500 nM U-99194A) and D4R-ant (50 nM L-750,667) to first reveal the D2R-type response (1) and then reveal the D3R-type response (2) after washout of the D3R-ant. Compared with the effects of its inactive analog, U-73343 (10 μM; A), the PLC inhibitor U-73122 (10 μM; B) greatly diminished both D2R- and D3R-type modulations. Similarly, compared with the effects of its inactive analog, α-3M3-FBS (25 μM; C), the PLC activator m-3M3-FBS (25 μM; D) mimicked the action of the agonist and occluded both D2R- and D3R-type modulations. E: histogram summarizes changes in D3R-type modulation under the conditions described above. *P < 0.05; **P < 0.01.

![Diagram](https://via.placeholder.com/150)

Fig. 4. Protein phosphatase 2B (PP-2B), but not ERK, mediates D3R-type modulation of Ca2+ current. All experiments were carried out in neurons from DA-depleted striata exposed to 100 μM quinolone in the presence of D3R-ant (500 nM U-99194A) and D4R-ant (50 nM L-750,667) to first reveal the D2R-type response (1) and then reveal the D3R-type response (2) after washout of the D3R-ant. Ca2+ current modulation by D2R and D3R types is shown after PP-2B inhibition with 1 μM FK506 (A) and after ERK inhibition with 20 μM SL327 (B). C: histogram summarizes changes in D3R-type modulation under the described conditions. Compared with D3R-type modulation obtained under control conditions (without drugs, see Fig. 1D), there were significant reductions when cells were exposed to FK506 only (**P < 0.05).

analog U-73343 (10 μM; n = 4), blocked most D3R- and D2R-type modulations (Fig. 3, A, B, E; *P < 0.01). Second, to further support the participation of PLC in the signaling pathway activated by D3R, we used a selective PLC activator: m-3M3-FBS (25 μM; n = 5) (Bae et al. 2003). This activator, but not the inactive analog α-3M3-FBS (25 μM; n = 4), mimicked D2R-class modulation on Ca2+ currents (Prieto et al. 2009): current was reduced by 57 ± 6% (P < 0.02). Finally, when tested after m-3M3-FBS, D3R and D2R modulations were greatly occluded (Fig. 3, C–E; P < 0.05). Therefore, as previously observed in MSNs from naive rats (Hernandez-Lopez et al. 2000), a slow cascade initiated by PLC mediates Ca2+ current modulation by D3R and D2R activation in neurons from DA-depleted striata.
nervated animals (about one-half the modulation mediated by D₃R). Accordingly, we hypothesized that it could correspond to the extra D₃R action induced by DA depletion (Fig. 1).

Next, we investigated whether the fraction of D₃R modulation, not controlled by PP-2B, was regulated by the MAPK-ERK pathway (Ahlgren-Beckendorf and Levant 2004), which is known to be enhanced by DA depletion (Cai et al. 2000; Gerfen et al. 2002). Ca²⁺ current modulation was studied while ERK activation was being inhibited by 20 μM SL327. Neither D₂R nor D₃R actions were prevented (Fig. 4, B and C; n = 5), suggesting that Ca²⁺ current modulation by D₂R class is not mediated by MAPK-ERK.

Clearly, then, D₃R modulates Ca²⁺ channels through a slow PLC-dependent pathway. However, in neurons from the lesioned side, about one-half of the D₃R-type modulation cannot be explained by either PP-2B or ERK regulation. Importantly, this unexplained modulation may correspond to that appearing after DA depletion, because neurons from both control and lesioned animals had the same amount of PP-2B modulation.

Recently, it was shown that endogenous membrane PIP₂ helps in maintaining the function of high-voltage-activated Ca²⁺ channels, and stimuli that activate PLC deplete PIP₂ and reduce Ca²⁺ channels currents (Perez-Burgos et al. 2010; Rousset et al. 2004; Suh et al. 2010; Wu et al. 2002). Given that D₃R activates PLC in MSNs (Fig. 3), we hypothesized that D₃R modulates Ca²⁺ channels by depleting PIP₂. Neurons were exposed to physiological levels of PIP₂ (10 μM) (McLaughlin and Murray 2005; Suh and Hille 2008) intracellularly (through the recording pipette) so that a constant level of the phosphoinositide was attained independently of PLC activation. Comparison of Fig. 5, A and B, shows that in MSNs from control side, D₂R and D₃R actions were not significantly affected by exposure to intracellular PIP₂. In contrast, D₃R modulation was significantly reduced to 15 ± 5% (about one-half D₃R total modulation) by intracellular PIP₂ in neurons from DA-depleted striatum (Fig. 5, D and E; n = 5; P < 0.05), whereas D₂R modulation was unaffected. Histograms in Fig. 5, C and F, summarize these results: only D₃R actions were found enhanced in neurons from DA-depleted striatum, and only this enhancement was abolished by PIP₂ exposure.

To support the contribution of both PP-2B activation and PIP₂ depletion to the D₃R-mediated modulation of Ca²⁺ channels, we examined the effect of intracellular PIP₂ on D₃R-type modulation in control and DA-depleted neurons. Experiments were carried out in neurons from control (A–C) and DA-depleted striatum (D–F). All experiments were carried out in neurons exposed to 100 μM quinelorane in the presence of D₃R-ant (500 nM U099194A) and D₃R-ant (50 nM L-750,667) to first reveal the D₂R-type response (1) and then reveal the D₃R-type response (2) after subsequent washout of D₃R-ant. A: D₂R- and D₃R-type responses in a control neuron. B: intracellular 10 μM PIP₂ blocked neither D₂R- nor D₃R-type responses in control neurons. C: histogram summarizes results from neurons from control side with and without intracellular PIP₂ (no significant difference, NS). D: D₂R- and D₃R-type responses in a neuron from DA-depleted striatum. E: intracellular 10 μM PIP₂ reduced D₃R- but not D₂R-type response. F: histogram summarizes results from a sample of neurons from DA-depleted striatum with and without intracellular PIP₂ (10 min). D₃R-type modulation was significantly reduced to about one-half by PIP₂, without affecting D₂R-type modulation (*P < 0.05).
nels in MSNs from the DA-depleted side, we carried out parallel experiments by activating D3R directly, applying 100 μM quinolorene in the presence of D2R- and D4R-type antagonists (cf. Fig. 1, D and E). Again, D3R modulation was partially, but significantly, inhibited by 1 μM FK506; modulation was reduced to 17 ± 2% (Fig. 6A; n = 10). Importantly, when 1 μM FK506 was added to the extracellular solution and 10 μM PIP2 was present in the recording pipette, D3R modulation was completely inhibited (Fig. 6B; n = 7). The histogram in Fig. 6C summarizes these results. Overall, these series of experiments clearly show that in neurons from DA-depleted striatum, PP-2B regulates about one-half, and PIP2 depletion of experiments clearly show that in neurons from DA-depleted C

Thus suggesting that they express the same amount of CaV1.5 channels (Prieto et al. 2009). As expected, blockage of CaV1.5 channels left a remaining D3R modulation in neurons from D3R-mediated inhibition was clearly observed in neurons dissociated from control striatum (Fig. 7, A and B; n = 4), and it was not affected by 50 μM wortmannin (Fig. 7, C and D; n = 4): 85 ± 6% of recovery was observed in control conditions compared with 69 ± 11% in the presence of wortmannin (Fig. 7E; NS). Neurons from the DA-depleted side also exhibited a reversible D3R-mediated inhibition of Ca2+ current (Fig. 7, F and G; n = 3; see also Fig. 1D), but when exposed to 50 μM wortmannin, current only recovered partially (Fig. 7, H and I; n = 4): 81 ± 7% of recovery was attained in control conditions compared with 29 ± 10% in the presence of wortmannin (Fig. 7J; P < 0.05). In most neurons, wortmannin treatment caused a current inhibition on its own, a phenomenon that may suggest a role for the tonic PI 3-kinase activity in maintaining the function of Ca2+ channels.

Overall, the results from the two independent protocols described above (Figs. 6 and 7) show that D3R modulation in MSNs from the lesioned side can be completely explained by the sum of PP-2B action and PIP2 depletion. The first mechanism is present in neurons from both control and DA-depleted striata, whereas the second mechanism is only evident after DA depletion and explains in part the D3R supersensitivity. To further test this hypothesis, we then studied the D3R modulation in neurons in which the Ca2+ channels targeted by PP-2B, CaV1.5 (Hernandez-Lopez et al. 2000), were previously blockaded by 10 μM nitrendipine. Reduction of Ca2+ current by nitrendipine was 22 ± 7 and 26 ± 5% in MSNs from control and DA-depleted striata, respectively (Supplemental Fig. S6; NS), thus suggesting that they express the same amount of CaV1.5 channels (Prieto et al. 2009). As expected, blockage of CaV1.5 channels left a remaining D3R modulation in neurons from DA-depleted striata (19 ± 1%; Fig. 8A). However, Fig. 8B shows that intracellular PIP2 blockaded this remaining modulation (n = 7; P < 0.001). The histogram in Fig. 8C summarizes these results: in MSNs from the lesioned side, PIP2 depletion

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Fig. 6. Signaling through PP-2B and PIP2 mediates whole Ca2+ current modulation by D3R type in neurons from DA-depleted striatum. A and B: D3R-type response was isolated by adding 100 μM quinolorene after a prolonged exposure (10 min) to D2R-ant (50 nM L-741,626) and D4R-ant (50 nM L-750,667) in neurons from the lesioned side exposed to 1 μM FK506 (A) and in neurons exposed to both 1 μM FK506 and 10 μM PIP2 (B). C: histogram summarizes changes in D3R-type modulation under the described conditions. Compared with D3R-type modulation obtained under control conditions (without drugs, see Fig. 1H), there were significant reductions when cells were exposed to FK506 alone (*P < 0.05) and to FK506 + PIP2 (***P < 0.001). Remaining D3R modulation after FK506 exposure was inhibited by coadministration of PIP2 (*P < 0.05) so that both maneuvers abolished all D3R-type action.

DA-depleted striata (19 ± 1%; Fig. 8A). However, Fig. 8B shows that intracellular PIP2 blockaded this remaining modulation (n = 7; P < 0.001). The histogram in Fig. 8C summarizes these results: in MSNs from the lesioned side, PIP2 depletion
modulated Ca\(^{2+}\) current not blocked by nitrendipine. Therefore, the fraction of D3R modulation enhanced after DA deple-
tion, and blocked by PIP2 administration, does not target
CaV1 channels. Next, we wanted to find out which Ca\(^{2+}\) channel was regulated by enhanced D3R mod-
ulation after DA depletion.

Enhanced D3R-mediated modulation induced by DA deple-
tion targets CaV2.1 Ca\(^{2+}\) channels. MSNs express a variety of
Ca\(^{2+}\) channels (CaV1.2,3-LC,D; CaV2.1–3-N, P/Q, R) (Bargas
et al. 1994; Olson et al. 2005; Perez-Burgos et al. 2008;
Salgado et al. 2005). D2R-class receptors preferentially target
CaV1 and CaV2.1 channels (Hernandez-Lopez et al. 2000;
Salgado et al. 2005). CaV2.1 channels are modulated by PIP2
depletion induced by muscarinic M1 receptor activation (Perez-
Burgos et al. 2010). Therefore, a main suspect to explain
D3R-type modulation induced by DA depletion was the CaV2.1
channel. We used 400 nM \(\omega\)-agaTx-TK to block CaV2.1
channels to see whether this blockade would reduce D3R-type
modulation to levels similar to those found before DA deple-
tion. \(\omega\)-agaTx-TK blocked a similar amount of Ca\(^{2+}\) current:
39 \pm 5 and 33 \pm 4\% in neurons from control and DA-depleted
striata, respectively (Supplemental Fig. S6; NS). This result
confirms that MSNs from both control and DA-depleted sides
express the same amount of CaV2.1 channels (Prieto et al.
2009). Moreover, \(\omega\)-agaTx-TK did not significantly modify
D2R or D3R modulations (Fig. 9A; \(n = 7\); NS) in control
neurons.

In contrast, enhanced D3R modulation seen after DA
deployment was abolished by \(\omega\)-agaTx-TK (Fig. 9B). Remain-
ing modulation (12 \pm 4\% from 33\% modulation without the
toxin; Fig. 9C; \(n = 7\); \(P < 0.01\)) was not significantly
different from that observed in MSNs from the control side
(non-DA-depleted, 13 \pm 2\%), supporting the notion that
extra D3R modulation induced by DA depletion targets
CaV2.1 channels, making this modulation as important as
that exerted on CaV1 channels (Hernandez-Lopez et al.
2000).

It was therefore demonstrated that CaV1 and CaV2.1 chan-
nels comprise all modulated current by D3R in MSNs from
DA-depleted striata. CaV1 channels are regulated by PP-2B in
neurons from both control and DA-depleted striata (Hernandez-
Lopez et al. 2000; Perez-Burgos et al. 2008), whereas CaV2.1
channel regulation by PIP2 depletion is only manifested after
DA depletion.
DISCUSSION

Novel findings of this work include the following. 1) D2R-class supersensitivity following DA depletion, measured as increased Ca2+ current modulation, can in part be attributed to an enhanced D3R-type activity, present in a large fraction of indirect pathway neurons and a small percentage of direct pathway neurons. 2) Enhanced D3R-type activity unveiled a signaling branch of the PLC pathway that had not previously been described as being associated with dopaminergic modulation in this system: the depletion of PIP2, CaV2.1 channels were the targets of PIP2 signaling. 3) Although supersensitivity of other receptor types (D2R and D4R) after DA depletion has previously been suggested (Gerfen et al. 1990; Newman-Tancredi et al. 2001; Prieto et al. 2009; Qin et al. 1994), the amount of Ca2+ current modulated by them is not altered in the 6-OHDA-denervated striatum. All increase in the $E_{\text{max}}$ for Ca2+ current modulation by quinelorane after DA depletion (Prieto et al. 2009) is virtually due to enhanced D3R-type modulation. Supersensitivity contributed by other receptor types belonging to the D2R class deserves its own study to relate their signaling with some excitability parameter (Azdad et al. 2009).

Enhanced D3R-type modulation. In addition to exhibiting an increased sensitivity to the D2R-class general agonist quinlorane (a leftward shift in the whole C-R plot), the C-R plot for Ca2+ current modulation in MSNs from the 6-OHDA-lesioned side exhibited several components, one of which corresponds to a site with nanomolar sensitivity (Prieto et al. 2009) and an associated increase in $E_{\text{max}}$. We tried to isolate the actions of the various DA receptor types (Surmeier et al. 1996) to determine whether this sensitive site corresponds to a particular receptor type. We found evidence that it corresponds to D3R. Indeed, reported affinity of D3R for quinlorane (about 1 nM) (Coldwell et al. 1999; Gackenheimer et al. 1995) closely matches the IC50 of the sensitive site unveiled by DA depletion: 0.8 nM (Prieto et al. 2009). Different experimental evidence supports the pharmacological and electrophysiological separation of D3R responses from other dopamine receptors, in particular, D2R. First, D3R actions could be observed in neurons that had no D2R response, including neurons from the BAC D1R eGFP mice. Second, a signaling pathway and a Ca2+ channel class (CaV2.1) associated with D2R activity. Third, two different combinations of selective receptor antagonists administered at concentrations at least 20 times over its reported dissociation constant yielded the same result: only the D3R response was enhanced following DA depletion. However, pharmacological techniques have limitations. Therefore, further evidence employing other techniques is necessary to better support the present data. At this time, there is complementary biochemical evidence of enhanced D3R activity after DA depletion in different brain regions that had no D2R activity.
areas, including the striatum (Gurevich et al. 1999; Ishibashi et al. 2002; Levant 1995; Ridray et al. 1998; Sato et al. 1994).

Previous reports were contradictory because they tried to evaluate a change in expression when a mixture of D1R sources was present in the striatal preparation: both MSNs and DA terminals express the receptor (Hurley et al. 1996; Mercuri et al. 1997; Quik et al. 2000; Sokoloff et al. 1990; Stanwood et al. 2000). Therefore, enhanced expression in one source (e.g., striatal neurons) could be compensated with less protein from the other source (fewer DA terminals after denervation). To avoid this problem, we directly recorded D1R function from one of the sources, MSNs. The result was that an increase in activity of D1R was demonstrated.

In our hands, neither the expression (current density) nor the function of the molecular effectors, CaV2.1 channels, were altered as a result of DA depletion (Prieto et al. 2009). Hence, functional changes observed could solely be attributed to D1R adjustments. DA depletion induced an imbalance in the expression of D1R and its splice variant, D1nf: the latter was decreased in the lesioned side. Changes in the expression of D1nf could be associated with the reduced activation of D1R after DA depletion, as has been suggested for D2R activity, which regulates the expression of its splicing isoforms D2LR and D2SR (Sasabe et al. 2011). In support of this hypothesis, it was recently reported that D1nf levels in the brain are influenced by DA signals: D1R mRNA expression was not modified after its blockade, although D1nf mRNA was reduced (Richtand et al. 2010), leading to an increase in the D1R-D1nf mRNA ratio during the “hypodopaminergic state” (Richtand et al. 2010). This result closely matches our biochemical and pharmacological findings. Interestingly, complementary evidence was reported in chronic schizophrenia (a “hypodopaminergic state”), where expression of D1nf is increased (Schmauss 1996). Therefore, the hypothesis of D3nf as a negative regulator of D1R (Elmhurst et al. 2000) is attractive, suggesting that a reduced expression of D3nf may contribute to the D1R hyperactivity (Fig. 11). Nonetheless, the detailed molecular mechanism involved in the enhancement of the D1R activity in the DA-depleted striatum remains to be investigated (Elmhurst et al. 2000; Karpa et al. 2000).

Among other functions, CaV2.1 channels control GABA release in adult MSN terminals (Salgado et al. 2005). D1R-class negative modulation of GABA release from MSN terminals has been documented (Guzmán et al. 2003; Salgado et al. 2005; Tecuapetla et al. 2007), as well as D1R-class positive modulation among direct pathway MSN synapses (Guzmán et al. 2003; Tecuapetla et al. 2007). Similarly, in the pallidionigral pathway, D1R activation decreases inhibition, whereas D1R blockade increases inhibition (Aceves et al. 2011). That is, D1R tonically turns up inhibition to the required level by sensing extracellular DA. However, after DA depletion, high affinity and supersensitivity of D1R in the presence of very low DA concentrations may greatly decrease inhibition among striatal MSNs (Taverna et al. 2008; Tecuapetla et al. 2007), thus impairing microcircuit dynamics (Carrillo-Reid et al. 2008). In contrast, a subpopulation of low threshold spike (LTS) interneurons greatly increase their inhibitory output after DA depletion, eliciting sudden episodes of giant inhibitory synaptic potentials (Dehorster et al. 2009). Coincident with these circuit events in monkeys rendered parkinsonian with l-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a partial agonist of D1R, induces a reduction in dyskinesias, perhaps by controlling LTS output, and at the same time stops interfering with l-dihydroxyphenylalanine (l-DOPA) therapeutic
actions on the other receptor types (Bezard et al. 2003), thus restoring microcircuits dynamics (Carrillo-Reid et al. 2008; Jáidar et al. 2010). To conclude, when in vitro and in vivo data are taken together, they suggest that a control of D₃R supersensitivity is essential for improving L-DOPA actions, movement performance, and the control of dyskinesias, as has been observed by employing more selective D₃R partial agonists (e.g., rotigotine and pramipexole) (Scheller et al. 2009). By contrast, D₃R antagonists worsen both Parkinsonian and dyskinetic states (Bezard et al. 2003). In other words, although most dopamine receptor functions can be restored with L-DOPA, in need of L-DOPA (Aceves et al. 2011; Taverna et al. 2008). These arguments illustrate that detailed studies of the different components of denervation-induced supersensitivity are necessary to improve therapeutics.

**PIP₂ depletion: signaling mechanism for D₃R-type modulation of Ca²⁺ current**. A detailed study of the participation of D₃R type within D₂R-class supersensitivity has not been reported, therefore we focused on signaling and molecular targets of D₃R-type modulation. An array of signaling molecules has been linked to D₃R-class and, in particular, to D₃R type: adenyllyl cyclase, PKA, PKB/Akt, PKC, PLC, PLD, PP-2B, and ERK (Ahlgren-Beckendorf and Levant 2004; Beaulieu et al. 2007; Chen et al. 2009; Everett and Senogles 2004; Griffon et al. 1997; Pedrosa et al. 2004). The enhanced D₃R activity demonstrated in this study was found to be associated with an alternative branch of the PLC cascade, PIP₂ depletion, thus adding another member to the list. The target of this pathway was the Caᵥ₂.1 channel, a channel sensitive to PIP₂ depletion in MSNs (Perez-Burgos et al. 2010). PIP₂ depletion by D₃R has not been previously associated with dopaminergic signaling in control/naive striata; we found that this mechanism is unveiled only after DA depletion.

In neuronal cell lines, D₃R-mediated inhibition of calcium channels is prevented by pertussis toxin treatment, thus involving a Gₛₒ-dependent mechanism (Kuzhikandathil and Oxford 1999; Seabrook et al. 1994). Coupling of D₃R to Gₛₒ proteins does not exclude the activation of PLC, since it has been observed after the activation of D₃R-class in medium spiny neurons (Hernandez-Lopez et al. 2000). In our study, U-73122, a PLC inhibitor, and m-3M3-FBS, a PLC activator, significantly antagonized D₃R-induced Ca²⁺ current inhibition, thus indicating that D₃R action depends on PLC activation. In MSNs, the canonical pathway following PLC activation by D₂R-class (diacylglycerol-IP₃/Ca²⁺ release/Ca²⁺/CaM) leads to PP-2B activation and the subsequent inhibition of Caᵥ₁ channels (Hernandez-Lopez et al. 2000). After inhibition of PP-2B with FK506, a substantial D₃R-mediated modulation of Ca²⁺/CaM channels was still observed in neurons from the DA-depleted side. Notably, the remaining FK506-insensitive D₃R modulation was found to be dependent on PIP₂ depletion.
Thus, when PP-2B was inhibited with FK506 and PIP2 levels were clamped by including 10 μM PIP2 in the recording pipette, all D3R action was blocked. The role of PIP2 depletion in the D3R-mediated Ca2+ current inhibition was further supported by the following findings: clamping intracellular PIP2 levels partially reduced D3R action, and when PIP2 synthesis was blocked by wortmannin-induced inhibition of PI 4-kinase, the D3R-mediated current inhibition was only partially reversible.

Once activated by GPCRs, PLC catalyzes the hydrolysis of PIP2. However, it has been proposed that PIP2 depletion can be prevented by the receptor-activated synthesis of PIP2 (Xu et al. 2003), thus suggesting that the mechanisms involved in the turnover of PIP2 might be impaired in the denervated striatum. Resynthesis of PIP2 might also be dampened in the 6-OHDA model as a consequence of an enhanced catabolism of membrane phospholipids. It has been suggested that D3R-class supersensitivity enhances the hydrolysis of phosphatidylinositol and phosphatidylethanolamine, the substrates of PLA2 (Hayakawa et al. 2001). Moreover, both PLA2 activity and expression have been found to be upregulated in the DA-depleted striatum (Lee et al. 2010). Because diacylglycerol is a common intermediate in the synthesis of phosphatidylinositol, phosphatidylethanolamine, and PIP2, an increased catabolism of membrane phospholipids might locally compromise their resynthesis and then contribute to the PIP2 depletion in specific microdomains following DA denervation.

U-73122 and m-3M3-FBS also significantly antagonized D3R-induced Ca2+ current inhibition in neurons from DA-depleted striatum, thus indicating that this D3R-class action depends on PLC activation. However, D3R modulation was not prevented in MSNs from either the control or the DA-depleted side when intracellular PIP2 was controlled. This finding emphasizes that PLC activation is necessary but not sufficient to deplete PIP2 after GPCRs activation. Hence, PIP2 depletion by GPCRs and its consequences in the cell might be dependent on different variables, including the activation of parallel signal transduction pathways (e.g., hydrolysis of other phospholipids, see above) and the localization of the receptors. Differential redistribution of D2R and D3R after denervation (Cangiano 1985) may specifically facilitate the PIP2 depletion induced by D2R, but not by D3R, and its action on CaV2.1 channels. Interestingly, it has been shown that D3R-induced D3R-type interaction alters the cellular distribution of D3R (Karpa et al. 2000), supporting a model whereby reduced expression of D3R after DA depletion allows the proximity of D3R type and CaV2.1 (Fig. 11).

Functional consequences. Homeostatic mechanisms intended to maintain DA signaling in the striatum undergoing denervation include the spraying of DA terminals (Finkielstein et al. 2000). This finding is in agreement with the axonal restoration and spraying of the nigrostriatal pathway induced by activation of striatal D2R after 6-OHDA-lesions (Van Kampen and Eckman 2006). In the present work, we found supersensitive D3R in MSNs from the DA-depleted striatum. Therefore, contralateral rotation induced by D3R-class agonists in 6-OHDA-lesioned rats (Cai et al. 2000) commonly attributed to D2R-class supersensitivity and its enhanced signaling may be a substantial component of D3R supersensitivity.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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