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The Kinase Fyn As a Novel Intermediate in L-DOPA-Induced Dyskinesia in Parkinson's Disease

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Abstract Dopamine replacement therapy with L-DOPA is the treatment of choice for Parkinson's disease; however, its long-term use is frequently associated with L-DOPA-induced dyskinesia (LID). Many molecules have been implicated in the development of LID, and several of these have been proposed as potential therapeutic targets. However, to date, none of these molecules have demonstrated full clinical efficacy, either because they lie downstream of dopaminergic signaling, or due to adverse side effects. Therefore, discovering new strategies to reduce LID in Parkinson's disease remains a major challenge. Here, we have explored the tyrosine kinase Fyn, as a novel intermediate molecule in the development of LID. Fyn, a member of the Src kinase family, is located in the postsynaptic density, where it regulates phosphorylation of the NR2B subunit of the N-methyl-D-aspartate (NMDA) receptor in response to dopamine D1 receptor stimulation. We have used Fyn knockout and wild-type mice, lesioned with 6-hydroxydopamine and chronically treated with L-DOPA, to investigate the role of Fyn in the induction of LID. We found that mice lacking Fyn displayed reduced LID, ΔFosB accumulation and NR2B phosphorylation compared to wild-type control mice. Pre-administration of saracatinib (AZD0530), an inhibitor of Fyn activity, also significantly reduced LID in dyskinetic wild-type mice. These results support that Fyn has a critical role in the molecular pathways affected during the development of LID and identify Fyn as a novel potential therapeutic target for the management of dyskinesia in Parkinson's disease.

Keywords Parkinson's disease · L-DOPA · Dyskinesias · Fyn · NR2B · Saracatinib

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Introduction

Levodopa (L-3,4-dihydroxyphenylalanine, L-DOPA) is still the gold standard treatment for Parkinson's disease (PD); however, its long-term use triggers undesired side effects, including abnormal involuntary movements (AIMs) known as dyskinesia. Levodopa-induced dyskinesia (LID) affects the majority of patients after 5 to 10 years of treatment and constitutes a clinically relevant therapeutic problem [1–3]. Improving the therapeutic window of L-DOPA by reducing dyskinesia currently represents one of the major challenges in the management of PD.

To attain this objective, it is crucial to understand the molecular and cellular processes that take place in a "dyskinetic brain" to identify potential targets for intervention. Dopamine (DA) D1 is a determinant actor in the development of LID [4], and it intensively crosstalks with glutamate N-methyl-D-aspartate (NMDA) receptors (R) to determine short- and long-term striatal effects. While L-DOPA restores DA signaling, the NMDA antagonist amantadine is the only available treatment

for LID, however, numerous side effects frequently prevent its prolonged use and its efficacy is limited [5].

In this scenario, the greatest clinical challenge is to reduce the development of LID without affecting the positive restorative effect of DA stimulation and the NMDAR is a desired target to achieve such a goal as amantadine alleviates LID by reducing glutamate signaling [6], without affecting DA response [5, 7]. In addition to the classical signaling downstream of the dopamine receptors, which likely participate in both the therapeutic and side effects of dopamine stimulation, the postsynaptic density (PSD) zone is determinant of plastic rearrangements and a candidate area aiming at dissecting both effects. D1 and NMDARs actively adapt to DA and glutamate stimulation influencing downstream effectors, in a process mediated by the scaffolding protein PSD-95 [8, 9] and the kinase Fyn [10, 11], among others.

Fyn is a Src tyrosine kinase that regulates the NR2A and NR2B subunits of the NMDAR increasing NMDA currents [11–13], and mediates the subcellular re-distribution of NMDA by D1 receptors, taking place in the dopamine-denervated striatum following L-DOPA treatment [10]. Moreover, PSD-95, which has been recently shown to mediate LID [8], promotes the phosphorylation of NMDARs by Fyn [14]. The NR2B subunit is critical in the regulation of the glutamate NMDAR and therefore in the development of LID [15, 16]. Indeed, a clinical trial with the NR2B antagonist CP-101,606 has been proven to reduce LID despite its side effects [17]. NR2B determines plastic changes related with long-term potentiation (LTP) and long-term depression (LTD) [18, 19], while DA stimulation increases its phosphorylation at tyrosine 1472 [18, 20, 21], which is in fact the target of Fyn [22].

We propose an alternative strategy based on modifying NMDA signaling by means of their intrinsic regulators, and to reach this goal, Fyn arises as a suitable candidate. In view of the well-described role of Fyn in normal brain plasticity, it is also likely to play a relevant role in plastic changes underlying dyskinesia; yet, the putative role of Fyn in LID has not been fully explored. Therefore, the aim of this study was to investigate in vivo the role of Fyn as a key mediator in the development of LID in a well-established mouse model of PD developing dyskinesia under L-DOPA treatment. To this end, we analyzed the development of LID and related molecular changes in Fyn knockout (Fyn-KO) mice and tested a pharmacological intervention with a Fyn inhibitor, in an attempt to reduce LID.

Materials and Methods

Animals

The study was performed on adult C57BL6 or Fyn knockout mice (Fyn-KO), https://www.jax.org/strain/002385 [23], from 3 to 6 months old and weighing 25–30 g. We used either female or male mice for the Fyn-KO experiments and only female for the experiment with saracatinib. All surgical procedures and experimental manipulations were performed in accordance with the UK Home Office and the European Directive 2010/63/EU and approved by the Ethics Committee of "Facultad de Farmacia y Bioquímica" (Universidad de Buenos Aires, Argentina).

Dopaminergic Depletion with 6-Hydroxydopamine

To generate the mouse model of PD, animals were anesthetized with 1.5% isoflurane, then placed into a mouse stereotaxic frame (Stöelting Co.), and injected with 1 μl of 6-OHDA (Sigma, USA cat no. H4381; 3.4 μg/μl, free-base diluted in 0.1% ascorbic acid) at a rate of 0.5 μl/min into the left medial forebrain bundle at coordinates (in mm): AP − 0.1, L + 0.11, V − 0.5 from bregma, ensuring that bregma and lambda were at the same horizontal point, according to the Mouse Brain Atlas [24]. Mice received intense nursing care during the ensuing 3 weeks to reduce weight loss, avoid suffering, and increase survival rates. Twice daily, mice received rehydration therapy (up to 1 ml of 5% dextrose in water, s.c.) and wet mash diet according to individual needs. Wet pellets were supplemented with sunflower-raisins, multicereal preparation ("Nestum", Nestle, Argentina), and yeast gelatine preparation. This diet started 1 week prior to surgery to adapt the animals to the taste of this food supplement and to improve both their weight and physical condition. After surgery, the mice were kept at warmer temperature (23 to 26 °C) and weighed every 2 days. Animals showing no weight recovery or signs of suffering were sacrificed. The injection of 6-OHDA at the level of the MFB has been very well characterized [25]. The sham-operated mice is an independent group of mice that were subjected to the entire surgical procedure with the exception of the 6-OHDA injection.

Pharmacological Treatments

To model LID in rodents, 28 wild-type (WT) and 30 Fyn-KO mice were treated with L-DOPA for 16 days starting 3 to 4 weeks after the 6-OHDA lesion at a dose of 6/12 mg/kg/ day of L-DOPA/benserazide (Sigma, USA cat no. D9628 & B7283) on the first 5 days of treatment and then 12/12 mg/kg/ day i.p. diluted in sterile saline solution. WT mice treated with saracatinib (AZD0530, Cayman Chemical, USA cat no. 11497) received 12/12 mg/kg/day L-DOPA/benserazide throughout the experiment.

Saracatinib was dissolved in 0.5% hydroxypropyl methyl cellulose (HPMC)/0.1% Tween 80 (w/v) and a dose volume of 200 μl was administered by oral gavage, twice a day, at doses of 10 mg/kg/day. The dosage and administration of saracatinib to mice was that described by Kaufman et al., who established the conditions for the use of this drug to reach the central nervous system [26]. To determine the effect of saracatinib

on LID, wild-type (WT) mice were lesioned with 6-OHDA, selected with the cylinder test, and randomly assigned to each of the treatment groups.

Behavior

All behavioral experiments were conducted in a quiet room with controlled temperature, which was separate from the housing area. Animals were handled in advance of the experiments to reduce stress during the testing sessions.

Rotarod

WT and Fyn-KO mice were given 24 h to adjust to their new surroundings prior to being individually placed in a neutral position on the immobile rotarod treadmill (Ugo Basile, Italy). Following 1 min of habituation, the speed was increased to 16 rpm. Mice were given a 2-min training session during which they were repositioned on the rotarod after each fall. Mice were tested 1 h later for 3 min. Latency to the first fall was recorded (in seconds) as an indicator of motor coordination performance. Rotarod test was performed on healthy mice before the 6-OHDA lesion to evaluate if Fyn-KO mice have any motor impairment that might interfere in the development of AIMs.

Cylinder Test

Two weeks after a 6-OHDA lesion, rodents were challenged in the cylinder test to evaluate motor impairment due to dopaminergic degeneration [27]. Briefly, rodents were placed in a plastic transparent cylinder (diameter × height for mice 10 × 20 cm) and the number of wall contacts made with each forelimb (or both together) was measured for 3 min. With these, values of the percentage use of the contralateral forelimb were calculated.

Abnormal Involuntary Movements

Evaluations were performed following standard protocols [28–31] by a blinded observer every 3 days after the first administration of L-DOPA. On each testing day, animals were observed and scored for 1 min at 20-min intervals for 2 h after the administration of L-DOPA. At that time, no further AIMs were detectable in all animals. Briefly, three categories of AIMs were observed [32]: (1) orofacial, discrete vertical (open and close) jaw movements toward the contralateral side, with eventual tongue protrusion; (2) forelimb, 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; and (3) dystonic, lateral deviation of the trunk, neck, and head toward the contralateral side, leading to a loss of orthostatic equilibrium. The frequency and intensity of each AIM were evaluated using a standard scale: 0 = absent; 1 = present for less than half of the observation period; 2 = present for

more than half of the observation period; 3 = present constantly, but suspended by a tactile stimulus; and 4 = present constantly, irrespective of a stimulus. Values for each category were totalled per animal for each day, obtaining the mean per day for each treatment or genotype.

Immunohistochemistry

From the totality of mice included into the analysis of AIMs, 20 per genotype were perfused transcardially with 4% paraformaldehyde 1 h after the last dose of L-DOPA (the remaining 10/genotype were prepared for WB). The brains were processed, and immunodetection performed in both the striatum and substantia nigra as previously reported [33, 34]. Briefly, 30-μm free-floating coronal sections were incubated with the following primary antibody: rabbit anti-tyrosine hydroxylase (Pel-Freez Biologicals USA cat no. P40101 RRID:AB_2313713; TH, 1/1000) and rabbit anti-FosB/ΔFosB (Santa Cruz Biotechnology cat no. sc-48 RRID:AB_631515; 1/10000). The secondary biotinylated antibody used was anti-rabbit IgG (Vector Laboratories cat no. BA-1000 RRID:AB_2313606; 1/250). Sections were developed with the avidin-biotin peroxidase system (Vector Laboratories cat no. PK-6100; 1/125), and diaminobenzidine (Sigma, USA cat no. D5905). Images were captured using a Nikon Eclipse 50i microscope (×4 objective) equipped with a Nikon Digital Sight DS-L1 camera. To analyze both TH or FosB, 4–5 images of the striatum were taken from 1.50 to − 0.10 mm anteroposterior from the bregma [REF Paxinos] for each mouse. The analysis of the images was performed using the ImageJ software (http://rsbweb.nih.gov/ij/, NIH, USA). We have either analyzed the optical density of the staining as reported before [33] or cell counting of FosB/ΔFosB-positive cells using the Plugin ITCN_1_6 (Image-based Tool for Counting Nuclei).

Western Blot

Mice were sacrificed 1 h after the last L-DOPA administration, and the entire left (ipsilateral) striata was immediately dissected and frozen. General procedures were performed as previously reported [23, 34]. Briefly, tissues were gently homogenized in a glass Teflon homogenizer in 150 μl of lysis buffer complemented with HaltTM protease and phosphatase inhibitor cocktail EDTA-free (Pierce Biotechnology, USA cat no. 78441). Total protein content was determined using the Bradford reagent Coomassie brilliant blue G250 (MP Biomedicals, USA cat no. 6104-58-1). For WB, 50–60-μg protein was resolved in 12% Tris-glycine SDS-PAGE gels. The primary antibodies used were the following: anti-Fyn (Santa Cruz Biotechnology cat no. sc-16 RRID: AB_631528; 1/1000), anti-TH (Pel-Freez Biologicals USA cat no. P40101 RRID:AB_2313713; 1/1000), anti-FosB/ΔFosB (Santa Cruz Biotechnology cat no. sc-48 RRID:AB_631515; 1/500), anti-pNR2B-Tyr1472 (Pel-Freez Biologicals cat no. P43301–0, RRID:AB 476111; 1/400), anti-NR2B (UC Davis/

NIH NeuroMab Facility cat no. 75-097, RRID:AB_10673405; 1/400), and anti-β-actin (Sigma-Aldrich cat no. A2066, RRID: AB_476693; 1/2000). As secondary antibodies, we used horseradish peroxidase-conjugated anti-rabbit IgG (H + L) (Cell Signaling Technology cat no. 7074, RRID:AB_2099233; 1/2000) or anti-mouse IgG (H + L) (Cell Signaling Technology cat no. 7076, RRID:AB_330924, 1/3000). Enzymatic chemiluminescence reactions were developed with Western blotting substrate (Pierce Biotechnology, USA cat no. 32106). Immunoreactive bands were detected using an Image Quant 350, and the intensity of bands was determined using Alpha Ease FluorChem v4.1.0 software (Alpha Innotech Corporation, USA).

Statistics

Results are presented as mean ± SEM. Differences between biological conditions were determined using two-tailed unpaired Student's t test and two-way ANOVA with repeated measures using Graphpad Prism 6.0 software. Posthoc comparisons were made using Tukey's test, and significance was set at $p < 0.05$.

Results

Evaluation of LID in Fyn-KO Mice

To evaluate the role of Fyn in LID, we designed a protocol to test the development of AIMs following 6-OHDA lesioning and L-DOPA treatment in Fyn-KO mice, in comparison with their WT controls (Fig. 1a). We found that, at a similar level of lesion, treated Fyn-KO mice developed significantly lower AIMs scores than WT, in a consistent manner along sessions (Fig. 1d). On average, Fyn-KO mice showed a 30% reduction in AIMs scores. In each individual session, we observed a
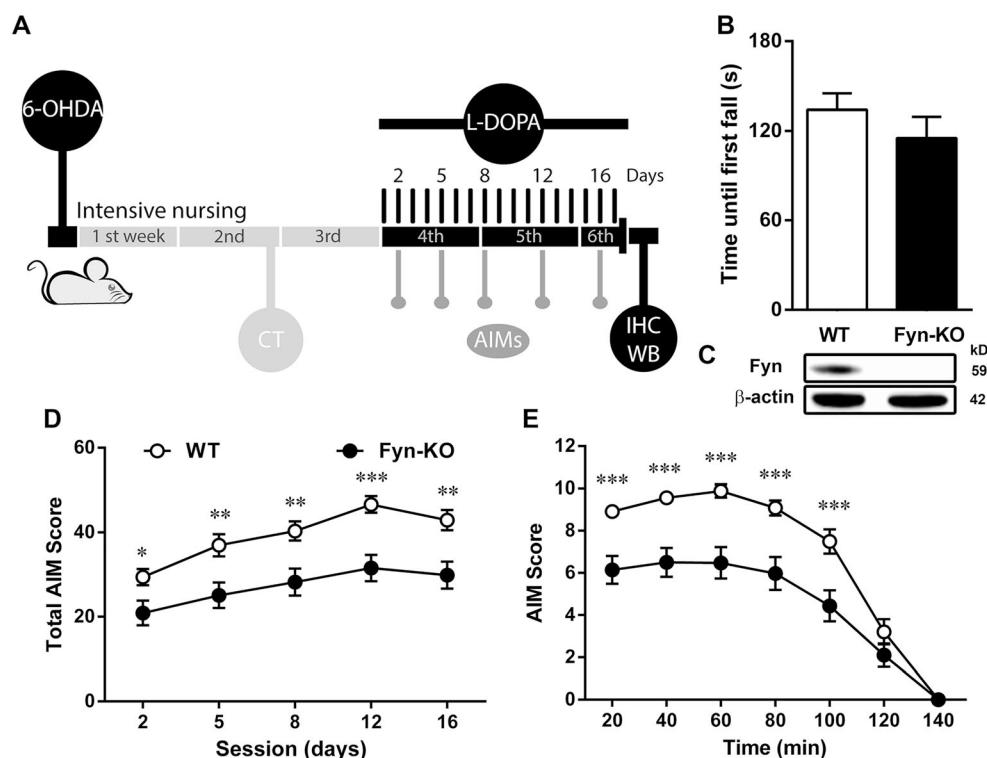
Fig. 1 Evaluation of AIMs in Fyn-KO vs WT mice. **a** Schematic representation of the paradigm of dyskinesia used to evaluate the development of LID in Fyn-KO compared with that of WT. Briefly, Fyn-KO or WT (obtained from Fyn +/- breedings) were lesioned with 6-OHDA into the MFB, received intense care for 2 weeks, and selected by the cylinder test. One week later, they were challenged with L-DOPA to induce dyskinesia. Abnormal involuntary movements (AIMs) were determined on days 2, 5, 8, 12, and 16. Postmortem, we conducted histological or biochemical analysis. **b** Rotarod analysis of Fyn-KO vs. WT mice. Data are mean ± SEM ($n = 6$ mice/group), compared by unpaired Student's t test ($p = 0.3170$). **c** Illustrative WB of Fyn detection to reconfirm genotype. **d** Sum of values for axial dystonia, orolingual, and limb dyskinesia per day throughout treatment. Data are

mean ± SEM ($n = 30$ for Fyn-KO and $n = 28$ for WT). Two-way ANOVA with repeated measures showed no interaction effect (time × genotype) [$F_{(4, 318)} = 0.4017$; $p = 0.8074$], a significant effect of genotype [$F_{(1, 318)} = 51.73$; ***$p < 0.0001$] and time [$F_{(4, 318)} = 8.261$; ***$p < 0.0001$]. Independent Student's t tests were run for each day: day 2 *$p = 0.0118$; day 5 **$p = 0.0047$; day 8 **$p = 0.0036$; day 12 ***$p < 0.0001$; day 16 **$p = 0.0014$. **e** Representative session day corresponding to day 12. Two-way ANOVA with repeated measures showed no interaction effect (time × genotype) [$F_{(5, 318)} = 1145$; $p = 0.3366$], a significant effect of genotype [$F_{(1, 318)} = 75.29$; ***$p < 0.0001$], and time [$F_{(5, 318)} = 29.49$; ***$p < 0.0001$]. Independent Student's t test was run for each time interval, ***$p < 0.0001$ between 20 and 100 min

similar percentage reduction in the intensity of AIMs (Fig. 1e). Each type of AIM showed a similar profile during daily sessions; therefore, data is presented as the sum of all types of AIMs for each day. No sex-related differences were detected, so data from males and females were pooled (WT, $p = 0.62$; KO, $p = 0.83$, determined by unpaired Student's t test).

We determined the performance of Fyn-KO mice on the rotarod in order to assess their locomotor coordination. We found no differences between genotypes, showing that Fyn-KO mice have no evident motor impairment (Fig. 1b). The absence of Fyn in the Fyn-KO animals was reconfirmed by WB (Fig. 1c).

Histological and Biochemical Determinations

Both groups of animals had equal levels of dopaminergic denervation as measured by TH immunohistochemistry in both the striatum and substantia nigra. We determined the minimum level of DA loss necessary to develop LID, in our hands. In the group of mice analyzed by immunohistochemistry, we observed that, according to the detection system used, a minimum of 95% loss of striatal dopaminergic terminals was needed to develop AIMs in WT mice; therefore, only WT and Fyn-KO mice showing
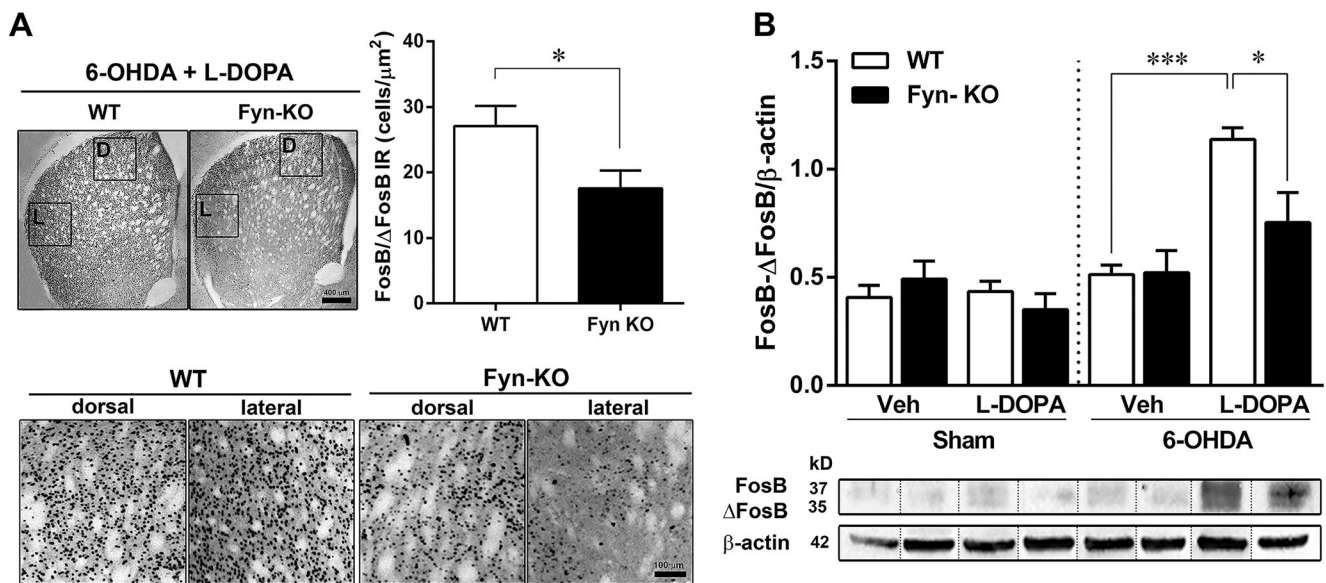
DA denervation above this level were included in the behavioral and histological analysis (Fig. 2a, b). A separate group of animals was used for WB analysis to quantify the amount of striatal proteins, including remaining TH (Fig. 2c). As expected, we observed a very important loss of nigral and striatal TH both in WT- and Fyn-KO-lesioned mice. Similarly, when striatal TH was determined by WB, we set a threshold point of 85% of DA loss necessary in the WT group to induce LID and then excluded from the analysis any animal (either WT or Fyn-KO) below that point. Both WT and Fyn-KO mice included in the study exhibited an equivalent loss of TH content (Fig. 2b, c). Remarkably, the efficiency in lesioning was similar irrespective of the genotype, being 76% for the WT group ($n = 51$) and 68% for the Fyn-KO ($n = 47$, Student's t test, $p = 0.53$).

The immediate early gene FosB is strongly linked downstream of the dopamine and NMDA receptors to the development of LID [35], and the current consensus postulates that ΔFosB accumulates in a linear fashion during the development of dyskinesia [3]. We examined FosB/ΔFosB in different groups of animals and by two different approaches. Immunohistochemical analysis determined that the Fyn-KO group showed a 33% reduction in the number of cells stained with FosB/ΔFosB in the striatum (Fig. 3a). WB showed a
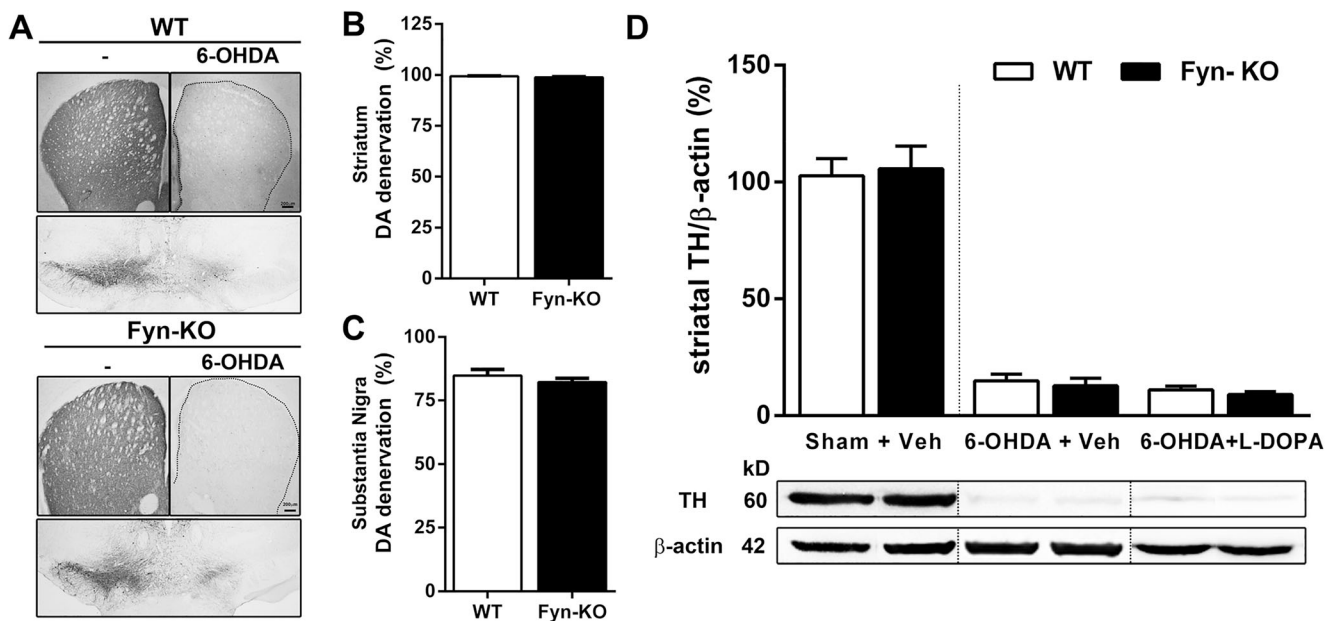
Fig. 2 Tyrosine hydroxilase immunodetection in sham- and 6-OHDA-lesioned Fyn-KO and WT mice. a Illustrative photomicrographs of striatal and nigral TH immunostaining. Scale bar = 200 μm. b Quantification of striatal TH immunoreactivity expressed as percentage of dopaminergic denervation relative to contralateral side. Data are mean ± SEM ($n = 18$ mice/group), compared by unpaired Student's t test ($p = 0.5270$). c Quantification of nigral TH immunoreactivity. Data are mean ± SEM ($n = 9$ mice/group), compared by unpaired Student's t test ($p = 0.3483$). d TH protein levels determined by WB from ipsilateral striatal homogenates of Fyn-KO and WT mice. Percentage of TH/ß-actin normalized to sham + veh WT mice. Data are mean ± SEM (5 to 10 striata/group). In sham + veh-treated mice, TH levels were not different between genotypes, compared by unpaired Student's t test ($p = 0.8090$). There was no effect of genotype [$F(1, 34) = 0.6636$, $p = 0.4210$] or treatment (veh vs. L-DOPA) [$F(1, 34) = 2.417$, $p = 0.1293$] in TH levels between 6-OHDA-lesioned mice, nor was there any interaction effect (treatment × genotype) [$F(1, 34) = 0.0010$; $p = 0.9746$], determined by two-way ANOVA

significant increase of FosB/ΔFosB in WT dyskinetic group (6-OHDA + L-DOPA) compared to 6-OHDA-lesioned mice, and no effect of treatment (veh vs. L-DOPA) in WT sham-operated animals as it was expected. In addition, Fyn-KO dyskinetic mice showed a significant reduction (~ 30%) in the accumulation of FosB/ΔFosB compared to the WT dyskinetic group (Fig. 3b). Considering the consistent and linear association of this transcription factor with LID, we interpret these results as being a strong biochemical support of the behavioral data.

To further investigate the mechanism involving Fyn in LID, we explored the amount and phosphorylation status of the glutamate NMDA receptor subunit NR2B at Tyr1472 which is the target of Fyn. We found a significant effect of the genotype in p-NR2B levels in both sham- and 6-OHDA-operated mice groups, indicating reduced amounts of p-NR2B in Fyn-KO mice irrespective of the lesion or treatment (Fig. 4). As the phosphorylation at Tyr1472 increases NMDAR permeability and enhances NMDA-mediated Ca^{2+} influx [11, 13, 36], this result suggests that the absence of Fyn likely reduces LID through the reduction of NMDAR signaling. It is interesting to note that pioneering detection of total tyrosine phosphorylation of NR2B showed a similar reduction in Fyn-KO mice [12].

Evaluation of a Fyn Inhibitor on the Development of LID

The results presented herein support our hypothesis that Fyn participates in the development of LID. Therefore, we further explored the possibility that a pharmacological inhibitor of Fyn would be able to prevent or revert LID. We selected the Src family inhibitor saracatinib (AZD0530), which has been demonstrated to inhibit Fyn activity in the brain [26]. 6-OHDA-lesioned WT mice, treated with L-DOPA, were administered either saracatinib or vehicle twice a day using two different treatment paradigms: (1) starting 8 days before L-DOPA administration (prevention) or (2) starting 8 days after L-DOPA administration (reversion) (Fig. 5a). In both cases, saracatinib treatment was continued until the end of the protocol. Under these experimental conditions, we found that saracatinib was not effective in reversing already established dyskinesia (Fig. 5b). However, in the prevention paradigm, administration of saracatinib resulted in a significant reduction of the development of LID of approximately 30% (Fig. 5c). The difference between both groups was maintained over time. To illustrate this, we plotted the profile of the dyskinetic score for the first and the last day (Fig. 5d, e). It is worthy of note that after 19 days, saracatinib remained as effective as in the beginning. All animals, irrespective of the protocol to which they were assigned, had equivalent levels of
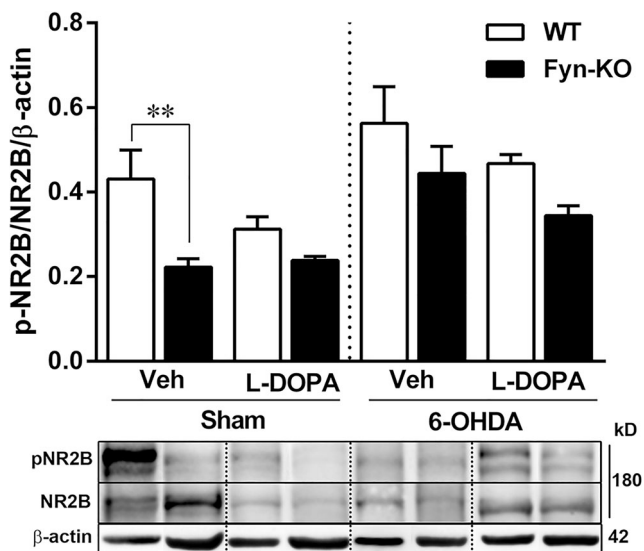
Fig. 4 Striatal protein level of phosphorylated NMDAR subunit NR2B at Tyr1472 in sham and 6-OHDA-lesioned Fyn-KO and WT mice. Amounts of phospho-Tyr1472 of NR2B relative to total NR2B levels normalized to β-actin from ipsilateral striatal homogenates bands. Data are mean ± SEM (n = 5 to 9 striata/group). Two-way ANOVA determined that both sham- and 6-OHDA-treated groups had a significant reduction of p-NR2B levels due to the genotype [$F(1, 17)$ = 13.74, p = 0.0018] and [$F (1, 25)$ = 5.056; p = 0.0336], respectively. There have not been detected in any group an effect of the treatment (sham [$F(1, 17)$ = 1.821, p = 0.1949]; 6-OHDA [$F(1, 25)$ = 3.290, p = 0.0817]) nor of its interaction (treatment × genotype) sham [$F (1, 17)$ = 3.110, p = 0.0958]; 6-OHDA [$F(1, 25)$ = 0.0019, p = 0.9656). **p = 0.0073, by Tukey's test. Bottom panel: representative western blot

dopaminergic denervation (Fig. 5f). The immunohistochemical analysis of FosB/ΔFosB determined that the saracatinib group showed a 28% reduction in their immunoreactive staining in the striatum in comparison with the vehicle-treated group (Fig. 5g).

Discussion

In this study, we report for the first time that the kinase Fyn mediates LID in the 6-OHDA mouse model of PD on the basis of three different evidences: (i) Fyn-KO mice develop less dyskinesia than WT mice; (ii) FosB/ΔFosB accumulates less in Fyn-KO mice than it does in WT mice in the same paradigm; and (iii) the pharmacological inhibition of Fyn using saracatinib reduces the development of LID and the accumulation of FosB/ΔFosB.

We favor the hypothesis that the plastic maladaptive rearrangements that take place in the postsynaptic density (PSD) zone under chronic L-DOPA administration are critical to the genesis of dyskinesia. Among the multiple components, PSD-95 attracts particular attention because of its role mediating the crosstalk between NMDA and D1 receptors, which is determinant for the induction of LID [8]. Fyn has been also singled out as a key component between these two receptors [10], and

recently, it has been demonstrated that PSD-95 also modulates the regulatory element DREAM, that has been shown to mediate LID [37]. Moreover, long-term structural plasticity of the PSD zone is strongly involved in the development of LID, affecting the spine morphology and synaptic transmission, in a different manner in striatonigral or striatopallidal subtypes of medium spiny neurons (MSNs) [38, 39].

The relationship between DA and Fyn in the striatum has been addressed by different in vivo and in vitro approaches but not fully studied in models of PD or dyskinesia. The pivotal paper by Dunah et al. [10] elegantly demonstrated the role of Fyn as an intermediate between D1R effects on NMDAR trafficking. These authors also used Fyn-KO mice and reported that they found evidence of reduced tyrosine phosphorylation of NR2A and NR2B subunits of the NMDAR in vitro, while D1R agonists failed to induce the subcellular distribution of NMDAR in Fyn-KO mice. Yet, they did not explore the behavioral correlations of these findings arguing that Fyn-KO mice "were resistant to a neurotoxic lesion with 6-OHDA". Similarly, Panicker et al. [40] reported attenuated DA loss in the Fyn-KO mice intrastriatally injected with 6-OHDA. The authors attributed that result to a reduced inflammatory response in Fyn-KO may be neuroprotective. On the contrary, we found that the efficiency of the lesion in WT and Fyn-KO mice was similar in our experimental conditions. We believe that the differences between these two reports and our results could be attributed to the very different dynamic of dopaminergic lesion induced by 6-OHDA depending on the injection site: while 6-OHDA injected into the medium forebrain induces an acute dopaminergic degeneration [25, 27, 41], intrastriatal lesions induce a slow and progressive degeneration during 2 to 3 weeks, allowing the action of neuroprotective agents and showing a dopaminergic recovery even in the absence of any treatment [42, 43]. We have analyzed a large number of mice and we can confirm that in our experimental conditions, there were no differences in the extent of dopaminergic lesion between genotypes.

In line with our results, Mao and Wang [21] showed that D1R stimulation by the selective agonist SKF81297 specifically activates Fyn, and not Src, increasing the phosphorylation of NR2B at Tyr1472. Because Fyn and Src are among the Src family kinases (SFK), those enriched at synaptic sites [44, 45], and Fyn is highly expressed in striatal tissue in comparison to Src [46], therefore, Fyn appears to be the putative member of the SFK mediating D1R and NMDAR interactions in striatal neurons.

These evidences are consistent and complementary with our results, summarized in the model presented (Fig. 6). Considering our previous results showing upregulation of the PTN-RPTPζ/β pathway in L-DOPA-treated rats [34] and that RPTPζ/β modulates Fyn phosphorylation in response to PTN [47], it is also tempting to speculate a role for these molecules in L-DOPA-induced plasticity, probably through the modulation of Fyn activity.
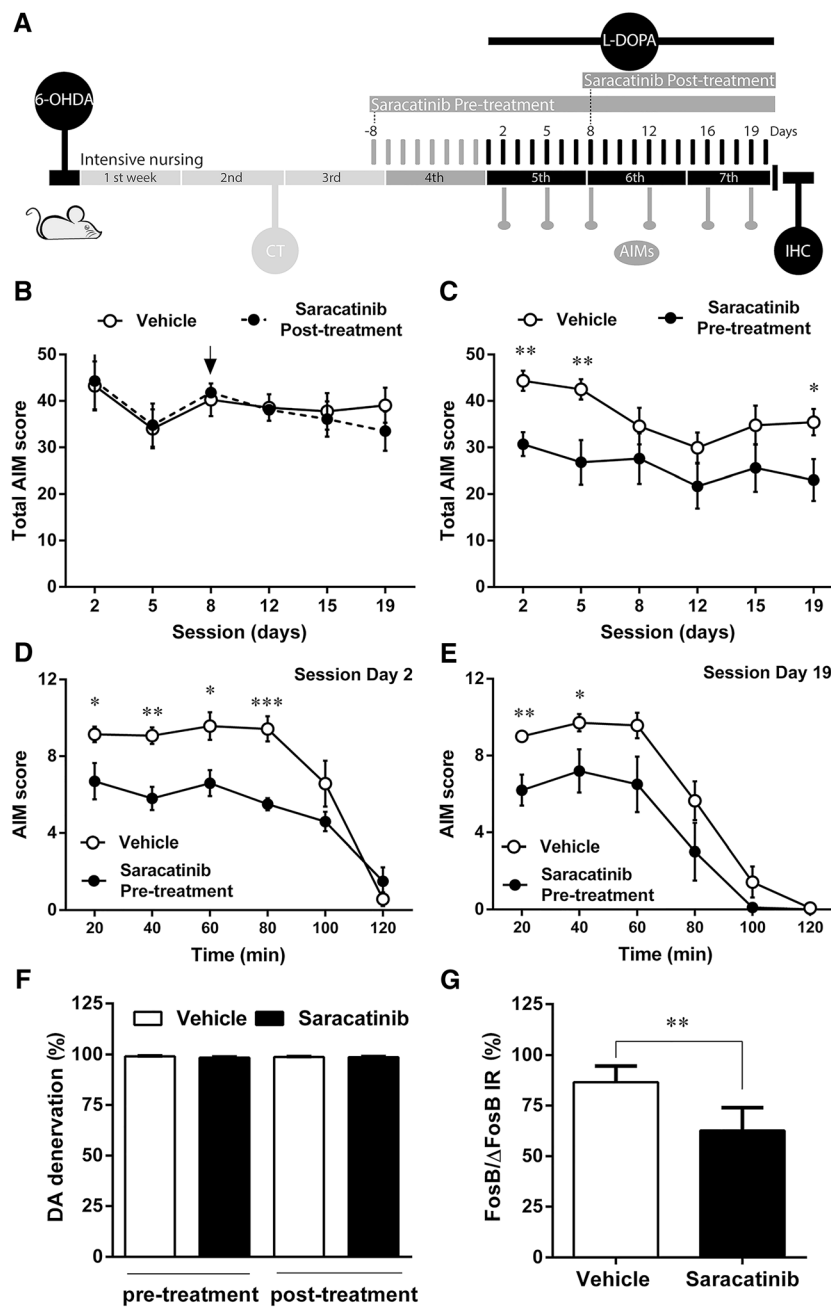
Fyn-KO mice might have a reduced activity of NMDAR, suggested by the reduced phosphorylation of the NR2B subunit at Tyr1472, which can therefore explain the reduced dyskinesia observed in these animals. Recent studies showed that the NR2B subunit antagonist CP-101,606 reduces LID in animal models [18, 48] and patients, but at the expense of inducing several side effects [17]. On the other hand, its use has been controversial as higher doses exacerbate dyskinesia [49]. This bimodal paradoxical response has not been fully addressed but can be attributed to a side effect of the drug rather than the target, therefore, the NR2B subunit is still a desirable target to prevent LID (reviewed in [3]).
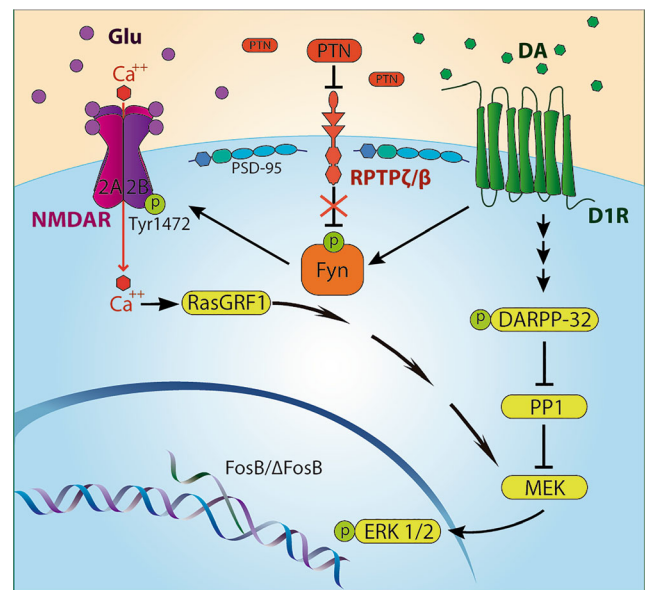
Even though there are several evidences connecting D1R and Fyn in striatonigral neurons from the direct pathway, we cannot rule out a role of Fyn in striatopallidal neurons from the indirect pathway. Fyn is expressed by both types of MSNs, and it has been recently shown that D2R modulates metabotropic glutamate receptor 5 (mGluR5) by Fyn phosphorylation [50]. Taking into account the role of Fyn in spine morphology [51, 52], the recovery of dendritic spines in striatopallidal—but not striatonigral—MSNs of dyskinetic mice [38], and the fact that D2 stimulation itself is also involved in the development of LID under some circumstances [30, 53], it is suitable to hypothesize that Fyn could be mediating dyskinesia by modulating protein activity also in striatopallidal neurons.

◄ Fig. 5 Evaluation of AIMs on saracatinib-treated WT mice. **a** Schematic representation of the paradigm of dyskinesia used to evaluate the effect of saracatinib on LID. Briefly, WT C57 mice were lesioned with 6-OHDA into the MFB, received intense care for 2 weeks, were selected by the cylinder test, and randomly assigned to be treated or not with saracatinib in two pharmacological schemes: starting 8 days before L-DOPA treatment (pre-treatment) or 8 days after L-DOPA administration (post-treatment). Abnormal involuntary movements (AIMs) were determined on days 2, 5, 8, 12, 15 and 19. **b** Sum of values for axial dystonia, orolingual, and limb dyskinesia per day throughout the L-DOPA treatment of mice that received saracatinib starting 8 days after the first L-DOPA administration (post-treatment). Data are mean ± SEM (veh: $n = 6$; saracatinib: $n = 5$). Two-way ANOVA with repeated measures showed no interaction (time × treatment) [$F(5, 52) = 0.2320$; $p = 0.9468$], no effect of time [$F(5, 52) = 1.423$; $p = 0.2317$], and neither of treatment [$F(1, 52) = 0.1004$; $p = 0.7526$]. **c** Similar to **b** but treatment with saracatinib starting 8 days before L-DOPA (pre-treatment) (veh: $n = 7$; saracatinib: $n = 5$). Two-way ANOVA with repeated measures showed no interaction effect (time × treatment) [$F(5, 60) = 0.3985$; $p = 0.8479$], no effect of time [$F(5, 60) = 2339$; $p = 0.0525$], and a significant effect of treatment [$F(1, 60) = 24.91$; ***$p < 0.0001$]. Independent Student's t tests were run for each day: day 2 **$p = 0.0023$, day 5 **$p = 0.0078$; day 19 *$p = 0.0332$. **d, e** Sum of values for axial dystonia, orolingual, and limb dyskinesia on days 2 and 19 throughout one behavioral session of the pre-treated group. Data are mean ± SEM. Independent Student's t tests were run for each time interval. **d** Two-way ANOVA with repeated measures showed interaction effect (time × treatment) [$F(5, 60) = 2.980$; $p = 0.0181$], significant effect of time [$F(5, 60) = 29.95$; ***$p < 0.0001$], and significant effect of treatment [$F(1.60) = 31.73$; ***$p < 0.0001$]. Minute 20 *$p = 0.0240$; minute 40 **$p = 0.0010$; minute 60 *$p = 0.0162$; minute 80 ***$p = 0.0008$. **e** Two-way ANOVA with repeated measures showed no interaction effect (time × treatment) [$F(5, 60) = 1.026$; $p = 0.4103$], significant effect of time [$F(5.60) = 44.05$; ***$p < 0.0001$], and significant effect of treatment [$F(1.60) = 20.00$; ***$p < 0.0001$]. Minute 20 **$p = 0.0035$; minute 40 *$p = 0.0415$. **f** Quantification of striatal TH immunoreactivity expressed as percentage of dopaminergic denervation relative to contralateral side. Data are mean ± SEM, pre-treatment (veh: $n = 7$; saracatinib: $n = 5$); $p > 0.05$; post-treatment (veh: $n = 6$; saracatinib: $n = 5$); $p > 0.05$, compared to those of unpaired Student's t test. **g** Quantification of FosB/ΔFosB immunoreactive (IR) area in the striatum of vehicle and saracatinib pre-treated mice. Data are mean ± SEM (veh: $n = 6$; saracatinib: $n = 4$); $p = 0.0043$, compared by unpaired Student's t test

Fig. 6 Molecular regulation at the postsynaptic density zone in LID. Figure illustrates a D1R-expressing MSN dendritic spine. Fyn regulates the crosstalk between D1R and NMDAR by phosphorylation of the NR2B subunit of NMDAR at Tyr1472. The kinase activity of Fyn can be also modulated by PTN through inactivating phosphatase activity of RPTPζ/β

We are aware that Fyn-KO mice are fearless and show less exploratory behavior in the open field test compared to WT mice [54]. Since these phenotypes could result from impaired locomotion, and therefore interfere with the expression of AIMs, we undertook comparative rotarod testing of these mice. Our analysis found no differences between Fyn-KO and WT mice. There is also clinical evidence from patient observations that dyskinesia can develop even in the context of reduced motor activity as evidenced by the concomitant presence of elevated UPDRS motor scores [7]. These arguments suggest that the reduced locomotor activity of Fyn-KO mice does not constitute a significant motor impairment capable of interfering with our interpretation of the LID results.

We observed a 30% reduction in the development of AIMs in Fyn-KO compared to that of WT mice. This effect is similar

in magnitude to that reported previously with similar approaches using other constitutive KO mice, lacking the expression of other related molecules such as Ras-GRF1 [55] or MSK1 [56]. The necessary and sufficient condition for a single molecule to account for certain behavioral changes is difficult to determine in a constitutive KO model, as compensatory mechanisms could take place during development that might limit the full expression of the effects of proteins with important signaling roles, such as Fyn.

To circumvent this issue and to further test our hypothesis, we performed an experiment using a pharmacological approach. Although there is no specific inhibitor of Fyn, the Src and Abl family kinases1 inhibitor saracatinib has been shown to inhibit Fyn activity in the mouse brain, providing proof of its potential use in the therapy of Alzheimer's disease [26]; and a clinical trial with this drug is ongoing [57, 58]. Also, there is evidence that DA stimulation increases Fyn phosphorylation and not Scr [21] and that Fyn is the preferred member of the SFK expressed in the striatum [46]; it is likely that the effect of saracatinib is due to Fyn inhibition rather than the inhibition of other members of the family. However, the development of a selective Fyn antagonist is desirable. Mice responding to saracatinib show a reduction of LID scores and FosB/ΔFosB staining in the order of 30% compared to controls, which is comparable to what has been reported with other drugs that have previously shown efficacy against LID in the same animal model, such as amantadine or riluzole [6, 28]. We may conclude that, in agreement with our findings in Fyn-KO mice, there seems to be a plateau of therapeutic effect

with every drug that has been tested, giving further support to the hypothesis that several mechanisms are involved in the development of LID.

The treatment of LID with saracatinib represents a proof of concept that this type of drugs can be used to reduce the development of LID and Fyn could be a valid pharmacological target to achieve such a goal. The use of saracatinib may require further refinement indeed, such as dosage and adequate timing of administration to establish the optimal conditions for its therapeutic use. Alternatively, highly specific inhibitors for Fyn can be developed and tested. In our experimental conditions, we observed no effect when saracatinib was administered once LID was established. Even if this lack of response must be further explored reducing L-DOPA doses and/or increasing saracatinib doses, that primary evidence suggests a stronger early role of Fyn in the genesis of LID. This can be probably attributed to the participation of Fyn during early plastic rearrangements, likely at the level of the PSD zone as it has been shown in the establishment of LTP [59] and in the formation of dendritic spines [51]. This finding raises novel insights into the development of dyskinesia, which is of significant clinical importance. Although further analysis is necessary, it is tempting to speculate that a potential therapeutic strategy might be preferred targeted at preventing the development of LID by combining Fyn inhibition at the start of L-DOPA therapy.

In conclusion, our results demonstrate that Fyn is a pivotal molecule in the development of dyskinesia likely through a reduction of the NMDA signaling pathway, positioning it as a novel key component in the molecular mechanism of LID. We therefore propose Fyn as a new potential target molecule to be considered for pharmacological intervention to manage L-DOPA-induced dyskinesia in PD.

Compliance with Ethical Standards All surgical procedures and experimental manipulations were performed in accordance with the UK Home Office and the European Directive 2010/63/EU and approved by the Ethics Committee of "Facultad de Farmacia y Bioquímica" (Universidad de Buenos Aires, Argentina).

Conflict of Interest The authors declare that they have no conflict of interests.

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