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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