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# Sustained increase of PKA activity in the post-commissural putamen of dyskinetic monkeys

Regular research article

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**Abstract**

Levodopa-induced dyskinesias (LID) are a frequent complication of Parkinson´s disease pharmacotherapy that causes significant disability and narrows the therapeutic window. Pharmacological management of LID is challenging partly because the precise molecular mechanisms are not completely understood. Here, our aim was to determine molecular changes that could unveil targetable mechanisms underlying this drug complication. We examined the expression and downstream activity of dopamine receptors (DR) in the striatum of 1-methyl-4-phenyl-1,2,3,6 tetrahydropiridine (MPTP)-lesioned monkeys with and without L-DOPA treatment. Four monkeys were made dyskinetic and other four received a shorter course of L-DOPA and did not develop LID. Our results show that L-DOPA treatment induces an increase in DRD2 and DRD3 expression in the post-commissural putamen, but only DRD3 is correlated with the severity of LID. Dyskinetic monkeys show a hyperactivation of the canonical DRD1-signalling pathway, measured by an increased phosphorylation of protein kinase A (PKA) and its substrates, particularly DARPP32. In contrast, activation of the DRD2-signalling pathway, visible in the levels of Akt phosphorylated on Thr308 and GSK3β on Ser9, is associated with L-DOPA treatment, independently of the presence of dyskinesias. Our data clearly demonstrate that dyskinetic monkeys present a dysregulation of the DRD3 receptor and the DRD1-pathway with a sustained increase of PKA activity in the post-commissural putamen. Importantly, we found that all signaling changes related to long-term L-DOPA administration are exquisitely restricted to the post-commissural putamen, which may be related to the recurrent failure of pharmacological approaches.

Key words: L-DOPA, Dyskinesias, DRD3, PKA, DARPP32

## Introduction

Levodopa (L-3,4-dihydroxyphenylalanine, L-DOPA) remains the most efficacious treatment for alleviating the motor symptoms of Parkinson's disease (PD). However, long-term administration leads to the development of fluctuations in the motor response and, frequently, to the appearance of involuntary movements, known as L-DOPA-induced dyskinesias (LID) (Luquin et al., 1992). LID are difficult to manage pharmacologically (Huot et al., 2013) and negatively impact on quality of life (Pechevis et al., 2005), even if some patients tolerate them in the context of improved mobility (Hung et al., 2010).

To date the precise molecular mechanisms underlying LID are not completely understood but possibly involve an imbalance of dopamine (DA) transmission. Other neurotransmitter systems have also been implicated in LID. These include glutamatergic (ionotropic and metabotropic), serotonergic, adenosine, adrenergic, peptidergic and cholinergic neurotransmission. In fact, in recent years, great effort has been made to target these non-DA systems to prevent or treat this complication (for review see Bargiotas and Konitsiotis, 2013). However, the majority of the pharmacological agents have failed to fulfill expectations.

Work in animal models indicates that LID could develop in response to the activation of sensitized DRD1 receptors of the direct pathway (for review see Murer and Moratalla, 2011). Indeed an increased level of phosphorylated (p) dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP32) on Thr34 in the striatum is consistently considered a molecular marker of LID in experimental models, both in rat (Picconi et al., 2003; Santini et al., 2007; Aristieta et al., 2012; Azkona et al., 2014) and non-human primates (Gerfen et al., 2008; Santini et al., 2010). The phosphorylation of DARPP32 is

regulated by protein kinase A (PKA), downstream DRD1 activation. In rodents, PKA activity is increased during peak-dose dyskinesias (Martinez et al., 2012). Moreover, the reduction of PKA activity attenuates LID (Oh et al., 1997; Lebel et al., 2010; Martinez et al., 2012). To the best of our knowledge, the activity of PKA in the striatum has not been characterized in dyskinetic monkeys.

Post-synaptic DRD2-class receptors on striato-pallidal neurons also appear to be involved in LID, although they are classically regarded as less relevant than DRD1 receptors. DA acts on the Akt/GSK3 signalling cascade through interaction with striatal DRD2-class receptors (Beaulieu et al., 2007b) and it has been reported that chronic L-DOPA administration enhances the activity of the Akt/GSK3 pathway (Bychkov et al., 2007; Morissette et al., 2010). DRD3 receptor, which is mainly expressed in striatal limbic and associative regions (Morissette et al., 1998), is overexpressed in the striatum in both rat and primate models of LID (Bordet et al., 1997; Bezard et al., 2003; Aristieta et al., 2012; Azkona et al., 2014). Even if DRD3 receptor is a DRD2-class receptor, it has been shown that DRD3 stimulation potentiates DRD1-mediated behaviours by a different mechanism than DRD2 stimulation (Marcellino et al., 2008).

1-methyl-4-phenyl-1,2,3,6 tetrahydropiridine (MPTP)-lesioned monkeys represent the best model of PD for translational studies. The animals manifest all cardinal motor symptoms and the full extent of motor complications associated with chronic dopaminergic replacement (Bezard and Przedborski, 2011).

In this study we took advantage of this primate model to dissect DA receptor signalling pathways in the striatum, to unveil targetable mechanisms underlying this drug complication.

## Materials and Methods

### Animals

Fourteen cynomolgus male monkeys (*Macaca fascicularis*; R.C. HARTELUST, Holland) were included in the study. Animals were housed under standard conditions of humidity (50 ± 5%), temperature (22 ± 1ºC), air flow (16L/min) and, light (12h cycles) with *ad libitum* food and water. All reasonable efforts were made to minimise animal suffering and the number of animals per group. Thus, the three control brains were obtained from healthy, naive, age-matched animals included in an unrelated study in our institute. Experiments were carried out following European (2010/63/UE) and Spanish (RD 1201/2005) regulations for the care and use of laboratory animals.

Animals were sacrificed 24 hours after the last saline or L-DOPA dose. Animals recieved a sodium pentobarbital overdose (150 mg/kg, i.v.) under anesthesia (ketamine; 10 mg/kg and midazolam; 1 mg/kg; i.m.), and were perfused intracardially with saline buffer. Brains were rapidly removed, divided into 3 blocks, frozen *in situ* on dry ice, and stored at -80 °C until processing. For the experiments, 20 or 40 μm coronal sections were obtained using a cryostat (Microm, HM550)

### Experimental groups and design

The design and timeline of the experiment is shown in Figure 1*a*, based on our previous experimental schedule (San Sebastian et al., 2007; Vazquez-Claverie et al., 2009). Eleven monkeys received one weekly intravenous injection of MPTP (0.25–0.55 mg/Kg in 0.9% Na Cl; i.v.; Sigma-Aldrich) until they developed bilateral parkinsonian features (about 6 months) (Table 1). Four weeks after the last MPTP administration, 4 MPTP-lesioned monkeys were treated with L-DOPA/Benserazide (12.5 mg/kg/day Madopar® divided in 2 doses), dissolved in orange juice, for 2 months and developed moderate to severe dyskinesias. Four MPTP-monkeys received a shorter course of L-DOPA (4

weeks) and did not develop dyskinesias. They were classified as non-dyskinetic animals. During the following two years dyskinetic monkeys were on a maintenance regime with L-DOPA/Benserazide (10 mg/kg Madopar®, twice a week). After this period, dyskinetic and non-dyskinetic animals were given a daily dose of L-DOPA/Benserazide (10 mg/kg Madopar®) during 5 consecutive days, with the last dose given 24h before sacrifice. Three MPTP monkeys that did not receive L-DOPA served as parkinsonian controls and another 3 animals that did not receive any MPTP or L-DOPA were used as control. The groups are referred to as Control (C; n = 3), Parkinsonian, MPTP + vehicle (P; n = 3), Non-dyskinetic, MPTP + L-DOPA (ND; n = 4) and Dyskinetic MPTP + L-DOPA (D; n = 4). Dyskinetic, non-dyskinetic and MPTP + vehicle animals were sacrificed 2 years after the last MPTP dose.

**Behavioural assessment**

Motor deficits induced by MPTP were assessed according to a non-human primate disability rating scale, which independently scores parkinsonian features such as tremor (intensity and duration), balance, feeding and freezing from 0 (normal) to 3 (maximum disability); bradykinesia and posture from 0 (normal) to 4 (maximum disability), and the reduction in spontaneous activity from 0 (normal) to 5 (maximum disability), thus giving a total maximum score of 28 (Luquin et al., 1999). All motor assesment were performed by the same investigator. Evaluations were made before MPTP intoxication, one month after the last dose of MPTP, and once a month until the animals were sacrificed. Motor activity induced by placebo and L-DOPA was assessed by direct observation every 15 min for 3 consecutive hours, which is the estimated active period of the L-DOPA. Motor activities were also video-recorded and scored by an investigator blind to the treatment.

Dyskinesias were rated using a specific disability score that independently evaluated the severity, the topography (focal or generalized) and the duration of the abnormal movement with respect to the duration of the therapeutic L-DOPA response (Sanchez-Pernaute et al., 2007).

**Western blot**

Punches from the pre and post-commissural caudate nucleus and putamen were collected from 40 μm-thick frozen coronal sections on an ice-cold surface. Western blotting was performed as previously described (Azkona et al., 2010) using the following primary antibodies: mouse anti-TH (1:1000; Millipore), anti-DRD3 (1:1000), anti-Akt (1:1000), anti-STEP (1:1000, Santa Cruz Biotechnology), anti-pERK (pT202/pY204, 1:1000), anti-ERK1/2 (1:1000), anti-GSK3 (1:1000; BD Biosciences), anti-pGSK3 (Y279/Y216, 1:1000; ECM Bioscience), and anti-α-tubulin (1:50000, Sigma-Aldrich) and, rabbit anti-DRD1 (1:1000; Sigma-Aldrich), anti-DRD2 (1:1000; Lifespan Biosciences), anti-pDARPP32 (Thr34) (1:1000), anti-DARPP32 (1:1000), anti-p(Ser/Thr)PKA substrates (1:1000), anti-pPKAc Thr197 (1:1000), anti-pGSK3 (S21/9, 1:1000; Cell Signaling Technology), anti-pSTEP61/46 (Ser211/Ser49, 1:1000, Milipore), anti-PKAc (1:1000), anti-pAkt (T308, 1:1000, Santa Cruz Biotechnology), and anti-GAPDH (V-18) HRP (1:1000; Santa Cruz Biotechnology). Detection was based on secondary binding chemiluminescence detection system. Quantification was made by densitometric analysis of non-saturated films using ImageJ software 1.42q (NIH, USA). The levels of each phosphoprotein were normalized to the amount of the corresponding non-phosphorylated protein and GAPDH or α-tubulin ratio.

**qRT-PRCR**

The RNeasy kit (Qiagen) was used following the manufacturer's instructions for RNA extraction. DNase was applied to remove DNA contamination. RNA integrity was

confirmed by gel electrophoresis, and RNA concentration and purity was assessed spectrophotometrically. For reverse transcription-polymerase chain reaction (RT-PCR), up to 2 μg of total RNA was transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit with RNAse Inhibitor (4374966, Applied Biosystems) and random primers. For quantitative analysis of gene expression, real-time PCR using SYBR green I was performed using the StepOne Plus Real-Time PCR System (Applied Biosystems). Standard curves with serial 10-fold cDNA dilutions were built to validate the primers sets. The fluorescent signals from specific qPCR products were normalized against GAPDH and relative expression was calculated using the ΔΔCt method (Livak et al., 2000). Samples were run in triplicates. The sequence of primers used was taken from (Xiang et al., 2008). qRT-PCR data were normalized to GAPDH.

**Statistical analysis of data**

Experimental data were analyzed using the computer program GraphPad Prism (v. 5.01, GraphPad Software, Inc). Given the small sample size, non-parametric tests were applied. For multiple comparisons, a non-parametric Kruskal-Wallis test was used to estimate the overall significance followed by a non-parametric Mann-Whitney *U* test for independent groups with significance defined as $p \leq 0.05$. Both the effect of L-DOPA (dyskinetic and non-dyskinetic) and that of dyskinesias were examined. Graphical data are presented as group medians ± range and in text mean ± SD. To examine correlations we used Spearman's rank correlation coefficient.

## Results

### Characterization of the model

Animals received weekly injections of MPTP until they developed a moderate-severe parkinsonian syndrome. Length of the treatment period and cumulative dose was adjusted to individual susceptibility, which is known to be rather variable (Potts et al., 2013). Individual data are provided in **Table 1**. Stable disability scores were obtained 4 weeks after the last MPTP injection. Average score was 13.16 ± 0.75 (Table 1). Eight animals were started on chronic L-DOPA administration (12.5 mg/kg/day Madopar®). Acute L-DOPA administration (10 mg/kg) improved motor symptoms in all animals with an average reduction of 30-40% (Fig 1*b*). The improvement of motor symptoms was accompanied in the 4 dyskinetic animals by the appearance of abnormal involuntary movements (AIMs) that peaked between 30 and 90 min, and lasted 1.5 to 3.5 hours. The severity at maximum intensity was similar for the 4 animals, average: 9.625 ± 0.25 (scale 0-12) (Fig 1*c*). As the duration of AIMs was variable, we also calculated the area under the curve (AUC, average: 1046 ± 128), which provides an integrated index of severity and duration, for correlation with molecular data obtained from the different regions (Fig 1*d*).

The severity of DA denervation was verified by western blot in the posterior striatum (Fig 1*e*). In our experience, there is a good correlation between striatal TH levels determined by immunohistochemistry and western blot in this model (Ordonez et al., 2013). TH expression analysis showed a severe reduction in all MPTP-lesioned groups with no significant differences between groups in either the post-commissural caudate (P = 91.5 ± 1.2%, ND = 90.2 ± 1.8% and D = 89.1 ± 1.2% reduction) or the post-commissural putamen (P = 89.2 ± 1.1%, ND = 91.3 ± 2.3% and D = 89.6 ± 0.04%).

**DRD2 and DRD3 levels are increased in the post-commisural putamen of L-DOPA treated monkeys**

In order to analyze the persistent changes associated with LID, we analysed the brains 24 h after the last saline or L-DOPA dose, a time point when animals were not actively dyskinetic. We first determined by western blot the expression of the DA receptors, DRD1, DRD2 and DRD3 in four regions: pre-commissural and post-commissural putamen and caudate nucleus. In the pre-commissural caudate and putamen neither MPTP-lesion nor L-DOPA treatment induced significant changes in the expression of DA receptors (Fig S1). Likewise, we did not observe significant changes in the expression of DRD1 receptors in the post-commissural striatum (Fig 2*a*). Although, there were no significant differences in DRD2 levels between groups (Fig 2*b*), taking together all L-DOPA treated animals, non-dyskinetic and dyskinetic, we found a significant increase in DRD2 levels compared with untreated monkeys ($p < 0.05$).

Dyskinetic monkeys presented higher levels of DRD3 compared to parkinsonian animals (116.46 ± 38.8% vs 53.46 ± 5.44%, $p < 0.05$) and a trend towards significance compared with non-dyskinetic monkeys ($p = 0.09$), in the post-commissural putamen (Fig 2*c*). Besides, the intensity of AIMs in dyskinetic monkeys was correlated with DRD3 levels in the post-commissural striatum ($R = 0.65$, $p < 0.05$).

In order to determine whether the increase in DRD2 and DRD3 protein expression in the post-commissural putamen was due to transcriptional activation, we performed quantitative RT-PCR in extracts from both post-commissural striatal nuclei. We also analyzed the expression profile of the three DA receptors in the external and internal globus pallidus (GPe and GPi) and the frontal and temporal cortices (FCx and TCx, respectively). There were not significant changes in DRD1 and DRD2 expression. In the post-commissural putamen we observed that dyskinetic monkeys presented an

increase in DRD3 mRNA level, and this increase reached statistical significance when compared with non-dyskinetic monkeys (ND: 0.5 ± 0.13 vs D: 1.3 ± 0.07, $p < 0.05$). As found for protein expression, DRD3 mRNA levels correlated with AIMs severity ($R = 0.93$, $p < 0.01$) (Table 2). The lower DRD3 mRNA levels in ND monkeys probably reflect a tight transcriptional regulation of DRD3 in response to the treatment (5 consecutive days before sacrifice). We did not observe any significant differences in the rest of the brain areas examined in this study (Table S1).

Collectively, these results demonstrate that L-DOPA treatment modified DRD2 levels and that dyskinetic monkeys presented DRD3 over-expression. These changes were only detected in the post-commissural putamen.

**DRD1 signalling pathway is activated in the post-commissural striatum of dyskinetic monkeys**

It has been suggested that LID are associated with changes downstream DRD1 activation (Murer and Moratalla, 2011). Therefore, to determine whether DRD1 pathway is abnormally activated we analyzed the phosphorylation profile of proteins involved in this pathway.

We first examined PKA activity in the post-commissural caudate and putamen by using an antibody that detects serine/threonine substrates when phosphorylated at the PKA consensus region. In the post-commissural putamen, dyskinetic monkeys presented a higher PKA activity compared with control animals (154 ± 25.7% vs 100 ± 14.9%, $p < 0.05$; Fig 3*a*), whereas parkinsonian and non-dyskinetic monkeys did not present significant changes. To further explore the increased activation of PKA, we analyzed the levels of phosphorylation of Thr197 in the activation loop of the catalytic subunit of PKA (PKAc), which has been shown to be an essential step for its proper biological function (Cheng et al., 1998). Dyskinetic monkeys presented a 2-fold increase in the

level of pPKA Thr197 compared with control monkeys (205 ± 23.7% vs 100 ± 7.2%, $p < 0.01$; Fig 3*b*). Interestingly, this increased PKA activity was also specific for the post-commissural putamen, since we did not observe any significant changes of these proteins in the post-commissural caudate (Fig S2).

Next, we examined the phosphorylation levels of two specific PKA substrates, DARPP32 and STEP61 (striatal-enriched protein tyrosine phosphatase 61), in both post-commissural striatal nuclei. The western blot results showed that, in the post-commissural putamen, dyskinetic monkeys had higher levels of pDARPP32, reaching statistical significance when compared with MPTP-lesioned animals (D: 165.31 ± 7.04% vs P: 79.43 ± 16.62%, $p < 0.05$; Fig 3*c*). Indeed, pDARPP32 levels in this region were correlated with AIMs severity ($R = 0.77$, $p < 0.01$). The increase in pDARPP32 was once again region specific since we did not observe significant differences in the caudate nucleus (Fig S2). In contrast, we did not find any differences in pSTEP61 levels in either the caudate nucleus (Fig S2) or the putamen (Fig 3*d*).

Finally, we analyzed pERK2 levels, that can be activated downstream DRD1, but these were not significantly different across groups in either putamen (Fig 3*e*) or caudate (Fig S2). However, taking together all treated animals, there was a significant effect of chronic L-DOPA administration with an increase of pERK2 levels ($p < 0.05$) in the post-commissural putamen.

In summary, these results clearly demonstrate that dyskinetic monkeys present a persistent DRD1 pathway activation, indicated by a higher PKA activity and increased pDARPP32 levels, selectively at the post-commissural putamen. On the other hand, we observed that L-DOPA treatment increases pERK2 levels in the same brain region.

**DRD2 signalling pathway is altered in the post-commissural striatum of L-DOPA treated monkeys**

DRD2 stimulation activates the Akt/GSK3 signalling cascade. Our western blot results showed that dyskinetic monkeys presented lower levels of Akt phosphorylated on Thr308 compared with control animals (C: 100 ± 5.6% vs D: 50.9 ± 10%, $p < 0.05$; Fig 4*a*). Next, we analyzed the phosphorylation state of GSK3β in two residues, Tyr216, which increases the kinase activity, and Ser9, which inhibits it. Results showed that whereas pTyr216 levels were not significantly different between groups (Fig 4*b*), dyskinetic monkeys presented lower levels of pSer9 compared with MPTP lesioned monkeys (P: 159.1 ± 94.5% vs D: 41.9 ± 10.8%, $p < 0.05$; Fig 4*c*). Similar modifications were observed in GSK3α isoform, with decrease phosphorylation in Ser21 (data not shown). Interestingly, we did not observe any significant changes in the post-commissural caudate (Fig S3). However, these changes were not selective for dyskinetic animals, since we found a robust effect of treatment when comparing monkeys treated or not with L-DOPA (pAkt Thr308; $p < 0.01$ and pGSK3 Ser9/Ser21; $p < 0.001$).

These results indicate that L-DOPA treated monkeys present a persistent DRD2 pathway activation at the post-commissural putamen, independently of the dyskinetic status.

## Discussion

In this study we found that dyskinetic monkeys present persistent changes in the post-commissural putamen that clearly point to a deregulation of DRD1 signalling and confirm previous findings of an aberrant overexpression of DRD3. Interestingly, we observed that chronic L-DOPA treatment produces a long-lasting DRD2 pathway activation in the same region. Therefore, one of our major findings is that systemic L-DOPA administration produces selective changes in DA transmission that are anatomically restricted to the post-commissural putamen.

Dyskinetic monkeys presented increased DRD3 levels only in the post-commissural putamen, which correlated with AIMs severity, as previously reported (Bezard et al., 2003). Even if DRD3 comprises less than 1% of DA receptors in the motor striatum (Sokoloff et al., 1990; Levesque et al., 1992), several studies have documented that in rodent and non-human primate monkey models of LID, behavioural sensitization correlates with ectopic expression of DRD3 (Bordet et al., 1997; Bezard et al., 2003; Morissette et al., 2010; Aristieta et al., 2012; Azkona et al., 2014). Because dopamine has a higher affinity for DRD3 than for other receptors, adaptive changes may occur faster at the DRD3 (Richtand et al., 2003). In fact, intrastriatal administration of oligonucleotide antisense to DRD3 mRNA reduced rotational behaviour in 6-OHDA-lesioned rats (van Kampen and Stoessl, 2003). On the other hand, administration of a selective DRD3 agonist induced dyskinesias (Blanchet et al., 1997), whereas DRD3 antagonists and partial agonists exert potent anti-dyskinetic effects (Bordet et al., 1997; Bezard et al., 2003; Kumar et al., 2009a; Kumar et al., 2009b). Thus, targeting the ectopically expressed DRD3 or (even better) preventing DRD3 expression in the post-commissural putamen could efficiently control the development of LID.

An interesting point is that *in vivo* studies have demonstrated functionally active DRD1-DRD3 heteromers in the striatum (Marcellino et al., 2008). Furthermore, DRD3 receptor stimulation potentiates DRD1-mediated behaviours in mice (Marcellino et al., 2008). In dyskinetic animals DRD3 could mediate DRD1-signalling increased activity (Berthet et al., 2009; Bagetta et al., 2012), given that there is an abnormal DRD1 localization at the membrane of MSNs that is actively maintained by DRD3 (Berthet et al., 2009). Moreover, we have recently observed that a DRD3 antagonist is able to normalize pDARPP32 levels in the striatum of dyskinetic rats (Azkona et al., 2014).

Activation of DRD1 receptors is coupled to adenyl cyclase and increases cAMP production stimulating PKA activity. In dyskinetic rats the striatal injection of a PKA inhibitor effectively reduces AIMs (Oh et al., 1997; Lebel et al., 2010; Martinez et al., 2012). In our study, dyskinetic monkeys presented an increased PKA activity. Besides, we observed that this increase in PKA activity results in enhanced phosphorylation levels of some, but not all, PKA substrates. Thus, whereas pSTEP61 levels were unchanged, dyskinetic monkeys presented increased pDARPP32 on Thr34 levels in the post-commissural putamen. This result suggests that PKA differentially phosphorylates its substrates, maybe related to their subcellular localization, i.e. in the membrane (STEP61) or in the cytoplasm (DARPP32). Therefore, PKA inhibitors may not be adequate drug candidates in spite of the reported benefit in rodent models (Lebel et al., 2010).

Increased pDARPP32 levels observed in dyskinetic monkeys correlated with AIMs severity, confirming previous work in rat and monkey models of LID (Picconi et al., 2003; Santini et al., 2007; Lebel et al., 2010; Santini et al., 2010; Azkona et al., 2014). Thus, pDARPP32 levels in the post-commissural putamen are a reliable marker of LID. On the other hand, it is not clear whether the same applies to pERK1/2 levels. Some

studies (Pavon et al., 2006; Santini et al., 2007; Westin et al., 2007; Darmopil et al., 2009), but not others (Lindenbach et al., 2013; Azkona et al., 2014), have reported a good correlation between pERK2 levels and AIMs. In the primate model pERK2 enhancement was associated with the induction of LIDs (Santini et al., 2010). In our hands, increased pERK2 levels were associated with L-DOPA treatment, independently of the presence of dyskinesias. Nonetheless, it is worth noticing that most likely the monkeys classified as non-dyskinetic in this study would have developed dyskinesias under longer treatment periods, given that in PD patients there is a clear direct correlation between time on L-DOPA and the frequency of LIDs (Schrag and Quinn, 2000). Taken together our results suggest that the over-expression of DRD3 could be instrumental in the activation of DRD1-downstream signalling, and indicate that DRD1-cAMP-PKA-DARPP32 signalling in the post-commissural putamen plays a pivotal role in the manifestation of dyskinesias. However, *in vivo* imaging studies would be helpful to confirm the contribution of each of these receptors in PD patients.

DRD2 agonists have been investigated as an alternative to L-DOPA, aiming to delay the onset of dyskinesias. One advantage is the availability of compounds with long half-life, since one of the problems of L-DOPA is the pulsatile stimulation of the receptors (Huot et al., 2013). In addition, it has been suggested that chronic treatment with the D2R agonist could normalize the up-regulation of NMDAR/AMPA receptor (AMPAR) ratio (Bagetta et al, 2012). At the molecular level, DRD2-class receptors activate the Akt/GSK3 signalling cascade (for review see (Beaulie et al., 2007a). In dyskinetic monkeys changes in this pathway in the post-commissural striatum have been correlated with the development of AIMs (Morissette et al., 2010). However, some of the changes that these authors observed in the putamen, in particular the increase in pGSK3 Ser9, appear paradoxical since DRD2 activation is associated with an increase in the activity

of GSK3 (Beaulieu et al., 2011). Indeed, we observed a clear reduction in both pAkt and pGSK3 Ser21/9 corresponding to canonical DRD2-signalling activation in dyskinetic monkeys. It is true that analysis at this time point does not allow detecting the molecular changes that occur during peak-dose dsykinesias. On the other hand, the activation of Akt/GSK3 signalling cascade through DRD2 corresponds to the second way of this signalling cascade activation, mediated by beta arrestin (Beaulieu et al., 2007a). These type of changes as well as long-lasting modifications in signalling cascades maybe better appreciated in this paradigm. Nevertheless, when we analyzed all the animals for L-DOPA treatment, we concluded that DRD2 activity was related to the treatment and not to the presence of dyskinesias.

Finally, we believe that the most remarkable finding of our study is that L-DOPA-induced molecular changes were limited to the post-commissural putamen, even when TH levels were equivalent in both striatal nuclei. This regional effect could be dependent on the anatomo-functional organization of the basal ganglia. Whereas the anterior region and the medial part of the posterior region of the caudate nucleus and the rostral regions of the putamen correspond to associative and limbic territories, at the post-commissural level, nearly the entire putamen and the lateral part of the caudate nucleus are related to sensoriomotor territories (Parent and Hazrati, 1995). Thus, our results point out that molecular events underlying LID are taking place primarily in the more caudal sensoriomotor territories of the striatum, the post-commissural putamen. Furthermore, although levels of TH were similar in both nuclei, it is plausible that there are regional differences in proximal dendritic remodelling, spine density, receptor redistribution, cortico-striatal synapses and other plastic and/or toxic changes that we have not addressed in this study. Likewise, regional specific changes in peptidergic transmission attributable to different cellular processing, have been recently reported in

this model (Bourdenx et al., 2013). These studies suggest that the failure of current pharmacological approaches can be partly due to their lack of regional selectivity. Indeed it may be required to take this variable into account, in addition to targeting specific receptor subtypes to normalize striatal DA signalling pathways, in order to develop more efficacious therapies.

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**Statement of Interest**

None.

**Figure Legends**

**Figure 1. Experimental design and characterization of AIMs in monkeys.** (*a*) Schematic representation of experimental treatments and groups. (*b*) L-DOPA induced improvement in motor disability is shown as percentage change after 10 mg/kg of Madopar®. (*c*) Evaluation of the time course (240 min) of dyskinesias after a single injection of L-DOPA (10 mg/kg) in the four dyskinetic animals included in the study. (*d*) Representative picture of a brain slice at the level of the post-commissural striatum. Abbreviations: Cd: caudate, P: putamen, GPe: external globus pallidus, GPi: internal globus pallidus, FCx: frontal cortex and TCx: temporal cortex. (*e*) Western blot representative images (upper panel) and quantification (lower panel) showing TH expression of putamen (upper panel) and caudate (lower panel). Groups: Control (C; n = 3), MPTP-lesioned saline (P; n = 3), Non-dyskinetic (ND; n = 4) and Dyskinetic (D; n = 4). Data represent median and ranges. * $p < 0.05$.

**Figure 2**. **Dopamine receptor protein expression in the post-commissural striatum.** Western blot representative images (upper panel) and quantification (lower panel) of (*a*) DRD1, (*b*) DRD2 and (*c*) DRD3 expression in the caudate (right) and the putamen (left). Groups: Control (C; n = 3), MPTP-lesioned saline (P; n = 3), Non-dyskinetic (ND; n = 4) and Dyskinetic (D; n = 4). Data represent median and ranges. * $p < 0.05$.

**Figure 3. PKA and its substrates in the post-commissural putamen.** Western blot representative images (upper panel) and quantification (lower panel). (*a*) p(Ser/Thr) PKA substrates, (*b*) pPKAc, (*c*) pDARPP32, (*d*) pSTEP61 and (*d*) pERK2 levels. Groups: Control (C; n = 3), MPTP-lesioned saline (P; n = 3), Non-dyskinetic (ND; n = 4) and Dyskinetic (D; n = 4). Data represent median and ranges. * $p < 0.05$, ** $p < 0.01$.

**Figure 4. DRD2 signalling pathway in the post-commissural putamen.** Western blot representative images (upper panel) and quantification (lower panel). (*a*) pAkt Thr308,

(*b*) pGSK3 Tyr216 and, (*c*) pGSK3 Ser9 levels. Groups: Control (C; n = 3), MPTP-lesioned saline (P; n = 3), Non-dyskinetic (ND; n = 4) and Dyskinetic (D; n = 4). Data represent median and ranges. * $p < 0.05$.

**Supplementary Figure 1**. **Dopamine receptor protein expression in the pre-commissural striatum.** Western blot representative images (upper panel) and quantification (lower panel) of (*a*) DRD1, (*b*) DRD2 and (*c*) DRD3 expression in the caudate (right) and the putamen (left). Groups: Control (C; n = 3), MPTP-lesioned saline (P; n = 3), Non-dyskinetic (ND; n = 4) and Dyskinetic (D; n = 4). Data represent median and ranges.

**Supplementary Figure 2**. **PKA and its substrates phosphorylation in the post-commissural caudate.** Western blot representative images (upper panel) and quantification (lower panel). (*a*) pSer(Thr) PKA substrates, (*b*) pPKAc, (*c*) pDARPP32, (*d*) pSTEP61 and (*d*) pERK2 levels. Groups: Control (C; n = 3), MPTP-lesioned saline (P; n = 3), Non-dyskinetic (ND; n = 4) and Dyskinetic (D; n = 4). Data represent median and ranges.

**Supplementary Figure 3. DRD2 signalling pathway in the post-commissural caudate.** Western blot representative images (upper panel) and quantification (lower panel). (*a*) pAkt Thr308, (*b*) pGSK3 Tyr216 and, (*c*) pGSK3 Ser9 levels. Groups: Control (C; n = 3), MPTP-lesioned saline (P; n = 3), Non-dyskinetic (ND; n = 4) and Dyskinetic (D; n = 4). Data represent median and ranges.

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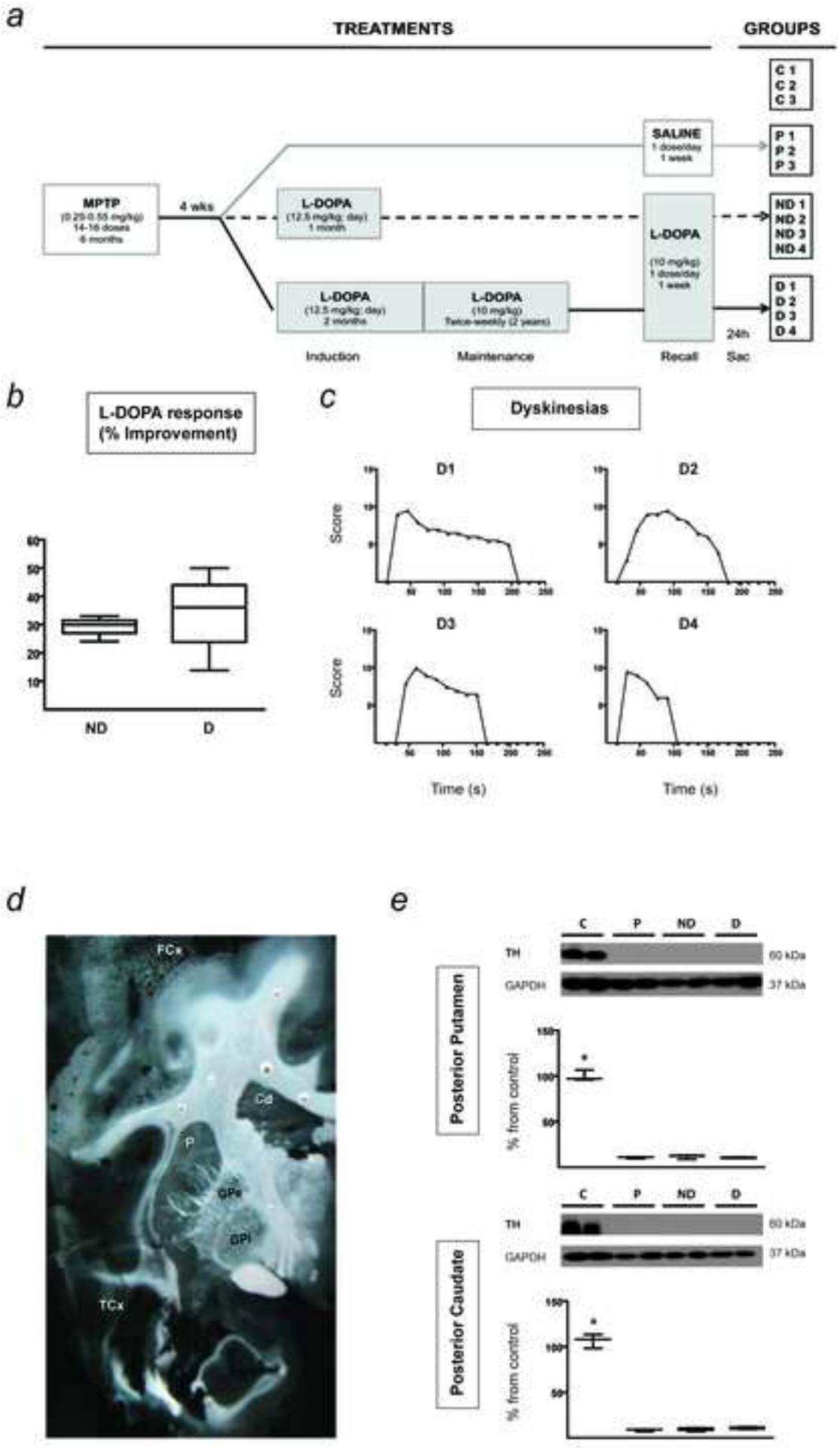

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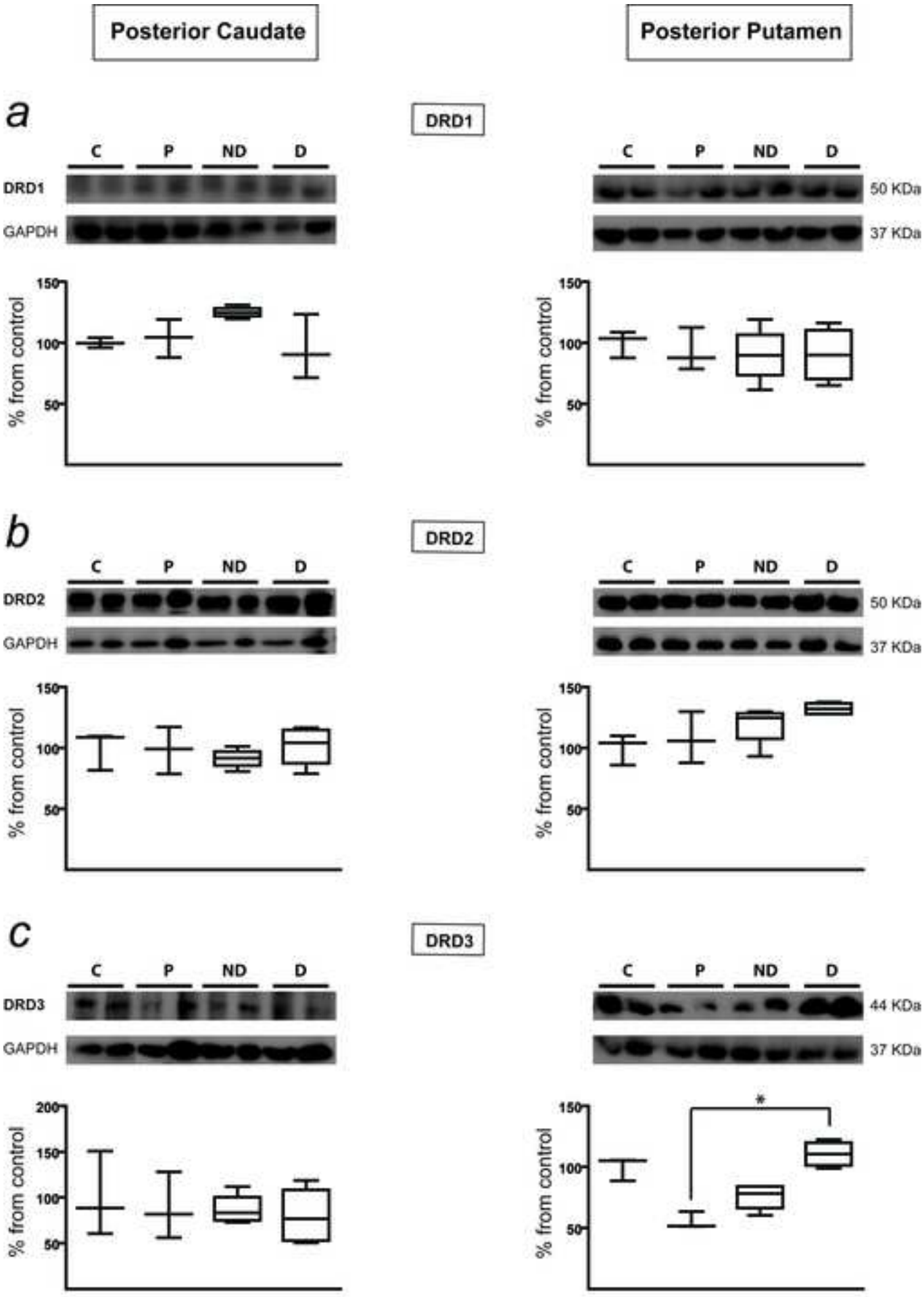

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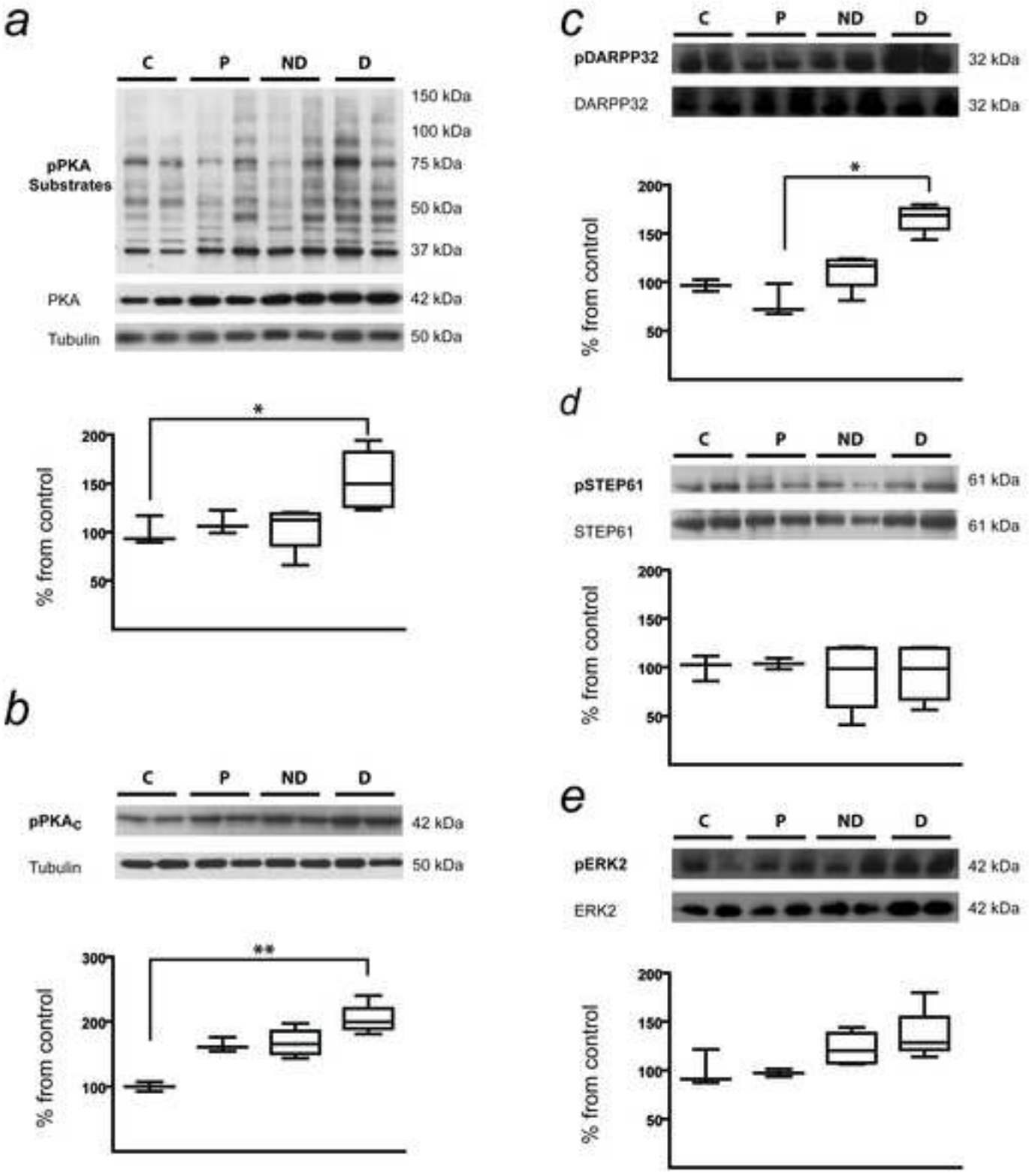

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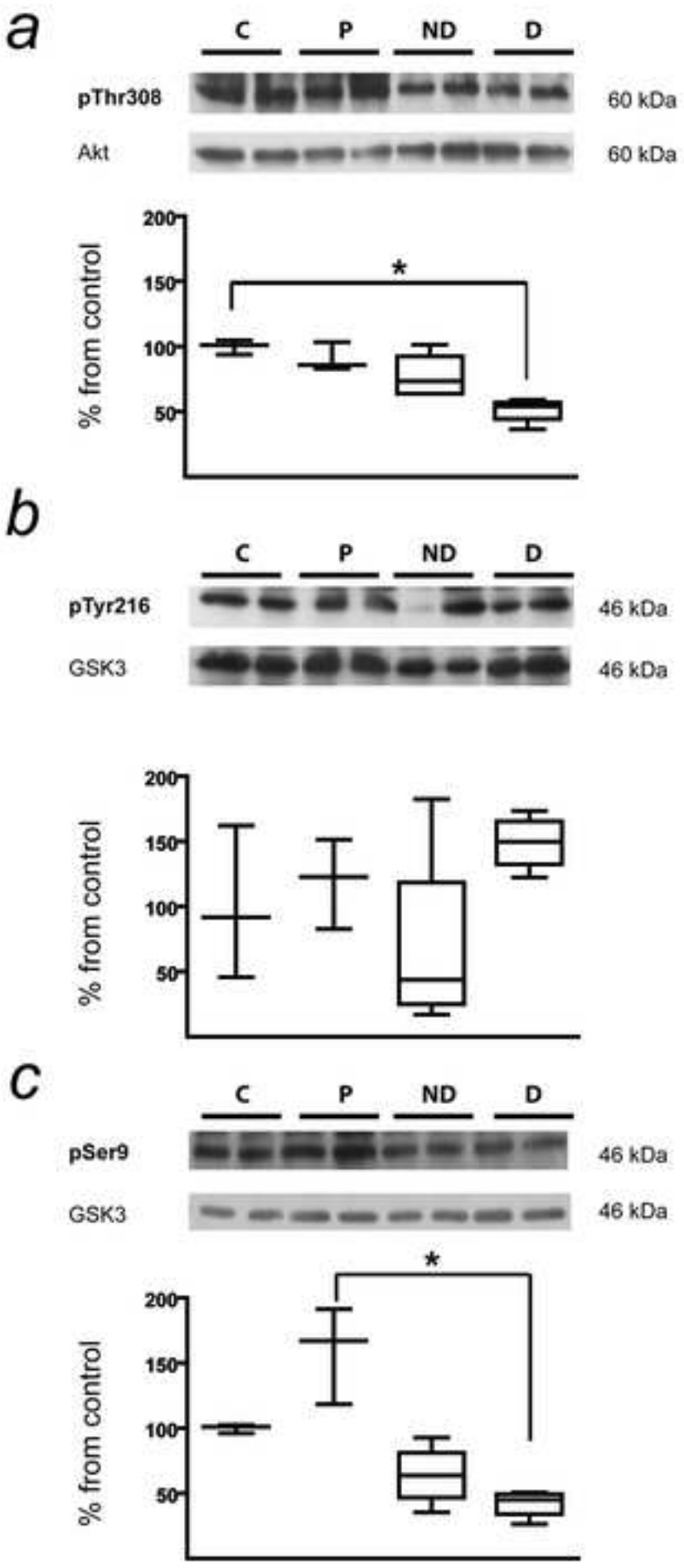

Supplementary Material Fig 1

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Supplementary Material Table 1

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Table 1
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**Table 1. MPTP administration and average parkinsonian stable scores (4 weeks after the last MPTP dose).**

| Animal | Weight (kg) | | MPTP (0.25-0.6 mg/kg/week) | | Disability Score (0-24) |
|---|---|---|---|---|---|
| | Study start | Study end | Nº Doses | Cumulative dose (mg) | |
| P1 | 2.4 | 3.6 | 10 | 7.53 | 13.5 |
| P2 | 2.7 | 4.2 | 13 | 12.55 | 14 |
| P3 | 2.7 | 3.6 | 15 | 17.56 | 14 |
| ND1 | 2.5 | 6.2 | 17 | 16.83 | 13.5 |
| ND2 | 2.8 | 5.8 | 13 | 12.76 | 12.5 |
| ND3 | 3.4 | 6.7 | 12 | 18.37 | 13 |
| ND4 | 3.6 | 6.4 | 35 | 89.02 | 12.5 |
| D1 | 3.1 | 5.1 | 16 | 19.50 | 12 |
| D2 | 2.6 | 6.3 | 17 | 18.89 | 11.5 |
| D3 | 2.9 | 6.4 | 14 | 18.95 | 13 |
| D4 | 2.8 | 5.6 | 15 | 19.96 | 13 |

Table 2
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**Table 2. Dopamine receptor mRNA expression in the post-commissural striatum.** Groups: Control (C; n =3), MPTP-lesioned saline (P; n = 3), Non-dyskinetic (ND; n = 4) and Dyskinetic (D; n = 4). Data are presented as group mean ± SD. * $p < 0.05$ dyskinetic vs. non-dyskinetic.

| **Group** | **Post-commissural Caudate** | | | **Post-commissural Putamen** | | |
|---|---|---|---|---|---|---|
| | DRD1 | DRD2 | DRD3 | DRD1 | DRD2 | DRD3 |
| C | 1 ± 0.38 | 1 ± 0.31 | 1 ± 0.15 | 1 ± 0.55 | 1 ± 0.12 | 1 ± 0.11 |
| P | 0.79 ± 0.27 | 1.04 ± 0.69 | 0.84 ± 0.22 | 1.05 ± 0.26 | 1.21 ± 0.47 | 0.93 ± 0.36 |
| ND | 0.96 ± 0.34 | 1 ± 0.6 | 0.66 ± 0.13 | 1.23 ± 0.3 | 1.22 ± 0.61 | 0.52 ± 0.13 |
| D | 1.33 ± 0.31 | 1.95 ± 0.09 | 1.35 ± 0.43 | 0.98 ± 0.14 | 1.63 ± 0.16 | **1.31 ± 0.07*** |