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 deple-
tion (Creese et al. 1977; Gerfen et al. 1990, 2002). Both classes of DA receptors seem to be involved: the D₁ receptor (D₁R) class (including the D₁R and D₃R types associated to Gi/o) (Neve et al. 2004).

Increased receptor expression associated with D₁R-class supersensitivity in unilaterally DA-depleted animals has been correlated with an increase in turning behavior induced by D₁R-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) and extracellular signal-regulated kinase (ERK) (Cai et al. 2000), and modulation of Ca²⁺ currents in neurons from the globus pallidus (Stefani et al. 2002).

D₁R-class activity depresses striatal medium spiny neuron (MSN) excitability (Hernandez-Lopez et al. 2000) and synaptic GABA release (Guzmán et al. 2003; Tecuapetla et al. 2007) in neurons obtained from control animals. These actions are in part due to Ca²⁺ current inhibition (Olson et al. 2005; Salgado et al. 2005). Previously, we reported that Ca²⁺ current inhibition mediated by D₁R-class activation reflected an enhanced sensitivity in the DA-denervated striatum from unilaterally 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 quinolinol, a D₁R-class agonist. It was found that the concentration-response relationship (C-R plot) from DA-depleted striata exhibited a considerable lef-
tward shift (change in IC₅₀) and presented an increased maximal response (Eₘₐₓ) compared with the C-R plot from control striata. In addition, the C-R plot from control striata was better fit with a two-site model, whereas the C-R plot obtained from DA-depleted striata was better fit by a three-site model. The additional site detected in DA-denervated striata represented about 20% of the whole inhibition and showed a high sensitivity for quinolenol (0.8 nM) (Prieto et al. 2009). Accordingly, we hypothesized that the enhanced Ca²⁺ 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 D₁R class, present in MSNs (Surmeier et al. 1996), the present work used quinolenol in the presence of very selective antagonists for different receptor types.

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; 4.8 mm caudal, 1.6

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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 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 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 thio urea, and 0.2 ascorbic acid; pH 7.4 adjusted with HCl; 300 ± 5 mosM with glucose; saturated with 95% O2 and 5% CO2). The dorsal neostriatum was dissected from 300-µm-thick sagittal slices 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 firepolished Pasteur pipettes. Cell suspension was plated into a petri dish formed 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 by 10.220.33.5 on June 22, 2017 http://jn.physiology.org/ Downloaded from

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 current 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, Foster City, CA) and controlled and monitored with pClamp software (version 8) with a 125-kHz direct memory access interface (Axon Instruments, Foster City, CA). Cell suspension was plated into a petri dish formed on medium-sized neostriatal neurons (main diameter 10–12 µm, whole cell capacitance 6–7 pF) (Bargas et al. 1994). The 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, Foster City, CA). Cell suspension was plated into a petri dish formed on medium-sized neostriatal neurons (main diameter 10–12 µm, whole cell capacitance 6–7 pF) (Bargas et al. 1994). The 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, Foster City, CA)

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). Ca2+ 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 Ca$$^{2+}$$ 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 Ca$$^{2+}$$ current modulation, we exposed neurons to the D2R-class agonist quinolone in the presence of antagonists for the different receptor types. Quinolone was used at 100 μM, given that when Ca$$^{2+}$$ 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 D2R class (>20–1,000 times compared with the other D2R 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). Quinolone-responsive (D2R-class responsive) neurons from both control and DA-depleted sides were 71% of tested neurons (n = 107/151).

Afterward, quinolone was administered together with 500 nM U-99194A, a selective D2R-type antagonist at this concentration (Waters et al. 1993), plus 50 nM L-750,667, a selective D4R-type antagonist at this concentration (Patel et al. 1996), so that responses induced by quinolone were putative-D2R 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 quinolone during D2R- and D4R-type blockade; that is, they were D2R-type responsive, coincident with the reported number of neurons belonging to the indirect pathway plus neurons that colocalize D3R and D2R types (Sutin et al. 1996).

In these conditions, and once the remaining Ca$$^{2+}$$ current was stable, D3R-type antagonist was washed out to disclose D2R-type modulation of remaining Ca$$^{2+}$$ current (Fig. 1, A and B). D2R-type responsiveness was comparable when samples were compared from both sides, and results were then pooled. D2R-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$$^{2+}$$ current. D4R-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 Ca$$^{2+}$$ current by themselves (data not shown). Notice that not all neurons responding to the D2R-class agonist (quinolone) 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 striata 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, D4R-responsive neurons were obtained in n = 7/11 (64%) and n = 10/17 (59%) control and DA-depleted striata, respectively (Fig. 1C; NS). However, Ca$$^{2+}$$ 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 D3R.

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 Ca$$^{2+}$$ 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 quinolone exhibits its high affinity for all D2R-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 quinolone 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 D3R 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 D4R (Fig. 1, F and G). First, the cells were exposed to both D2R- and D4R-type antagonists (used at concentrations that block their respective receptors: 50 nM
et al. 1996), and then quinelorane (100 nM L-741,626) would first act on D3R. 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. 1A), in complete agreement with previous observations (Kulagowski et al. 1996; Patel et al. 1996), and that quinelorane (100 µM) was introduced to act only on D3R. Note that in these experiments, underestimation of actions would rely on D2R and D4R, because the high agonist concentration would first act on D3R. This alternative isolation procedure to isolate receptor-type actions showed 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 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 modulation enhancement with respect to the whole Ca2+ current (16%) matches the E_max.
increment (13%) associated with the D_{2R}-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 D_{3R} 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 D_{1R} and D_{3R} 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 D_{3R} activation did not respond to D_{1R} activation (n = 2/6), suggesting that some direct pathway neurons may be D_{3R} 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 D_{1R} type (BAC D_{1R} eGFP; Supplemental Fig. S4A) can be used to identify direct pathway neurons, we used these mice to confirm that a D_{3R} response can be obtained independently or in the absence of a D_{1R} response. Notably, a D_{3R} response was not only obtained in isolation, but it was similarly enhanced by DA depletion in rat D_{2R}-nonresponsive and mouse BAC D_{1R} eGFP neurons (n = 4/15; Supplemental Fig. S4, B and C). BAC D_{1R} eGFP mice were only used in these experiments. It was found that about 30% of BAC D_{1R} MSNs exhibited a D_{3R} 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 D_{3R} 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 D_{3R} after DA depletion.

**Molecular mechanisms underlying Ca^{2+} current modulation by D_{3R}.** Ca^{2+} 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 G_{i/o} 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 Ca^{2+} chelators and mediated by second messengers (Tedford and Zamponi 2006). In MSNs, modulation of Ca^{2+} channels by D_{3R}-class agonists, such as quinpirole and quinelorane, is mediated by a voltage-independent (slow) mechanism: it is affected by Ca^{2+} chelators and second messengers but not by strong depolarizing prepulses (Hernandez-Lopez et al. 2000; Perez-Burgos et al. 2008; Sallago et al. 2005). It was then expected that D_{3R} modulation of Ca^{2+} channels was not voltage regulated. To test this hypothesis, we compared the modulation of currents evoked by a step to 0 mV before and after a strong (+80 mV) depolarization (Fig. 2A, inset) while D_{1R} was activated with quinelorane (100 μM) in the presence of D_{2R} plus D_{4R}-type blockade (see 2nd protocol described above, Fig. 1, F–H). Modulation by D_{3R} was not reversed by strong depolarizations in neurons from the DA-depleted side (Fig. 2A). Ca^{2+} current amplitude reductions were 38 ± 5 and 36 ± 1%, respectively, before and after a prepulse command to 80 mV (Fig. 2B; n = 6; NS). Equivalent results were found in control neurons (not shown). This result discards voltage G_{i/o}-dependent regulation as the mechanism to explain D_{3R} modulation and confirmed that D_{3R}-class receptors use a slow pathway to modulate Ca^{2+} channels.

It has been described that D_{3R}-class activation in MSNs inhibits Ca^{2+} 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 Ca_{v}1.1 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 Ca^{2+} current in neurons obtained from the control side of hemiparkinsonian rats: inhibition of PP-2B with FK506 (1 μM) significantly reduced Ca^{2+} current modulation by 10.2 ± 0.33.5 on June 22, 2017 http://jn.physiology.org/ Downloaded from D_{3R} current modulation (%)

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Fig. 2. D_{3R}-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 quinolone in the presence of D_{3R}-ant (50 nM L-741,626) and D_{1R}-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).
analog U-73343 (10 μM; n = 4), blocked most D2R- and D3R-type modulations (Fig. 3, A, B, E; P < 0.01). Second, to further support the participation of PLC in the signaling pathway activated by D2R, we used a selective PLC activator: 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 (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 D2R and D3R activation in neurons from DA-depleted striata.

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. 4A; 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-
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, D3R and D2R 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+ chan-

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**Fig. 5.** D3R-type modulation is influenced by intracellular phosphatidylinositol 4,5-bisphosphate (PIP2) after DA deple-

tion but not under control conditions. 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 D4R-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: D3R- and D4R-type responses in a control neuron. B: intracellular 10 μM PIP2 blocked neither D3R- nor D4R-type responses in control neurons. C: histogram summarizes results from neurons from control side with and without intracellular PIP2 (no significant difference, NS). D: D3R- and D4R-type responses in a neuron from DA-depleted striatum. E: intracellular 10 μM PIP2 reduced D3R- but not D4R-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 one-half by PIP2, without affecting D2R-type modulation (*P < 0.05).
channels in MSNs from the DA-depleted side, we carried out parallel experiments by activating D₃R directly, applying 100 µM quinelorane 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 strata, respectively (Supplemental Fig. S6; NS), dipine was 22% when 10 µM nitrendipine. Reduction of Ca²⁺ current by nitrendipine was 82 ± 7% (Fig. 6F; n = 4): 81 ± 7% 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 4-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 (Hernandez-Lopez et al. 2000), were previously blocked 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 channels (Prieto et al. 2009). As expected, blockage of Caᵥ₁ channels left a remaining D₃R modulation in neurons from

| D₃R PLASTICITY AFTER DENERVATION |

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 quinelorane 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 blocked 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 D_{3}R 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 D_{3}R modulation after DA
depletion.

Enhanced D_{3}R-mediated modulation induced by DA depletion

targets CaV_{2.1} Ca^{2+} channels. MSNs express a variety of
Ca^{2+} channels (CaV_{1.2,3-LC,D}, CaV_{2.1–3-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 receptors preferentially target
CaV_{1} and CaV_{2.1} channels (Hernandez-Lopez et al. 2000;
Salgado et al. 2005). CaV_{2.1} channels are modulated by PIP2
depletion induced by muscarinic M_{1} receptor activation (Perez-
Burgos et al. 2010). Therefore, a main suspect to explain
D_{3}R-type modulation induced by DA depletion was the CaV_{2.1}
channel. We used 400 nM ω-agaTx-TK to block CaV_{2.1}
channels to see whether this blockade would reduce D_{3}R-type
modulation to levels similar to those found before DA depletion.
ω-agaTx-TK blocked a similar amount of Ca^{2+} current:
39 ± 5 and 33 ± 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 CaV_{2.1} channels (Prieto et al.
2009). Moreover, ω-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 ω-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 from that observed in MSNs from the control side
(non-DA-depleted, 13 ± 2%), supporting the notion that
extra D_{3}R modulation induced by DA depletion targets
CaV_{2.1} channels, making this modulation as important as
that exerted on CaV_{1} channels (Hernandez-Lopez et al.
2000).

It was therefore demonstrated that CaV_{1} and CaV_{2.1} channels
comprise all modulated current by D_{3}R in MSNs from
DA-depleted striata. CaV_{1} 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 CaV_{2.1}
channel regulation by PIP2 depletion is only manifested after
DA depletion.

Fig. 7. Recovery of Ca^{2+} current after D_{3}R
modulation is affected by the inhibition of
phosphoinositide (PI) kinases in medium
spiny neurons (MSNs) from DA-depleted
striatum only. D_{3}R-type response was iso-
lated by adding 100 μM quinelorane after
exposure to D_{2}R-ant (50 nM L-741,626) and
D_{4}R-ant (50 nM L-750,667) in neurons from
control (A–E) and lesioned sides (F–J). A: inhi-
bition of Ca^{2+} current was recovered
after washout of quinelorane in a neuron from
control striatum. The time course of this ex-
periment is shown in B. C: a similar result
was observed when neurons were exposed to
50 μM wortmannin, a PI 3-kinase and PI
4-kinase inhibitor. The time course is shown
in D. E: histogram summarizes the amount
(%) of reversal of the D_{3}R modulation in
neurons from the control side with and with-
out wortmannin (NS). F: inhibition of Ca^{2+}
current by D_{3}R was also fully recovered after
washout 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 D_{3}R action on Ca^{2+} current in neurons
from the DA-depleted side (H). The time
course of this experiment is shown in I. J:
histogram summarizes the results obtained
in neurons from the DA-depleted side. *P <
0.05. Wortma, wortmannin.

(1): D_{2}R-ant + D_{4}R-ant
(2): +quinelorane
(3): wash out quinelorane

(1): Control
(2): Control
(3): Control

(1): Control
(2): Control
(3): Control

(1): Control
(2): Control
(3): Control

(1): Control
(2): Control
(3): Control

(1): Control
(2): Control
(3): Control

(1): Control
(2): Control
(3): Control

(1): Control
(2): Control
(3): Control

*P < 0.05. Wortma, wortmannin.
explain physiological differences. Nonetheless, expression of a D3R splice variant, D3nf (~68 kDa) (Liu et al. 1994), was diminished in DA-depleted striata (Fig. 10, B and C; \( P < 0.05 \)). D3nf does not bind DA, but it is known to physically interact with D3R and downregulate 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 the following. 1) D2R-class supersensitivity following DA depletion, measured as increased Ca\(^{2+}\) 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 (D3R 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 Ca\(^{2+}\) current modulated by them is not altered in the 6-OHDA-denervated striatum. All increase in the \( E_{\text{max}} \) for Ca\(^{2+}\) current modulation by quinolene after DA depletion (Prieto et al. 2009) is virtually due to enhanced D3R-type modulation. Supersensitivity contributed by other receptor types belonging to the D3R class deserves its own study to relate their signaling with some excitability parameter (Azdad et al. 2009). 4) Downregulation of D3nf variant in striatum supported the enhanced D3R-type activity.

**Enhanced D3R-type modulation.** In addition to exhibiting an increased sensitivity to the D3R-class general agonist quinolene (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 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 quinolene (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 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 Ca\(^{2+}\) channel class (CaV2.1) associated with D3R activity unveiled a supersensitive activity at the site with nanomolar sensitivity (Prieto et al. 2009) is virtually due to enhanced D3R-type modulation. Supersensitivity contributed by other receptor types belonging to the D3R class deserves its own study to relate their signaling with some excitability parameter (Azdad et al. 2009). 4) Downregulation of D3nf variant in striatum accompanied the enhanced D3R-type activity.

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 naive, control, and DA-depleted striata (Fig. 10, A and C; NS). Thus variation in expression levels of D3R cannot
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 D3R 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 D3R function from one of the sources, MSNs. The result was that an increase in activity of D3R was demonstrated.

In our hands, neither the expression (current density) nor the function of the molecular effectors, Ca2+ channels, were altered as a result of DA depletion (Prieto et al. 2009). Hence, functional changes observed could solely be attributed to D3R adjustments. DA depletion induced an imbalance in the expression of D3R and its splice variant, D3nf, the latter was decreased in the lesioned side. Changes in the expression of D3R could be associated with the reduced activation of D3R after DA depletion, as has been suggested for D2R activity, which regulates the expression of its splicing isoforms D2LA and D2SB (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 reduced (Richtand et al. 2010), leading to an increase in the D3R-to-D3nf mRNA ratio during the "hypodopaminergic state" (Richtand et al. 2010). This result closely matches our biochemical and pharmacological findings. Interestingly, complementatory evidence was reported in chronic schizophrenia (a "hypodopaminergic 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, Ca2+ channels control GABA release in adult MSN terminals (Salgado et al. 2005). D3R-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 D3R-class positive modulation among direct pathway MSN synapses (Guzmán et al. 2003; Tecuapetla et al. 2007). Similarly, in the pallidonoigral 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 subpopulation of low threshold spike (LTS) interneurons greatly increase their inhibitory output after DA depletion, eliciting sudden episodes of giant inhibitory synaptic potentials (Dehorer et al. 2009). Coincident with these circuit events in monkeys rendered parkinsonian with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a partial agonist of D3R, 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 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 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, 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, whereas transmission from direct pathway neurons almost completely collapses due to the lower affinity of D3R, 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: adenyl 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 Gi/o-dependent mechanism (Kuzhikandathil and Oxford 1999; Seabrook et al. 1994). Coupling of D3R to Gi/o 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 D3R-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+ 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.

**DA-depleted**

Fig. 11. A model that may explain the enhanced D3R-type modulation of Ca2+ current after DA depletion. Under normal DA levels (top), D3R type modulates CaV1 (L-type) channels through a PLC/IP3/Ca2+/CaM/calmodulin/PP-2B signaling pathway. In this condition there is an interaction of D3R type with its D3nf variant. After chronic DA depletion (bottom), D3R type-D3nf interaction is reduced as a result of the D3nf downregulation (1). Membrane redistribution of the “D3nf-free” D3R type after DA depletion would situate it in close proximity to CaV2.1 channels (2), thus allowing channels to sense PIP2 depletion and reduce their opening after PLC activation by D3R. These changes lead to an enhanced D3R-type activity: D3R type retains its ability to inhibit CaV1 channels, but, in addition, a CaV2.1 (P/Q-type) channel modulation is also manifested.
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, phosphatidylethanolamine, and PIP2, an increased calcium influx, phosphatidylethanolamine, and PIP 2, an increased calcium influx by 102, 340, 270, and 130%, respectively. 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 these phospholipids after D3R-class activation 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 D3R and D3R after denervation (Cangiano 1985) may specifically facilitate the PIP2 depletion induced by D3R, but not by D2R, and its action on Ca2+ channels.

Interestingly, it has been shown that D1R-D3R-type interaction alters the cellular distribution of D3R (Karpa et al. 2000), supporting a model whereby reduced expression of D1R after DA depletion allows the proximity of D3R type and Ca2+ channels (Fig. 11).

**Functional consequences.** Homeostatic mechanisms intended to maintain DA signaling in the striatum undergoing denervation include the spraying of DA terminals (Finkelstein et al. 2000). This finding is in agreement with the axonal restoration and spraying of the nigrostriatal pathway induced by activation of striatal D3R 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 D3R-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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