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L-DOPA activates ERK signaling and phosphorylates histone H3 in the striatonigral medium spiny neurons of hemiparkinsonian mice

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Abstract

In the dopamine-depleted striatum, extracellular signal-regulated kinase (ERK) signaling is implicated in the development of L-DOPA-induced dyskinesia. To gain insights on its role in this disorder, we examined the effects of L-DOPA on the state of phosphorylation of ERK and downstream target proteins in striatopallidal and striatonigral medium spiny neurons (MSNs). For this purpose, we employed mice expressing enhanced green fluorescent protein (EGFP) under the control of the promoters for the dopamine D_2 receptor (*Drd2*-EGFP mice) or the dopamine D_1 receptor (*Drd1a*-EGFP mice), which are expressed in striatopallidal and striatonigral MSNs, respectively. In 6-hydroxydopamine-lesioned *Drd2*-EGFP mice, L-DOPA increased the phosphorylation of ERK, mitogen- and stress-activated kinase 1 and histone H3, selectively in EGFP-negative MSNs. Conversely, a complete co-localization between EGFP and these phosphoproteins was observed in *Drd1a*-EGFP mice. The effect of L-DOPA was prevented by blockade of dopamine D_1 receptors. The same pattern of activation of ERK signaling was observed in dyskinetic mice, after repeated administration of L-DOPA. Our results demonstrate that in the dopamine-depleted striatum, L-DOPA activates ERK signaling specifically in striatonigral MSNs. This regulation may result in ERK-dependent changes in striatal plasticity leading to dyskinesia.

Keywords: bacterial artificial chromosome, L-DOPA-induced dyskinesia, mitogen- and stress-activated kinase 1, Parkinson's disease, striatum, transgenic mice.

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The progressive loss of the dopaminergic input to the dorsal striatum represents the pathological hallmark of Parkinson's disease (PD) and results in dysfunction of neurotransmission within the basal ganglia, ultimately leading to severe motor impairment. Evidence emerging from studies performed in various experimental models of PD and on postmortem material from parkinsonian patients indicates that dopamine depletion is accompanied by dramatic changes in the ability of striatal medium spiny neurons (MSNs) to respond to dopaminergic drugs. These alterations affect various components of the signal transduction machinery and are in large part related to the development of sensitized dopamine D_1 receptor (D_1R) transmission. Thus, loss of striatal dopamine is accompanied by increased levels and efficiency of the G_{olf} protein (Corvol *et al.* 2004; Aubert *et al.* 2005), which couples D_1Rs to activation of adenylyl cyclase (Zhuang *et al.* 2000; Corvol *et al.* 2001), and by the resultant enhanced cAMP-dependent protein kinase (PKA)-mediated phosphorylation (Picconi *et al.* 2003; Santini *et al.* 2007).

Dopamine depletion also results in a remarkable enhancement in the ability of dopaminergic drugs to activate the

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Abbreviations used: 6-OHDA, 6-hydroxydopamine; AIMs, abnormal involuntary movements; D_1R, dopamine D_1 receptor; D_2R, dopamine D_2 receptor; DARPP-32, dopamine- and cAMP-regulated phosphoprotein, 32 kDa; EGFP, enhanced green fluorescent protein; ERK, extracellular signal-regulated kinase; LID, L-DOPA-induced dyskinesia; MSK1, mitogen- and stress-activated kinase 1; MSNs, medium spiny neurons; PD, Parkinson's disease; P-ERK, phospho-ERK; PKA, cAMP-dependent protein kinase; TBS, Tris-buffered saline.

extracellular signal-regulated kinase (ERK) transduction pathway, which is involved in basic physiological processes and in synaptic plasticity (Thomas and Huganir 2004). Activation of D_1Rs increases ERK phosphorylation in the dorsal striata of rats lesioned with 6-hydroxydopamine (6-OHDA) (Gerfen *et al.* 2002). A similar response is observed in the mouse, following genetic inactivation of tyrosine hydroxylase, which prevents dopamine synthesis (Kim *et al.* 2006). In line with these observations it has been reported that administration of L-DOPA, the most common antiparkinsonian drug, activates ERK signaling in various experimental models of PD (Pavon *et al.* 2006; Santini *et al.* 2007; Westin *et al.* 2007; Nicholas *et al.* 2008; Schuster *et al.* 2008) and that this effect is exerted through activation of D_1Rs (Westin *et al.* 2007). Recent studies indicate that the inability of striatal MSNs to normalize ERK phosphorylation during the course of chronic L-DOPA administration leads to the development of serious motor side effects, or L-DOPA-induced dyskinesia (LID) (Santini *et al.* 2007; Schuster *et al.* 2008). The exact cellular localization of abnormal ERK regulation is therefore of primary importance to identify specific subsets of striatal neurons implicated in LID.

Striatal MSNs can be divided into two roughly equivalent populations, which form the striatonigral, or direct pathway, and the striatopallidal, or indirect pathway (Gerfen 1992). These two groups of MSNs exert opposing regulations on the output stations of the basal ganglia and can be distinguished based on their ability to express D_1Rs (striatonigral MSNs) or dopamine D_2 receptors (D_2Rs) (striatopallidal MSNs) (Gerfen 1992). Previous work performed in 6-OHDA-lesioned rats reported that LID is associated with a large increase in ERK phosphorylation in both striatonigral and striatopallidal neurons (Westin *et al.* 2007). In this study, we have examined whether a similar regulation also occurs in a commonly employed mouse model of PD and LID (Lundblad *et al.* 2004, 2005). Using transgenic mice expressing enhanced green fluorescent protein (EGFP) under the control of the promoter for the D_2R (*Drd2*-EGFP mice), or the D_1R (*Drd1a*-EGFP mice) (Gong *et al.* 2003), we show that, following lesion with 6-OHDA, a single L-DOPA injection activates ERK selectively in the D_1R expressing MSNs of the striatonigral pathway, without affecting striatopallidal MSNs. An identical regulation is exerted by L-DOPA on the mitogen- and stress-activated kinase-1 (MSK1), a major nuclear target of ERK (Deak *et al.* 1998; Brami-Cherrier *et al.* 2005), and on histone H3 which is phosphorylated in response to activation of MSK1 (Soloaga *et al.* 2003; Brami-Cherrier *et al.* 2005; Chwang *et al.* 2007). Moreover, after chronic treatment with L-DOPA, the persistent elevation in ERK, MSK1, and histone H3 phosphorylation, which occurs in dyskinetic mice but not in non-dyskinetic mice (Santini *et al.* 2007), remains restricted to the striatonigral neurons.

Materials and methods

Animals

Male C57BL/6J mice (30 g) were purchased from Taconic (Tornbjerg, Denmark). Bacterial artificial chromosomes transgenic mice expressing EGFP under the control of the promoter for the D_2R (*Drd2*-EGFP) or the dopamine D_1R (*Drd1a*-EGFP) were generated by the Gene Expression Nervous System Atlas program at the Rockefeller University (Gong *et al.* 2003) and were crossed on a C57BL/6 background for three generations. The animals were housed in groups of five under standardized conditions with 12 h light/dark cycle, stable temperature (20°C), and humidity (40–50%).

Drugs

Drugs (Sigma-Aldrich Sweden AB, Stockholm, Sweden) were dissolved in physiological saline (0.9% NaCl) and injected intraperitoneally in a volume of 10 mL/kg of body weight. L-DOPA was injected at a dose of 20 mg/kg together with the peripheral DOPA decarboxylase inhibitor benserazide hydrochloride (12 mg/kg). The D_1R antagonist, SCH23390 (0.125 mg/kg), and the D_2R antagonist, raclopride (0.25 mg/kg), were administered 10 min before L-DOPA. When mice were not treated with drugs, they received equivalent volumes of vehicle.

6-Hydroxydopamine lesion

Mice were anesthetized with a mixture of fentanyl citrate (0.315 mg/mL), fluanisone (10 mg/mL) (VetaPharma, Leeds, UK), midazolam (5 mg/mL) (Hameln Pharmaceuticals, Gloucester, UK), and water (1 : 1 : 2 in a volume of 10 mL/kg) and mounted in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) equipped with a mouse adaptor. 6-OHDA-HCl (Sigma-Aldrich Sweden AB) was dissolved in 0.02% ascorbic acid in saline at a concentration of 3 μg of free-base 6-OHDA/μL. Each mouse received two unilateral injections of 6-OHDA (2 μL/injection) into the right striatum as previously described (Santini *et al.* 2007), according to the following coordinates (mm) (Franklin and Paxinos 1997): AP +1, ML −2.1, DV −3.2 and AP +0.3, ML −2.3, DV −3.2. Animals were allowed to recover for 3 weeks, before behavioral evaluation and drug treatment. This procedure leads to a decrease in striatal tyrosine hydroxylase immunoreactivity ≥ 80% (Santini *et al.* 2007) and to the loss of D_2R expressing midbrain dopaminergic neurons in the substantia nigra pars compacta.

Abnormal involuntary movements

Drd2-EGFP mice were treated for 10 days with one injection per day of L-DOPA /benserazide. Abnormal involuntary movements (AIMs) were assessed after the last injection (day 10) using a previously established AIM scale (Lundblad *et al.* 2004). Twenty minutes after L-DOPA administration, mice were placed in separated cages and individual dyskinetic behaviors (i.e., AIMs) were assessed for 1 min every 20 min, over a period of 140 min. AIMs were classified into four subtypes: locomotive AIMs (contralateral turns), axial AIMs (dystonic posturing of the upper part of the body toward the side contralateral to the lesion), limb AIMs (abnormal movements of the forelimb contralateral to the lesion), and orolingual AIMs (vacuous jaw movements and tongue protrusion). Each subtype was scored on a severity scale from 0 to 4: 0, absent; 1, occasional; 2, frequent; 3, continuous; and 4, continuous and not interruptible by outer stimuli.

Western blotting

Mice were treated with L-DOPA plus benserazide alone, or in combination with SCH23390 or raclopride and killed by decapitation 30 min later. The heads of the animals were immediately immersed in liquid nitrogen for 6 s. The brains were then removed and the striata were dissected out within 20 s on an ice-cold surface, sonicated in 750 μL of 1% sodium dodecyl sulfate, and boiled for 10 min. The effectiveness of this extraction procedure in preventing protein phosphorylation and dephosphorylation, hence ensuring that the level of phosphoproteins measured ex vivo reflects the *in vivo* situation, has previously been tested (Svenningsson *et al.* 2000). Aliquots (5 μL) of the homogenate were used for protein determination using a bicinchoninic acid assay kit (Pierce Europe, Oud Beijerland, The Netherlands). Equal amounts of protein (30 μg) for each sample were loaded onto 10% polyacrylamide gels. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred overnight to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Uppsala, Sweden) (Towbin *et al.* 1979). The membranes were immunoblotted using phospho-Thr202/Tyr204-ERK1/2 (1 : 2000; Cell Signaling Technology, Beverly, MA, USA) antibodies. Antibodies reacting with ERK1/2 independently of its phosphorylation state (1 : 1500; Cell Signaling Technology) were used to estimate the total amount of proteins. In some experiments, an antibody against tyrosine hydroxylase (1 : 1000, Chemicon International, Temecula, CA, USA) was used to assess the severity of 6-OHDA lesions. Detection was based on fluorescent secondary antibody binding and quantified using a Li-Cor Odyssey infrared fluorescent detection system (Li-Cor, Lincoln, NE, USA). The levels of each phosphoproteins were normalized for the amount of the corresponding total protein detected in the sample.

Tissue preparation and immunofluorescence

Mice were rapidly anesthetized with pentobarbital (60 mg/kg, i.p., Apoteksbolaget, Umeå, Sweden) and perfused transcardially with 4% (weight/vol) *p*-formaldehyde in 0.1 M sodium phosphate buffer, pH 7.5. Brains were post-fixed overnight in the same solution and stored at 4°C. Thirty micron-thick sections were cut with a vibratome (Leica, Nussloch, Germany) and stored at −20°C in 0.1 M sodium phosphate buffer containing 30% (vol/vol) ethylene glycol and 30% (vol/vol) glycerol, until they were processed for immunofluorescence. Brain regions were identified using a mouse brain atlas (Franklin and Paxinos 1997), and coronal sections equivalent to the following bregma coordinates were taken (mm): AP +0.86 (dorsal striatum) and AP −3.08 (substantia nigra). Free-floating sections were rinsed in Tris-buffered saline (TBS; 0.25 M Tris and 0.5 M NaCl, pH 7.5), incubated for 5 min in TBS containing 3% H_2O_2 and 10% methanol (vol/vol), and then rinsed three times for 10 min each in TBS. After 20 min incubation in 0.2% Triton X-100 in TBS, sections were again rinsed three times in TBS. Finally, they were incubated overnight at 4°C with the different primary antibodies. For detection of phosphorylated proteins, 0.1 mM NaF was included in all buffers and incubation solutions. Phosphorylation of several proteins was analyzed using rabbit polyclonal antibodies against phospho-Thr202/Tyr204-ERK1/2 (1 : 400; Cell Signaling Technology), phospho-Thr581-MSK1 (1 : 750; Cell Signaling Technology, Danvers, MA, USA), and phospho-Ser10-acetyl-Lys14-H3 (1 : 500; Upstate Ltd., Milton Keynes, UK). In the double labeling experiments, a monoclonal antibody against EGFP (1 : 500; Invitrogen, Carlsbad, CA, USA) was used to detect EGFP fluorescence in *Drd2*- and *Drd1a*-EGFP mice. In the triple labeling experiments, a monoclonal antibody against the dopamine- and cAMP-regulated phosphoprotein, 32 kDa (DARPP-32; 1 : 1000) was used (Snyder *et al.* 1992). Following incubation with primary antibodies, sections were rinsed three times for 10 min in TBS and incubated for 45 min with goat Cy3 coupled (1 : 400; Jackson Laboratory, Bar Harbor, ME, USA) and goat A633 (1 : 400; Invitrogen) secondary antibodies. In the triple labeling experiment, after incubation with the secondary antibodies, the slices were then rinsed three times for 10 min in TBS and incubated overnight with a polyclonal antibody against EGFP directly coupled to A488 (1 : 500; Invitrogen). Sections were rinsed for 10 min twice in TBS and twice in Tris buffer (0.25 M Tris) before mounting in 1,4-diazabicyclo-[2,2,2]-octane (Sigma-Aldrich Sweden AB).

Immunofluorescence analysis

Single-, double-, and triple-labeled images from each region of interest were obtained using sequential laser scanning confocal microscopy (Zeiss LSM, Carl Zeiss, Jena, Germany). Neuronal quantification was performed in 562 × 562 μm images by counting nuclear EGFP fluorescence (for assessment of D_2R-positive cells) and nuclear Cy3 immunofluorescence (for each marker analyzed).

Statistics

Data were analyzed using one-way or two-way ANOVA, where treatment and time were the independent variables, followed by Bonferroni–Dunn *post hoc* test for specific comparisons.

Results

L-DOPA-induced phosphorylation of ERK is restricted to striatonigral MSNs

The selective expression of EGFP in the striatopallidal MSNs of *Drd2*-EGFP mice and in the striatonigral MSNs of *Drd1a*-EGFP mice has been previously described (Gong *et al.* 2003; Bertran-Gonzalez *et al.* 2008). To study the specific neuronal populations in which signaling pathways are activated by L-DOPA in 6-OHDA-lesioned mice, we crossed these strains on a C57BL/6 background, in which the effects of this lesion have been previously studied (Santini *et al.* 2007). In these backcrossed animals, the distribution of EGFP was similar to that previously described in the parental line (data not shown).

We first investigated the effect of acute L-DOPA (20 mg/kg) treatment on phospho-ERK (P-ERK) immunoreactivity in the dorsal striata of sham- and 6-OHDA-lesioned *Drd2*-EGFP mice. Lesion with 6-OHDA did not affect the levels of total (data not shown) or phosphorylated ERK (Fig. S1). As previously shown (Santini *et al.* 2007), L-DOPA did not affect ERK phosphorylation in sham-lesioned animals (Figs S1 and 1a). In contrast, following lesion with 6-OHDA, the same dose of L-DOPA produced a large increase

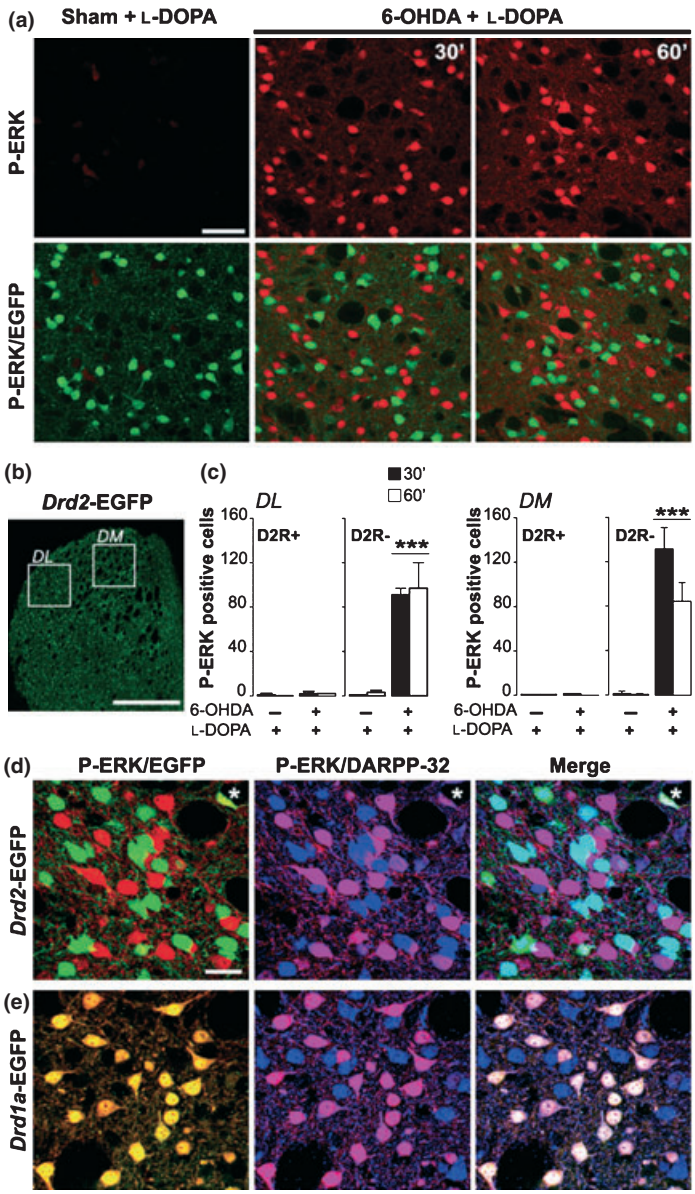

Fig. 1 L-DOPA-induced phosphorylation of ERK occurs exclusively in striatonigral MSNs, in the dopamine-depleted striatum. (a) Upper panels: Immunofluorescence detection of phospho-ERK (P-ERK) in the dorsal striata of sham- or 6-OHDA-lesioned *Drd2*-EGFP mice treated with L-DOPA (20 mg/kg) and perfused after 30 or 60 min (30′ and 60′). Lower panels: Double labeling with anti-EGFP and anti-P-ERK antibodies. Note the absence of co-localization of P-ERK and EGFP at both 30 and 60 min. Scale bar, 40 μm. (b) Coronal section of the dorsal striatum of a *Drd2*-EGFP mouse labeled with an antibody against EGFP, showing the regions selected for the quantification: *DL*, dorsolateral; *DM*, dorsomedial. Scale bar, 500 μm. (c) Quantification of P-ERK positive cells among EGFP-positive (D_2R+) and EGFP-negative (D_2R−) neurons, in the DL and DM striata of sham- and 6-OHDA-lesioned *Drd2*-EGFP mice, 30 min (filled bars) and 60 min (open bars) after administration of L-DOPA. Data are expressed as mean ± SEM (n = 3–4). Two-way ANOVA showed a significant effect of the treatment in EGFP-negative (D_2R−) neurons [$^{***}p < 0.001$; $F_{(1,8)} = 93.05$ for DL striatum and $F_{(1,10)} = 67.3$ for DM striatum], no significant effect of time and no significant effect of treatment × time interaction. (d and e) Triple labeling of P-ERK (red), EGFP (green), and DARPP-32 (blue) in the striatum of a 6-OHDA-lesioned *Drd2*- (d), or *Drd1a*-EGFP mouse (e) treated with L-DOPA. Note the co-localization between P-ERK and DARPP-32, indicating that activation of ERK occurs in MSNs. The absence of EGFP in the vast majority of P-ERK-positive MSNs in (d) indicates that they are almost exclusively D_2R negative. The asterisk indicates one single EGFP-positive (D_2R+) neuron with increased ERK phosphorylation. In the *Drd1a*-EGFP mouse (e), P-ERK immunoreactivity is instead exclusively localized in DARPP-32- and EGFP-positive MSNs. Scale bar, 20 μm.

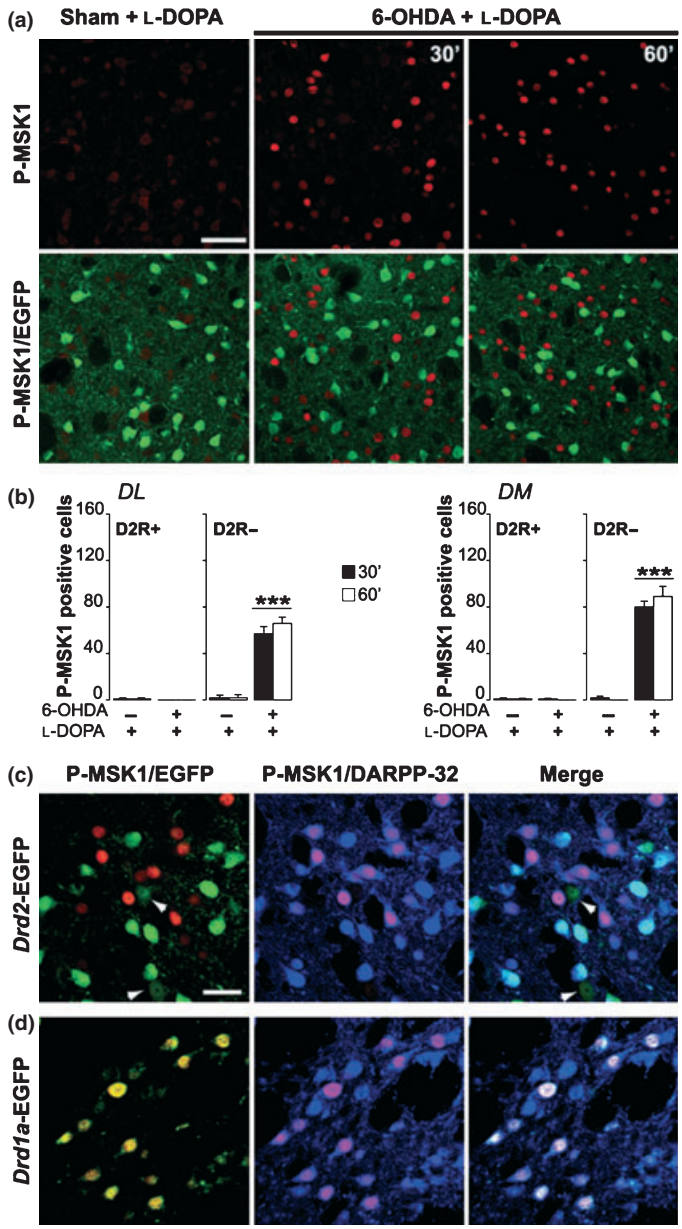

Fig. 2 L-DOPA-induced phosphorylation of MSK1 occurs exclusively in striatonigral MSNs, in the dopamine-depleted striatum. (a) Upper panels: Immunocytochemical detection of phospho-MSK1 (P-MSK1) in the dorsal striata of sham- or 6-OHDA lesioned *Drd2*-EGFP mice treated with L-DOPA (20 mg/kg) and perfused after 30 or 60 min (30′ and 60′). Lower panels: Double labeling with anti-EGFP and anti-P-MSK1 antibodies. Note the absence of co-localization of P-MSK1 and EGFP at both 30 and at 60 min. Scale bar, 40 μm. (b) Quantification of P-MSK1 positive cells among EGFP-positive (D_2R+) and EGFP-negative (D_2R–) neurons, in the dorsolateral (DL) and dorsomedial (DM) striata (cf. Fig. 1b) of sham- and 6-OHDA-lesioned *Drd2*-EGFP mice, 30 (filled bars) and 60 min (open bars) after administration of L-DOPA. Data are expressed as mean ± SEM (n = 3–4). Two-way ANOVA showed a significant effect of the treatment in EGFP-negative (D_2R–) neurons [$^{***}p < 0.001$; $F_{(1,10)} = 180.6$ for DL striatum and $F_{(1,10)} = 312.4$ for DM striatum], no significant effect of time and no significant effect of treatment × time interaction. (c and d) Triple labeling of P-MSK1 (red), EGFP (green) and DARPP-32 (blue) in the striatum of a 6-OHDA-lesioned *Drd2*- (c), or *Drd1a*-EGFP mouse (d) treated with L-DOPA. (c) Note the co-localization between P-MSK1 and DARPP-32 and the absence of EGFP, indicating that activation of MSK1 occurs in EGFP negative (D_2R–) MSNs. Arrowheads indicate large, D_2R containing (i.e. EGFP-positive) neurons devoid of DARPP-32. In these cells, which most likely represent large aspiny cholinergic interneurons, L-DOPA does not increase P-MSK1 immunoreactivity. (d) In the *Drd1a*-EGFP mouse, P-MSK1 immunoreactivity is exclusively localized in DARPP-32- and EGFP-positive MSNs. Scale bar, 20 μm.

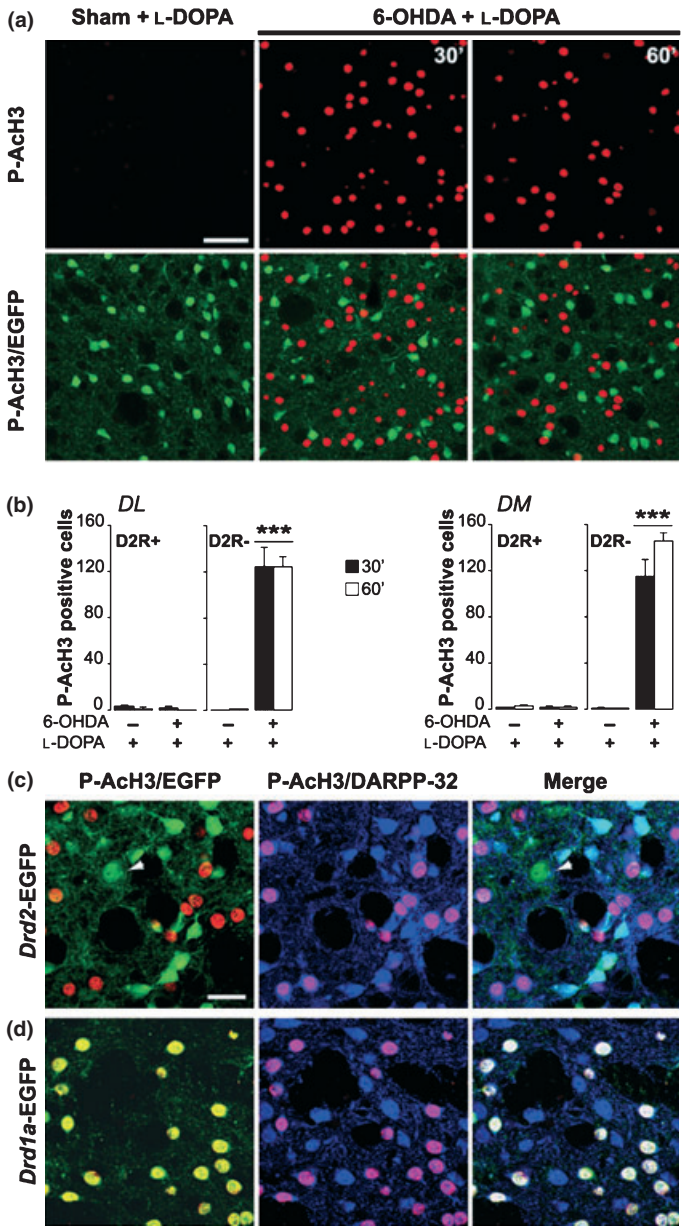

Fig. 3 L-DOPA-induced phospho-acetylation of histone H3 occurs exclusively in striatonigral MSNs, in the dopamine-depleted striatum. (a) Upper panels: Immunocytochemical detection of phospho-acetylated histone H3 (P-AcH3) in the dorsal striata of sham- or 6-OHDA lesioned *Drd2*-EGFP mice treated with L-DOPA (20 mg/kg) and perfused after 30 or 60 min (30′ and 60′). Lower panels: Double labeling with anti-EGFP and anti-P-AcH3 antibodies. Note the absence of co-localization of P-AcH3 and EGFP at both 30 and at 60 min. Scale bar, 40 μm. (b) Quantification of P-AcH3 positive cells among EGFP-positive (D_2R+) and EGFP-negative (D_2R–) neurons in the dorsolateral (DL) and dorsomedial (DM) striata (cf. Fig. 1b) of sham- and 6-OHDA-lesioned *Drd2*-EGFP mice, 30 (filled bars) and 60 min (open bars) after administration of L-DOPA. Data are expressed as mean ± SEM (n = 3–4). Two-way ANOVA showed a significant effect of the treatment in EGFP-negative (D_2R–) neurons [$^{***}p < 0.001$; $F_{(1,10)} = 131.8$ for DL striatum and $F_{(1,10)} = 201$ for DM striatum], no significant effect of time and no significant effect of treatment × time interaction. (c and d) Triple labeling of P-AcH3 (red), EGFP (green) and DARPP-32 (blue) in the striatum of a 6-OHDA-lesioned *Drd2*- (c), or *Drd1a*-EGFP mouse (d) treated with L-DOPA. (c) Note the co-localization between P-AcH3 and DARPP-32 and the absence of EGFP, indicating that phosphorylation of acetylated histone H3 occurs in EGFP negative (D_2R–) MSNs. Arrowheads indicate large, D_2R containing (i.e. EGFP-positive) neurons devoid of DARPP-32. In these cells, which most likely represent cholinergic interneurons, L-DOPA does not increase P-AcH3 immunoreactivity. (d) In the *Drd1a*-EGFP mouse, P-AcH3 immunoreactivity is exclusively localized in DARPP-32- and EGFP-positive MSNs. Scale bar, 20 μm.

in the number of P-ERK-positive cells, which lasted for at least 1 hr (Fig. 1a and c). The effect of L-DOPA was exerted at the level of MSNs, as demonstrated by the presence of DARPP-32 in the P-ERK-positive neurons (Fig. 1d). Most importantly, P-ERK immunoreactivity was detected only in EGFP-negative MSNs, with virtually no detectable double-labeling in D_2R expressing MSNs [Fig. 1a (lower panels), c and d]. One single EGFP- and P-ERK-positive neuron is shown in Fig. 1d, possibly representing one of the very few MSNs (≈5%) that express both D_1Rs and D_2Rs (Gong *et al.* 2003; Bertran-Gonzalez *et al.* 2008). We also analyzed the distribution of L-DOPA-induced P-ERK in the striata of 6-OHDA-lesioned *Drd1a*-EGFP mice. In this case, we found a complete co-localization between P-ERK and EGFP-immunoreactivity (Fig. 1e). These results demonstrated the selective restriction of P-ERK immunoreactivity to D_1Rs expressing neurons. We therefore concluded that administration of L-DOPA increases ERK phosphorylation in the MSNs of the striatonigral direct pathway, with no apparent regulation in striatopallidal MSNs.

L-DOPA-induced phosphorylation of MSK1 and histone H3 is restricted to striatonigral MSNs

In 6-OHDA-lesioned rats, the activation of ERK produced by acute L-DOPA is accompanied by increased phosphorylation of MSK1 at Ser376 (Westin *et al.* 2007). Here, we extend this observation by showing that L-DOPA increases MSK1 phosphorylation at Thr581, a site known to be a direct target of activated ERK (Deak *et al.* 1998). Similarly to ERK activation, this increased MSK1 phosphorylation was observed in the MSNs and enhanced for at least 1 hr after drug administration (Fig. 2a and b). As for P-ERK, we observed a complete segregation between EGFP and phospho-MSK1 immunoreactivity in the striata of *Drd2*-EGFP mice [Fig. 2a (lower panels), b, and c], indicating that the effect of L-DOPA was exerted selectively in the D_1R containing striatonigral MSNs. This conclusion was supported by the observation of co-localization between phospho-MSK1 and EGFP-immunoreactivity in the striata of 6-OHDA-lesioned *Drd1a*-EGFP mice treated with L-DOPA (Fig. 2d). Similar results were obtained when we examined the regulation exerted by L-DOPA on the state of phosphorylation of the Lys14-acetylated form of histone H3, which has been shown to represent an important target for MSK1 in striatal MSNs (Brami-Cherrier *et al.* 2005; Santini *et al.* 2007; Stipanovich *et al.* 2008) (Fig. 3). Triple labeling experiments showed that H3 phosphorylation was restricted to EGFP-negative MSNs, in *Drd2*-EGFP mice (Fig. 3c) and to EGFP-positive MSNs, in *Drd1a*-EGFP mice (Fig. 3d). Overall, these results indicate that administration of L-DOPA to 6-OHDA-lesioned mice produces a strong activation of ERK signaling in the striatum, leading to phosphorylation of MSK1 and histone H3 in a selected group of MSNs belonging to the direct, striatonigral pathway.

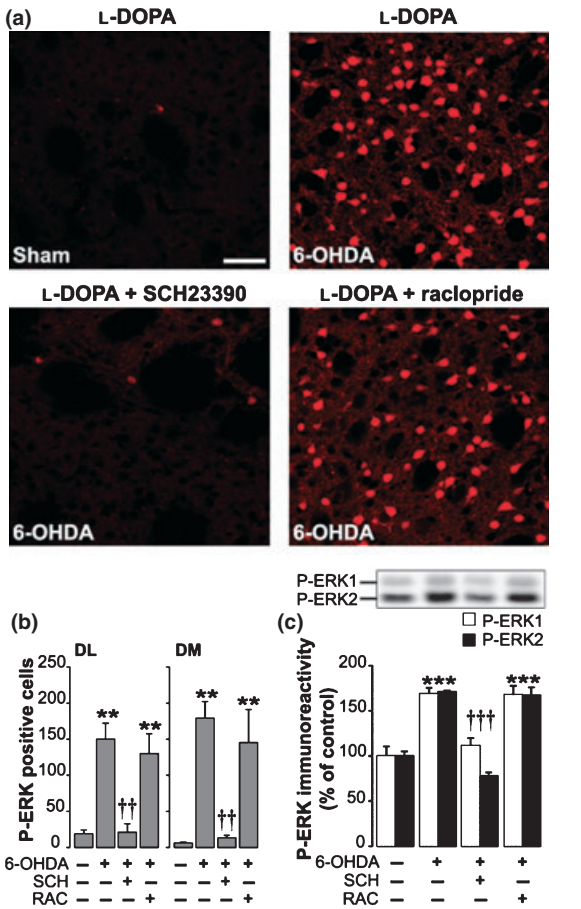

Fig. 4 The increase in ERK phosphorylation produced by L-DOPA in the dopamine-depleted striatum requires activation of D_1Rs but not D_2Rs. Sham- or 6-OHDA-lesioned C57BL/6 mice were treated with SCH23390 (0.125 mg/kg), raclopride (0.25 mg/kg), or vehicle 10 min prior administration of L-DOPA (20 mg/kg) and killed 30 min later. (a) Immunofluorescent detection of phospho-ERK (P-ERK) in the dorsal striata of a sham-lesioned mouse treated with L-DOPA and of 6-OHDA-lesioned mice treated with L-DOPA, L-DOPA + SCH23390, or L-DOPA + raclopride. Note the reduction of ERK phosphorylation in the SCH23390-pre-treated mouse. Scale bar, 40 μm. (b) Quantification of P-ERK positive cells in the dorsolateral (DL) and dorsomedial (DM) striatum (cf. Fig. 1b) of sham- and 6-OHDA-lesioned mice treated with L-DOPA alone, or in combination with SCH23390 or raclopride. Data are expressed as mean ± SEM ($n = 3$). $^{**}p < 0.01$ versus sham treated with L-DOPA and $^{\dagger\dagger}p < 0.01$ versus 6-OHDA treated with L-DOPA; one-way ANOVA [$F_{(3,8)} = 13.7$ and $F_{(3,8)} = 12.2$, respectively] followed by Bonferroni–Dunn test. (c) Quantification of phospho-ERK1 (P-ERK1, open bars) and phospho-ERK2 (P-ERK2, filled bars) by western blotting. Upper panel, representative autoradiogram. Lower panel, quantification of autoradiograms. Data are expressed as mean ± SEM ($n = 7$) in % of controls. $^{***}p < 0.001$ versus sham treated with L-DOPA and $^{\dagger\dagger\dagger}p < 0.001$ versus 6-OHDA treated with L-DOPA; one-way ANOVA [$F_{(3,36)} = 9.6$ for P-ERK1 and $F_{(3,36)} = 52.4$ for P-ERK2], followed by Bonferroni–Dunn test.

L-DOPA-induced phosphorylation of ERK, MSK1 and histone H3 depends on D_1R activation

We next examined the involvement of D_1Rs and D_2Rs in the L-DOPA-mediated activation of the ERK signaling cascade. As shown in Fig. 4, the ability of L-DOPA to increase ERK phosphorylation in the dorsal striatum was prevented by the administration of a D_1R antagonist, SCH23390 (0.125 mg/kg), but not by the administration of a D_2R antagonist, raclopride (0.25 mg/kg). Western blotting analysis revealed that SCH23390 abolished the increase in phosphorylation of both the 42 kDa (ERK1) and the 44 kDa (ERK2) isoforms of ERK (Fig. 4c). These results, which confirm previous data obtained in hemiparkinsonian rats (Westin *et al.* 2007), are in line with the ability of L-DOPA to activate ERK specifically in D_1R containing MSNs. The importance of D_1Rs in the regulation of ERK signaling, following 6-OHDA lesion, was confirmed by parallel experiments, showing that SCH23390, but not raclopride, prevented the increases in phospho-MSK1 (Fig. 5a and b) and phospho-Ser10-acetyl-Lys14-histone H3 (Fig. 5c and d) produced by L-DOPA.

L-DOPA-induced dyskinesia is associated with activation of ERK signaling in striatonigral, but not striatopallidal MSNs

It has been shown that persistent activation of ERK, produced by repeated administration of L-DOPA, leads to the development of dyskinesia (Santini *et al.* 2007). We therefore examined the effects on ERK phosphorylation produced, in the striata of *Drd2*-EGFP mice and *Drd1a*-EGFP mice, by 10 consecutive daily injections of L-DOPA. As previously reported (Lundblad *et al.* 2004; Santini *et al.* 2007), this procedure resulted in the appearance, in the majority of the mice, of severe AIMs, which were assigned a

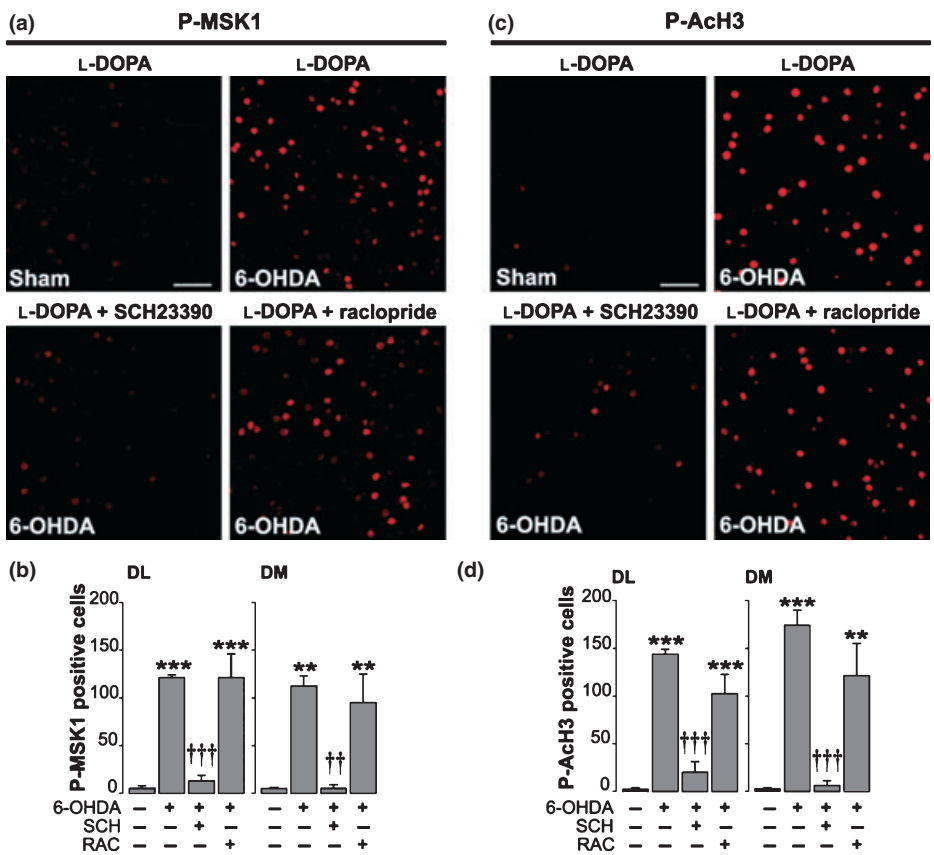

Fig. 5 The increase in phospho-MSK1 and phospho-acetylated histone H3 produced by L-DOPA in the dopamine-depleted striatum requires activation of D_1Rs but not D_2Rs. Sham- or 6-OHDA-lesioned C57BL/6 mice were treated with SCH23390 (0.125 mg/kg), raclopride (0.25 mg/kg), or vehicle 10 min prior administration of L-DOPA (20 mg/kg) and killed 30 min later. (a and c) Immunofluorescent detection of phospho-MSK1 (P-MSK1) (a) and phospho-acetylated histone H3 (P-AcH3) (c) in the dorsal striata of sham-lesioned mice treated with L-DOPA and of 6-OHDA-lesioned mice treated with L-DOPA, L-DOPA + SCH23390, or L-DOPA + raclopride. Note the reduction of P-MSK1 and P-AcH3 in SCH23390-treated mice. Scale bar, 40 μm. (b and d) Quantification of P-MSK1 (b) and P-AcH3 (d) positive cells in the dorsolateral (DL) and dorsomedial (DM) striata of sham- and 6-OHDA-lesioned mice treated with L-DOPA alone, or in combination with SCH23390 or raclopride. Data are expressed as mean ± SEM ($n = 3$). $^{**}p < 0.01$ and $^{***}p < 0.001$ versus sham treated with L-DOPA; $^{\dagger\dagger}p < 0.01$ and $^{\dagger\dagger\dagger}p < 0.001$ versus 6-OHDA treated with L-DOPA; one-way ANOVA [(b) $F_{(3,8)} = 20.82$ for DL striatum and $F_{(3,8)} = 12.2$ for DM striatum; (d) $F_{(3,8)} = 29.4$ for DL striatum and $F_{(3,8)} = 20.6$ for DM striatum] followed by Bonferroni–Dunn test.

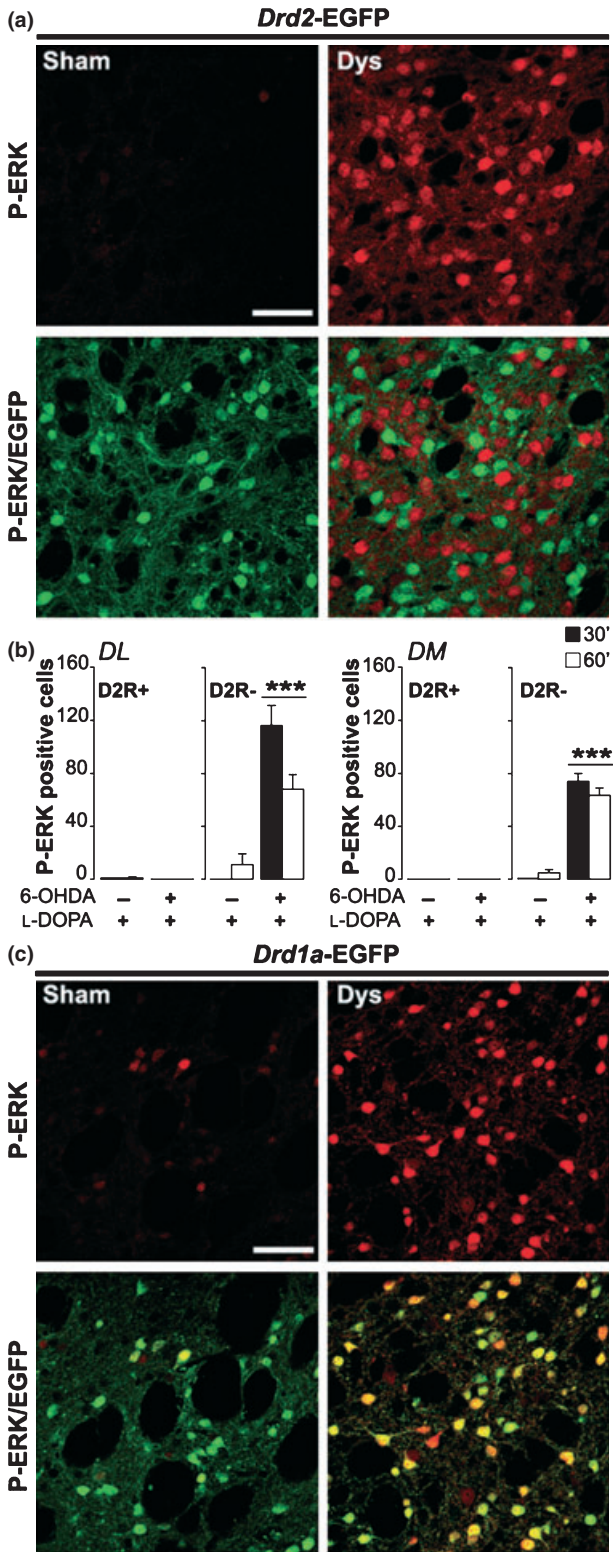

Fig. 6 L-DOPA-induced dyskinesia is associated with phosphorylation of ERK in striatonigral neurons. Sham- and 6-OHDA-lesioned *Drd2*- and *Drd1a*-EGFP mice were treated for 10 days with 20 mg/kg of L-DOPA, which produced dyskinesia in the lesioned mice (see text) and were perfused 30 (a–c) or 60 min (b) after the last drug administration. (a) Upper panels: Immunofluorescent detection of phospho-ERK (P-ERK) in the dorsal striata of sham-lesioned and dyskinetic (Dys) *Drd2*-EGFP mice. Lower panels: Double labeling with anti-EGFP and anti-P-ERK antibodies. Note the absence of co-localization of P-ERK and EGFP. Scale bar, 40 μm. (b) Quantification of P-ERK positive cells among EGFP-positive (D_2R+) and EGFP-negative (D_2R-) neurons in the dorsolateral (DL) and dorsomedial (DM) striata (cf. Fig. 1b) of sham-lesioned and Dyskinetic (Dys) *Drd2*-EGFP mice, 30 (filled bars), and 60 min (open bars) after the last administration of L-DOPA. Data are expressed as mean ± SEM ($n = 3–4$). Two-way ANOVA showed a significant effect of the treatment in EGFP-negative (D_2R-) neurons [$^{***}p < 0.001$; $F_{(1,8)} = 80.59$ for DL striatum and $F_{(1,8)} = 194.9$ for DM striatum], no significant effect of time and no significant effect of treatment × time interaction. (c) Upper panels: Immunofluorescent detection of P-ERK in the dorsal striata of sham-lesioned and dyskinetic (Dys) *Drd1a*-EGFP mice. Lower panels: Double labeling with anti-EGFP and anti-P-ERK antibodies. Note the co-localization of P-ERK and EGFP. Scale bar, 40 μm.

score ranging from 56 to 31 (total AIMs score during a period of 2 h). The few non-dyskinetic mice showed negligible activation of ERK and were not analyzed. We found that, in the dyskinetic mice, the pattern of ERK activation was indistinguishable from that observed after a single injection of L-DOPA (cf. Figs 1 and 6). P-ERK immunoreactivity was strongly induced in the EGFP-negative MSNs of *Drd2*-EGFP mice at 30 and 60 min following the last administration of L-DOPA (Fig. 6a and b). Conversely, in dyskinetic *Drd1a*-EGFP mice, virtually all P-ERK -positive cells were also immunoreactive for EGFP (Fig. 6c). Similar results were obtained on the phosphorylation of MSK1 (Fig. 7a, c, and e) and histone H3 (Fig. 7b, d, and f), with the only exception that the phosphorylation of histone H3 returned to basal levels 1 hr after the last injection of L-DOPA (Fig. 7c).

Discussion

The main finding of this study is that, in a mouse model of PD and LID, administration of L-DOPA results in concomitant phosphorylation of ERK, MSK1, and histone H3 at the level of the MSNs of the striatonigral pathway, without affecting ERK signaling in striatopallidal MSNs. This conclusion is mainly based on results obtained using transgenic mice that express EGFP under the control of the promoter for the D_2R. There is extensive evidence indicating that, in *Drd2*-EGFP mice, analysis of EGFP immunoreactivity allows the specific detection of striatopallidal MSNs. First, in the dorsal striatum D_2Rs are almost invariably expressed in striatopallidal MSNs and virtually absent from striatonigral MSNs (Gerfen 1992). Second, as shown previously (Gong *et al.*

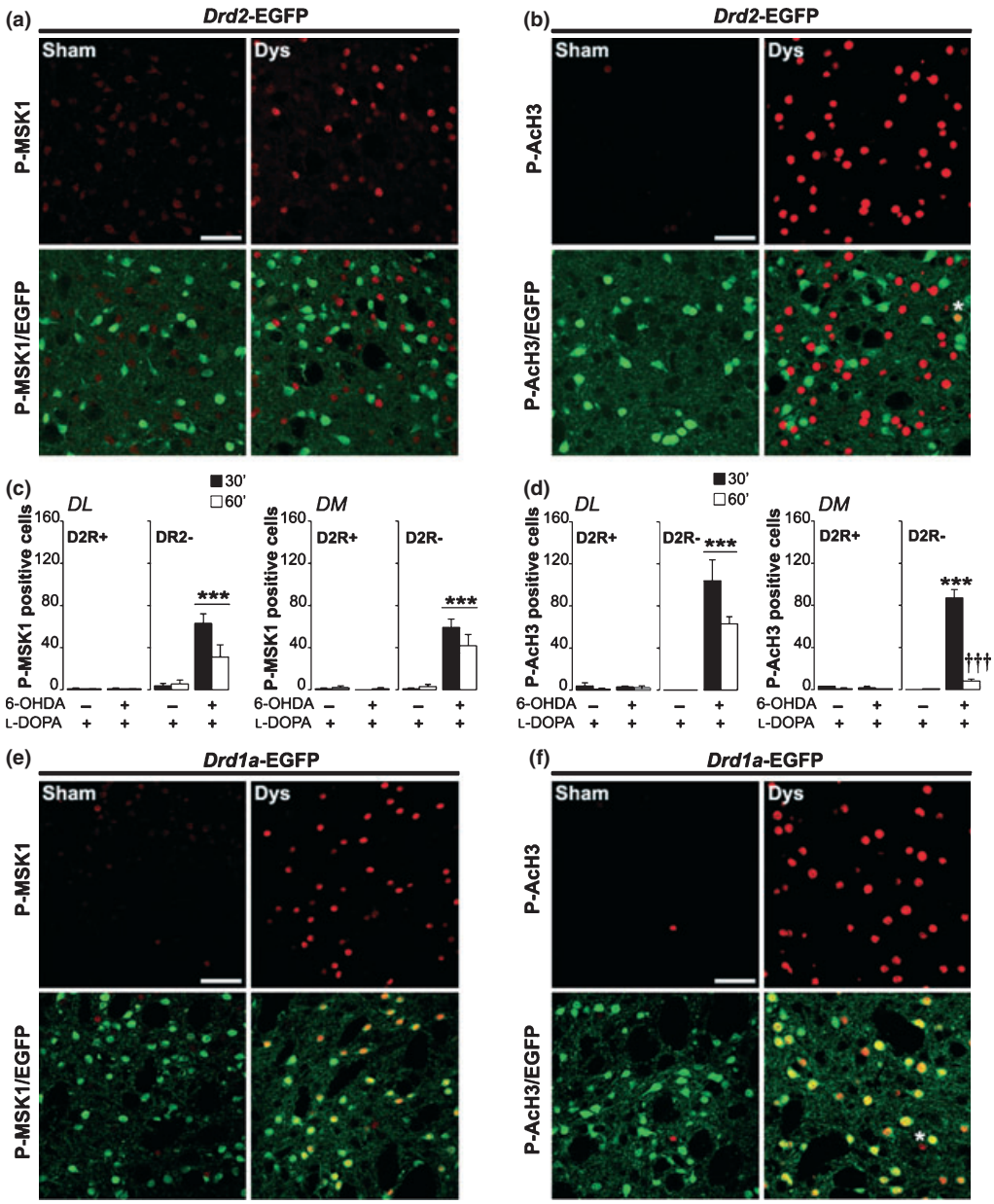

Fig. 7 L-DOPA-induced dyskinesia is associated with phosphorylation of MSK1 and acetylated histone H3 in striatonigral neurons. Sham- and 6-OHDA-lesioned *Drd2*- and *Drd1a*-EGFP mice were treated for 10 days with 20 mg/kg of L-DOPA, which produced dyskinesia in the lesioned mice (see text), and were perfused 30 (a–f) or 60 min (c and d) after the last drug administration. (a and b) Upper panels: Immunofluorescent detection of phospho-MSK1 (P-MSK1) (a) and phospho-acetylated histone H3 (P-AcH3) (b) in the dorsal striata of sham-lesioned or dyskinetic (Dys) *Drd2*-EGFP mice. Lower panels: Double labeling with anti-EGFP and anti-P-MSK1 (a) or anti-P-AcH3 (b) antibodies. The asterisk in (b) (cf. lower panel on the right) indicates one single EGFP-positive (D_2R+) neuron with increased histone H3 phosphorylation. Scale bar, 40 μm. (c and d) Quantification of P-MSK1 (c) and P-AcH3 (d) positive cells among EGFP-positive (D_2R+) and EGFP-negative (D_2R−) neurons in the dorsolateral (DL) and dorsomedial (DM) striata of sham- and 6-OHDA-lesioned *Drd2*-EGFP mice, 30 (filled bars) and 60 min (open bars) after the last administration of L-DOPA. Data are expressed as mean ± SEM (n = 3–4). In EGFP-negative (D_2R−) neurons, two-way ANOVA showed a significant effect of the treatment [$^{***}p < 0.001$; (c) $F_{(1,10)} = 30.9$ for DL striatum and $F_{(1,10)} = 54.1$ for DM striatum; (d) $F_{(1,11)} = 53.8$ for DL striatum and $F_{(1,11)} = 110.84$ for DM striatum]. A significant effect of time [$F_{(1,11)} = 77.6$] and of treatment × time interaction [$F_{(1,11)} = 79.3$] was found for P-AcH3 in the DM striatum. $^{***}p < 0.001$ versus sham treated with L-DOPA and $^{†††}p < 0.001$ versus 6-OHDA treated with L-DOPA at 30 min; Bonferroni–Dunn test. (e and f) Upper panels: Immunofluorescent detection of P-MSK1 (e) and P-AcH3 (f) in the dorsal striata of sham-lesioned or Dys *Drd1a*-EGFP mice. Lower panels: Double labeling with anti-EGFP and anti-P-MSK1 (e) or anti-P-AcH3 (f) antibodies. The asterisk in (f) (cf. lower panel on the right) indicates one single EGFP-negative (D_1R−) neuron with increased histone H3 phosphorylation. Scale bar, 40 μm.

2003; Bertran-Gonzalez *et al.* 2008) and confirmed in the present study (data not shown), EGFP immunoreactivity in *Drd2*-EGFP mice is specific to the projections that originate from the MSNs of the indirect pathway and innervate the globus pallidus. Third, it has been shown that, in *Drd2*-EGFP mice, EGFP-immunoreactive MSNs represent about 50% of the total number of MSNs (Bertran-Gonzalez *et al.* 2008), indicating that the vast majority of D_2R containing striatopallidal neurons are positively identified. In connection with this last point, it should be noted that, in this study, an antibody against EGFP was employed to maximize the detection of D_2R expressing neurons.

Based on the above considerations, our results, showing a nearly complete absence of P-ERK immunoreactivity in the EGFP-positive MSNs of *Drd2*-EGFP mice, indicate that the influence of L-DOPA on ERK signaling takes place selectively on the other large group of striatal projection neurons, which correspond to the D_1R expressing MSNs of the direct pathway. This conclusion is supported by experiments using *Drd1a*-EGFP mice, in which EGFP is selectively expressed in the D_1R containing neurons of the striatonigral pathway (Gong *et al.* 2003; Bertran-Gonzalez *et al.* 2008). In these animals, we found a complete co-localization of P-ERK and EGFP immunoreactivity.

The distribution of P-ERK overlaps with that of phosphorylated MSK1 and histone H3 which, following L-DOPA administration, appear to be exclusively localized in the EGFP-negative (i.e. D_1R containing) MSNs of *Drd2*-EGFP mice. Previous work showed that, in 6-OHDA-lesioned mice, L-DOPA-induced activation of ERK mediates the sequential phosphorylation of MSK1 and histone H3 (Santini *et al.* 2007). This study demonstrates that the activation of this signaling cascade occurs specifically in striatonigral MSNs.

The L-DOPA-induced phosphorylation of ERK, MSK1, and histone H3 is abolished by blockade of D_1Rs. It is known that activation of this subtype of dopamine receptor leads to stimulation of a G_{olf} protein, which is positively coupled to adenylyl cyclase and cAMP synthesis (Corvol *et al.* 2001). In fact, administration of L-DOPA to 6-OHDA-lesioned rats and mice increases the phosphorylation of DARPP-32 at the PKA site (Picconi *et al.* 2003; Santini *et al.* 2007), leading to inhibition of protein phosphatase-1 (Hemmings *et al.* 1984). Interestingly, PKA-dependent phosphorylation of DARPP-32 has been implicated in the activation of ERK signaling induced by dopaminomimetic drugs, such as cocaine and L-DOPA (Valjent *et al.* 2005; Santini *et al.* 2007). Whereas the precise localization in the striatum of L-DOPA-induced DARPP-32 phosphorylation remains to be established, the requirement of D_1Rs for this effect strongly suggests that the activation of the ERK/MSK1/histone H3 cascade observed in striatonigral MSNs is functionally coupled to the stimulation of the cAMP/PKA/DARPP-32 pathway. Therefore, following the loss of striatal dopaminergic inputs, the MSNs of the direct pathway emerge as a neuronal population critically affected by multiple and coordinated changes in signal transduction properties.

Our data indicate that the pattern of activation of ERK signaling produced by acute administration of L-DOPA is preserved even after repeated L-DOPA administration. Thus, in *Drd2*-EGFP mice, 10 consecutive injections of L-DOPA, which lead to the development of a robust dyskinesia, are associated with persistent increase in the phosphorylation of ERK, MSK1, and histone H3 in EGFP-negative, i.e. striatonigral MSNs. These results are in contrast with a recent report documenting the ability of L-DOPA to produce a similar activation of ERK in the striatonigral and striatopallidal MSNs of dyskinetic rats (Westin *et al.* 2007). This difference might be because of the animal model utilized (rat vs. mouse in this study). It still remains to be assessed whether or not ERK activation in striatopallidal MSNs leads to concomitant phosphorylation of downstream targets. Therefore, it will be important to determine the cellular localization of L-DOPA-induced MSK1 and histone H3 phosphorylation in the striata of 6-OHDA-lesioned rats.

In dyskinetic mice, the increase produced by L-DOPA on phospho-acetylated-histone H3 is more sustained in the dorsolateral than in the dorsomedial striatum. In this regard, it is interesting that the dorsolateral striatum has been specifically implicated in the development and expression of non-locomotive AIMs (Andersson *et al.* 1999). Increased phosphorylation of histone H3 is involved in chromatin decondensation and regulation of nucleosomal response (Cheung *et al.* 2000; Soloaga *et al.* 2003). Thus, it is likely that the ability of L-DOPA to enhance the levels of phospho-acetylated-histone H3 in striatonigral MSNs is implicated in the multiple changes in gene expression that occur specifically in this neuronal population. For instance, administration of L-DOPA to 6-OHDA-lesioned rats results in increased levels of the immediate-early gene product, FosB, and of mRNA for prodynorphin (Andersson *et al.* 1999), a marker for D_1R containing striatonigral MSNs (Gerfen 1992). Furthermore, repeated administration of L-DOPA has been shown to enhance the expression of the D3 subtype of dopamine receptor, specifically in the D_1R expressing MSNs of 6-OHDA-lesioned rats (Bordet *et al.* 2000). Finally, a persistent increase in mRNA for the immediate-early gene Zif268 has been described to occur selectively in striatonigral MSNs (Carta *et al.* 2005). Each of these alterations has been proposed to play a role in the development of LID (Santini *et al.* 2008). Future studies will be necessary to determine the implication of increased ERK/MSK1/histone H3 signaling in these abnormal responses.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Fig. S1 Quantification of phospho-ERK1 (P-ERK1, open bars) and phospho-ERK2 (P-ERK2, filled bars) in sham- or 6-OHDA-lesioned mice treated with vehicle or L-DOPA.

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