Up-regulation of D$_2$-class signaling in dopamine-denervated striatum is in part mediated by D$_3$ receptors acting on CaV2.1 channels via PIP$_2$ depletion


Instituto de Fisiología Celular-Neurociencias. Universidad Nacional Autónoma de México, Mexico City, Mexico.

Running head: D$_3$R plasticity after denervation

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Address correspondence to: José Bargas, MD, PhD, Instituto de Fisiología Celular-Neurociencias. Universidad Nacional Autónoma de México (UNAM). Mexico City DF. PO Box 70-253. Mexico 04510. Tel.: (5255) 5622-5670, Fax: (5255) 5622-5607; E-mail: jbargas@ifc.unam.mx

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The loss of dopaminergic neurons in the substantia nigra compacta followed by striatal dopamine depletion is a hallmark of Parkinson’s disease. After dopamine depletion, dopaminergic D2-receptor class (D2R-class) supersensitivity develops in striatal neurons. The supersensitivity results in an enhanced modulation of Ca\[^{2+}\] currents by D2R-class receptors. However, the relative contribution of the D2R, D3R, and D4R types to the supersensitivity, as well as the mechanisms involved have not been elucidated. Here, whole-cell voltage-clamp recordings were performed to study Ca\[^{2+}\] current modulation in acutely dissociated striatal neurons obtained from rodents with unilateral 6-hydroxydopamine lesions in the substantia nigra compacta. Selective antagonists for D2R-, D3R- and D4R-types were used to identify whether the modulation by one of these receptors experiences a selective change after dopaminergic denervation. It was found that D3R-mediated modulation was particularly enhanced. Increased modulation targeted Ca\_\text{v}2.1 (P/Q) Ca\[^{2+}\]-channels via the depletion of phosphatidylinositol-4,5-biphosphate (PIP\_2); an intracellular signaling cascade hard to detect in control neurons and hypothesized as being amplified by dopamine depletion. An imbalance in the striatal expression of D3R and its splice variant D3nf accompanied enhanced D3R activity. Because Ca\_\text{v}2.1 Ca\[^{2+}\]-channels mediate synaptic \(\gamma\)-aminobutyric acid (GABA) release from the terminals of striatal neurons, reinforcement of their inhibition by D3R may explain in part the profound decrease in synaptic strength in the connections among striatal projection neurons observed in the dopamine-depleted striatum.
Keywords: supersensitivity, medium spiny neurons, D3nf, 6-hydroxydopamine, Parkinson’s disease.

INTRODUCTION

Parkinson’s disease is accompanied 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 depletion (Creese et al. 1977; Gerfen et al. 1990; Gerfen et al. 2002). Both classes of DA receptors seem to be involved: The D1-receptor class (including the D1R- and D5R-types associated to Gs/olf), and the D2-receptor class (D2R-class) (including the D2R-, D3R- and D4R-types associated to Gi/o) (Neve et al. 2004).

Increased receptor expression associated with D2R-class supersensitivity in unilaterally DA-depleted animals has been correlated with an increase in turning behavior induced by D2R-class agonists (Schwarting and Huston 1996), a greater coupling of G-proteins to the receptors (Cai et al. 2002; Newman-Tancredi et al. 2001), an augmentation in the activation of phospholipase A2 (PLA2) (Hayakawa et al. 2001), extracellular signal-regulated kinase (ERK) (Cai et al. 2000), and modulation of Ca2+ currents in neurons from the globus pallidus (Stefani et al. 2002).

D2R-class activity depresses striatal medium spiny neurons (MSNs) excitability (Hernandez-Lopez et al. 2000) and synaptic GABA release (Guzman et al. 2003; Tecuapetla et al. 2007) in neurons obtained from control animals. These actions are in
part due to Ca\(^{2+}\) current inhibition (Olson et al. 2005; Salgado et al. 2005). Previously, we reported that Ca\(^{2+}\) current inhibition mediated by D\(_2\)-R-class activation reflected an enhanced sensitivity in the DA-denervated striatum from unilateral 6-OHDA-lesioned rats (Prieto et al. 2009). In this previous study, MSNs were exposed to different concentrations (ranging from 0.1 nM to 100 μM) of quinelorane, a D\(_2\)-R-class agonist. It was found that the concentration–response relationship (C–R plot) from DA-depleted striata exhibited a considerable leftward shift (change in IC\(_{50}\)) and presented an increased maximal response (E\(_{\text{max}}\)) compared to the C–R plot from control striata. In addition, the C–R plot from control striata was better fit with a two sites model, whereas the C–R plot obtained from DA-depleted striata was better fit by a three sites model. The additional site detected in DA-denervated striata represented about 20% of the whole inhibition and showed a high sensitivity for quinelorane (0.8 nM) (Prieto et al. 2009). Accordingly, we hypothesized that the enhanced Ca\(^{2+}\) current inhibition observed under DA-deprived conditions was mainly associated to one particular receptor. Therefore, to dissect the participation in supersensitivity of each receptor type belonging to the D\(_2\)-R-class, present in MSNs (Surmeier et al. 1996), the present work used quinelorane in the presence of very selective antagonists for different receptor types.

METHODS

6-OHDA lesion of the nigrostriatal pathway

Studies were approved by the UNAM Committee of Bioethics and followed the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.
(NIH approval number A5281-01). DA depletion after 6-OHDA has been previously described (Ungerstedt and Arbuthnott 1970). Briefly, anesthetized (ketamine-xylazine 87 and 13 mg/kg i.p., respectively) male Wistar rats (180–200g) 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-eGFP transgenic mice (30 days old) on an FVB background developed by the GENSAT project (Heintz 2004) were injected stereotaxically with 1 μg of 6-OHDA (SN: 3.40 mm caudal, 1.1 mm lateral to bregma, 4.4 mm ventral to the skull surface). Coordinates for rat and mice were set according to the atlas of Paxinos and Watson (Paxinos and Watson 1996), and Paxinos and Franklin (Paxinos and Franklin 2004), respectively. 6-OHDA solution (4 μg/μl saline with 0.2 mg/ml of ascorbic acid) was ejected at a rate of 0.1 μl/min. It was confirmed that this procedure efficiently reduces (>95%) the dopaminergic inervation in the striatum, as measured by TH staining (Fig S1). In addition, lesion of the SN did not induce an apparent non-specific damage in other neighboring nuclei. After 14-17 days, animals were selected based on their rotational behavior in response to amphetamine (4 mg/kg i.p; 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 weeks 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 thiourea, 0.2 of ascorbic acid; pH=7.4/HCl; 300±5 mOsm/l with glucose; saturated with 95% O₂ and 5% CO₂). The dorsal neostriatum was dissected from 300 μm thick sagittal slices and it was then incubated with 1 mg/ml of 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, 130 NaCl, 3 KCl, 5 BaCl₂, 2 MgCl₂, 10 HEPES, 10 glucose; pH=7.4/NaOH; 300±5 mOsm/l with glucose). eGFP-positive neurons were visualized using an UV lamp (X-Cite, EXFO, Ontario, Canada).

Voltage clamp recordings of calcium currents

Recordings were performed on medium-sized neostriatal neurons (10-12 μm main diameter; 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 Corp., Novato, CA) and fire polished prior to use. Whole-cell recordings were obtained with an Axopatch-200B patch-clamp amplifier (Axon Instruments, Foster City, CA) and controlled and monitored with pClamp program (version 8) with a 125 kHz DMA interface (Axon Ins.). 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, 0.1 leupeptin (pH=7.2/H₂SO₄; 280±5 mOsm/l).
Currents were recorded (carried by Ba\textsuperscript{2+}, a potent potassium channel blocker) while blocking Na\textsuperscript{+}-channels (1 µM tetrodotoxin). Current was carried by Ba\textsuperscript{2+}, however, as Ba\textsuperscript{2+} flows through Ca\textsuperscript{2+} channels, along the text it was referred as Ca\textsuperscript{2+} current. Intracellular potassium was replaced by 180 mM N-methyl-D-glucamine. Current-voltage relationships (I-V plots) were built, before and after drug applications, with both 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 D.C.-controlled microvalve system (Lee, Essex, CT). This method allowed reversible drug applications (Perez-Burgos et al. 2008).

Drugs

Chemicals used were: tetrodotoxin (TTX) (Alomone Labs, Jerusalem, Israel), ω-agatoxin TK (ω-agaTx-TK) (Peptides International, Louisville, KY), nitrendipine, quinelorane, L741,626, U99194A, L750,667 (Sigma), FK 506 (A.G. Scientific Inc., San Diego, CA), SL327, m-3M3-FBS, o-3M3-FBS (Tocris Cookson, Ellisville, MO) U73122, U73343 and PIP\textsubscript{2} (sn-1-stearoyl-2-arachidonoyl phosphatidylinositol-4,5-bisphosphate, Calbiochem, La Jolla, CA). Most active substances were dissolved in water to get stock solutions and after thawed added to the superfusate to give the final concentration. Nitrendipine, FK 506 SL327 and U73122 were prepared in 1% DMSO; m-3M3-FBS in 0.1% DMSO; PIP\textsubscript{2} in 0.1% chloroform. In each case control saline also contained DMSO or chloroform at the same final concentration.
Data analysis

Mean±S.E.M. of peak (maximum value) Ca\(^{2+}\) 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 non-paired 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 inhibitors cocktail (Roche Applied Science, Indianapolis, IN) by using a tissue grinder with teflon pestle. Homogenate was centrifuged at 2000 g for 5 min; supernatant was recovered and centrifuged at 10,000 g for 1h. Pellet was dissolved in the same solution and stored at -70°C. The protein content in the brain membrane preparation was determined by the Bradford assay using BSA as standard. Protein extracts (30-50 µg) were submitted to 10% SDS–PAGE. Resolved proteins were transferred onto nitrocellulose or PVDF membranes (Bio-Rad, Hercules, CA) and immunoblotted with the following antibodies: anti-D3R (1:500 dilution; Calbiochem), anti-D3nf (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-sodium/potassium ATPase alpha-1 (1:250; Affinity Bioreagents, Rockford, IL). Specificity of anti-D3R and anti-D3nf was demonstrated
using lysates from rat eyes (Fig. S2), tissue that does not express D₃R (Fujieda et al. 2003). Immune complexes were revealed by using appropriate peroxidase-conjugated secondary antibodies (Jackson Immuno-Research, 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, 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 (Chemicon, Temecula, CA; dilution 1:1000) in PBS containing 2.5% BSA and 0.1% Triton X-100. After washing 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. (Fig. S1).
RESULTS

Enhanced Ca\(^{2+}\) current modulation during D\(_2\)-R-class supersensitivity is in part due to the enhanced activity of D\(_3\)-R-type

Neurons were obtained from the non-injured 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). Ba\(^{2+}\) currents were evoked with depolarizing voltage commands using steps and ramp protocols (Fig. S3A, B). The match between both protocols after constructing the I-V plots suggests acceptable space-clamp and voltage control (Fig. S3C). For clarity, figures will only show I-V plots obtained with ramp commands (Perez-Rosello et al. 2005). Average Ca\(^{2+}\) current amplitude in neurons from control striatum was 375±22 pA (n=70) and it was 360±17 pA (n=126) in neurons from the DA-depleted side (NS). In agreement with previous data (Prieto et al. 2009), there were no significant differences in current density (current/whole cell capacitance) comparing the results from control and DA-depleted striata: 60±4 pA/pF and 62±3 pA/pF, respectively.

In a previous work, D\(_2\)-R-class supersensitivity was detected when using 0.1 nM – 100 \(\mu\)M quinelorane (Prieto et al. 2009). Under these conditions, D\(_2\)-R, D\(_3\)-R and D\(_4\)-R can be activated (Coldwell et al. 1999; Gackenheimer et al. 1995; Newman-Tancredi et al. 1997). Therefore, to dissect the role of D\(_2\)-R, D\(_3\)-R and D\(_4\)-R on Ca\(^{2+}\) current modulation, neurons were exposed to the D\(_2\)-R-class agonist quinelorane but in the presence of antagonists for the different receptor types. Quinelorane was used at 100 \(\mu\)M given that,
when measuring Ca\textsuperscript{2+} channels modulation, 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 D\textsubscript{2}R-class (> 20-1000 times compared to the other D\textsubscript{2}R-class). Based on 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 to block 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 (D\textsubscript{2}R-class responsive) neurons from both control and DA-depleted sides were 71% of tested neurons (n = 107/151).

Afterwards, quinelorane was administered together with 500 nM U99194A, a selective D\textsubscript{3}R-type antagonist at this concentration (Waters et al. 1993), plus 50 nM L750,667, a selective D\textsubscript{4}R-type antagonist at this concentration (Patel et al. 1996), so that responses induced by quinelorane were putative-D\textsubscript{2}R-type only (Figs. 1A,B). As percentages of responsive neurons in samples from both sides were comparable (see Fig 1C), measurements were pooled together. It was found that 68% of rat MSNs (n=19/28) responded to quinelorane during D\textsubscript{3}R- and D\textsubscript{4}R-type blockade; that is, they were D\textsubscript{2}R-type responsive, coincident with reported number of neurons belonging to the indirect pathway plus neurons that co-localize D\textsubscript{1}R- and D\textsubscript{2}R-types (Surmeier et al. 1996).

In these conditions, and once the remaining Ca\textsuperscript{2+} current was stable, D\textsubscript{3}R-type
antagonist was washed out to disclose D3R-type modulation of remaining Ca\textsuperscript{2+} current (Figs. 1A,B). D3R-type responsiveness was comparable when comparing samples from both sides and then results were pooled. D3R-type responsiveness was attained in n=17/28 of tested neurons (61%, Fig. 1C). Finally, D4R-type antagonist was also washed out to finally disclose D4R-type action on the remaining Ca\textsuperscript{2+} current. D4R-type responsiveness was also comparable when comparing samples from both sides; it was obtained in n=15/28 of tested neurons (54%, Fig 1C). Antagonists used during these experiments, 500 nM U99194A and 50 nM L750,667, did not significantly modify Ca\textsuperscript{2+} current by themselves (data not illustrated). Notice that not all neurons responding to the D2-class receptor 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 comparing control and DA-depleted sides (Fig. 1C). Percentages of responding neurons to each receptor type agree with scRT-PCR studies (Surmeier et al. 1996).

In particular, D3R-responsive neurons were: n=7/11 (64%) and n=10/17 (59%) in control and DA-depleted striata, respectively (Fig. 1C, NS). However, Ca\textsuperscript{2+} current inhibition by the putative D3R-type was significantly larger in neurons from DA-depleted striatum as compared to 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 D_3R.

A representative time course of one experiment is shown in Figure 1E. D_3R-type action was clear, reversible and reproducible in responsive neurons (dark gray background). It was also clearly independent from D_2R-type reversible action (light gray background, on and off arrows; n=10). Although agonist-induced D_2R desensitization has been described in dopaminergic neurons of the ventral tegmental area (Bartlett et al. 2005), in our Ca^{2+} current recordings D_2R 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 exhibit high affinity for all D_2R-class (< 100 nM) (Aceves et al. 2011; Coldwell et al. 1999; Gackenheimer et al. 1995; Newman-Tancredi et al. 1997), it was plausible that when using 100 μM quinelorane, D_3R and D_4R were only partially blocked by 500 nM U99194A and 50 nM L750,667, respectively. However, a clear disclosure of the action of these receptor types was obvious in each experiment after D_2R have been occupied by high agonist concentrations. Moreover, even if D_3R and D_4R actions are underestimated, the interest here relies 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, a different and independent protocol was used to stimulate D_3R (Figs. 1F, G): first, the cells were exposed to both D_2R- and D_4R-type antagonists (used at concentrations that block their respective
receptors: 50 nM L741,626 plus 50 nM L750,667) (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 procedure to isolate receptor-type actions yielded that 67% of tested neurons were D3R-responsive (n=10/15, from both control and DA-depleted sides), in complete agreement with the first protocol (see above), suggesting 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 Ca\(^{2+}\) current was significantly stronger in neurons obtained from DA-depleted striatum as compared to the controls (Fig. 1H) and in the same proportion.

Histograms in Figures 1D, H summarize the above results. Using the first protocol (Fig. 1D), percentages of D2R- D2R- and D4R-Ca\(^{2+}\) current inhibition distributed among the three receptor types were as follows: D2R: 33±4% in control side (n=8) and 35±3% in DA-depleted side (n=11; NS); D3R: 17±2% in control side (n=7) and 33±4% in DA-depleted side (n=10; P<0.05); D4R: 13±2% in control side (n=8) and 19±2% in DA-depleted side (n=7; NS). Noticeably, the only significant enhancement occurred for D3R-type modulation. Using the second protocol (Fig. 1H), putative D3R-type mediated modulation increased from 17±2% in the control side to 33±6% in neurons from the DA-depleted side (n=10; P<0.05). Noticeably, this D3R-type modulation enhancement with respect to the whole Ca\(^{2+}\) current (16%) matches the maximal response (E\(_{\text{max}}\)) increment (13%) associated with the D2R-class supersensitivity described in a previous work (Prieto et al. 2009). This percentage may seem small as compared to 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.

But, 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 D₃R activation did not respond to D₂R activation (n=2/6), suggesting that some direct pathway neurons may be D₃R-responsive (Hopf et al. 2003; Mizuno et al. 2007; Surmeier et al. 1996). Because bacterial artificial chromosome transgenic mice expressing the enhanced green fluorescent protein under the control of the promoter for D₁R-type (BAC D₁R eGFP, Fig. S4A) can be used to identify direct pathway neurons, we used these mice to confirm that a D₃R response can be obtained independently or in the absence of a D₂R response. Notably, D₃R response was not only obtained in isolation, but it was similarly enhanced by DA depletion in rat D₂R-unresponsive and mice BAC D₁R eGFP neurons (n=4/15; Fig. S4B,C). BAC D₁R eGFP mice were only used in these experiments. It was found that about 30% of BAC D₁R MSNs exhibited a D₃R 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 D₃R action are in complete agreement after using three different independent protocols that employ different drugs and animal species. The next step was to study signaling
mechanisms and functions of D3R after DA-depletion.

Molecular mechanisms underlying Ca\textsuperscript{2+} current modulation by D3R

Ca\textsuperscript{2+} channels can be modulated by G protein-coupled receptors (GPCRs) through two different mechanisms. One is a voltage-dependent mechanism (also referred 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 G\textsubscript{i/o} proteins, but insensitive to calcium chelators. By contrast, other mechanisms (also referred as the “slow” pathway) are voltage-independent, sometimes insensitive to pertussis toxin but sensitive to calcium chelators and mediated by second messengers (Tedford and Zamponi 2006). In MSNs, modulation of Ca\textsuperscript{2+} channels by D2R-class agonists, such as quinpirole and quininelorane, is mediated by a voltage-independent (“slow”) mechanism: it is affected by Ca\textsuperscript{2+} chelators and second messengers, but not by strong depolarizing pre-pulses (Hernandez-Lopez et al. 2000; Perez-Burgos et al. 2008; Salgado et al. 2005). It was then expected that D3R modulation of Ca\textsuperscript{2+} channels was not voltage regulated. To test this hypothesis the modulation of currents evoked by a step to 0 mV before and after a strong (+80 mV) depolarization (Fig. 2A, top) were compared, while D3R was activated with quininelorane (100 μM) in the presence of D2R- plus D4R- type blockade (see protocol 2 above, Fig. 1F-H). Modulation by D3R was not reversed by strong depolarizations in neurons from the DA-depleted side (Fig. 2A). Ca\textsuperscript{2+} current amplitude reductions were 38±5% and 36±1%, before and after a pre-pulse command to 80 mV, respectively (Fig. 2B; n = 6; NS). Equivalent results were found in control neurons (not illustrated). This result discards voltage G\textsubscript{βγ}-dependent
regulation as the mechanism to explain D₃R modulation, and confirmed that D₂R-class
use a “slow” pathway to modulate Ca²⁺ channels.

It has been described that D₂R-class activation in MSNs inhibits Ca²⁺ current by a
PLCβ-dependent signaling pathway which 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 Caᵥ1 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 FK 506 (1 µM) significantly reduced Ca²⁺ current modulation mediated by
both D₂R and D₃R (Fig. S5; n=6; P<0.05). Moreover, we now demonstrate 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 U73122 (10
µM; n=5), but not the inactive analog U73343 (10 µM; n=4), blocked most D₂R- and
D₃R-type modulations (Fig. 3A, B, E; P<0.01). Second, to further support the
participation of PLC in the signaling pathway activated by D₃R, a selective PLC activator
was used: m-3M3-FBS (25 µM; n=5) (Bae et al. 2003). This activator, but not the
inactive analog o-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, D₂R and D₃R modulations were greatly occluded (Figs. 3C, D,
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 Ca\textsuperscript{2+} current modulation by D\textsubscript{2}R and D\textsubscript{3}R activation in neurons from DA-depleted striata.

Following the previously described steps of this “slow” pathway, we next investigated whether D\textsubscript{3}R-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 naïve animals as it is in neurons from the control side (Fig. S5). Here we demonstrate that both D\textsubscript{2}R and D\textsubscript{3}R PP-2B modulations were inhibited by 1 \mu M of FK 506 (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. 4A; P<0.05).

Nonetheless, the action of D\textsubscript{3}R was reduced to 16±4% (Figs. 4A, C; n=6; P<0.05), so that the remaining FK 506-insensitive D\textsubscript{3}R-type modulation is substantial in denervated animals (about half the modulation mediated by D\textsubscript{3}R). Accordingly, we hypothesized that it could correspond to the extra D\textsubscript{3}R action induced by DA depletion (Fig. 1).

Next, we investigated whether the fraction of D\textsubscript{3}R 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). Ca\textsuperscript{2+} current modulation was studied while ERK activation was being inhibited by 20 \mu M SL327. Neither D\textsubscript{2}R nor D\textsubscript{3}R actions were prevented (Fig. 4B, C; n=5), suggesting that Ca\textsuperscript{2+} current modulation by D\textsubscript{2}R-class is not mediated by MAPK-ERK.

Clearly then, D\textsubscript{3}R modulates Ca\textsuperscript{2+} channels through a “slow” PLC-dependent pathway. However, in neurons from the lesioned side about half of D\textsubscript{3}R-type modulation cannot be explained neither by PP-2B nor ERK regulations. Importantly, this unexplained modulation may correspond to that appearing after DA depletion because both neurons
from control and lesioned animals had the same amount of PP-2B modulation. Recently, it has been shown that endogenous membrane phosphatidylinositol-4,5-bisphosphate (PIP$_2$) helps in maintaining the function of high-voltage activated Ca$^{2+}$ channels, and stimuli that activate PLC deplete PIP$_2$ and reduce Ca$^{2+}$ channels currents (Perez-Burgos et al. 2010; Rousset et al. 2004; Suh et al. 2010; Wu et al. 2002). Given that D$_3$R activates PLC in MSNs (Fig. 3), we hypothesized that D$_3$R modulates Ca$^{2+}$ channels by depleting PIP$_2$. Neurons were exposed to physiological levels of PIP$_2$ (10 $\mu$M; (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 Figures 5A,B shows that in MSNs from control side, D$_2$R and D$_3$R actions were not significantly affected by exposure to intracellular PIP$_2$. In contrast, D$_3$R modulation was significantly reduced to 15±5% (about half D$_3$R total modulation) by intracellular PIP$_2$ in neurons from DA-depleted striatum (Fig. 5D, E; n=5; P<0.05), while D$_2$R modulation was unaffected. Histograms in Figures 5C,F summarize these results: only D$_3$R actions were found enhanced in neurons from DA-depleted striatum and only this enhancement was abolished by PIP$_2$ exposure.

To support the contribution of both PP-2B activation and PIP$_2$ depletion to the D$_3$R-mediated modulation of Ca$^{2+}$ channels in MSNs from DA-depleted side, parallel experiments were carried out by activating D$_3$R directly: applying quinelorane 100 $\mu$M in the presence of D$_2$R- and D$_4$R-types antagonists (cf., Figs. 1D, E). Again, D$_3$R modulation was partially, but significantly, inhibited by 1 $\mu$M of FK 506; modulation was reduced to 17±2% (Fig. 6A; n=10). Importantly, when 1 $\mu$M of FK 506 was added to the extracellular solution and 10 $\mu$M PIP$_2$ was present in the recording pipette D$_3$R
modulation was completely inhibited (Fig. 6B; n=7). Histogram in Figure 6C summarizes the results. On an overall, these series of experiments clearly show that in neurons from DA-depleted striatum PP-2B regulates about half, and PIP2 depletion about the other half of D3R modulation of Ca\(^{2+}\) current (see above), the PP-2B mediated modulation is present in both neurons from control and DA depleted striata, while PIP2 mediated modulation only appears in neurons from DA depleted striata.

Hydrolysis and synthesis of PIP2 is a dynamical cellular process. For the synthesis of PIP2, phosphatidylinositol is phosphorylated at the inositol 4- and 5- positions by the PI-4-kinase and PIP-5-kinase, respectively. Recent evidence indicates that rephosphorylation of phosphoinositides (i.e. PIP2 synthesis) is necessary to attain reversibility of current when the modulation depends on PIP2 depletion by PLC (Perez-Burgos et al. 2010; Suh and Hille 2002; Suh et al. 2010). Therefore, to further support the involvement of PIP2 depletion as a mechanism for the D3R-mediated inhibition of Ca\(^{2+}\) channels, the recovery of current was evaluated when PIP2 re-synthesis was blocked by 50 \(\mu\)M wortmannin, inhibitor of PI-3-kinase and PI-4-kinase at this concentration (Nakanishi et al. 1995; Suh and Hille 2002). Recovery of current after D3R-mediated inhibition was clearly observed in neurons dissociated from control striatum (Fig. 7A, B; n=4), and it was not affected by 50 \(\mu\)M wortmannin (Fig. 7C, D; n=4): 85±6% of recovery was observed in control conditions, compared to 69±11% in the presence of wortmannin (Fig. 7E; NS). Neurons from DA-depleted side also exhibit a reversible D3R-mediated inhibition of Ca\(^{2+}\) current (Fig. 7F, G; n=3; see also Fig. 1D) but, when exposed to 50 \(\mu\)M wortmannin current only recovers partially (Fig. 7H, I; n=4): 81±7% of recovery was attained in control conditions, compared to 29±10% in the presence of...
wortmannin (Fig. 7J; P<0.05). In most neurons, wortmannin treatment caused a current 468 inhibition by its own, a phenomenon that may suggest a role for the tonic PI4-K activity 469 in maintaining the function of Ca\textsuperscript{2+} channels.

On an overall, the results from the two independent protocols above (Figs. 6 and 471 7) show that D\textsubscript{3}R modulation in MSNs from the lesioned side can be completely 472 explained by the sum of PP-2B action and PIP\textsubscript{2} depletion. The first mechanism is present 473 in neurons from both control and DA-depleted striata whereas the second mechanism is 474 only evident after DA depletion and explains in part the D\textsubscript{3}R-supersensitivity. To further 475 test this hypothesis, we then studied the D\textsubscript{3}R modulation in neurons in which the Ca\textsuperscript{2+} 476 channels targeted by PP-2B, Ca\textsubscript{V}1 (Hernandez-Lopez et al. 2000), were previously 477 blocked by 10 \textmu M nitrendipine. Reduction of Ca\textsuperscript{2+} current by nitrendipine was 22±7\% 478 and 26±5\%, in MSNs from control and DA-depleted striata, respectively (Fig. S6; NS). 479 Thus suggesting that they express the same amount of Ca\textsubscript{V}1 channels (Prieto et al. 2009). 480 As expected, blockage of Ca\textsubscript{V}1 channels left a remaining D\textsubscript{3}R modulation in neurons 481 from DA-depleted striata (19±1\%, Fig. 8A). However, Figure 8B shows that intracellular 482 PIP\textsubscript{2} blocked this remaining modulation (n=7; P<0.001). Histogram in Figure 8C 483 summarizes these results: in MSNs from the lesioned side, PIP\textsubscript{2}-depletion modulates Ca\textsuperscript{2+} 484 current not blocked by nitrendipine. Therefore, the fraction of D\textsubscript{3}R modulation enhanced 485 after DA depletion, and blocked by PIP\textsubscript{2} administration, does not target Ca\textsubscript{V}1 channels. 486 Then, we wanted to find out what Ca\textsuperscript{2+}-channel was regulated by enhanced D\textsubscript{3}R 487 modulation after DA depletion.
Enhanced $D_3$R-mediated modulation induced by DA depletion targets $Ca_{V2.1} Ca^{2+}$ channels

MSN express a variety of $Ca^{2+}$ channels ($Ca_{V1.2,3-L,C,D}, Ca_{V2.1-3-N,P/Q,R}$) (Bargas et al. 1994; Olson et al. 2005; Perez-Burgos et al. 2008; Salgado et al. 2005). $D_2$R-class preferentially target $Ca_{V1}$ and $Ca_{V2.1}$ channels (Hernandez-Lopez et al. 2000; Salgado et al. 2005). $Ca_{V2.1}$ channels are modulated by PIP$_2$-depletion induced by muscarinic $M_1$ receptors activation (Perez-Burgos et al. 2010). Therefore, a main suspect to explain $D_3$R-type modulation induced by DA depletion was the $Ca_{V2.1}$ channel. 100 nM $\omega$-agaTx-TK were used to block $Ca_{V2.1}$ channels to see whether this blockade reduces $D_3$R-type modulation to levels similar to those found before DA depletion. $\omega$-agaTx-TK blocked a similar amount of $Ca^{2+}$ current: 39±5% and 33±4%, in neurons from control and DA-depleted striata, respectively (Fig. S6; NS). This result confirms that MSNs from both control and DA-depleted sides express the same amount of $Ca_{V2.1}$ channels (Prieto et al. 2009). Moreover, $\omega$-agaTx-TK did not significantly modify $D_2$R or $D_3$R modulations (Fig. 9A; n=7; NS) in control neurons.

In contrast, enhanced $D_3$R modulation seen after DA depletion was abolished by $\omega$-agaTx-TK (Fig. 9B). Remaining modulation (12±4% from 33% modulation without the toxin; Fig. 9C; n=7; $P<0.01$), was not significantly different to that observed in MSNs from control side (non-DA-depleted, 13±2%), supporting the notion that extra $D_3$R modulation induced by DA depletion targets $Ca_{V2.1}$ channels, making this modulation as important as that exerted on $Ca_{V1}$ channels (Hernandez-Lopez et al. 2000).

It was therefore demonstrated that $Ca_{V1}$- and $Ca_{V2.1}$-channels 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 channels regulation by PIP2-depletion is only manifested after DA depletion.

Expression of D3R-type following DA depletion.

Immunoblots for D3R were performed using striatal membrane lysates. In agreement with the expected molecular mass of the rat D3R, a similar level of expression of the peptide at ~45 kDa was observed in naïve, control and DA-depleted striata (Fig. 10A, C; NS). Thus, variation in expression levels of D3R cannot explain physiological differences. Nonetheless, expression of a D3R splice variant, D3nf (~68 kDa) (Liu et al. 1994), was diminished in DA-depleted striata (Figs. 10B, C; P<0.05). D3nf does not bind DA but it is known that physically interacts with D3R and down-regulates its activity (Elmhurst et al. 2000; Karpa et al. 2000), suggesting that a decrease in this regulator may enhance D3R action after DA depletion.

DISCUSSION

Novel findings of this work include: 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 in a small percentage of direct pathway neurons. 2) Enhanced D3R-type action unveiled a signaling branch of the PLC-pathway that had not previously been described as
associated with dopaminergic modulation in this system: the depletion of PIP_2, Ca_v2.1 channels were the targets of PIP_2-signaling. 3) Although supersensitivity of other receptor types (D_2R and D_4R) 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 Ca^{2+} current modulated by them is not altered in the 6-OHDA-denervated striatum. All increase in the maximal response (E_max) for Ca^{2+} current modulation by quinelorane after DA depletion (Prieto et al. 2009) is virtually due to enhanced D_3R-type modulation. Supersensitivity contributed by other receptor types belonging to D_2R-class deserve their own studies to relate their signaling with some excitability parameter (Azdad et al. 2009). 4) Down-regulation of D_3nf variant in striatum accompanied the enhanced D_3R-type activity.

Enhanced D_3R-type modulation

In addition to exhibiting an increased sensitivity to the D_2R-class general agonist quinelorane (a leftward shift in the whole C-R plot), the C-R plot for Ca^{2+} current modulation in MSNs from the 6-OHDA-lesioned side exhibit several components, one of them corresponds to a site with nanomolar sensitivity (Prieto et al. 2009) and an associated increase in the maximal response (E_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 D_3R. Indeed, reported affinity of D_3R for quinelorane (about 1 nM) (Coldwell et al. 1999; Gackenheimer et al. 1995) closely matches the IC_{50} of the sensitive site unveiled by DA depletion: 0.8 nM (Prieto et al. 2009). Different experimental evidences support the
pharmacological and electrophysiological separation of D$_3$R responses from other dopamine receptors, in particular, D$_2$R: First, D$_3$R actions could be observed in neurons that had no D$_2$R response, including neurons from the eGFP D$_1$R BAC mice. Second, a signaling pathway and a Ca$^{2+}$ channel class (CaV2.1) associated with D$_3$R responses were not associated with D$_2$R 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 D$_3$R 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 D$_3$R activity after DA depletion in different brain 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 D$_3$R 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 (less DA-terminals after denervation). To avoid this problem, we directly recorded D$_3$R function from one of the sources: MSNs. The result was that an increase in activity of D$_3$R was demonstrated.

In our hands, neither the expression (current density) nor the function of the molecular effectors: Ca$^{2+}$ channels, are altered as a result of DA depletion (Prieto et al. 2009). Hence, functional changes observed can solely be attributed to D$_3$R adjustments.
DA depletion induced an imbalance in the expression of D₃R and its splice variant D₃nf: the latter is decreased in the lesioned side. Changes in the expression of D₃nf could be associated with the reduced activation of D₃R after DA depletion, as it 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 D₃nf levels in the brain are influenced by DA signals: D₃R mRNA expression was not modified after its blockade although D₃nf mRNA was found reduced (Richtand et al. 2010), leading to an increase in the D₃R/D₃nf 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 “hyperdopaminergic state”), where expression of D₃nf is increased (Schmauss 1996). Therefore, the hypothesis of D₃nf as a negative regulator of D₃R (Elmhurst et al. 2000) is attractive, suggesting that a reduced expression of D₃nf may contribute to the D₃R hyperactivity (Fig. 11). Nonetheless, the detailed molecular mechanism involved in the enhancement of the D₃R 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 MSNs terminals (Salgado et al. 2005). D₂R-class negative modulation of GABA release from MSNs terminals has been documented (Guzman et al. 2003; Salgado et al. 2005; Tecuapetla et al. 2007), as well as D₁R-class positive modulation among direct pathway MSNs synapses (Guzman et al. 2003; Tecuapetla et al. 2007). Similarly, in the pallidonigral pathway D₃R activation decreases inhibition whereas D₃R blockade increases inhibition (Aceves et al. 2011). That is, D₃R tonically turns up inhibition to the
required level by sensing extracellular DA. However, after DA depletion, high affinity
and supersensitivity of D₃R 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 sub-
population of low threshold spike (LTS) interneurons greatly increase their inhibitory
output after DA depletion eliciting sudden episodes of giant inhibitory synaptic potentials
(Dehorter et al. 2009). Coincident with these circuit events, in monkeys rendered
parkinsonian with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a partial
agonist of D₃R induces a reduction in dyskinesias, perhaps by controlling LTS output,
and at the same time, stops interfering with L-DOPA therapeutic actions on the other
receptor types (Bezard et al. 2003), thus restoring microcircuit 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 it has been
observed employing a D₃R more selective 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, while most
dopamine receptors functions can be restored with L-DOPA, a special and partial
agonistic action is necessary to control D₃R supersensitivity. When L-DOPA is not
present, low doses of DA are enough to partially decrease inhibition via D₃R in indirect
pathway neurons, while transmission from direct pathway neurons almost completely
collapse due to the lower affinity of D₁R; 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 in order to improve therapeutics.

**PIP2-depletion: signaling mechanism for D3R-type modulation of Ca^{2+} current**

A detailed study of the participation of D3R-type within D2R-class supersensitivity has not been reported, therefore, here we focused on signaling and molecular targets of D3R-type modulation. An array of signaling molecules have been linked to D2R-class and, in particular, to D3R-type: adenylyl 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 here 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 Ca_{V2.1} 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/naïve 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 G_{i/o}-dependent mechanism (Kuzhikandathil and Oxford 1999; Seabrook et al. 1994). Coupling of D3R to G_{i/o} proteins does not exclude the activation of PLC, as it has been observed after the activation of D2R-class in medium spiny neurons (Hernandez-Lopez et al. 2000). In our study, U73122, a PLC inhibitor, and m-3M3-FBS, a PLC activator, significantly antagonized D3R-induced Ca^{2+} current inhibition, thus indicating that this 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 FK 506, a substantial D₃R-mediated modulation of Ca²⁺ channels was still observed in neurons from the DA-depleted side. Notably, the remaining FK 506-insensitive D₃R modulation was found to be dependent on PIP₂ depletion. Thus, when PP-2B was inhibited with FK 506 and PIP₂ levels were clamped by including PIP₂ 10 μM 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 PI4-K, 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. Re-synthesis 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 D₂R-class supersensitivity enhances the hydrolysis of phosphatidycholine and phosphatidylethanolamine, the substrates of PLA₂ (Hayakawa et al. 2001). Moreover, both PLA₂ activity and expression have been found up-regulated in the DA-depleted striatum (Lee et al. 2010). As diacylglycerol is a common intermediate in the synthesis of phosphatidylcholine, phosphatidylethanolamine and PIP₂, an increased catabolism of these phospholipids after D₂R-class activation might
locally compromise their re-synthesis and then contribute to the PIP$_2$ depletion in specific microdomains following DA denervation.

U73122 and $m$-3M3-FBS (PLC inhibitor and PLC activator, respectively) also significantly antagonized D$_2$R-induced Ca$^{2+}$ current inhibition in neurons from DA-depleted striatum, thus indicating that this D$_2$R-action depends on PLC activation. However, neither in MSNs from the control nor from the DA-depleted side D$_2$R modulation was prevented when intracellular PIP$_2$ was controlled. This finding emphasizes that PLC activation is necessary but not sufficient to deplete PIP$_2$ after GPCRs activation. Hence, PIP$_2$ 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 D$_2$R and D$_3$R after denervation (Cangiano 1985) may specifically facilitate the PIP$_2$ depletion induced by D$_3$R, but not by D$_2$R, and its action on Ca$\gamma$2.1 channels. Interestingly, it has been shown that D$_{3nf}$-D$_3$R-type interaction alters the cellular distribution of D$_3$R (Karpa et al. 2000), supporting a model whereby reduced expression of D$_{3nf}$ after DA-depletion allows the proximity of D$_3$R-type and Ca$\gamma$2.1 (Fig. 11).

**Functional consequences**

Homeostatic mechanisms intended to maintain DA signaling in the striatum undergoing denervation include the sprouting of DA terminals (Finkels tein et al. 2000). This finding is in agreement with the axonal restoration and sprouting of the nigrostriatal pathway induced by activation of striatal D$_3$R 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 D2R-class agonists in 6-
OHDA-lesioned rats (Cai et al. 2000) commonly attributed to D2R-class supersensitivity
and its enhanced signaling, may have a substantial component of D3R supersensitivity.

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**FOOTNOTES**

**FIG. 1.** D3R-type modulation of Ca\(^{2+}\) current is enhanced in neurons from DA-depleted striatum. (A, B) I-V plots from medium sized neostriatal neurons acutely dissociated from rat brain, in control conditions (A), and after DA-depletion (B). Experiments were carried out in neurons exposed to 100 μM quinelorane in the presence of antagonists for D3R-type and D4R-type (“D3R-ant”= 500 nM U99194A and “D4R-ant”= 50 nM L750,667, respectively) to first reveal the D2R-type response (1), thereafter reveal the D3R-type response (2) after subsequent wash out of the D3R-antagonist and finally disclosed a D4R-type response (3) after wash out of D4R-antagonist. These particular neurons responded to the activation of all three D2R-class receptor types - but
not all neurons did. Notice a larger D₃R-type effect in neurons obtained from DA-depleted striatum. (C) Histogram summarizes the percentages of neurons that exhibited a response associated with each receptor-type. Similar percentages were found when comparing neurons from control and DA-depleted striatum. (D) Histogram summarizes the Ca²⁺ current modulation by each receptor-type in samples subject to protocol depicted in A, B. Only D₃R-type response was significantly larger in DA-depleted cells (*P<0.05). (E) Representative time-course of an experiment such as that in A- B: quinelorane modulates Ca²⁺ current via D₂R-type (arrows and light gray background) and D₃R-type (dark gray background). Actions were independent, reproducible and reversible. Bars indicate time intervals with D₂R- or D₄R-type selective antagonists. (F, G) D₃R-type response was also isolated by adding quinelorane (100 μM) during a prolonged exposure (10 min) to the D₂R-type antagonist (“D₂R-ant”= 50 nM L741,626) and “D₄R-ant” (50 nM) in control (F) and DA-depleted cells (G). Again, a more prominent D₃R-type action was observed in neurons from DA-depleted striatum. (H) Histogram summarizes the Ca²⁺ current modulation by putative D₃R-type in samples subject to protocol in F, G (*P<0.05).

**FIG. 2.** **D₃R-type modulation is not voltage-dependent.** (A) Neurons dissociated from ipsilateral striata of 6-OHDA-lesioned rats were subjected to a standard double pulse protocol (top). Current amplitude evoked by depolarizing commands to 0 mV before and after a 80 mV prepulse is the same (black trace). After exposure to 100 μM quinelorane in the presence of D₂R- and D₄R-type antagonists (50 nM L741,626 and L750,667,
respectively), current reduction was the same before and after the pre-pulse (gray trace). (B) Histogram summarizes the results of experimental sample (n = 6).

**FIG. 3.** **D3R-type modulation depends on PLC activation.** All experiments were carried out in neurons from DA-depleted striata exposed to 100 μM quinelorane in the presence of antagonists for D3R-type and D4R-type ("D3R-ant" = 500 nM U99194A and "D4R-ant" = 50 nM L750,667, respectively) to first reveal the D2R-type response (1), and then disclose the D3R-type response (2) after washing out of the D3R-antagonist. Compared to the effects of its inactive analog U73343 (10 μM, A), the PLC inhibitor U73122 (10 μM, B) greatly diminished both D2R- and D3R-type modulations (***P<0.01). Similarly, compared to the effects of its inactive analog o-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 (*P<0.05). (E) Histogram summarizes changes in D3R-type modulation under conditions described above.

**FIG. 4.** **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 quinelorane in the presence of antagonists for D3R-type and D4R-type ("D3R-ant" = 500 nM U99194A and "D4R-ant" = 50 nM L750,667, respectively) to first reveal the D2R-type response (1), and then reveal the D3R-type response (2) after subsequent wash out of the D3R-antagonist. Ca2+ current modulation by D2R- and D3R-types after PP-2B inhibition with 1 mM FK 506 (A) and after ERK inhibition with 20 mM SL327 (B). (C) Histogram summarizes changes in D3R-type modulation under described conditions: compared to
D₃R-type modulation obtained under control conditions (without drugs: see Fig. 1D), there were significant reductions when cells were exposed to FK 506 only (*P<0.05).

**FIG. 5. D₃R-type modulation is influenced by intracellular PIP₂ after DA-depletion, but not under control conditions.** Experiments were carried out in neurons from control (A-C) and DA-depleted striatum (D-F) as follows: All experiments were carried out in neurons exposed to 100 μM quinelorane in the presence of antagonists for D₃R-type and D₄R-type (“D₃R-ant”= 500 nM U99194A and “D₄R-ant”= 50 nM L750,667, respectively) to first reveal the D₂R-type response (1), and then reveal the D₃R-type response (2) after subsequent wash out of D₃R-antagonist. (A) D₂R- and D₃R-type responses in a control neuron. (B) Intracellular 10 μM PIP₂ neither blocked D₂R- nor D₃R-type responses in control neurons. (C) Histogram summarizes results from neurons from control side with and without intracellular PIP₂ (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 striata with and without intracellular PIP₂ (10 min). D₃R-type modulation was significantly reduced to about half by PIP₂ (*P<0.05), without affecting D₂R-type modulation.

**FIG. 6. Signaling through PP2B and PIP₂ mediates whole Ca²⁺ current modulation by D₃R-type in neurons from DA-depleted striatum.** (A, B) D₃R-type response was isolated by adding quinelorane (100 μM) after a prolonged exposure (10 min) to antagonists for D₂R-type (“D₂R-ant”= 50 nM L741,626) and for D₄R-type (“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 to 1 μM FK 506 and 10 μM PIP2 (B). (C) Histogram
summarizes changes in D3R-type modulation under described conditions: compared to
D3R-type modulation obtained under control conditions (without drugs: see Fig. 1H),
there were significant reductions when cells were exposed to FK 506 alone (*P<0.05) and
to FK 506 plus PIP2 (**P<0.001). Remaining D3R modulation after FK 506 was
inhibited by co-addition of PIP2 (*P<0.05) so that both maneuvers abolished all D3R-type
action.

FIG. 7. Recovery of Ca2+ current after D3R modulation is affected by the inhibition
of phosphoinositides kinases in MSNs from DA-depleted striatum only. D3R-type
response was isolated by adding quinelorane (100 μM) after the exposure to antagonists
for D2R-type (“D2R-ant”= 50 nM L741,626) and for D4R-type (“D4R-ant”= 50 nM L-
750,667) in neurons from control (A-E) and lesioned (F-J) sides. (A) Inhibition of Ca2+
current was recovered after the wash out of quinelorane in a neuron from control
striatum. The time course of this experiment is shown in (B). (C) A similar result was
observed when neurons were exposed to 50 μM wortmannin, a PI-3K and PI-4K
inhibitor. The time course is shown in (D). (E) Histogram summarizing the amount (%)
of reversion of the D3R modulation in neurons from control side with and without
wortmannin (NS). (F) Inhibition of Ca2+ current by D3R was also fully recovered after
wash out of quinelorane in a neuron from DA-depleted striatum. The time course of this
experiment is shown in (G). However, 50 μM wortmannin partially prevented
reversibility of D3R action on Ca2+ current in neurons from DA-depleted side (H). The
time course of this experiment is shown in (I). (J) Histogram summarizing the results obtained in neurons from DA-depleted side (*P<0.05).

**FIG. 8. Enhanced D3R-type Ca\(^{2+}\) current modulation induced by DA-depletion does not involve Ca\(_{\text{V}1}\) Ca\(^{2+}\)-channels.** Ca\(_{\text{V}1}\) channels were blocked with 10 μM nitrendipine. Experiments illustrated were carried out in neurons from the lesioned side exposed to 100 μM quinelorane in the presence of antagonists for D3R-type and D4R-type ("D3R-ant"= 500 nM U99194A and "D4R-ant"= 50 nM L750,667, respectively) to first reveal the D2R-type response (1), and then reveal the D3R-type response (2) after subsequent wash out of D3R-antagonist. (A) Ca\(_{\text{V}1}\) channels blockade reduced both D2R- and D3R-type modulations (*P<0.05). (B) PIP\(_2\) (10 μM) abolished the D3R-type modulation not blocked by nitrendipine (***P<0.001) without affecting remaining D2R-type modulation. (C) Histogram separates D2R- from D3R-type modulations and shows that after blockade of Ca\(_{\text{V}1}\) channels only D3R-type modulation is significantly reduced by PIP\(_2\) (*P<0.05).

**FIG. 9. D3R-type activation targets Ca\(_{\text{V}2.1}\) Ca\(^{2+}\)-channels after DA depletion.** 400 nM ω-agaTx-TK were used to block Ca\(_{\text{V}2.1}\) (P/Q) channels. Experiments were carried out in neurons from control (A) and DA-depleted (B) striatum exposed to 100 μM quinelorane in the presence of antagonists for D3R-type and D4R-type ("D3R-ant"= 500 nM U99194A and "D4R-ant"= 50 nM L750,667, respectively) to first reveal the D2R-type response (1), and then reveal the D3R-type response (2) after subsequent wash out of the D3R-antagonist. (A) Neither D2R- nor D3R-type modulations were significantly affected by Ca\(_{\text{V}2.1}\) blockade in neurons from control striata. (B) In neurons from DA-depleted
striata, blockade of CaV2.1 channels occluded the fraction of D3R-type modulation enhanced by DA-depletion. (C) Histogram shows that once CaV2.1 channels are blocked, D3R-type modulation is similar to the control non-DA-depleted neurons (NS). However, it is significantly reduced as compared to the modulation observed without ω-agaTx-TK (**P<0.01).

**FIG. 10. Immunoblotting detection of D3R and D3nf in striatal membrane lysates.**

(A-B) Protein extracts (30-50 mg) from naïve, control and DA-depleted striata were resolved in 10% SDS-PAGE, transferred to a nitrocellulose membrane and probed with an anti-D3R polyclonal antibody (A), or to a PVDF membrane and probed with an anti-D3nf antibody (B). Lysates from olfactory bulb were used as positive controls in (A). Signal from ATPase (~110 kDa) was used as a loading control for densitometry. (C) Relative levels of expression of D3R-type (left) and D3nf (right); *P<0.05. Results shown are representative from five independent experiments for each case.

**FIG. 11. A model that may explain the enhanced D3R-type modulation of Ca2+ current after DA depletion.** Under normal DA levels (upper), D3R-type modulates CaV1 (L-type) channels through a PLC/IP3/Ca2+/Ca2+Calmodulin/PP-2B signaling pathway. In this condition there is an interaction of D3R-type with its D3nf variant. After chronic DA depletion (lower), D3R-type-D3nf interaction is reduced as a result of the D3nf down-regulation (1). Membrane re-distribution of the “D3nf-free” D3R-type after DA depletion would situate it in close proximity to CaV2.1 channels (2), thus allowing that channels sense PIP2 depletion and reduce their opening after PLC activation by D3R
These changes lead to an enhanced D₃R-activity: D₃R-type retains its ability to inhibit Caᵥ1 channels but, in addition, a Caᵥ2.1 (P/Q-type) channels modulation is also manifested.

Supporting information

FIG. S1. Tyrosine hydroxylase immunoreactivity. To verify unilateral DA depletion, tyrosine hydroxilase (TH) staining was performed in a subset of 6-OHDA-lesioned rats 14-17 days after 6-OHDA injection. Figure shows that TH-immunoreactivity is reduced (>95%, see Methods) in the ipsilateral striatum.

Figure S2. Specificity of the anti-D₃R and anti-D₃nf antibodies. Proteins extracts (30-50 μg) from striata and eye obtained from at least two rats were resolved in 10% SDS-PAGE, transferred to a nitrocellulose membrane and probed with an anti-D₃R polyclonal antibody (A), or to PVDF membrane and probed with an anti-D₃nf antibody (B). Signal from ATPase (∼110 kDa) was used as a loading control. Note the predominant detection of a single band at ∼45 and ∼68 kDa in (A) and (B), respectively, and the absence of signal in the lane corresponding to eye sample.

FIG. S3. Ca²⁺ currents in medium sized neostriatal neurons. (A) Representative family of currents evoked by 20 ms voltage commands (on top) from –80 to 50 mV, in 10-mV steps in an acutely dissociated striatal neuron. Arrow indicates the time where measurements were taken. (B) Representative current evoked with a depolarizing ramp
command from -80 to 50 mV (on top: 180-ms; 0.7-mV/ms) in the same neuron. (C) Current-voltage relationships (I-V plots) built with measurements from A (symbols) and B (continuous line). Superimposition shows a match between both protocols. Whole cell capacitance = 6 pF. Similar results were obtained in neurons dissociated from DA-depleted striata and from non-injured animals (naïve striatum) (not shown but see Prieto et al. 2009)

FIG. S4. D₃R-type modulation of Ca²⁺ current is present and also enhanced in D₂R-type non-responding neurons as attested by the use of BAC D₁ eGFP neurons from DA-depleted striata. (A) Example of an acutely dissociated BAC D₁ eGFP neostriatal neuron (arrow). (B, C) Acutely dissociated BAC D₁R eGFP neostriatal neurons from control (B) and DA-depleted mice striata (C) were exposed to 100 μM quinelorane in the presence of antagonists for D₃R-type ("D₃R-ant", 500 nM U99194A) and D₄R-type ("D₄R-ant", 50 nM L750,667). Notice that these neurons had not observable D₂R-type response similarly to some rat neurons (1). However, wash out of D₃R-antagonist clearly revealed a D₃R-type response (2). In these particular cells, D₄R-antagonist wash out also exhibited a D₄R-type response (3). Only a minority of BAC D₁R eGFP neurons exhibited any D₂R-class response under this protocol. Still, notice a larger D₃R-type response in neurons from DA-depleted striatum.

FIG. S5. Modulation of Ca²⁺ current by both D₂R and D₃R is inhibited by FK506 in neurons from control side. Neurons from control side were exposed to 100 μM quinelorane in the presence of antagonists for D₃R-type and D₄R-type ("D₃R-ant"= 500
nM U99194A and “D₄R-ant”= 50 nM L750,667, respectively) to first reveal the D₂R-type response (1), and then reveal the D₃R-type response (2) after subsequent wash out of the D₃R-antagonist. (A) D₂R- and D₃R-type responses. (B) Inhibition of PP-2B with 1 mM FK 506 significantly reduced (~50%) the modulation by both D₂R- and D₃R-types. (B) Histogram summarizing the results obtained for the D₃R modulation in neurons from control side with (n=5) and without (n=10) FK506 (*P<0.05).

**Figure S6. Contribution of L- and P/Q-type currents to whole Ca²⁺ current does not change after DA depletion.** Histogram shows the amount of Ca²⁺ current blocked by 10 μM nitrendipine (nitren) and 400 nM ω-agatoxin-TK (ω-agaTx) in neurons obtained from control and DA-depleted striata. There were no differences comparing these conditions (NS).
(1): quinelorane + D₃R-ant + D₄R-ant
(2): D₃R-ant wash out
(3): D₄R-ant wash out

A

control side

B

DA-depleted side

C

responsive neurons (%)

D

control
DA-depleted

E

D₂R-action
D₃R-action

F

control side

G

DA-depleted side

H

Ca²⁺ current reduction (%)
Prieto et al. Fig.2

A

-80 mV

current (pA)

0 100 200

time (ms)

without pre-pulse

after pre-pulse

B

\[ \text{Ca}^{2+} \text{ current reduction (\%)} \]

-80 mV 0 mV 80 mV

 Prieto et al. Fig.2

A

-80 mV

current (pA)

0 100 200

time (ms)

without pre-pulse

after pre-pulse

B

\[ \text{Ca}^{2+} \text{ current reduction (\%)} \]

-80 mV 0 mV 80 mV

 Prieto et al. Fig.2
(1): quinelorane + D₃R-ant + D₄R-ant
(2): D₃R-ant wash out

A, B: w/ U73343
C, D: w/ U73122

E: Ca²⁺ current reduction by D₃R-type action (%)
(1): quinelorane + D₃R-ant + D₄R-ant
(2): D₃R-ant wash out

A

B

C

Prieto et al. Fig. 4
(1): quinelorane + D₃R-ant + D₄R-ant
(2): D₃R-ant wash out

**A**
control side

**B**
control side w/PIP₂

**C**

control
control w/PIP₂

**D**
DA-depleted side

**E**
DA-depleted side w/PIP₂

**F**

DA-depleted
DA-depleted w/PIP₂

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(1): quinelorane + D₃R-ant + D₄R-ant
(2): D₃R-ant wash out

**Prieto et al. Fig. 5**
Prieto et al. Fig.6

A

DA-depleted side w/ FK506

B

DA-depleted side w/ FK506 + PIP₂

C

- DA-depleted
- DA-depleted w/FK506
- DA-depleted w/FK506 + PIP₂

(1): + D₂R-ant + D₄R-ant
(2): + quinelorane

Ca²⁺ current reduction by D₃R-type action (%)
(1): D2R-ant + D4R-ant
(2): +quinelorane
(3): wash out quinelorane

Prieto et al. Fig. 7
(1): quinelorane + D₃R-ant + D₄R-ant
(2): D₃R-ant wash out

Prieto et al. Fig. 8
(1): quinelorane + D₃R-ant + D₄R-ant  
(2): D₃R-ant wash out

A control side

B DA-depleted side

C

Prieto et al. Fig.9

- **Ca²⁺ current reduction (%)**
- control w/ ω-agaTx
- DA-depleted w/ ω-agaTx
- DA-depleted

D₂R  D₃R
Prieto et al. Fig. 10

A

(kDa)

55-

D₃R

150-

ATPase

B

(kDa)

72-

D₃nf

150-

ATPase

C

Expression level

naive

control

DA-depleted

D₃R-type

D₃nf

*
不做假，图中展示了一个神经递质系统的过程。

**Naive**

1. **D3nf** downregulation
2. **CaV2.1** inhibition

**DA-depleted**

1. **D3nf** downregulation
2. **CaV2.1** inhibition