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Research article

Regulation of Pleiotrophin and Fyn in the striatum of rats undergoing L-DOPA-induced dyskinesia

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ABSTRACT

L-DOPA is the gold standard pharmacological therapy for symptomatic treatment of Parkinson's disease (PD), however, its long-term use is associated with the emergence of L-DOPA-induced dyskinesia (LID). Understanding the underlying molecular mechanisms of LID is crucial for the development of newer and more effective therapeutic approaches. In previous publications, we have shown that Pleiotrophin (PTN), a developmentally regulated trophic factor, is up-regulated by L-DOPA in the striatum of dopamine denervated rats. We have also shown that both mRNA and protein levels of RPTPζ/β, a PTN receptor, were upregulated in the same experimental condition and expressed in striatal medium spiny neurons. The PTN-RPTPζ/β intracellular pathway has not been fully explored and it might be implicated in the striatal plastic changes triggered by L-DOPA treatment. RPTPζ/β is part of the postsynaptic density zone and modulates Fyn, a Src tyrosine kinase that regulates the NR2A and NR2B subunits of the NMDA receptor and has been singled out as a key molecule in the development of LID. In this study, we evaluated the changes in PTN and Fyn protein levels and Fyn phosphorylation status in the 6-OHDA rat model of PD rendered dyskinetic with L-DOPA. We found an increase in the number of PTN immunoreactive neurons, no changes in the amount of total Fyn but a significant increase in Fyn phosphorylation in the dorsolateral striatum of dyskinetic rats. Our results support the idea that both PTN and Fyn may be involved in the development of LID, further contributing to the understanding of its molecular mechanisms.

1. Introduction

L-DOPA is the treatment of choice for Parkinson's Disease (PD), however, its prolonged use triggers undesired side effects, including L-DOPA-induced dyskinesia (LID), that affects the majority of patients after 5–10 years of treatment, and constitutes a clinically relevant therapeutic problem [1]. A great challenge in this area is to reduce the development of LID without affecting the positive restorative effect of dopamine (DA) stimulation, thus improving the therapeutic window of L-DOPA.

The precise mechanisms involved in the pathophysiology underlying LID are poorly understood. Dopamine D1 receptor (D1R) and glutamate NMDA receptor (N-methyl-D-aspartate; NMDAR) are concentrated at the postsynaptic density (PSD) zone, a highly organized subcellular fraction of dendrite spines, a main player in the development and maintenance of dyskinesia and abnormal synaptic plasticity. D1R mediates directly the effect of DA through the canonical signaling pathway [2] which is strongly linked to the development of LID but also to the restorative effect of L-DOPA [3], therefore these molecules are weak targets to reduce LID because any anti-dyskinetogenic effect might counteract the therapeutic benefit. On the other hand, NMDARs trigger action potentials and crosstalk with the canonical pathway modulating ERK and fosB [2]. Antagonists of NMDAR are effective in reducing LID in animal models [4] and patients, without compromising the restorative action of L-DOPA [5]. In fact, this represents the only available target against LID, even if its efficacy is partial and its long term use remains controversial [5].

DA depletion induces plastic rearrangements at the PSD zone upon

Abbreviations: 6-OHDA, 6-hydroxydopamine; AIMs, abnormal involuntary movements; DA, dopamine; L-DOPA, L-3,4-dihidroxiphenyl-alanine; LID, L-DOPA induced dyskinesia; MSNs, medium spiny neurons; NMDA, N-methyl-D-aspartate; NMDAR, NMDA receptor; PD, Parkinson's Disease; PSD, postsynaptic density; PTN, pleiotrophin; SFK, Src family kinase; SNpc, *substantia nigra pars compacta*; TH, tyrosine hydroxylase

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DA and glutamate stimulation through changes in the phosphorylation status of their components which are mainly mediated by the scaffolding protein PSD-95 [6] and Fyn [7,8]. These changes likely influence downstream effectors leading to LID.

We have previously screened for gene expression changes in the denervated striatum following L-DOPA treatment, and found that the neuropeptide Pleiotrophin (PTN), a cytokine expressed in striatal interneurons [9,10], was enhanced by L-DOPA treatment [11,12]. PTN mediates several functions during development such as angiogenesis, mitogenesis, neuronal growth and differentiation. In the adult brain, its expression is restricted to some areas and has been reported to be upregulated after injury and plasticity [13]. Concerning the nigroestriatal system, PTN has been found to be neuroprotective for mesencephalic dopaminergic neurons *in vitro* [14] and *in vivo* [15]. In the adult striatum, PTN is expressed by two sub-population of interneurons: the GABAergic interneurons that co-express nitric oxide synthase (NOS)/somatostatin (SST)/Neuropeptide Y, and the cholinergic interneurons [9,10]. We have found that the PTN receptor RPTPζ/β is upregulated by L-DOPA and expressed by striatal MSNs [11]. In neurons, RPTPζ/β is part of the PSD complex, where it interacts with PSD-95 [16], and modulates Fyn phosphorylation in response to PTN [17]. Fyn is a Src tyrosine kinase that regulates NMDAR function [7], and mediates the subcellular re-distribution of NMDAR by D1R, taking place in the DA denervated striatum, following L-DOPA treatment [8]. Fyn-KO mice showed reduced development of LID by a still unknown mechanism [18]. Moreover, PSD-95, which has been recently shown to mediate LID [6], promotes the phosphorylation of NMDAR by Fyn [19]. Despite the importance of tyrosine phosphorylation in the modulation of NMDA signaling, the role of the kinase Fyn in the development of LID has not been fully addressed. To attain this goal, we have analyzed the number of cells expressing PTN and the protein amounts and phosphorylation status of Fyn in the striatum of 6-OHDA-lesioned rats rendered dyskinetic by L-DOPA.

2. Material and methods

2.1. Animals

The study was performed on adult male Wistar rats from *Facultad de Farmacia y Bioquímica (Universidad de Buenos Aires, Argentina)*. All surgical procedures and experimental manipulations were performed in accordance with the European Directive 2010/63/EU and the Ethics Committee of *Facultad de Farmacia y Bioquímica*.

2.2. 6-hydroxydopamine lesion

Rats received a stereotaxic injection of 6-hydroxydopamine (6-OHDA) in the medial forebrain bundle (MFB) as previously described [20,21]. Briefly, under deep anesthesia with ketamine/xylazine 40/10 mg/kg i.p., (Ketamina 50, Holliday Scott, Argentina and Xylazine, Kensol, König, Argentina), rats received 8 µg of 6-OHDA (free base) (MP Biochemicals, USA) in 4 µl of 0.1% ascorbic acid, at a rate of 0.5 µl/min. Coordinates from bregma (mm) were: AP: −2.0, ML:1.5 and DV: 8.3, incisal bar: −3.3, according to the Rat brain Atlas [22]. Rats received desipramine 30–45 min before 6-OHDA injection (Sigma, USA; 25 mg/kg, i.p.).

2.3. Pharmacological treatments

One month after surgery, animals were treated daily with L-3,4-dihydroxyphenyl-alanine (L-DOPA) for 11 days to induce LID. We administered commercially-available L-DOPA (Lebocar, L-DOPA/carbidopa 250/50 mg, Pfizer, Argentina) once a day at doses of 50 mg/kg/day, diluted in tap water, by oral *gavage*. The dose of L-DOPA was determined in a dose response experiment (Fig. 1B) and already used by our group [21]. It was such that produced significant functional recovery of forelimb use in the cylinder test and a significant level of dyskinesia.

2.4. Behavioral evaluation

Motor impairment due to dopaminergic degeneration was assessed in the cylinder test, as reported [4,21]. Animals showing marked spontaneous behavioural deficit after 6-OHDA injection were selected for further pharmacological studies. Abnormal Involuntary Movements (AIMs) or 'dyskinesias' were measured following standard protocols as before [4,20,21] by a blinded observer every two days after the first administration of L-DOPA. On each testing day, animals were observed and scored for 2 min every 30 min until no further AIMs were detectable. Two categories of AIMs were observed and rated separately: 1) Forelimb Dyskinesia (FD): twitching or jerking movements of the forelimb contralateral to the lesion of a choreic (non-rhythmic, spasmodic) or ballistic (choreic movements of a larger amplitude) pattern. 2) Axial Dystonia (AD): lateral deviation of the trunk, neck, and head toward the contralateral side, leading to a loss of orthostatic equilibrium. The maximal scores of FD and AD recorded after a drug challenge were added, given a single AIM score per rat per drug challenge with values ranging from 0 to 8. Orolingual dyskinesia were not determined because, in our experience in rats, both FD and AD have a superior discriminating power to indicate the presence of dyskinesia while orolingual is easily mistaken, introducing variability [20].

2.5. Immunohistochemistry

Rats were perfused transcardially with 4% paraformaldehyde 30 min after the 11th L-DOPA administration, as previously reported [21]. Coronal tissue sections of striatum and *substantia nigra pars compacta* (SNpc) from 30-µm-thick respectively, were cut in a freezing microtome.

Immunodetection was performed as before [11,15]. Briefly, free-floating sections were incubated overnight at 4 °C either with rabbit anti-tyrosine hydroxylase (TH; 1:1000, #P40101-0; Pel-Freez Biologicals, USA) or goat anti-PTN (1:100; #SC-1394; Santa Cruz Biotechnology, USA), followed by anti-rabbit or anti-goat biotin-conjugated antibody (1:250; Vector Laboratories, USA), avidin-biotin peroxidase complex (1:125; Vectastain, ELITE ABC kit, Vector Laboratories, USA), and developed with 3,3′-diaminobenzidine (Sigma, USA). To evaluate the extent of dopaminergic denervation, we determined the presence of TH+ cells in the SNpc and striatal TH-immunoreactivity. As we have previously reported using this same model, immunohistochemistry confirmed an almost full depletion of TH+ cell bodies in the SNpc (< 10 TH+ cells per coronal mesencephalic section) and axon terminals in the striatum ipsilateral to the 6-OHDA injection site in all rats included in the study [21,23]. PTN positive neurons (PTN+) were counted on striatal sections using the Mercator Pro software (Explora Nova, France) in each experimental groups of rats (n = 6–7 animals per group). Data was expressed as the percentage of the sum of PTN+ neurons in the dorsolateral (DL) lesioned striatum with respect to the contralateral one.

2.6. Western blot

Striata were quickly dissected 30 min after the last L-DOPA administration as reported [11,18]. Tissues were gently homogenized in a glass Teflon homogenizer and processed following standard protocols [18]. Briefly, 50 µg of total protein extracts were loaded in 12% SDS-PAGE gels, run and transferred to 0.2-mm nitrocellulose membranes. Proteins of interest were detected using the following primary antibodies: rabbit anti-Fyn (1:500, #SC-16, Santa Cruz Biotechnology, USA), rabbit anti-FosB/ΔFosB (1:2000, #SC-48, Santa Cruz, USA), or rabbit anti-β-actin (1:2000, #A2066, Sigma, USA). They were developed with the HRP-conjugated anti-rabbit IgG (1:2000, #7074; Cell

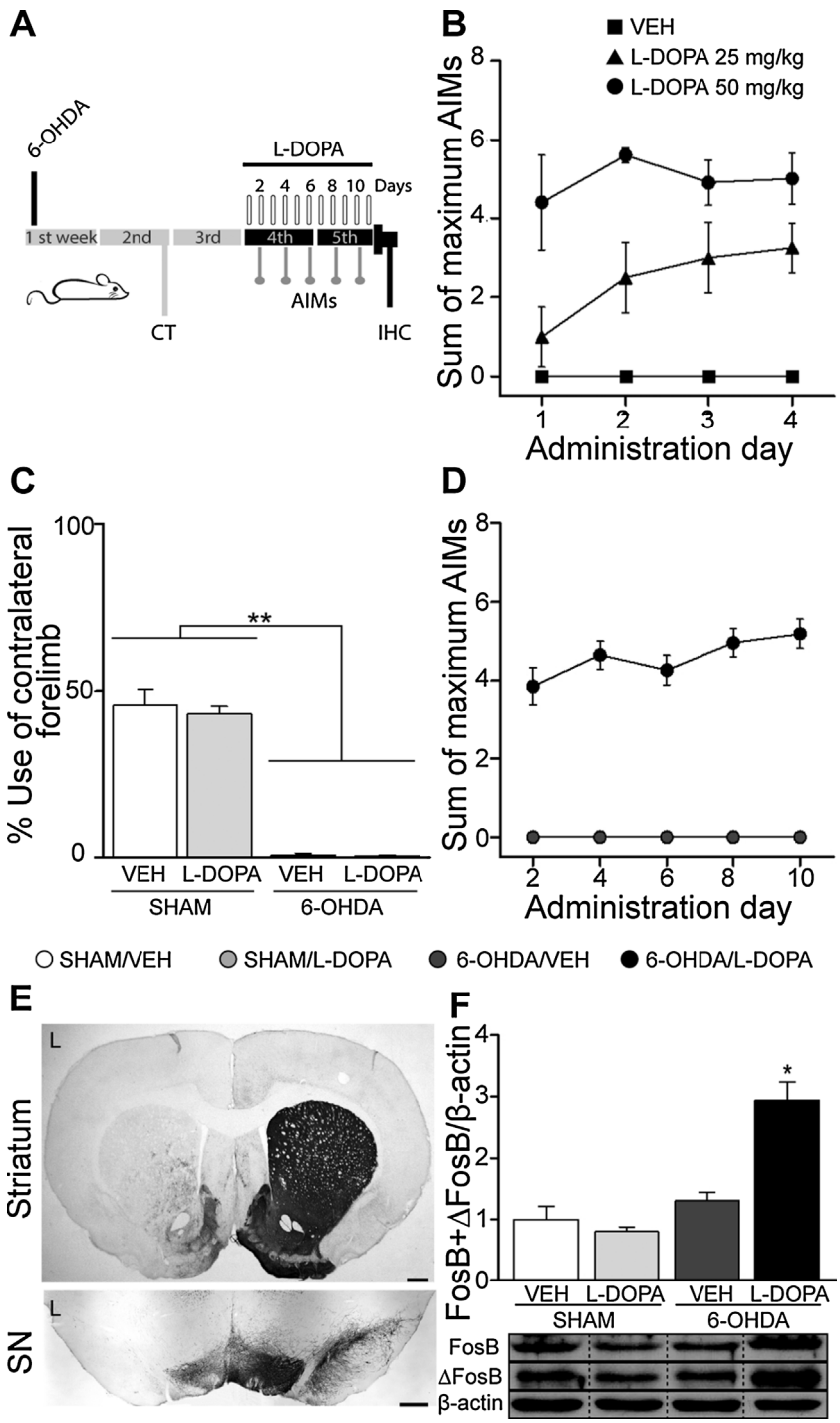

Fig. 1. (A) Schematic representation of the paradigm of LID in rats. (B) Dose response curves of abnormal involuntary movements (AIMs). (C) Cylinder test previous to start L-DOPA treatment. (D) Sum of maximum scores of AIMs of experimental rats. (E) Representative pictures of TH immunostaining of coronal sections of striatum (top) and *substantia nigra* (SN, bottom) of a 6-OHDA injected rat (L: lesioned hemisphere). Scale bar: 500 μm. (F) FosB/ΔFosB protein quantification determined by WB from ipsilateral striatal homogenates. Statistics. C): Two-way ANOVA: effect of lesion ($F_{(1,40)} = 236.41$, $p < 0.0001$), not for treatment ($F_{(1,40)} = 0.3183$, $p = 0.5758$) or interaction ($F_{(1,40)} = 0.2431$, $p = 0.6247$) n = 11. F) effect of treatment ($F_{(1,11)} = 13.15$, $p = 0.004$), lesion ($F_{(1,11)} = 38.67$, $p < 0.0001$) and interaction ($F_{(1,11)} = 21.78$, $p = 0.0007$). *$p < 0.001$, **$p < 0.0001$; n = 3–4 per group. Values are mean ± SEM.

Signaling, USA). Bands were detected by autoradiography using Pierce ECL Western Blotting Substrate (#32106, Pierce Biotechnology, USA) and quantified by densitometry analysis using ImageJ software (US National Institutes of Health, USA).

2.7. Immunoprecipitation

Immunoprecipitation experiments were designed to measure changes in the degree of phosphorylation of Fyn (p-Fyn). Briefly, 500 μg of total striatal proteins were diluted up to 500 μl in lysis buffer, and incubated overnight with 2 μg of rabbit IgG anti-Fyn antibody (200 μ g/ml, #SC-16, Santa Cruz Biotechnology, USA) at 4° C under gently agitation. We added 20 μl of protein A-agarose (#SC-2001, Santa Cruz Biotechnology, USA) and incubated again overnight at 4 °C. Immune complex (protein A/antibody anti-Fyn-IgG/Fyn) was isolated by centrifugation (5 min at 2000*g* and 4 °C). Pellets were washed 3 times with lysis buffer, resuspended in 40 μl of loading buffer (62.5 mM Tris (pH 6.8), 20% glycerol, 2% SDS, 5% β-Mercaptoethanol) and then heated 5 min at 99 °C to disrupt the mentioned complex. The proteins immunoprecipitated by anti-Fyn antibody were subsequently analyzed by Western blotting using a mouse anti-phosphotyrosine antibody (1:500, #05–321, Millipore, USA) and a horse anti-mouse IgG HRP-conjugated secondary antibody (1:4000, #7076; Cell Signaling, USA). Next, blots were stripped and incubated with anti-rabbit HRP-conjugated antibody (1:4000, #7074; Cell Signaling, USA) as a control for loading variability, as has been reported elsewhere [24,25]. Therefore, p-Tyr signals

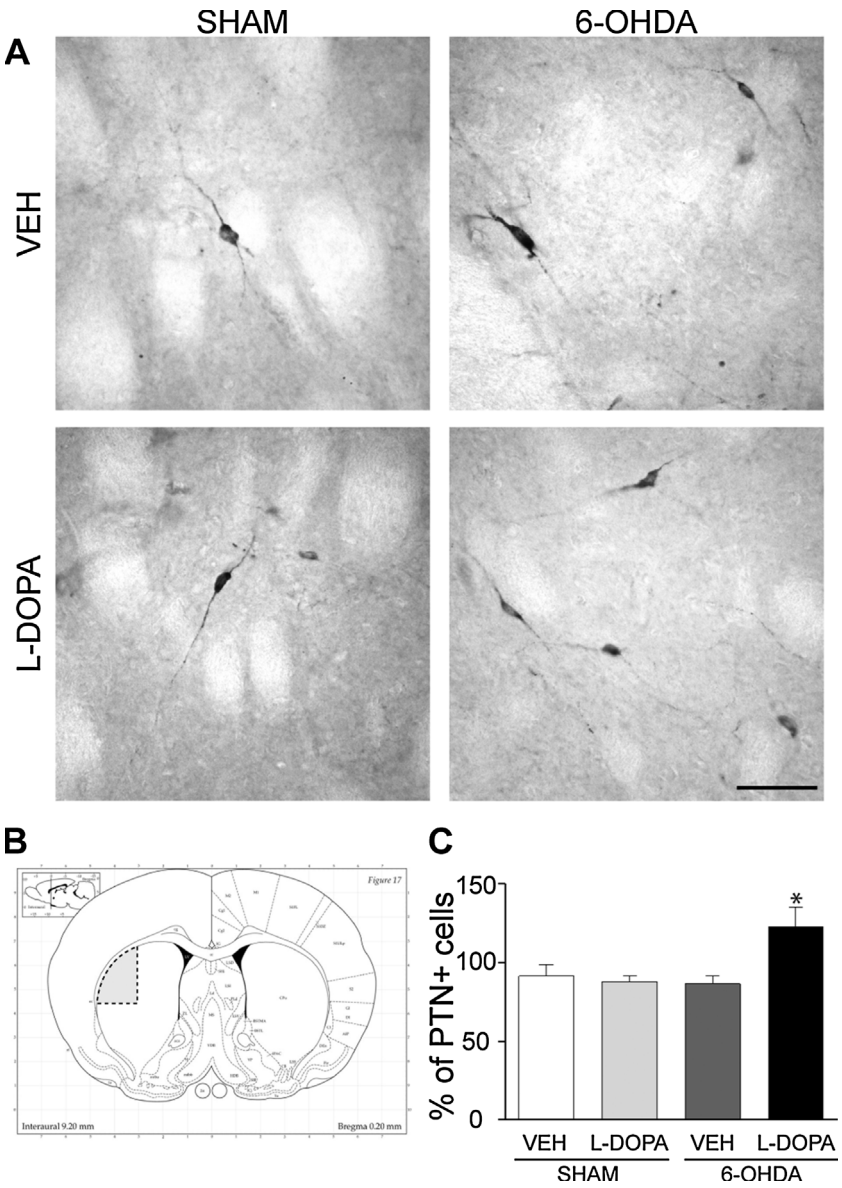

Fig. 2. (A) Representative pictures of PTN immunolabelling at dorsolateral (DL) striata. Scale bar: 50 μm (B) Schematic representation of the analysed area at DL striatum **(C)** Percentage of PTN immunoreactive cells at DL striatum related to the homologous non-lesioned area. Values are mean ± SEM (n = 6-7). Two-way ANOVA: interaction ($F_{(1,21)} = 5.26$, $p = 0.0322$), treatment ($F_{(1,21)} = 3.56$, $p = 0.0731$) and lesion ($F_{(1,21)} = 2.93$, $p = 0.1016$). Bonferroni *post-hoc* comparison determined a significant increase in dyskinetic group (* $p < 0.05$).

were normalized to those of the rabbit IgG that was used in the immunoprecipitation step.

2.8. Statistics

Data are presented as mean ± SEM and analysed by two-way ANOVA, using Graphpad Prism 6.0 software. *Post hoc* comparisons were made using Bonferroni test, and significance was set at $p < 0.05$.

3. Results

3.1. Validation of the L-DOPA-induced dyskinesia rat model

To simulate an advanced stage of PD and LID, severe lesions of the nigrostriatal system were induced by injecting 6-OHDA in the MFB and rats were challenged with L-DOPA according to the protocol depicted in Fig. 1A. 6-OHDA-injected rats showed marked spontaneous deficit as assessed by the cylinder test (Fig. 1C). Only lesioned-rats treated with L-DOPA developed marked AIMs (Fig. 1B,D). *Postmortem* immunohistochemical analyses confirmed an almost complete depletion of TH+ neurons in the SNpc and dopaminergic terminals in the striatum of 6-OHDA lesioned animals (Fig. 1E) similar to what we have already reported with this model [20,21]. We also determined striatal levels of FosB/ΔFosB as a biochemical marker of LID, and found that they were significantly increased only in the striatum of dyskinetic rats (Fig. 1F), as previously reported [3].

3.2. PTN overexpression in LID

We explored whether the number of PTN+ cells was modified after 6-OHDA injection and/or L-DOPA treatment in rats developing LID. Using immunohistochemical staining we detected striatal PTN-expressing cells of large or medium size with fusiform or polygonal somata and without dendritic spines as reported in previous descriptions of striatal interneurons in rodents, and in agreement with previous publications that showed PTN expression in GABAergic NOS/SST/NPY-containing and cholinergic interneurons [9,10] (Fig. 2A). PTN immunostained cells were increased in the DL striatum by L-DOPA in 6-OHDA-lesioned rats (Fig. 2B and C).

3.3. Fyn phosphorylation in LID

In a previous work we showed that RPTPζ/β, a PTN receptor, is upregulated in the striatum following L-DOPA administration [11],

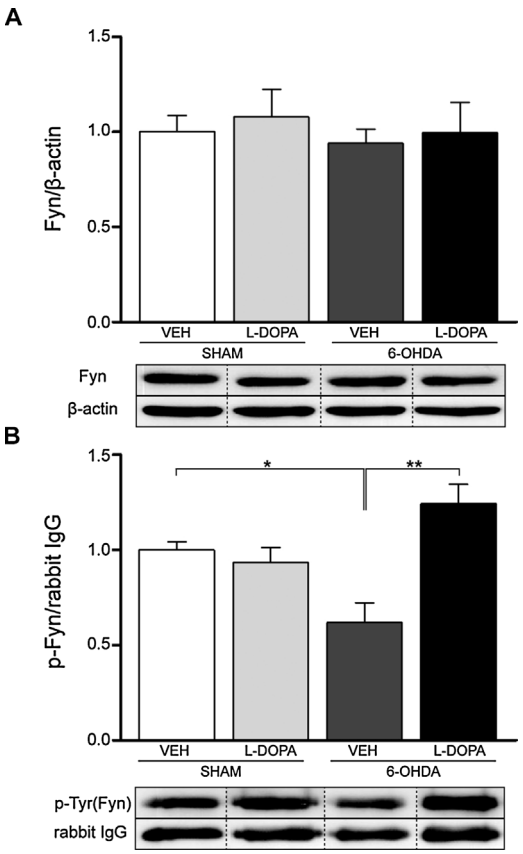

Fig. 3. (A) Total Fyn quantification by Western blot from ipsilateral striata. **(B)** Quantification of p-tyrosine (p-Tyr) from immunoprecipitated striatal Fyn. Two-way ANOVA: A) no effect of lesion, treatment or interaction ($F_{(1,8)} = 0.3451$, $p = 0.5731$; $F_{(1,8)} = 0.2852$, $p = 0.6079$; $F_{(1,8)} = 0.01045$, $p = 0.9211$; respectively). B) Effect of treatment ($F_{(1,12)} = 10.67$, $p = 0.0067$) and interaction ($F_{(1,12)} = 16.36$, $p = 0.0016$), but not of lesion ($F_{(1,12)} = 0.18$, $p = 0.6754$). All data are mean ± SEM. n = 3–4 striata per group. Bonferroni *post-hoc* comparison determined significances within groups: $^{*}p < 0.05$, $^{**}p < 0.001$.

suggesting a role for the PTN-RPTPζ/β pathway in L-DOPA induced plasticity. As Fyn is a downstream effector of PTN via RPTPζ/β [17], as well as key link in D1-NMDA crosstalk [8], we postulated that Fyn would also be modulated by L-DOPA treatment in a dyskinetic paradigm. To test this hypothesis, we determined the protein expression and phosphorylation status of Fyn on Western blots in the rat model of LID using immunoprecipitation.

We found that total striatal Fyn was unchanged irrespective of lesion or treatment (Fig. 3A). On the contrary, two-way ANOVA of Fyn phosphorylation levels showed that both treatment and interaction were significant. Fyn phosphorylation decreased after 6-OHDA injection and significantly increased following induction of dyskinesia (Fig. 3B). These results show that Fyn is strongly modulated by L-DOPA in 6-OHDA-lesioned rats and suggests that it may have an active role in the synaptic remodelling that takes place in striatal neurons in PD and LID.

4. Discussion and conclusions

In this study, we found that PTN and one of its downstream effectors, Fyn, were regulated in the striatum of rats in a paradigm of dyskinesia, further supporting a role of these molecules in L-DOPA induced plasticity, likely associated with the development of LID.

We found that cells expressing PTN in LID are increased in the DL striatum, in line with our previous results in partially denervated and L-DOPA treated rats [12]. The DL striatum is of particular interest in the development of AIMs as it is highly involved in locomotor control and learning [26]. Furthermore, Fyn phosphorylation is strongly regulated by DA depletion and L-DOPA treatment. PTN increases Fyn phosphorylation in tyrosine by inhibiting RPTPζ/β activity [17]. Even though the link between PTN and Fyn has not been shown in LID so far, the concomitant increase of PTN immunoreactivity and tyrosine phosphorylation of Fyn are consistent with such regulation.

The increased number of cells expressing PTN in the striatum of dyskinetic rats are very likely to be interneurons, suggested by the number, distribution, and morphology of the PTN-immunolabeled cells, and supported by previous studies [9,10]; in fact, it is tempting to hypothesize that they are the GABAergic-NOS/SST/NPY and cholinergic subtypes. This area is of increasing interest and further work is necessary to determine the types of striatal interneurons involved and their contribution to the development of LID.

The expression patterns of PTN and RPTPζ/β [9,11] support the hypothesis that these molecules could be involved in plastic changes of striatal neurons after dopaminergic loss and DA replacement treatment with L-DOPA. Notably, it has been speculated that PTN could also be involved in the mechanism of drug addiction as well as LID development [11,27], two molecular mechanisms that are closely related [28]. However, the putative role of PTN or any of its effectors have not been fully studied in LID. In addition to Fyn, it has been reported that upon stimulation of PTN, RPTPζ/β modulates two very important structural molecules such as beta-catenin [29] and beta-adducin [30]. These molecules are involved in many critical molecular functions including structural cytoskeletal changes. Moreover, some recent findings described increased structural plasticity in the striatum of MSNs in LID, remarkably, changes in spine density [31]. Thus, the cell adhesion molecule beta-catenin and the cytoskeletal protein beta-adducin could play a role in the formation, maturation, and stabilization of synaptic spines modified in LID. It is therefore tempting to speculate that PTN could participate in the modulation of the microarchitecture of synaptic remodeling in an orchestrated manner together with other intracellular factors. Further work will be necessary to confirm this hypothesis and to determine the importance of RPTPζ/β in LID.

Fyn, RPTPζ/β and PSD95 are constitutive components of the PSD zone and mediate the crosstalk between NMDAR and D1R in MSNs, which is determinant for the induction of LID [6,8,18]. The specific residues of Fyn specifically modulated by PTN via RPTPζ/β have not yet been determined. It would be of interest to understand how PTN regulates Fyn phosphorylation in LID and to develop a pharmacological intervention targeting this modulation.

Fyn is a critical link in the crosstalk between DA and NMDA receptors and seems to be very important in the regulation of NMDAR function, by preventing clathrin-mediated endocytosis [32]. Modulation of NMDAR function by a specific antagonist of any subunit or an upstream effector are very attractive options to manage LID [1]. Thus, PTN or Fyn become novel candidates to reduce LID through modulation of NMDAR activity.

In conclusion, our results suggest that PTN and Fyn contribute to the maladaptive changes that occur after DA depletion and replacement by L-DOPA, and could underlie the development of dyskinesia. Therefore, these findings point out the PTN/RPTPζ/β/Fyn pathway as novel components in the molecular mechanism of LID and further support Fyn as a target to be considered for pharmacological intervention to manage L-DOPA induced dyskinesia in PD.

Conflict of interest

The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contribution

MS, OG and JF conceived and designed the experiments. GG and MS

performed the experiments and analyzed the data. GG, MB, IT, JF and OG interpreted and discussed data. GG, MB and JF wrote the article. All the authors read and approved the final manuscript.

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