Genetic Inactivation of Dopamine D1 but Not D2 Receptors Inhibits L-DOPA–Induced Dyskinesia and Histone Activation

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Background: Pharmacologic studies have implicated dopamine D1-like receptors in the development of dopamine precursor molecule 3,4-dihydroxyphenyl-L-alanine (L-DOPA)-induced dyskinesias and associated molecular changes in hemiparkinsonian mice. However, pharmacologic agents for D1 or D2 receptors also recognize other receptor family members. Genetic inactivation of the dopamine D1 or D2 receptor was used to define the involvement of these receptor subtypes.

Methods: During a 3-week period of daily L-DOPA treatment (25 mg/kg), mice were examined for development of contralateral turning behavior and dyskinesias. L-DOPA-induced changes in expression of signaling molecules and other proteins in the lesioned striatum were examined immunohistochemically.

Results: Chronic L-DOPA treatment gradually induced rotational behavior and dyskinesia in wildtype hemiparkinsonian mice. Dyskinetic symptoms were associated with increased FosB and dynorphin expression, phosphorylation of extracellular signal-regulated kinase, and phosphoacetylation of histone 3 (H3) in the lesioned striatum. These molecular changes were restricted to striatal areas with complete dopaminergic denervation and occurred only in dynorphin-containing neurons of the direct pathway. D1 receptor inactivation abolished L-DOPA-induced dyskinesias and associated molecular changes. Inactivation of the D2 receptor had no significant effect on the behavioral or molecular response to chronic L-DOPA.

Conclusions: Our results demonstrate that the dopamine D1 receptor is critical for the development of L-DOPA-induced dyskinesias in mice and in the underlying molecular changes in the denervated striatum and that the D2 receptor has little or no involvement. In addition, we demonstrate that H3 phosphoacetylation is blocked by D1 receptor inactivation, suggesting that inhibitors of H3 acetylation and/or phosphorylation may be useful in preventing or reversing dyskinesia.

Key Words: Dopaminergic denervation, dynorphin, ERK1/2, FosB, Parkinson's disease, phosphoacetylated histone 3

Parkinson disease (PD) is caused by degeneration of midbrain dopaminergic neurons that project to the striatum. Despite extensive investigation and new therapeutic approaches, the dopamine precursor molecule 3,4-dihydroxyphenyl-L-alanine (L-DOPA) remains the most effective and most commonly used noninvasive treatment for PD. However, chronic treatment and disease progression lead to changes in the brain's response to L-DOPA, resulting in a lower therapeutic window and the appearance of abnormal involuntary movements. These movements, known as dyskinesias, interfere significantly with normal motor activity and are associated with changes in striatal gene expression.

Our hypothesis is that these changes are the result of intermittent stimulation of supersensitive dopamine receptors in denervated striatal neurons (1). These receptors have increased coupling to $G\alpha_{olf}$ (2), resulting in greater stimulation of adenylyl cyclase, which activates the extracellular signal-regulated kinase (ERK) pathway (3) and triggers posttranslational modification of histones (4), leading to gene transcription (5). All dopamine receptor (R) subtypes (D1-D5) are present in the striatum, although D1R and D2R are the most abundant. These two dopamine receptors exhibit opposite functions, and their expression is segregated: D1R and D2R are expressed in neurons of direct and indirect striatal output pathways, respectively. Some molecular changes correlated with dyskinesias such as increased FosB and dynorphin expression are confined to D1R-containing neurons, whereas p-ERK and Nurr1 expression have been described in both D1R- and D2R-containing neurons (6,7). Although dopamine receptors are clearly involved in dyskinesias, the contribution of each dopamine receptor subtype has not been demonstrated definitively, and the signaling pathways that trigger long-term changes that maintain dyskinesias are not fully defined.

Pharmacologic studies implicate both the D1/D5 and D2/D3 receptor families in the development of dyskinesias. In patients, chronic treatment with a nonselective dopamine agonist with a short plasma half-life is more likely to induce dyskinesia than treatment with D2R agonists with long plasma half-lives (8). In rodents, dyskinesias can be induced by D1-type (D1/D5) or D2-type (D2/D3) agonists (9–12) with D1/D5 agonists having the most powerful dyskinetogenic effect (7,12,13). Consistent with this, D1/D5 antagonists are more effective inhibitors of L-DOPA-induced dyskinesia than D2 antagonists (7,12–14).

Because D1 receptors greatly outnumber D5 receptors in the striatum, it is tempting to assume that the striatal actions of mixed D1/D5 ligands are due to the D1 receptor. However, there are several examples in which the less abundant dopamine receptor is the major player for specific functions. In the hippocampus, D5R are much more abundant than D1R, but the D5 receptors do not play a role in spatial learning or hippocampal long-term potentiation, whereas D1 receptors are critical in these processes (15). In addition, within the striatum itself, where D1 is predominant, D1 and D5 receptors are equally required for striatal

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synaptic plasticity and play different roles in corticostriatal long-term synaptic transmission (16). Thus, it is essential to test the hypothesis that D1 and not D5 receptors are responsible for the development of abnormal movements and molecular changes following L-DOPA. Currently, available pharmacologic tools do not distinguish between different subtypes within the same family (e.g., between D1 and D5 or between D2 and D3) (17,18). Using our mouse model of dyskinesias (3) and knockout technology, we investigated the specific roles of the D1R and D2R in the development of dyskinesia and associated molecular changes after chronic L-DOPA treatment.

Methods and Materials

Animals

This study was carried out in mice lacking D1R (15,16,19,20) or D2R (21–23) generated by homologous recombination as described previously. Wildtype (WT) and homozygote $D1^{-/-}$ and $D2^{-/-}$ (knockout [KO]) mice used in this study were derived from mating heterozygous mice. Genotype was determined by polymerase chain reaction analysis. The maintenance of animals followed guidelines from European Union Council Directive (86/609/European Economic Community) and experimental protocols were approved by the Consejo Superior de Investigaciones Científicas Ethics Committee.

Procedure for intrastriatal 6-hydroxydopamine (6-OHDA) administration was described previously (3,24). Mice recovered for 4 weeks after the lesion and then began L-DOPA methyl ester (Sigma-Aldrich, Madrid, Spain) treatment. During the 3 week treatment, 6-OHDA or sham-operated animals received a daily injection of 10 mg/kg benserazide hydrochloride (Sigma-Aldrich), a peripheral blocker of L-DOPA decarboxylase, followed 20 min later by an intraperitoneal injection of 25 mg/kg of L-DOPA. Control animals were injected with an equivalent amount of saline. Rotational behavior and dyskinesias were studied in the same group of animals, on alternate days, twice a week for each behavior as described previously (3).

Immunohistochemistry

Following behavioral analysis, animals were anesthetized and perfused with 4% paraformaldehyde in phosphate buffer (pH 7.4) 1 hour or 20 min (animals used for p-ERK immunohistochemistry) after the last L-DOPA/saline injection. Immunostaining was carried out in free-floating coronal brain sections (40 μm thick) using a standard avidin-biotin immunocytochemical protocol (25,26) with the following rabbit antisera: tyrosine hydroxylase (TH; 1:1000; Chemicon, Temecula, California), FosB (1:10,000, Santa Cruz Biotechnology, Santa Cruz, California), dynorphin-B (dyn-B; 1:10,000, Serotec, Oxford, United Kingdom), met-enkephalin (met-Enk; 1:1000, Incstar, Stillwater, Minnesota), phosphop44/42 mitogen-activated protein (MAP) kinase (Thr202/Tyr204; p-ERK1/2; 1:250; Cell Signaling Technology, Beverly, Massachusetts), and antiphospho (Ser10)-acetyl (Lys14)-Hystone 3 (p-AcH3; 1:500; Upstate, Cell Signaling Solutions, Lake Placid, New York) and mouse monoclonal met-Enk antisera (1:10; 27). Double-labeling immunohistochemistry protocols are described in Supplement 1.

Quantification of TH, FosB, dyn B, p-ERK, and p-AcH3 immunoreactivity was carried out using an image analysis system (AIS, Imaging Research, Linton, United Kingdom) as in Granado *et al.* (28). For both lesioned and unlesioned striatal sides, immunostaining intensity and number of immunolabeled nuclei/cells were determined in 4–6 animals per group using five serial rostrocaudal sections per animal and three counting frames (dorsal, dorsolateral, and lateral) per section (.091 mm^2 each frame). Images were digitized with Leica microscope under 40× lens. Before counting, images were thresholded at a standardized gray-scale level, empirically determined by two observers to allow detection of stained nuclei/cells from low to high intensity, with suppression of lightly stained nuclei (15). The data are presented as number of stained nuclei per mm^2 (mean ± standard error) in the lesioned and unlesioned striatum. Immunostaining intensities in the lesioned side are presented as fold increase over the value from the unlesioned striatum.

Statistical Analysis

Behavior was analyzed using repeated measures three-way analysis of variance (ANOVA; contralateral turns) or two-way ANOVA (dyskinesia scores) using time, lesion/treatment (sham/L-DOPA, lesion/saline, lesion/L-DOPA) and genotype (WT, KO) or time and genotype as the within and between-subject variables, respectively, followed by planned comparisons (a priori analysis). Quantifications of immunolabeling were analyzed by two-way ANOVA with genotype and lesion as between-subject variables followed by Scheffe post hoc test. Immunostaining intensities were compared by Student's *t* test. Data are expressed as mean ± standard error of mean. Statistical significance level was set at $p < .05$.

Results

Dopamine D1, but Not D2, Receptors Are Required for Rotational Sensitization Induced by L-DOPA in Hemiparkinsonian Mice

To establish the role of the D1 and D2 dopamine receptor subtypes in the development of behavioral sensitization and dyskinesias, we used genetically engineered mice lacking either the dopamine D1 or D2 receptor. Contralateral rotations were evaluated as a measure of behavioral sensitization to L-DOPA (25 mg/kg/day) for 3 weeks (3). Twice a week, we measured the total number of contralateral turns for 120 min. We also counted the contralateral turns during the first 20 min after L-DOPA administration to assess changes in time to onset of the response (Figure 1). As observed previously (3), in hemiparkinsonian WT littermates of both knockouts ($D1R^{WT}$ and $D2R^{WT}$), L-DOPA induced a gradual increase in contralateral turns in both the 20- and 120 min windows (Figure 1). However, in $D1R^{-/-}$ animals, contralateral rotations were completely abolished, for both time intervals (Figure 1A and 1B). In contrast, there was no significant difference between the rotational behavior of $D2R^{-/-}$ mice and $D2^{WT}$ mice at any point during chronic L-DOPA treatment (Figure 1C and 1D). Rotational behavior of the $D2^{WT}$ and $D1^{WT}$ animals was similar (Figure 1A and 1C).

Dopamine D1, but Not D2, Receptors Are Required for the Development of Dyskinesia Following L-DOPA Treatment in Hemiparkinsonian Mice

To measure L-DOPA-induced dyskinesias in hemiparkinsonian mice, we assessed orofacial, limb, and locomotive dyskinesia and axial dystonia. Dyskinetic symptoms were monitored twice a week over the 3-week course of the treatment as previously described (3). In WT animals, the time course and intensity of the L-DOPA response was similar to that found in an earlier study (3). Dyskinesias are already apparent at 2 days of treatment, with dyskinetic symptoms at maximal intensity 30 min and 60 min after L-DOPA, decreasing gradually to baseline values

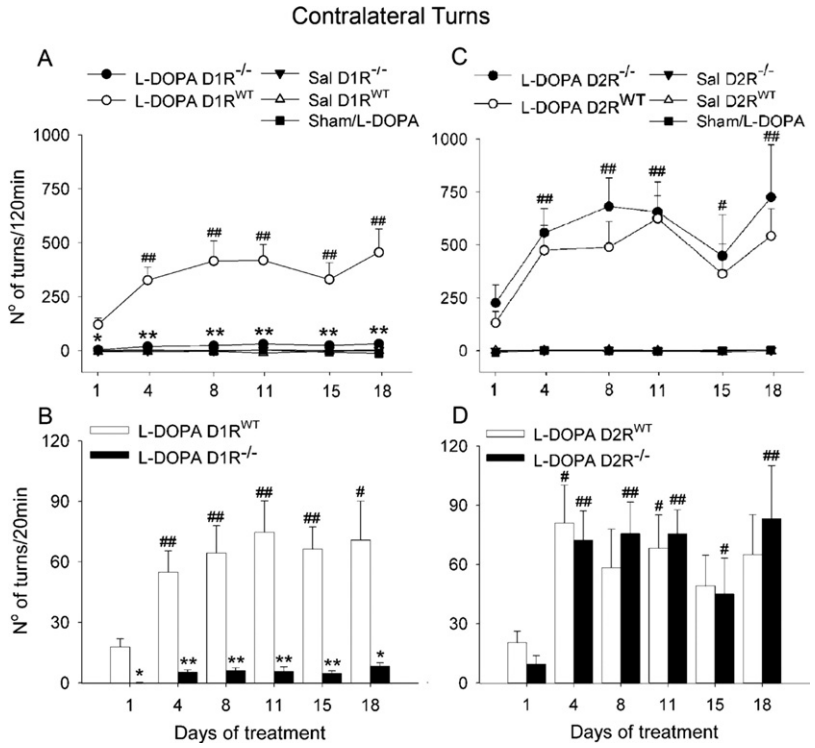

Figure 1. Genetic inactivation of dopamine D1 receptors blocked L-DOPA-induced dyskinesias in hemiparkinsonian mice. Time course for dopamine precursor molecule 3,4-dihydroxyphenyl-L-alanine (L-DOPA)-induced contralateral turning in hemiparkinsonian $D1R^{-/-}$ **(A)** and $D2R^{-/-}$ **(C)** mice and their wildtype (WT) littermates. Data points indicate the total number of contralateral turns (mean ± SEM) in 120 min following L-DOPA (25 mg/kg) administration. **(A)** Inactivation of D1R completely abolished turning behavior. Three-way analysis of variance (ANOVA) with repeated measures showed significant differences for genotype [$F(1,33) = 9.5$; $p = 4 \times 10^{-3}$], treatment [$F(2,33) = 15.8$; $p = 1.5 \times 10^{-5}$], genotype × treatment [$F(2,33) = 11.8$; $p = 1.4 \times 10^{-4}$] and treatment × time [$F(10,165) = 3$; $p = 1.8 \times 10^{-3}$]. **(C)** Inactivation of D2R has no effect on turning behavior. Statistical analysis showed significant differences for treatment [$F(2,36) = 21.7$; $p = 6.6 \times 10^{-7}$], time [$F(5,180) = 3.8$; $p = 2.5 \times 10^{-3}$], and treatment × time [$F(10,180) = 4.1$; $p = 4.7 \times 10^{-4}$]. All lesioned WT and $D2R^{-/-}$ mice treated with L-DOPA exhibit significantly more contralateral turns than control animals (sham/L-DOPA and 6-OHDA/saline) at all time points. **(B and D)** Histograms show the number of contralateral turns in the initial 20 min following L-DOPA administration. **(B)** Inactivation of D1R abolished behavioral sensitization. Two-way ANOVA with repeated measures showed significant differences for genotype [$F(1,15) = 14.1$; $p = 4 \times 10^{-3}$] and time [$F(5,75) = 2.7$; $p = 2.8 \times 10^{-2}$]. **(D)** Inactivation of D2R had no effect on behavioral sensitization. Statistical analysis showed significant differences for time [$F(5,80) = 7.8$; $p = 4.8 \times 10^{-6}$]. $^{*}p < .01$; $^{**}p < .0001$ versus wildtype $^{\#}p < .05$; $^{\#\#}p < .001$ versus first day of treatment after two or three-way ANOVA with repeated measures and planned comparisons, $n = 5$–10 per group. In Histogram B, differences versus first day of treatment are shown together for $D2R^{-/-}$ and $D2R^{WT}$ animals.

by 2 hours (data not shown). Because the dyskinesia scores at 30 and 60 min after L-DOPA administration were equivalent, we present only the 30 min scores (Figure 2; see also Figure 1 and 2A in Supplement 1). In contrast, $D1R^{-/-}$ animals showed a near-complete absence of orofacial and limb dyskinesias and very low-grade axial dystonia and locomotive dyskinesias (Figure 2). $D1R^{-/-}$ animals exhibit significantly less of all four dyskinetic symptoms at all time points, with the exception of orofacial dyskinesia on day 19 (Figure 2). In contrast, the L-DOPA-induced dyskinetic behaviors in $D2R^{-/-}$ and $D2R^{WT}$ animals were not significantly different from each other (Figure 1 in Supplement 1). The absence of dyskinetic symptoms observed in $D1R^{-/-}$ was also clearly evident in the posture of the animals, which was close to normal, compared with that of WT or $D2R^{-/-}$ animals (Figure 2 in Supplement 1), which showed great lateral deviation, twisted posture, and limb dyskinesia.

Dopamine D1, but Not D2, Receptors Are Required for the L-DOPA-Induced FosB and Dynorphin Expression

Increased FosB and dynorphin expression in the dorsolateral lesioned striatum correlates with the appearance of dyskinesia (3,4,29). We evaluated the effect of knocking out D1R or D2R on striatal FosB and dynorphin expression after chronic L-DOPA administration in hemiparkinsonian mice. In $D1R^{WT}$ and $D2R^{WT}$ animals, L-DOPA induced marked expression of FosB and dynorphin in the dorsolateral part of the lesioned striatum but not the unlesioned side. The anatomic distribution of FosB and dynorphin expression strictly overlaid striatal areas with complete denervation. The neurons enduring these molecular changes were completely denervated. An identical distribution of FosB and dynorphin expression was observed in hemiparkinsonian $D2R^{-/-}$ mice (Figure 3). In sharp contrast, there was no increase in FosB or dynorphin expression in $D1R^{-/-}$ mice, despite complete striatal denervation (Figures 3 and 4A).

We counted FosB- and dynorphin-positive neurons per mm^2 in the dorsolateral striatum. In $D1R^{WT}$, $D2R^{WT}$, and $D2R^{-/-}$ animals, L-DOPA induced approximately a 30-fold increase in FosB and a 10-fold increase in dynorphin-positive neurons in lesioned compared to the unlesioned striatum. Dynorphin staining intensity was also greater in the cytoplasm and neuropil of striatal areas with complete denervation: optical density measurements in L-DOPA-treated WT mice revealed a 160% increase in intensity compared to the comparable region on the unle-

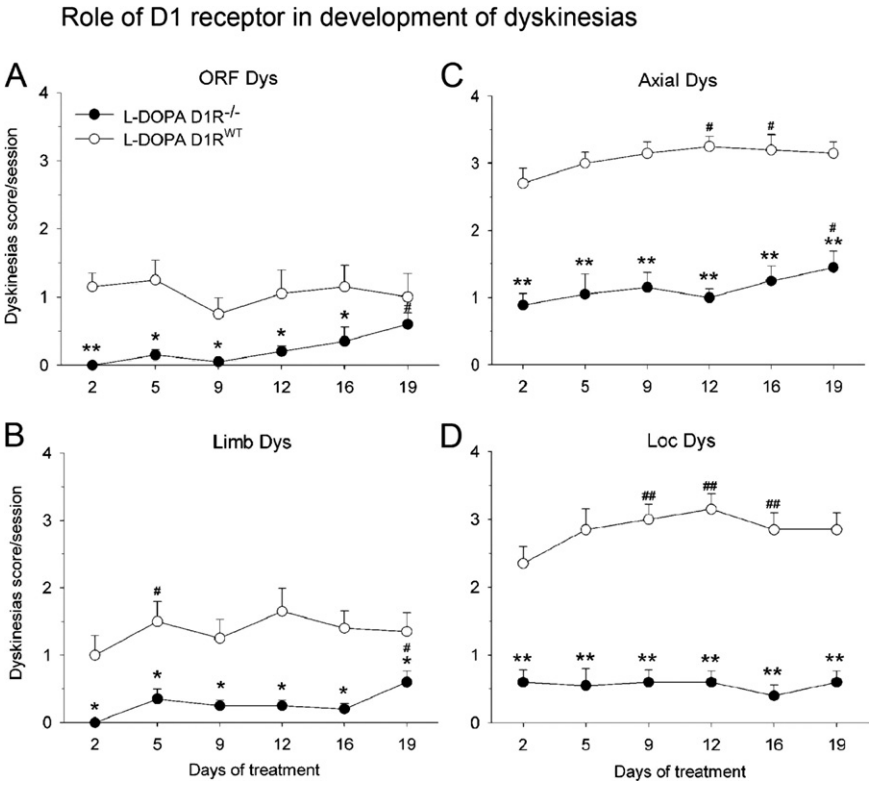

Figure 2. Genetic inactivation of dopamine D1 receptors blocked dopamine precursor molecule 3,4-dihydroxyphenyl-L-alanine (L-DOPA)-induced dyskinesias in hemiparkinsonian mice. Time course of appearance of dyskinetic symptoms: orofacial dyskinesias (ORF dys) **(A)**, limb dyskinesias (limb dys) **(B)**, axial dystonia (axial dys) **(C)**, and locomotor dyskinesias (loc dys) **(D)**. Movements were evaluated 30 min after L-DOPA (25 mg/kg) administration in $D1R^{WT}$ and $D1R^{-/-}$ hemiparkinsonian mice. Two-way ANOVA with repeated measures showed significant differences for genotype in all four types of dyskinetic symptoms: ORF dys [$F(1,18) = 10.8$, $p = 4.1 \times 10^{-3}$], loc dys [$F(1,18) = 95.3$, $p = 1.3 \times 10^{-8}$], limb dys [$F(1,18) = 21.5$, $p = 4 \times 10^{-4}$], axial dys [$F(1,18) = 104$, $p = 6.6 \times 10^{-9}$] and a significant effect on time for limb dys [$F(5,90) = 2.5$, $p = 3.6\ 10^{-2}$] and axial dys [$F(5,90) = 2.4$, $p = 4.5\ 10^{-2}$]. Data points represent the mean ± SEM. $^{*}p < .05$; $^{**}p < .0001$ versus $D1R^{WT}$; $^{\#}p < .05$, $^{\#\#}p < .005$ versus first day of treatment after two-way ANOVA with repeated measures and planned comparisons, $n = 10$ per group.

sioned side (Figure 4E). These L-DOPA-induced increases in FosB and dynorphin expression disappear in $D1R^{-/-}$ animals (Figure 4B, 4D, and 4E), although a few FosB-positive cells were observed in $D1R^{-/-}$ mice. We also quantified the area of FosB-immunoreactive (-ir) nuclei. L-DOPA treatment induced a twofold increase in the area of FosB-ir nuclei in the lesioned striatum of WT and $D2R^{-/-}$ mice, but no change in the area of FosB-ir nuclei in $D1R^{-/-}$ (Figure 4C).

Dopamine D1 but not D2 Receptors Blocked L-DOPA-Induced Phosphorylation of ERK and Phosphoacetylation of Histone Three in the Lesioned Striatum

We have shown previously that chronic L-DOPA-treatment in hemiparkinsonian animals greatly increased phosphorylation of ERK1/2 on Thr202 and Tyr204 (p-ERK) in the lesioned striatum (3). More recently, others have shown that this increase is directly correlated with the severity of dyskinetic symptoms (4,7). We examined p-ERK in the knockout animals and found that L-DOPA did not induce p-ERK in $D1R^{-/-}$ animals (Figure 5A), but in $D2R^{-/-}$ animals, p-ERK expression was similar to that in WT littermates (Figure 5A). These results are consistent with our behavior results implicating D1R, but not D2R, in the appearance of dyskinetic symptoms. Phosphorylation of ERK1/2 results in the sequential phosphorylation of MSK-1 and phospho acetylation of histone 3 (p-AcH3) (5,14,30). Chronic L-DOPA treatment increased phosphorylation of H3 on Ser10 and acetylation on Lys14 in the lesioned striatum with an identical expression-pattern to that described for FosB and dynorphin. As seen for induction of FosB and dynorphin, inactivation of D1R completely blocked L-DOPA-induced p-AcH3 in the depleted striatum, whereas inactivation of D2R had no significant effect (Figure 5A).

We counted p-ERK1/2- and p-AcH3-positive cells in both sides of the striatum in WT animals and found that L-DOPA produces a 15-fold increase in the number of p-ERK-ir cells and a 300-fold increase in p-AcH3-ir nuclei on the lesioned side compared with the unlesioned side (Figure 5B and 5D). p-ERK was also induced in the neuropil (Figure 5A and 5C), as reflected by an increase in optical density similar to the increase in the number of p-ERK-positive cells.

Phenotype of the Striatal Neurons Underlying the Molecular Changes Associated with Dyskinesia

To determine whether these molecular changes all occur in the same population of striatal neurons, we carried out double immunostaining assays. FosB and p-AcH3 were both coexpressed with dynorphin but not with enkephalin (markers of direct and indirect pathway neurons respectively). Similarly, the few scattered FosB nuclei observed in $D1R^{-/-}$ mice were in enkephalin-negative neurons (data not shown). These results were obtained using both classical DAB/DAB-nickel (Figures 6A, 6A′, 6B, 6B′ and 6C) and double immunofluorescence methods (Figure 6C′). Furthermore, FosB expression and p-AcH3 appeared only in those direct pathway neurons with increased dynorphin expression consequent to L-DOPA treatment (Figure 6A and A′, 6B and 6B′), suggesting that these changes were coordinated.

The Extent of the Dopaminergic Lesion Correlates with Dyskinesia Score, Contralateral Rotational Behavior and Induction of Molecular Changes

To confirm that the extent of the dopaminergic lesion did not differ between the various groups of animals studied here, we assessed the percentage of striatum with complete loss of TH-ir fibers for each group of animals. We found no significant differences between groups (44 ± 3% for $D1R^{WT}$; 52 ± 7% $D1R^{-/-}$; 41 ± 7% for $D2R^{WT}$; 31 ± 4% $D2R^{-/-}$; Figure 7A). In addition, we found that the percentage of striatal area with

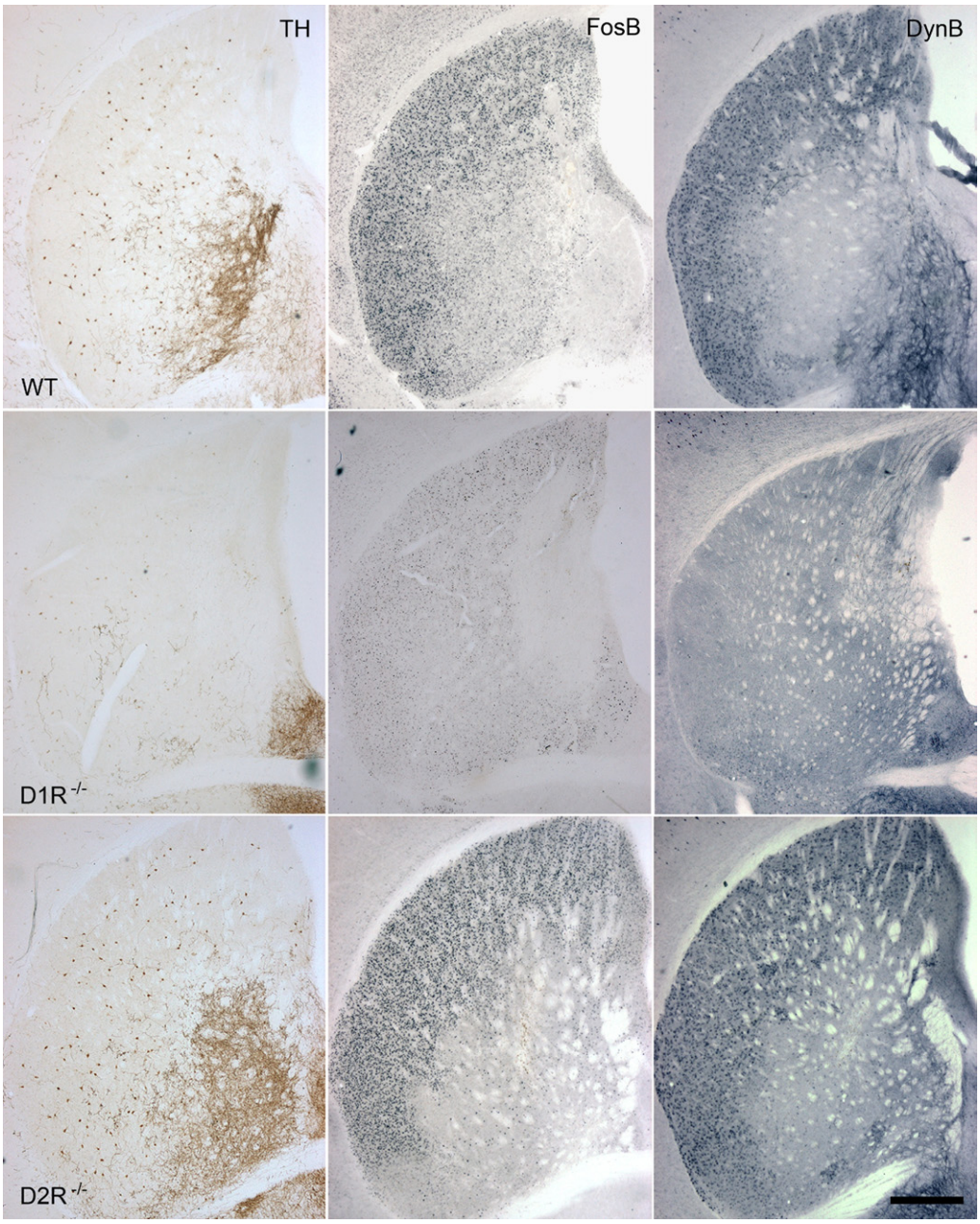

Figure 3. Genetic inactivation of dopamine D1 but not D2 receptors inhibits FosB and dynorphin (dyn) expression induced by chronic dopamine precursor molecule 3,4-dihydroxyphenyl-L-alanine (L-DOPA) treatment in the lesioned striatum. Photomicrographs of adjacent coronal striatal sections from wildtype (WT) (top), $D1R^{-/-}$ (middle) and $D2R^{-/-}$ (bottom) mice sacrificed 1 hour after the last L-DOPA injection and immunostained for tyrosine hydroxylase (TH), FosB, and dyn. Chronic L-DOPA treatment induced marked FosB and dyn expression in the striatal areas that are devoid of TH-immunoreactive fibers in WT and $D2R^{-/-}$ hemiparkinsonian mice. Note that in the striatum of $D1R^{-/-}$ hemiparkinsonian animals, L-DOPA does not induce FosB or dyn B expression. Scale bar = 500 μm.

complete dopaminergic lesion strongly correlated with the total dyskinesia score (Figure 7B, r = .91, $p > .001$) and with the total number of contralateral turns in 120 min (Figure 7C, r = .71, $p > .01$) on the last day of evaluation. Interestingly, all the molecular changes we observed in dyskinetic animals-increased FosB, dynorphin, p-ERK, p-AcH3-presented the same anatomic pattern of expression (Figure 7D and 7E) and were exclusively restricted to neurons and striatal areas with complete denervation (Figure 7). To strengthen this evidence, double immunostaining for TH and FosB revealed that the distribution of remaining TH fibers in the lesioned striatum is directly opposite to the distribution of L-DOPA-induced FosB expression (Figure 7F). Together, these findings indicate that complete denervation in these regions is required to trigger the molecular changes underlying dyskinesias.

Discussion

The findings we describe here strongly support a compulsory role for the D1R subtype in the development of dyskinesia and

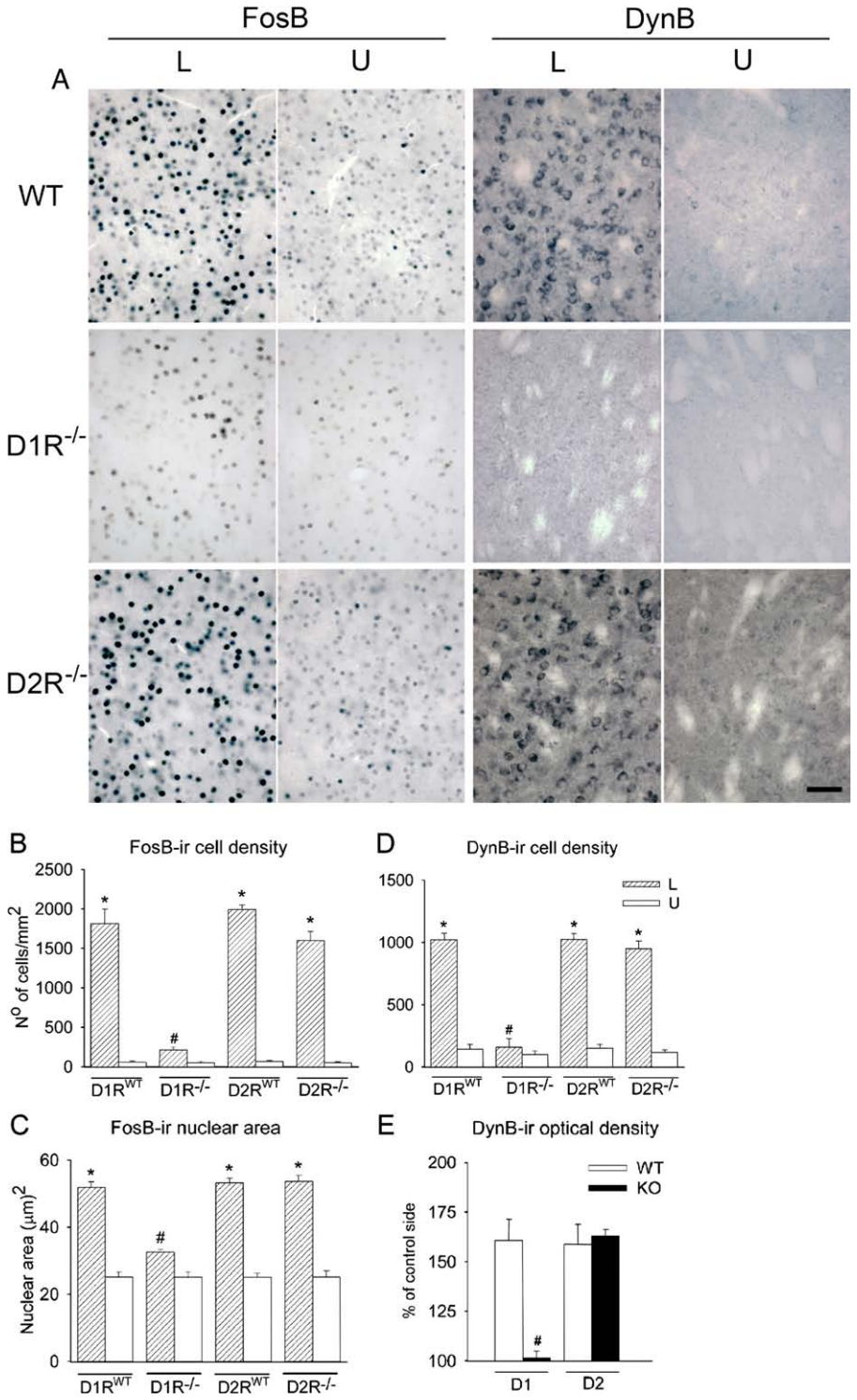

Figure 4. Role of dopamine D1 and D2 receptors in FosB and dyn expression in the striatum of hemiparkinsonian wildtype (WT), D1R$^{-/-}$, and D2R$^{-/-}$ mice following chronic dopamine precursor molecule 3,4-dihydroxyphenyl-L-alanine (L-DOPA) treatment. High power photomicrographs of coronal sections from the lesioned (L) and unlesioned (U) striatum of L-DOPA-treated WT, D1R$^{-/-}$ and D2R$^{-/-}$ mice sacrificed 1 hour after the last L-DOPA injection and stained for FosB or dyn **(A)**. Scale bar = 50 μm. Histograms represent quantification of **(B)** FosB- and **(D)** dynorphin-B (dyn-B)-positive cells (mean ± SEM) and nuclear area stained for FosB **(C)** in the lesioned and unlesioned striatum of hemiparkinsonian WT, D1R$^{-/-}$ and D2R$^{-/-}$ mice. Inactivation of D1R but not D2R abolished FosB and dyn expression induced by L-DOPA treatment. Two-way analysis of variance (ANOVA) showed significant differences between genotypes for D1R$^{-/-}$ mice for FosB- immunoreactive (ir) cell density [$F(1,52) = 101$], nuclear area [$F(1,56) = 72.2$], and dyn cell density [$F(1,32) = 171$] and between lesioned and unlesioned striatum for FosB-ir cell density: D1R^{WT} [$F(1,52) = 241$], D2R^{WT} [$F(1,52) = 253$], D2R$^{-/-}$ mice [$F(1,52) = 164$]; nuclear area: D1R^{WT} [$F(1,56) = 119$], D2R^{WT} [$F(1,56) = 123$], D2R$^{-/-}$ mice [$F(1,56) = 70.3$]; and dyn cell density: D1R^{WT} [$F(1,32) = 178$], D2R^{WT} [$F(1,32) = 177$], D2R$^{-/-}$ mice [$F(1,32) = 160$]. $^{*}p < 10^{-7}$ versus unlesioned side, $^{\#}p < 10^{-7}$ versus D1R^{WT} after two-way ANOVA followed by Scheffe post hoc test. **(E)** Optical density of dyn-B immunoreactivity in lesioned striatum expressed as a percentage of dyn-B-ir on the unlesioned side. $^{\#}p < .01$ versus D1R^{WT}, Student's *t* test.

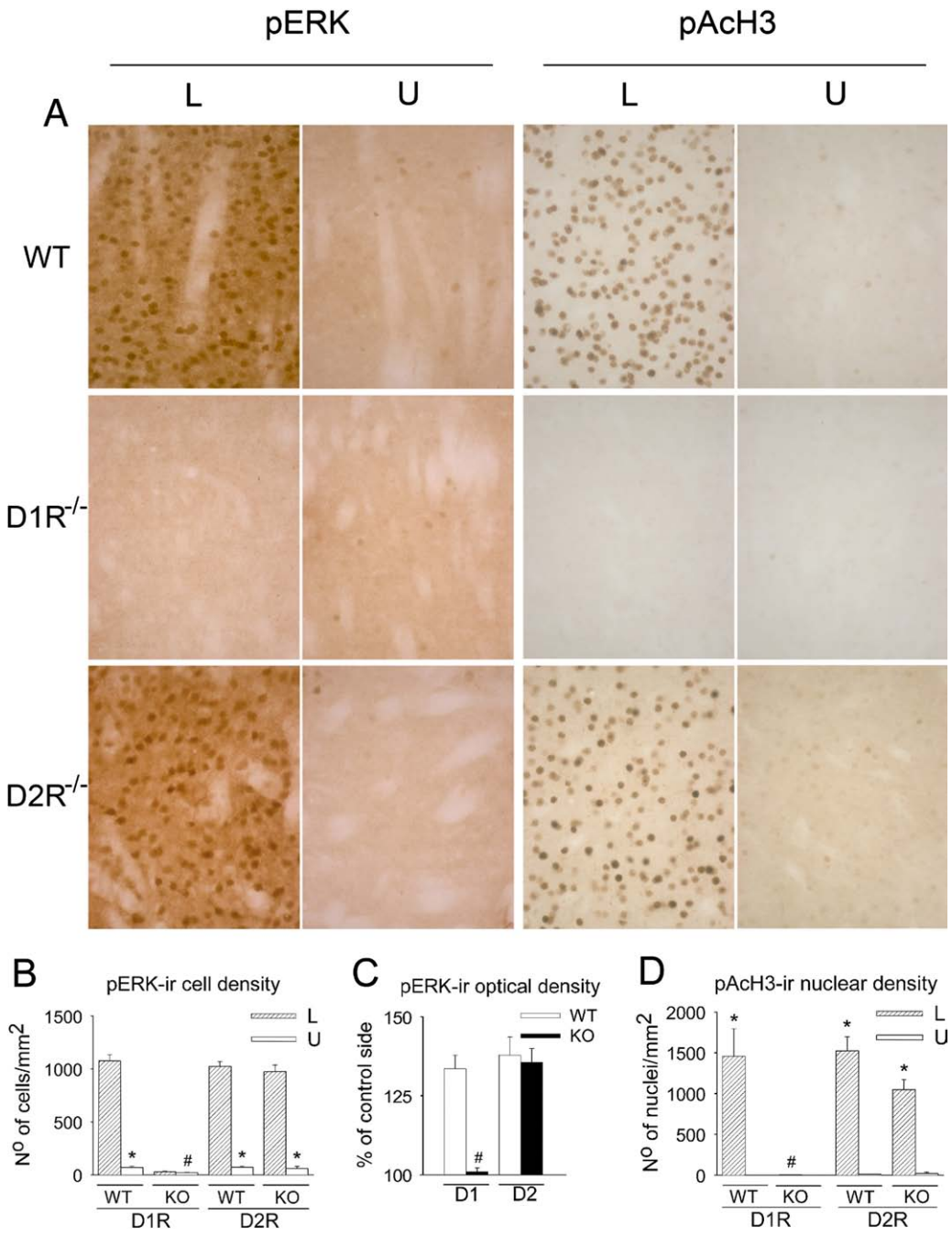

Figure 5. Genetic inactivation of dopamine D1 but not D2 receptors inhibits the phosphorylation of both extracellular signal-regulated kinase (ERK)1/2 and (Ser10)-acetyl (Lys14)-Hystone 3 (AcH3) induced by chronic dopamine precursor molecule 3,4-dihydroxyphenyl-L-alanine (L-DOPA) in the lesioned striatum. High-power photomicrographs of coronal sections from the lesioned (L) and unlesioned (U) striatum of L-DOPA-treated wildtype (WT), $D1R^{-/-}$, and $D2R^{-/-}$ hemiparkinsonian mice, sacrificed 1 hour after the last L-DOPA injection. Sections were immunostained for p-ERK1/2 or for p-AcH3 **(A)**. Scale bar = 50 μm. Histograms show the quantification of p-ERK1/2-positive cells **(B)**, phosphohistone AcH3-positive nuclei in the striatum **(D)** in WT, $D1R^{-/-}$, and $D2R^{-/-}$ hemiparkinsonian mice treated chronically with L-DOPA. Two-way analysis of variance (ANOVA) showed significant differences between genotypes for $D1R^{-/-}$ mice for p-ERK1/2-immunoreactive (ir) cell density [$F(1,32) = 174$] and p-AcH3-ir cell density [$F(1,24) = 81.4$] and between lesioned and unlesioned striatum for p-ERK1/2-ir cell density: $D1R^{WT}$ [$F(1,32) = 179$], $D2R^{WT}$ [$F(1,32) = 176$], $D2R^{-/-}$ mice [$F(1,32) = 161$]; and p-AcH3-ir density: $D1R^{WT}$ [$F(1,24) = 81.7$], $D2R^{WT}$ [$F(1,24) = 88.6$], $D2R^{-/-}$ mice [$F(1,24) = 40.9$]. Data represent mean ± SEM, $^{*}p < .001$ versus unlesioned side, $^{\#}p < 10^{-5}$ versus $D1R^{WT}$ after two-way ANOVA and followed by Scheffe posthoc test. **(C)** Optical density of p-ERK1/2-ir expressed as percent of staining in unlesioned striatum. $^{\#}p < .01$, versus $D1R^{WT}$, Student's *t* test.

rotational response following L-DOPA administration as well as in the molecular changes associated with these behaviors. In contrast, the D2R appears to have little effect on any of these. We demonstrate that these L-DOPA-induced molecular changes, including the phosphoacetylation of H3, occur in the direct pathway neurons within the fully denervated region of the striatum. Finally, we demonstrate a strong correlation between the extent of fully dopamine-denervated areas in the striatum and severity of dyskinesias.

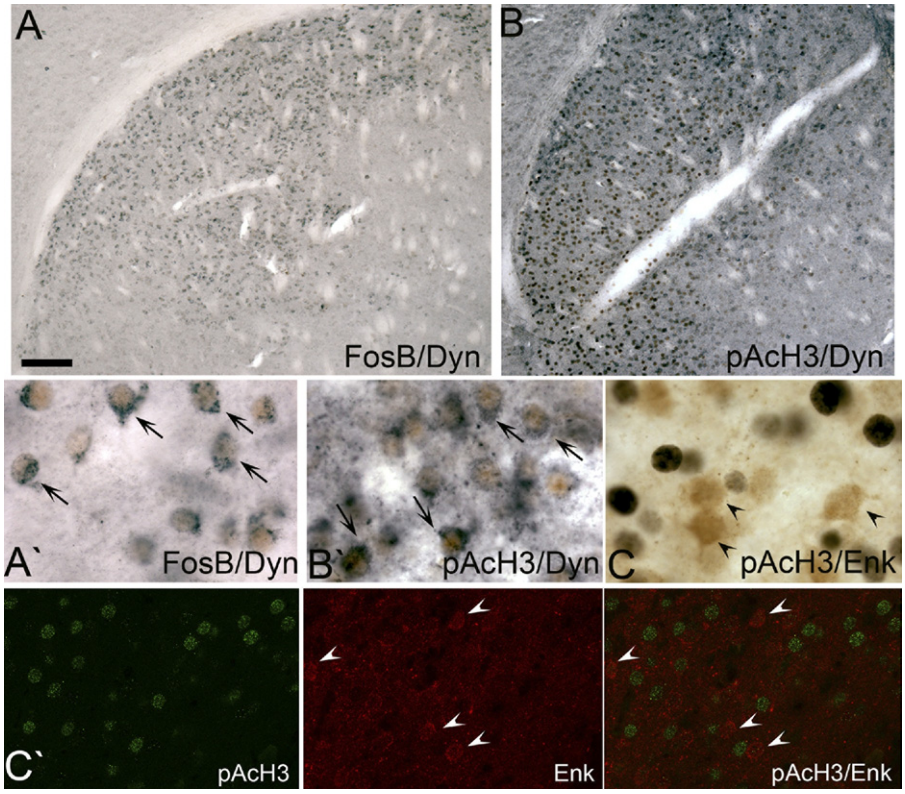

Figure 6. Phenotype of the striatal neurons underlying molecular changes associated with dyskinesia. High-magnification photographs showing colocalization of molecular changes in the same striatal denervated area **(A, B)** and in the same neurons **(A′, B′)** (arrows). Double immunostaining for **(B)** dynorphin-B (dyn-B; gray) and FosB (brown) **(A, A′)** and for dyn-B (gray) and p-AcH3 (brown) **(B, B′)**. **(C and C′)** p-AcH3 expression (gray) was not present in enkephalin (Enk)-immunoreactive (ir) neurons (brown, arrowheads) revealed by DAB/Ni double immunostaining **(C)** or by fluorescent double immunostaining with p-AcH3-ir (green) and Enk-ir (arrowheads) shown in red **(C′)**. Scale = 140 μm **(A, B)**, 20 μm **(A′, B′, C),** and 34 μm **(C′)**.

D1, but Not D2, Receptors Are Required for L-DOPA-Induced Rotational Response and Dyskinesia in Hemiparkinsonian Animals

Contralateral rotation response, a commonly used measure of behavioral sensitization to L-DOPA, is triggered by denervation-induced supersensitivity of dopamine receptors in the dopamine-depleted striatum. This molecular mechanism is associated with the appearance of L-DOPA-induced dyskinesias as well (31). This striking reduction in contralateral turns and dyskinesias in $D1R^{-/-}$ agrees with previous studies using D1/D5 agents. Pharmacologic studies in hemiparkinsonian rats supported a major role for the D1-type receptors in the development of contralateral turning (6,12); and dyskinesias (6,7,9,10,12,13) during L-DOPA treatment. Studies in humans and other primates concur that D1-type receptors are important for dyskinesias (32–35). However, these pharmacologic agents do not distinguish between D1 and D5 effects (18,36), thus our results are the first to specifically establish the critical role of D1R in the development of rotational response and dyskinesia.

In contrast, our data clearly indicate little role for the D2R in either rotational response or development of dyskinesia. This result contradicts previous data obtained using pharmacologic blockade (12) or stimulation (9) of D2 receptors, which suggested that dopamine D2R play a role in the development of rotational response. This discrepancy is likely because the D2 agents used in these previous studies are not specific: they act on other D2-like dopamine receptors (17,37–39). Although stimulation of D2-like receptors, especially D3R, can induce rotational behavior (12), our data indicate that this contribution is minor or dependent on D1R stimulation because no rotation develops in the absence of the D1R observed in our study or with D1/D5R blockage (40). Similarly, previous studies have reported that agonists acting preferentially at D2R vary in their ability to induce dyskinesia in rats (9–12,41). In humans and primates, dyskinetic responses were observed after prolonged treatment with D2 or D2/D3 agonists (35,42), whereas D2R-preferred antagonists, administered in combination with L-DOPA, reduced dyskinesia scores by approximately 50% (12,13). Again, the discrepancy between these previous findings and the results we report here is likely due to the lack of specificity of the D2 agents used, which also bind D3 and D4 receptors (17,37–39). Our data clearly show that D2R are not required for dyskinesias in hemiparkinsonian mice.

Despite the increased motor behavior displayed by $D1R^{-/-}$ in baseline conditions (15,16), in lesioned mice L-DOPA treatment does not induce dyskinetic movements. Thus, their hypermobility does not overcome blockade of dyskinesia. Similarly, the inability to move observed in $D2R^{-/-}$ mice (43) does not block the appearance of L-DOPA-induced dyskinetic movements in these mice. Therefore, differences in basal locomotor activity do not interfere with the appearance of dyskinesia. These results indicate that although normal locomotor activity and dyskinetic movements may involve the basal ganglia motor circuit, after denervation, different molecular mechanisms and anatomic substrates may be important.

D1, but Not D2, Receptor Is Required for Induction of Molecular Markers of Dyskinesia

Increased FosB expression after L-DOPA is causally linked with dyskinesia in rats (44) and mice (3,29). L-DOPA also induces expression of the opioid neuropeptide dynorphin in WT hemiparkinsonian mice as happens in PD patients (45) and in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine–treated monkeys (46). Dynorphin expression occurs downstream of L-DOPA-induced Fos activation in neurons of striatonigral pathway in rodents (3,7,44), altering the dynamic of this pathway, after repetitive stimulation of D1 receptors and thus underlying behavioral sensitization (19,47). Phosphorylation of ERK1/2 occurs upstream of FosB and dynorphin expression and seems to be

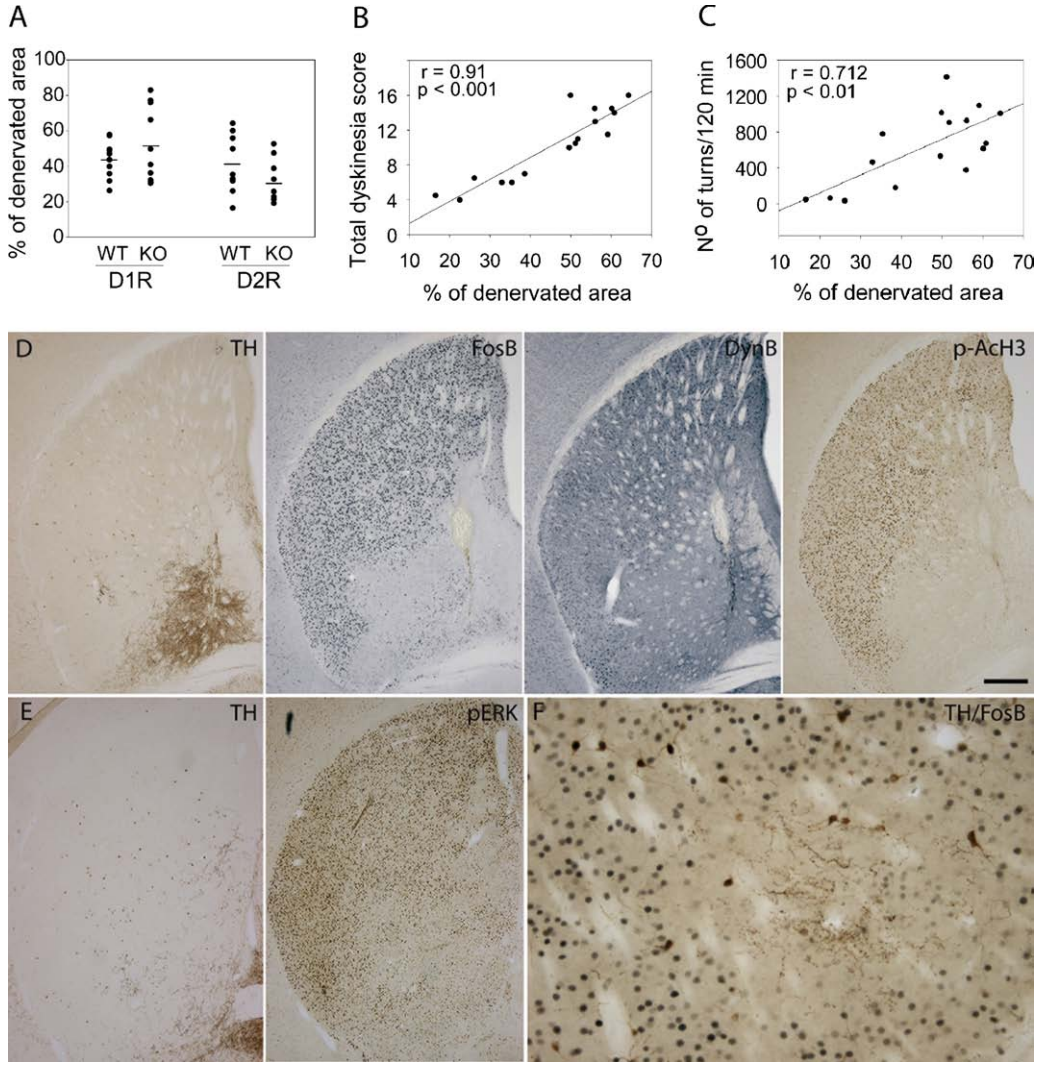

Figure 7. The extent of the striatal lesion correlates with development of dyskinesia and contralateral rotational response in wildtype (WT) animals. **(A)** Extent of striatal lesion in animals of each genotype as assessed by the percentage of total striatal area on the lesioned side that is completely denervated ($n = 10$ for each genotype). Any striatal area with optical density of tyrosine hydroxylase (TH)-immunoreactive (ir) fibers less than 5% was considered completely denervated. **(B and C)** Simple linear regression analysis illustrating the correlation between the percentage of total striatum that is completely denervated and the total dyskinesia score **(B)** or the number of turns in 120 min **(C)**. Behaviors were analyzed following the final dopamine precursor molecule 3,4-dihydroxyphenyl-L-alanine (L-DOPA) administration on Day 19 in WT animals. **(B)** $n = 16$; $r = .91$, $p < .001$; **(C)** $r = .71$, $p < .01$. **(D–F)** Histological pattern of L-DOPA-induced molecular changes in the lesioned striatum of WT animals. **(D)** Consecutive coronal sections through the lesioned striatum of mice sacrificed 1 hour after the last L-DOPA administration were immunostained for TH, FosB, dynorphin-B (dyn-B), antiphospho (Ser10)-acetyl (Lys14)-hystone 3 (p-AcH3). Scale = 400 μm. **(E)** Consecutive coronal sections through the lesioned striatum of mice sacrificed 20 min after the last L-DOPA injection were immunostained for TH and p- extracellular signal-regulated kinase 1/2 (p-ERK). Scale = 400 μm. **(F)** High-magnification photograph showing double immunostaining for TH (gray) and FosB (brown) showing an inhibition of FosB expression by remaining TH fibers. Scale = 120 μm.

limited to the same direct pathway neurons that overexpress FosB and dynorphin. p-ERK can trigger histone-phosphoacetylation and thus can have a cumulative effect on chromatin modification. In addition, FosB may interact with chromatin remodeling factors, providing a molecular basis for long-term alterations in gene expression (48,49). The increase in p-AcH3 we observed after L-DOPA treatment is consistent with one previous study (14) but conflicts with another (30) that found no change in p-H3 and decrease in Ac-H3 in MPTP-lesioned and L-DOPA-treated mice. These discrepancies may be due to differences in the mouse model used or differences in dose and duration of L-DOPA treatment, which would suggest that the threshold for histone modification by L-DOPA is more sensitive than some other molecular markers.

We now provide evidence that selective inactivation of D1R abolishes expression of all markers related to chronic L-DOPA treatment (FosB, dynorphin, p-ERK) including p-AcH3 in the lesioned striatum, whereas inactivation of D2R does not affect expression of these markers, in strict correspondence with our behavioral observations. Therefore, D1R activation is crucial for the stimulation of the MAPK- and cyclic adenosine monophosphate/PKA-dependent gene-expression pathways. These results are consistent with studies in rats in which administration of D1/D5 antagonists abolished L-DOPA induced FosB and p-ERK1/2 expression (7). The only alternative explanation of our results is that inactivation of D1R induced a dynorphin cell loss in the striatum of $D1R^{-/-}$ mice. Thus, our data add specificity to the pharmacologic data, indicating that the D1R, is required for

L-DOPA induced increases in FosB and dynorphin expression and p-ERK1/2 and extends these results to p-AcH3 in the lesioned striatum, strengthening the association between these molecular changes and the development of behavioral sensitization and dyskinesias.

The Severity of L-DOPA-Induced Dyskinesia Is Correlated with the Extent of Dopaminergic Depletion in the Lesioned Striatum

We found a strong correlation between the total dyskinesia score and the percentage of striatal area with complete dopamine depletion. The rotational response was also correlated with the extent of completely lesioned area but to a lesser extent. Although others (50) found that the magnitude of TH loss in the striatum is only a weak predictor of dyskinesias we consider that this disagreement with our data arises from the different approaches to determine the magnitude of TH-loss in the striatum. Whereas the previous study measured the average TH density in the whole striatum, our data are more specific and evaluate only the percentage of striatal area with complete denervation, verifying a high correlation between denervation and the intensity of dyskinesia. Our data agree with results showing that in denervated areas L-DOPA is converted to dopamine and released by striatal serotoninergic fibers (51), inducing dyskinesias (52,53) and FosB expression (51).

Moreover, our current and previous data (3) show that L-DOPA-induced increases in FosB, dynorphin, p-ERK, and p-AcH3 are all restricted to striatal areas completely free of TH fibers. A similar pattern of expression was observed for increased neurotensin and D3R mRNA in L-DOPA-treated rats (6). These results suggest that denervated striatal neurons hyperrespond to L-DOPA with a set of molecular changes in signaling and gene expression (54,55).

Phenotype of the Striatal Neurons Underlying the Molecular Changes Associated with Dyskinesia

Our data demonstrate that L-DOPA-induced p-AcH3 and FosB are localized in direct pathway neurons expressing D1R and only in those that are completely denervated and also overexpress dynorphin. These results are in line with the opposing functional roles of the two striatal output pathways. Whereas L-DOPA, which promotes movements, activates direct pathway neurons, D2R antagonists induce FosB in the indirect pathway neurons that mediate motor inhibition (56). In line with these results, the few FosB nuclei observed in $D1R^{-/-}$ were in enkephalin-negative neurons, indicating that these cells are direct pathway neurons or NOS interneurons (3).

Previous studies of L-DOPA-induced signaling in lesioned striatum were contradictory: Westin *et al.* (7) found p-ERK in both direct and indirect pathway neurons, whereas a more recent study found p-ERK expression only in direct pathway neurons (14). Our results confirm that p-ERK occurs exclusively in direct and significantly extend these findings by identifying that p-ERK and p-AcH3 occur only in those direct pathway neurons that are completely denervated and overexpress dynorphin. These results strongly suggest that any increase in p-ERK in indirect pathway neurons is not sustained for long enough to produce downstream changes. In addition, we demonstrate that inactivation of D1R completely blocks all the described molecular changes in these neurons, despite the presence of D5R (26) in these neurons.

Postranslational histone modification, including phosphorylation and acetylation, appears to modulate both the accessibility of chromatin for RNA transcription and how well chromatin serves as a template for transcription (49). Thus, H3 phosphoacetylation in dopamine-denervated cells may be a critical step in altering the response of these cells to L-DOPA and could provide the molecular basis for sustained chromatin modification and gene expression.

In conclusion, our data demonstrate that D1R are instrumental in the development of L-DOPA-induced dyskinesia in mice, without significant participation of D2R. Activation of D1R leads to expression of immediate early genes and downstream proteins in direct pathway neurons within the fully denervated region of the lesioned striatum. The p-AcH3 and the presumed modification of chromatin structure in these cells may be critical, opening the way for new gene expression in response to L-DOPA. Our data support this possibility and suggest that specific agents that inhibit histone modifications could help prevent or reverse the side effects of L-DOPA treatment in PD.

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Supplementary material cited in this article is available online.

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