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7-Nitroindazole down-regulates dopamine/DARPP-32 signaling in neostriatal neurons in a rat model of Parkinson's disease

Q4 J.E. Yuste, M.B. Echeverry, F. Ros-Bernal, A. Gomez, C.M. Ros, C.M. Campuzano, E. Fernandez-Villalba, M.T. Herrero*

Q1 *Clinical & Experimental Neuroscience (NICE) and Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Department of Human Anatomy and Psychobiology, School of Medicine, University of Murcia, Campus de Espinardo, 30100 Murcia, Spain*

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ABSTRACT

Neuronal nitric oxide synthase (nNOS) is involved in the regulation of diverse intracellular messenger systems in the brain. Nitric Oxide (NO) contributes to inducing signaling cascades that involve a complex pattern of phosphorylation of DARPP-32 (in Thr-34), which controls the phosphoproteins involved in neuronal activation. However, the role of NO in the pathophysiology of Parkinson's disease (PD) and its effect in striatal neurons have been scarcely explored. In the present work, we investigate the effects of a nitric oxide synthase (NOS) inhibitor, 7-nitroindazole (7-NI) in the nigrostriatal pathway of striatal 6-hydroxydopamine (6-OHDA) lesioned rats. Our quantitative histological findings show that treatment with 7-NI significantly reduced 6-OHDA-induced dopaminergic damage in the dorsolateral striatum and Substantia Nigra *pars compacta* (SNpc). Moreover, 6-OHDA lesioned rats show a significant increase of $nNOS^+$ and Phospho-Thr34-DARPP-32^+ cells, accompanied by a consequent decrease of total DARPP-32^+ cells, which suggests an imbalance of NO activity in the DA-depleted striatum, which is also reflected in behavioral studies. Importantly, these effects are reverted in the group treated with 7-NI. These results show a clear link between the state of phosphorylation of DARPP-32 and parkinsonism, which is regulated by nNOS. This new evidence suggests a prominent role for nitric oxide in the neurotransmitter balance within the basal ganglia in the pathophysiology of experimental parkinsonism.

1. Introduction

Idiopathic Parkinson's disease (PD) is a common age-related movement disorder characterized by bradykinesia, rigidity, resting tremor and postural instability. The neuropathological hallmarks of PD are the progressive loss of dopaminergic neurons in the Substantia Nigra *pars compacta* (SNpc) and the presence of intraneuronal cytoplasmic inclusions (Lewy bodies) in some surviving nigral dopaminergic (DA) neurons. The clinical manifestation of PD occurs when about 50% of nigral dopaminergic neurons and about 70% of striatal dopamine fibres are lost (Hornykiewicz and Kish, 1987; Von Bohlen Und Halbach, 2004). This loss of DA fibers in the striatum causes disorganization of the activity of the striatal neurons, accompanied by a subsequent alteration of the basal ganglia circuitry with detrimental effects for the patients concerned.

Nitric oxide (NO) has emerged as a key endogenous modulator of Q2 the neuronal function, but its role in the regulation of the activity of striatal neurons and its implication for PD are still unclear. NO is an intracellular and short-lasting molecule that is synthesized from L-arginine in several brain regions by a reaction catalyzed by NO synthase (NOS) (Ignarro, 1990; Dawson et al., 1991). NOS is located in neurons, perivascular nerves, glial cells and the endothelium (Bredt and Snyder, 1990; Murphy et al., 1993). The family of NOS, the enzymes that induce NO, consists of two different classes: the inducible and constitutive forms (Dawson and Snyder, 1994; Marletta, 1994). Additionally, among the constitutive forms, there are two calcium-dependent NOS (cNOS) isoforms: the neuronal NOS

Abbreviations: PD, Parkinson's disease; NOS, nitric oxide synthase; 7-NI, 7-Nitroindazole; DA, dopamine; NO, Nitric Oxide; 6-OHDA, 6-Hydroxydopamine; TH, Tyrosin Hydroxylase; CPu, Caudate–Putamen complex; SNpc, Substantia Nigra *pars compacta*; cNOS, calcium-dependent NOS; nNOS, neuronal Nitric Oxide synthase; eNOS, endothelial Nitric Oxide Synthase; NA, noradrenergic; Phospho-Thr34-DARPP-32, 32 kDa dopamine and cyclic AMP-regulated phosphoprotein in Thr-34; Total DARPP-32, 32 kDa dopamine and cyclic AMP-regulated total protein; EBST, Elevated Body Swing Test; cGMP, cyclic guanosine monophosphate; SDS, sodium dodecylsulfate; DAT, dopamine transporter; MAO-B, Monoamine oxidase B; sGC, soluble guanylyl cyclases; PP-1, Protein phosphatase-1; PKG, Protein kinase G; PKA, Protein Kinase A; MSN, Medium spiny neuron; LTD, long-term depression; LTP, long-term potentiation.

* Corresponding author. Tel.: +34 868 88 46 83; fax: +34 868 88 41 50.
E-mail address: mtherrer@um.es (M.T. Herrero).

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(nNOS) present in neurons, and the endothelial NOS (eNOS) present in both pyramidal cells and endothelial cells (Moncada et al., 1991).

Even if both NOS positive cell type have been found in basal ganglia (Vincent and Kimura, 1992), the function of nNOS in basal ganglia circuitry remains scarcely explored. In *ex vivo* striatal slices, it has been demonstrated that postsynaptic effects of DA are conferred to a 32 kDa dopamine- and cyclic AMP-regulated phosphoprotein (DARPP-32) (Nishi et al., 2005) in striatal projection neurons, which receive the bulk of DA innervation within the basal ganglia. Through interactions with multiple kinases and phosphatases, DARPP-32 regulates the phosphorylation levels, and thereby the activity of a broad range of target proteins (Greengard et al., 1999). In addition to DA, numerous neurotransmitters, peptides and neuromodulators have been shown to provide input to DARPP-32-associated signal transduction cascades (Greengard et al., 1999). This evidence highlights the importance of DARPP-32 as a critical component of signal integration in striatal projection neurons. Several recent studies have emphasized a crucial role for the nitrergic system in movement control and the pathophysiology of the basal ganglia (Pierucci et al., 2011). The NO produced is diffused into medium spiny neurons of the striatum, resulting in the formation of cGMP and activation of protein kinase G (PKG), where the DARPP-32, an excellent substrate for PKG and protein kinase A (PKA), is phosphorylated (Tsou et al., 1993; Nishi et al., 2005). These studies also suggest that the effects of nitric oxide on striatal neurons are partially mediated via cyclic guanosine monophosphate (cGMP) (West and Tseng, 2011).

However, the role of nNOS in the striatum pathway in parkinsonism *in vivo* is unknown and there are few studies concerning the influence of nNOS inhibition on the DA loss *in vivo* (Hantraye et al., 1996; Ferrante et al., 1999).

In the present work, we used a specific inhibitor of nNOS, 7-nitroindazole (7-NI) (Southan and Szabó, 1996), a relatively selective inhibitor of the neuronal isoform of nitric oxide synthase, in a model of PD induced by the intrastriatal injection of 6-OHDA (Di Matteo et al., 2009), observing that inhibition of nNOS has an important effect on the regulation of DARPP-32 signaling in the striatum of hemiparkinsonian rats. This suggests a prominent role for nNOS in the regulation of the activity of striatal neurons in parkinsonism.

2. Experimental procedures

2.1. Animals

Sixty-four adult male Sprague–Dawley rats weighing 250–300 g (purchased from Harlan) were housed in groups of four animals per cage in a temperature-controlled room (21 ± 1 °C), under a 12-h light–dark cycle, with free access to food and water. All animals experiments were carried out in accordance with the U.K. Animals (Scientific procedures) Act, 1986, and associated guidelines, and the European Communities Council Directive of 24 November 1986 (86/609/ECC) for the care and use of laboratory animals. All efforts were made to minimize animal suffering and to reduce the number of animals used in these experiments.

2.2. Surgical procedures: 6-OHDA microinjections

The rats were anesthetized with a mixture of ketamine/xylazine (50 mg/kg; 10 mg/kg i.p.) and placed on a stereotaxic frame (Kopf Texas Instruments). Unilateral striatal lesions were performed by microinjecting 6-OHDA with 0.02% ascorbic acid (4 μg/μl, RBI-Sigma) in 0.9% saline into the right striatum (2 microinjections of 2 μl each) with a 10 μl Hamilton syringe, at a rate of 1 μl/min. The concentration of 6-OHDA was selected on the basis of its successful use for inducing lesions restricted to the dorsolateral striatum in the rat, which rotated, in response to treatment with apomorphine (Schwarting and Huston, 1996). To prevent noradrenergic (NA) failure, 10 min before the 6-OHDA injection, the animals were treated with desmethylimipramine (inhibitor of the high affinity noradrenaline transport system, 25 mg/kg i.p., Sigma) and pargyline (inhibitor of the monoamino-oxidase enzyme, 40 mg/kg i.p., Sigma). The stereotaxic coordinates of the two injections, according to Paxinos & Watson's rat brain Atlas (1998), locating the tooth bar at −2.4 mm, were: (First injection) anterior: 1.20 mm; lateral: 2.5 mm; ventral: −4.5 mm; and (second injection) anterior: 0.48 mm; lateral: 3.4 mm; ventral: −5.0 mm from bregma. Sham-operated control animals ($n = 9$) were injected with saline (vehicle) instead of the neurotoxin using the same procedure. After the injection, the syringe was left in the target for an additional 2 min before removal.

2.3. Pharmacological treatment

The 7-NI (purchased from RBI-Sigma) administrations started 30 min before the stereotaxic surgery. The inhibitor was dissolved in peanut oil and administered for 5 days (one per day) at a therapeutic dose, previously tested in preliminary experiments, thus preventing cataleptic behavior in the animals (15 mg/kg, i.p., ×5) (Guevara et al., 2002; Padovan-Neto et al., 2009). Every group, Sham or lesioned, was divided into two subgroups, treated with vehicle (peanut oil) or treated with 7-NI: i) Sham + vehicle ($n = 9$), ii) Sham + 7-NI ($n = 9$), iii) 6-OHDA + vehicle ($n = 11$) and iv) 6-OHDA + 7-NI ($n = 11$).

The rats were placed in clean cages on warming pads bedding to recover from the surgery, after which they were returned the group housing (two or three rats per cage).

2.4. Behavioural tests

2.4.1. Evaluation of rotational behavior

To determine the success of unilateral striatal denervation, rats were injected with apomorphine hydrochloride (0.5 mg/kg s.c., RBI-Sigma) 15 days after the 6-OHDA lesions. The total number of (contralateral) rotations was recorded every 5 min for 45 min (Padovan-Neto et al., 2009). Turns were counted by a rotometer (Ungerstedt and Arbuthnott, 1970) [built in the CAID (*Centro de Apoyo a la Investigación y al Desarrollo)* of our university]. The behavioral evaluation was performed blindly by the observers. Given that after 6-OHDA lesion small depletions of striatal DA were associated with a lower intensity of rotations with apomorphine (Costall et al., 1976), only animals in the 6-OHDA/Veh group, which exhibited more than 15 total turns contralateral to the side of the 6-OHDA lesion, in 45 min, were included in this study.

2.4.2. Elevated body swing test (EBST)

An additional evaluation with EBST was made to verify the partial striatal lesion (Blandini et al., 2007) and to avoid the behavioral sensitization effects of repeated apomorphine injections. The EBST was performed to evaluate 6-OHDA-treated animals 35 days after the lesion as previously described (Borlongan and Sanberg, 1995). The animals were placed in a Plexiglas box (40 × 40 × 35.5 cm) for 2 min for habituation. Then, each rat was held 3 cm from the base of its tail and suspended approximately 3 cm above the table. In this position the animals start swinging to the right or left side and the events counted. The animal must return to the outstretched position for the next swing to be counted. At the beginning, a gentle pinch in the tail may stimulate the behavior. The number of left-biased (L) swings and right-biased (R) swings were counted during a period of 1 min. Biased swinging

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behavior was calculated as follows: L (% of total BS for left-biased swings) and R (% of total BS for right-biased swings), Total BS = L + R. All experimental tests were performed at the same time of day (Fig. 1, panel A.2).

2.4.3. Tissue preparation

To study the histological effects of the lesion, forty days after the last behavioral screening, 40 animals were deeply anesthetized with a mixture of ketamine/xylazine (50 mg/kg; 10 mg/kg i.p.) and transcardially perfused with PBS 0.01 M (300 ml; pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (250 ml; pH 7.4). The brains were rapidly removed and postfixed in the same fixative solution for 24 h (4 °C) and then cryoprotected in 30% sucrose/phosphate buffer. Brains were quickly frozen in cold isopentane (−40 °C; Sigma) and stored at −80 °C until the histochemical and immunocytochemical procedures were carried out.

2.4.4. Immunohistochemistry and histochemistry

Thirty μm-thick serial coronal sections were cut in a cryostat (Leica). After a period of time, adjacent brain sections containing Caudate–Putamen complex (CPu) nucleus or SNpc were immunostained for TH, DARPP-32 and nNOS. TH immunohistochemistry was performed as previously described (Barcia et al., 2004), and the same procedure was carried out for nNOS, total DARPP-32 and the same protein, phosphorylated at Thr-34. Briefly, tissue sections were washed and then incubated for 18–24 h with the primary antibody (TH, 1:1000, Calbiochem; NOS1, 1:1000, Santa Cruz; total DARPP-32 and DARPP-32 phospho T34, 1:400, Abcam ab51139 and ab51076, respectively). Sections were processed by the avidin-biotin immunoperoxidase method (Vectastain ABC kit, Vector Lab) and immunopositive cells were visualized by addition of the chromogen 3,3′-diaminobenzidine (DAB; Sigma, 1 mg/ml) and hydrogen peroxide (0.2%). For both DARPP-32, 1% Nickel was added to visualize the cells with greater contrast. Sections were mounted on gelatin-coated glass slides, dehydrated in ethanol, cleared in xylene and cover-slipped for microscopic observations. Cytoplasmic DARPP-32 positive cells could be visualized as a blue or dark blue reaction product, respectively, and nNOS and TH as a brown precipitate. In all experiments, tissues from Sham and all lesioned groups were always processed in the same assay.

2.4.5. Western blotting analysis

Ten Sham rats groups and fourteen 6-OHDA rats groups were killed by decapitation 30 min after the last 7-NI injection. The

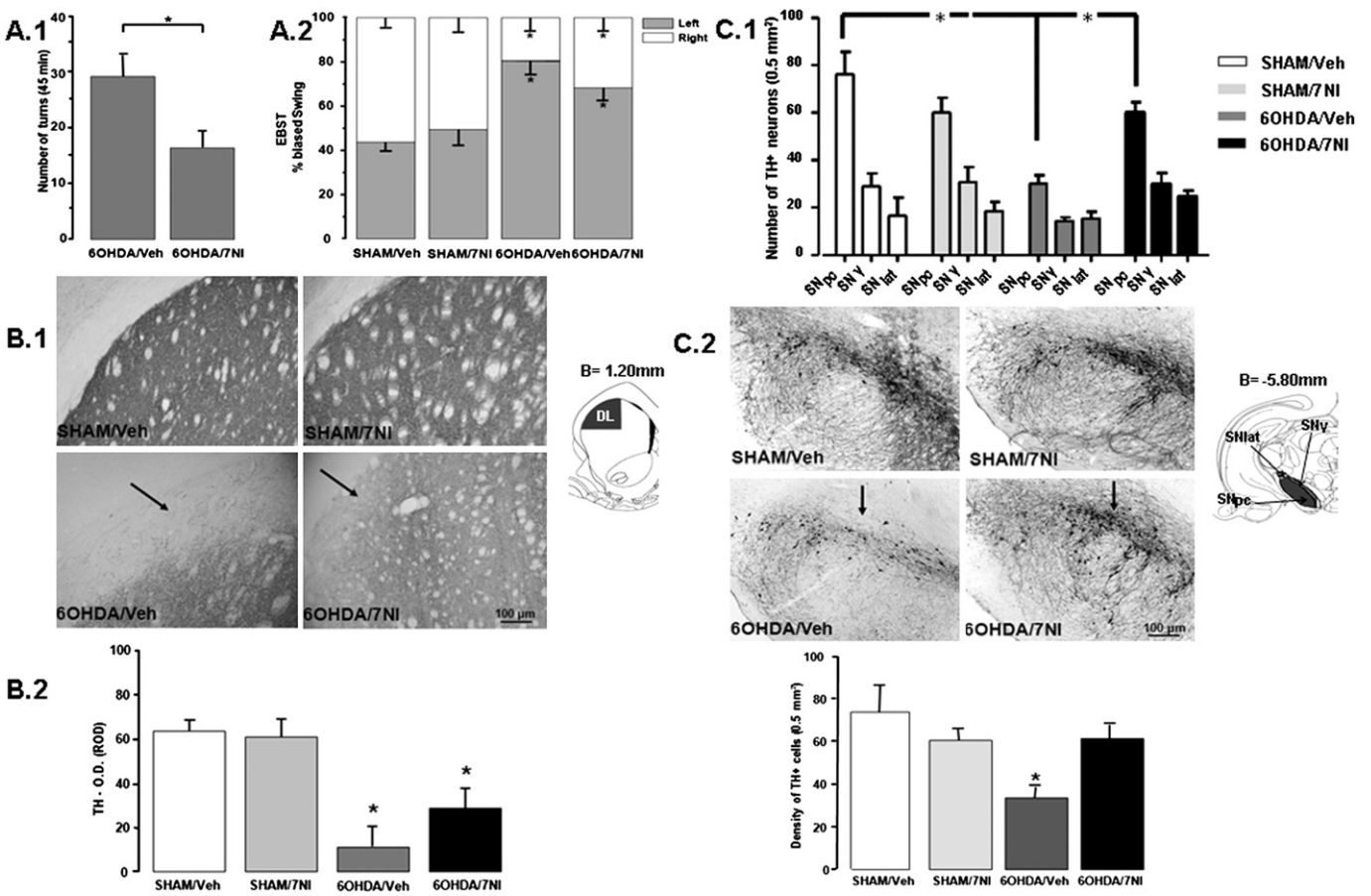

Fig. 1. Striatal injections of 6-OHDA causes a mild parkinsonism in rats which is reverted by 7-NI treatment. (A.1) The number of apomorphine-induced turns, 15 days after lesion, were significantly lower in the 6-OHDA/7-NI group than in the 6-OHDA/Veh group. (A.2) The left-biased swings measured by EBST 35 days after lesion in the 6-OHDA/Veh group were increased by 70% compared with Sham animals. *$P < 0.05$ lesioned animals *vs* Sham groups (ANOVA followed by the Duncan test). Photomicrographs show the distribution of TH^+ fibers in the dorsolateral striatum (B.1) and TH^+ neurons in the SNpc (C.2) in the different experimental groups, 45 days post-lesion. Schematics illustrations of the analyzed coronal section are shown on the right. Each bar of the histograms corresponds to mean ± SEM. The treatment with 7-NI moderately attenuated the loss of TH^+ fibers (B.2) and protected the cells (C.2) in both structures, respectively (striatum and SNpc), in 6-OHDA-lesioned animals. TH^+ neurons in the ventral mesencephalon in a section containing the intra-axial portion of the third craneal-nerve. The three different regions, analyzed of the SN are indicated in the scheme on the right to panel (C.2). In panel (C.1) a graph showing the count of TH+ neurons in the 3 areas of the SN (SNpc, SNγ and SN_{lat}). The AP localization of the analyzed Substantia Nigra area was AP −5.80 mm from bregma. We found only a significant difference in the SNpc with respect to other areas (ANOVA, $F_{(3,15)} = 3.24$, post-hoc Newman-Keuls). *$P < 0.05$, 6-OHDA/Veh *vs* Sham/Veh and 6-OHDA/7-NI. OD: Optical Density, ROD units: Relative Optical Density units.

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brains were removed, and the striata dissected out on an ice-cold surface. The structure was immediately immersed in liquid nitrogen. Samples were placed in homogenization buffer [phosphate buffered saline, 2% sodium dodecylsulfate (SDS) plus protease inhibitors (Roche, Germany) and phosphatase inhibitors Cocktail Set (Calbiochem, Germany)] and homogenized for 50 s prior to centrifugation at 6000 *g* for 20 min at 4 °C. Total protein concentrations were determined spectrophotometrically using the bicinchoninic acid method (Wiechelman et al., 1988). The optimum amount of protein to be loaded was determined in preliminary experiments by loading gels with increasing protein contents (25–100 μg) from samples of each experimental group. Equal amounts of protein (50 μg/lane) from each sample were loaded on a 10% SDS–polyacrylamide gel (SDS–PAGE), electrophoresed, and transferred onto a poly vinylidene difluoride (PVDF) membrane using a Mini Trans-Blot Electrophoresis Transfer Cell (Bio-Rad Lab., California, USA). Non-specific binding of antibodies was mitigated by incubating membranes with 1% bovine serum albumin (BSA) in tris buffer saline tween (TBST: 10 mM Tris–HCl, pH 7.6, 150 mM NaCl, and 0.05% Tween 20). Membranes were immunoblotted overnight using selective antibodies against Phospho-Thr34-DARPP-32 (1:1000; Cell Signaling Technology) and Total DARPP-32 (1:1000; Cell Signaling Technology). After extensive washings with TBST, the membranes were incubated for 1 h, at room temperature, with peroxidase-labeled secondary antibodies (anti-rabbit sc-2004 for DARPP-32 phosphorylated at Thr-34 and Total DARPP-32, at 1:2000 dilutions). After washing, immunoreactivity was detected with an enhanced chemiluminescent/chemifluorescent western blot detection system (ECL Plus, GE Healthcare, U.K) and visualized by a Typhoon 9410 variable mode Imager (GE Healthcare). Antibodies were stripped from the blots by incubation with stripping buffer (glycine 25 mM and SDS 1%, pH = 2), for 1 h at 37 °C. Anti β-actin was used as housekeeper (Cell Signaling, 45 kDa). The ratio of total DARPP-32/β-actin, phospho-DARPP-32/β-actin was plotted and analyzed.

2.5. Quantitative methods

2.5.1. Immunohistochemistry and histochemistry

The whole histological quantification was performed blindly. Neuroanatomical sites (Dorsolateral striatum and SNpc) were identified using the Paxinos and Watson (1998) atlas. The antero-posterior (AP) localization of the analyzed dorsolateral striatum area was AP 1.20 mm from bregma (Fig. 1, panel B.1). Stained neurons were counted using a computerized image analysis system. Images were captured from slices using a computer-assisted image analysis system (ImageJ 1.43 Analysis software from directory at rsb.info.nih.gov/ij/ with a Zeiss microscope connected to a digital camera (CoolSnap) through a Zeiss zoom set at 10× and a 0.1× adapter (Barcia et al., 2004)). The loss of dopaminergic fibers in the dorsolateral striatum was analyzed by measuring the optical density of the TH immunoreactivity with a computer analyzer (ImageJ System).

The optical density of a tissue area devoid of staining (corpus callosum) was measured, adjusting the light intensity to give a background density value. The background was subtracted from all subsequent measurements. A mean value for staining intensity was calculated and expressed on an arbitrary gray scale (relative optical density) units (ROD units; Echeverry et al., 2004). The boundary of SNpc was outlined under magnification of the 4× objective. Cells were counted with a 40× oil-immersion objective using a Nikon 80i microscope. Serial sections through the whole SNpc were placed 6 per slide (cut thickness of 30 μm) for systematic analysis of randomly placed counting frames (size 50 × 50 μm) on a counting grid (size of 190 × 130 μm) and sampled using an 18 μm optical dissector with 2 μm upper and lower guard zones. The nNOS labeled neurons were quantified in the dorsolateral region of the striatum (Fig. 2). For quantification of labeled neurons and cytoplasmic DARPP-32, an average number from a fixed size/area of the dorsolateral part of caudate nucleus was observed (Figs. 3–4). For each treatment, three sections from each animal were evaluated. A mean value for the number of cells in the three regions of the animal was then calculated.

2.5.2. Western blot analysis

All the quantifications for the immunoblot analysis corresponding to total DARPP-32 (32 kDa) and DARPP-32 phosphorylated at Thr-34 (32 kDa) bands were carried out by densitometry (AlphaImager, Nucliber, Madrid). We measured the integrated optical density of bands. The software generates quantitative data of band intensities. The values are in units of pixel gray levels and are proportional to the light intensity on that pixel during the exposure time of the image. The optical density was normalized to the background values. Relative variations between bands of experimental samples and control samples were calculated in the same image.

2.6. Statistical analysis

All values were expressed as mean ± S.E.M. Analysis of variance (ANOVA) for repeated measures was performed, taking as intrasubject factor the side of the brain, and the treatment as the factor between groups to analysis interaction between factors. In case of effect on treatment factor, differences between groups were analyzed using one-way ANOVA. The Duncan test was used for multiple comparison procedures. Differences between means were analyzed using one-way ANOVA. The Duncan test was used for multiple comparison procedures. Pearson *r* correlation was used to assess any correlation between the densitometry of TH *vs* rotational behavior, as well as between phospho-Thr34-DARPP-32 *vs* nNOS, TH *vs* nNOS and total DARPP-32 *vs* phospho-Thr34-DARPP-32. For Western blot analysis we used one-way ANOVA followed by the Newman–Keuls post-hoc test. A significance level of $P < 0.05$ was considered to be statistically significant.

3. Results

In the left side (contralateral to lesion) in dorsolateral striatum there was not any alteration by the lesion. (see Table 1).

3.1. 7-NI reverts parkinsonian behavior in 6-OHDA-lesioned rats

The treatment with apomorphine induced contralateral rotations in 6-OHDA-lesioned animals but not in control animals. However, a significant decrease in the number of rotations was seen in 7-NI compared to vehicle treated rats (control animals did not show any rotations (Sham/Vehicle, $n = 4$; Sham/7-NI, $n = 5$) (ANOVA, $F_{(3,19)} = 20.70$, $P < 0.001$) (Fig. 1, panel A.1)).

Although stereotyped behavior may be observed as a typical response to the lesion in the striatum of the rat (Iversen and Koob, 1977; Old et al., 1999), in our animals no significant differences were observed between the experimental groups. The percentage of left-biased swings measured by EBST five weeks after the striatal-lesion was significantly higher than in Sham rats (Duncan, $P < 0.05$). 6-OHDA/Vehicle animals showed more than a 70% increase in the number of left-biased swings compared with Sham animals. A decrease not significant, was observed in 6-OHDA/7-NI animals (Duncan, $P > 0.05$) (Fig. 1, panel A.2).

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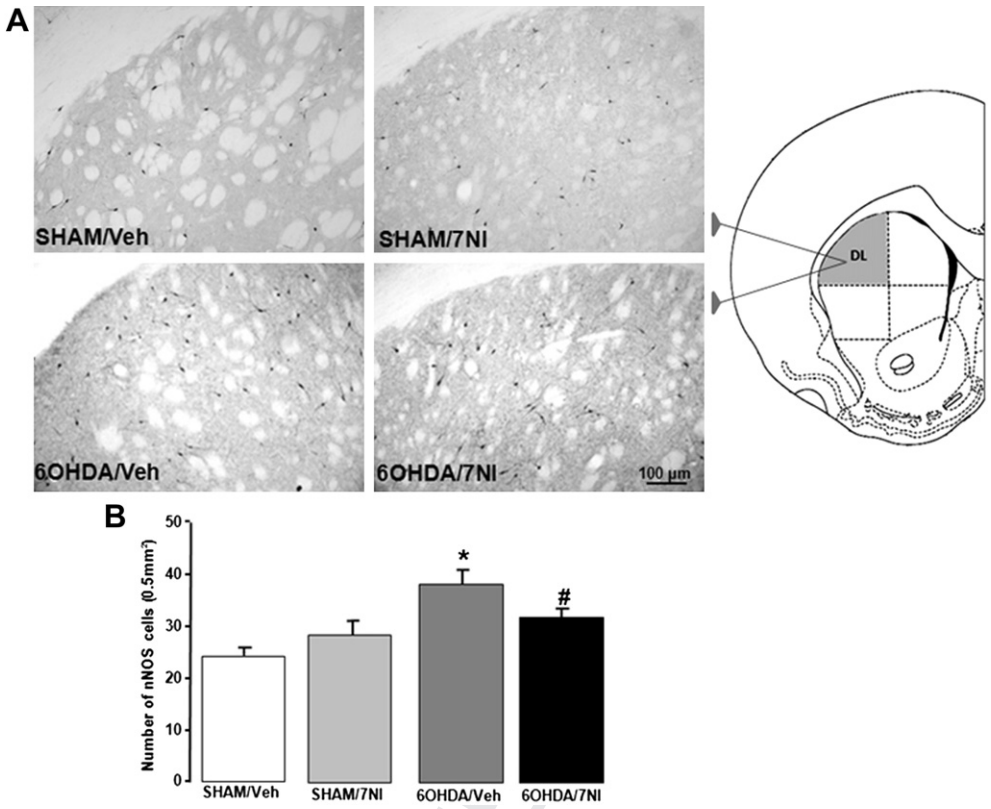

Fig. 2. Increase of striatal nNOS is reverted by 7-NI. (A) Photomicrographs show nNOS labeling in the dorsolateral striatum. An increase of nNOS$^+$ cells was observed in the 6-OHDA/Veh group with respect to Sham animals (B) and was slightly lower in the 7-NI treatment (6-OHDA/7-NI). *$P < 0.05$ 6-OHDA *vs* Sham groups, and #$P < 0.05$ 6-OHDA/7-NI *vs* Sham/Veh (ANOVA followed by the Duncan test).

3.2. 7-NI protects the nigrostriatal pathway in 6-OHDA-lesioned rats

Histological analyses were carried out in the dorsolateral striatum region of interest, and in the SNpc area (in the ipsilateral side to the injection). Previously, an analysis of both sides was performed. In the dorsolateral striatum region, an interaction (ANOVA Repeated Measures, $F(3,16) = 3.31$, $P = 0.047$), and effect in treatment factor was observed (ANOVA Repeated Measures, $F(3,16) = 5.08$, $P = 0.012$). Densitometry analysis revealed a significant reduction of TH$^+$ fibers in the lesioned-dorsolateral striatum of 6-OHDA rats. However, a significant effect was observed in the 6-OHDA/7-NI group (ANOVA, $F_{(3,19)} = 23.42$, $P < 0.001$) (Fig. 1, panel B.2). For lesion area, the ratio of the lesioned area (DA terminal loss) was calculated with ImageJ. The data were expressed in percentage of the total dorsolateral area. Sham/Veh: No lesion: 100%, Sham/7-NI: No lesion: 100%, 6-OHDA/Veh: No lesion: 15.5%; Lesion: 84.5% and 6-OHDA/7-NI: No lesion: 37.2%; Lesion: 62.8%. Besides, there was a significant negative correlation between the expression of striatal TH$^+$ fibers and rotational behavior ($r = -0.869$, $P < 0.001$). In the SNpc region (but not in SN_{lat} and SNγ), a decrease in TH$^+$ neurons was seen in 6-OHDA/Vehicle animals compared with Sham/vehicle and 6-OHDA/7-NI groups (ANOVA, $F_{(3,15)} = 3.24$, $P < 0.05$; Duncan, $P < 0.05$) (Fig. 1, panel C.2). Moreover, in the other areas of the Substantia Nigra (SN_{lat} and SNγ) we did not find significant difference in TH$^+$ neurons in any groups (ANOVA, $F_{(3,15)} = 3.24$, $P > 0.05$) (Fig. 1, panel C.1). These changes were observed in the SNpc, which projects toward the dorsolateral striatum.

3.3. 7-NI reduces nNOS striatal expression in 6-OHDA-lesioned rats

Effect on treatment factor was observed (ANOVA Repeated Measures, $F_{(3,15)} = 3.52$, $P = 0.041$). The density of nNOS-immunoreactive positive neurons in the 6-OHDA/Vehicle group increased in the lesioned-dorsolateral striatum compared with Sham groups but it was partially reverted in 6-OHDA/7-NI group (ANOVA, $F_{(3,16)} = 6.63$, $P < 0.005$) (Fig. 2, panel B). The density of nNOS cells was negatively correlated with the density of TH$^+$ fibers (Fig. 5, panel A).

3.4. 7-NI reduces DARPP-32 phosphorylation in 6-OHDA-lesioned rats

To accurately determine the increase in DARPP-32$^+$ cell numbers in pathophysiological stages in *postmortem* brain, it is important to establish a positive control for the quantification in a non-involved brain area. In this case, in all the sections we analyzed the motor cortex, immediately above the striatum (Figs. 3–4, panel A) (Ouimet et al., 1984, 1998), also stained for control area for both total DARPP-32 and phosphoThr-34-DARPP-32. Analysis with pospho-Thr34-DARPP-32 showed an interaction between both factors (ANOVA Repeated Measures, $F_{(3,16)} = 5.15$, $P = 0.011$). In lesioned-dorsolateral striatum, the number of pospho-Thr34-DARPP-32 cells was increased in the 6-OHDA/Vehicle group, which was significantly diminished in the 6-OHDA lesioned group treated with the nNOS inhibitor (ANOVA, $F_{(3,16)} = 4.62$, $P < 0.01$) (Fig. 4, panel D). An opposite effect was observed on the total DARPP-32$^+$ cell number. Previously, an interaction between both factors was observed (ANOVA Repeated Measures, $F_{(3,16)} = 21.53$, $P < 0.001$), and an effect on the treatment factor (ANOVA Repeated Measures, $F_{(3,16)} = 19.89$, $P < 0.001$). In lesioned-dorsolateral striatum, DARPP-32$^+$ cells decreased in the 6-OHDA/Vehicle group, whereas a significant increase was also found in the 6-OHDA/7-NI group (ANOVA, $F_{(3,17)} = 27.4$, $P < 0\ .001$; Duncan, $P < 0.05$) (Fig. 3, panel D). Furthermore, a significant correlation was seen between the levels of phospho-Thr34-DARPP-32 and total

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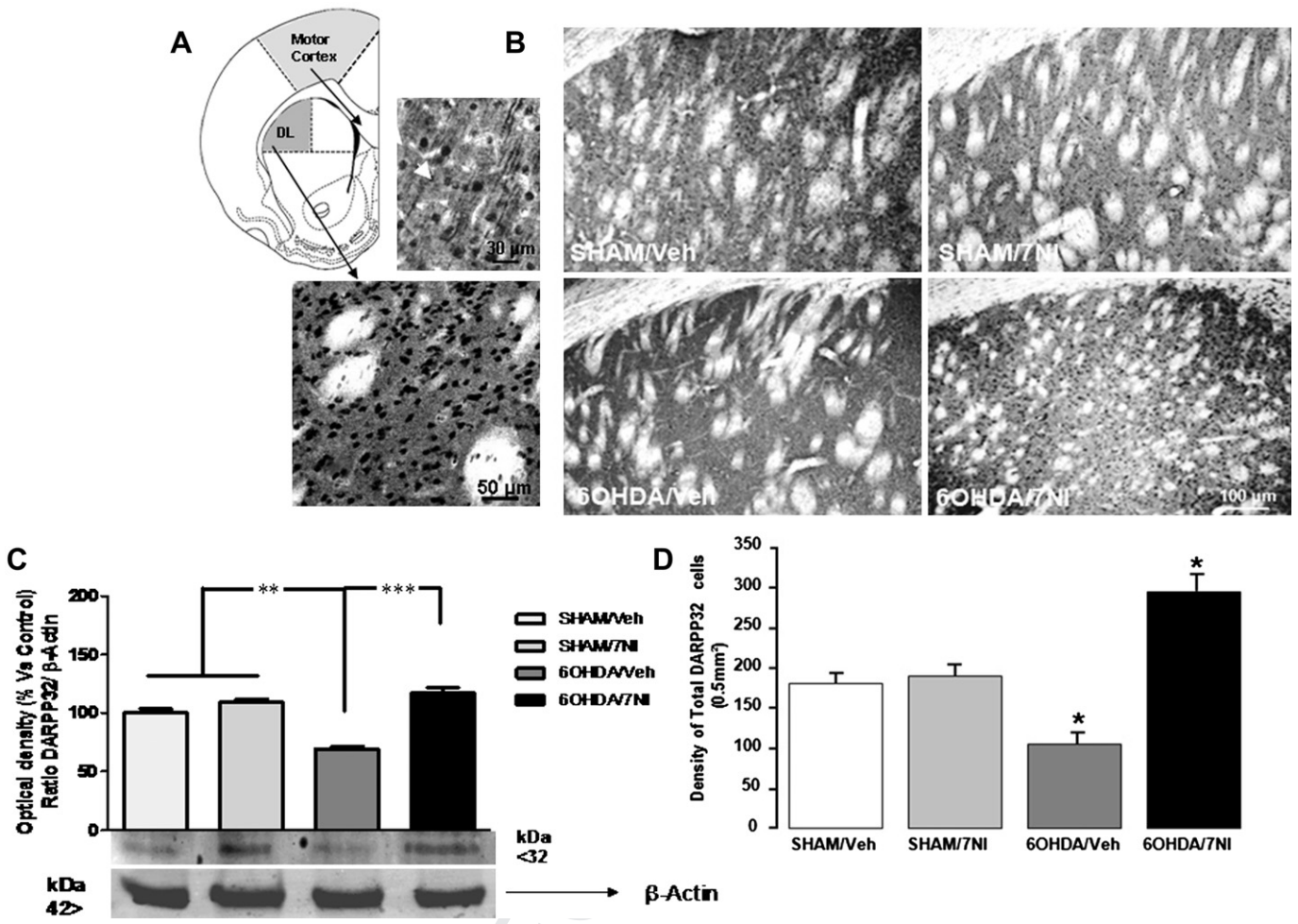

Fig. 3. Total DARPP-32 in the dorsolateral striatum is reduced by intrastriatal 6-OHDA injections and upregulated by 7-NI. (A) Photomicrographs showing cytosolic DARPP-32 labeling in Motor Cortex (arrowheads), and Caudate–Putamen. (B) Photomicrographs show an overall expression of total DARPP-32$^+$ cells in 6-OHDA/7-NI group compared with the other experimental groups. The quantification in the dorsolateral striatum showed a decrease in 6-OHDA/Veh and a striking increase in the 6-OHDA/7-NI group (D). $^*P < 0.05$ with respect to Sham groups (ANOVA followed by the Duncan test). (C) Normalized values with β-actin were compared with those of the Shams or 6OHDA treatment groups. $^{**}P < 0.01$, 6OHDA/Veh *vs* Shams groups (One way ANOVA followed Newman–Keuls post-hoc test) and $^{***}P < 0.001$, 6OHDA/Veh *vs* 6OHDA/7-NI (One way ANOVA followed Newman–Keuls post-hoc test).

DARPP-32 (Fig. 5 panel B) which suggests consumption of the total DARPP-32 phosphorylated by NO mediated mechanisms. This means that PKG (via NO) phosphorylated total DARPP-32 and that the protein turns into its phosphorylated form (phospho-Thr34-DARPP-32). In addition, a very significant positive correlation between nNOS labeling and the expression of pospho-Thr34-DARPP-32 was observed ($r = 0.556$, $P = 0.01$) (Fig. 5, panel C). Thus, these results demonstrate an association between the state of phosphorylation of DARPP-32 and nNOS cell number in the lesioned striatum.

3.5. 7-NI reduces values of phospho-DARPP-32 and increases total DARPP-32 in 6-OHDA-lesioned rats by western blot analysis

Phospho-Thr43-DARPP-32 and total DARPP-32 immunoblots in the striatum were examined by Western blot analysis in control treated with 6-OHDA/Vehicle and treated with 6-OHDA+ 7-NI rats. As regards DARPP-32, total and phospho-DARPP-32 was detected in a band located at ~32 kDa (Figs. 3–4, panel C). In rats treated with 6-OHDA/7-NI, the total DARPP-32 protein increased significantly with respect to controls groups (ANOVA, post-hoc Newman–Keuls, $P < 0.01$, Fig. 3, panel C). Similar results were obtained by immunohistochemistry. The opposite effect was observed in the phospho-Thr34-DARPP-32, which increased in the 6-OHDA/Vehicle group with respect to controls animals (One Way ANOVA, post-hoc Newman–Keuls, $P < 0.01$, Fig. 4, panel C). These data are in agreement with those obtained by immunohistochemistry, giving more consistency to the results.

4. Discussion

Based on quantitative immunohistochemical and behavioral analyses, the present study provides *in vivo* evidence for the upregulation of DARPP-32-signaling caused by a moderate DA depletion in the striatum, controlled by nNOS mediated mechanisms.

Our results show that a unilateral intrastriatal injection of 6-OHDA in rats induces a slight loss of TH$^+$ fibers in the striatum and TH$^+$ cells in the SNpc, associated with a low intensive rotation to apomorphine administration (Ungerstedt, 1971; Costall and Naylor, 1975; Herrera-Marschitz and Ungerstedt, 1984; Koob et al., 1984). Although drug-induced rotational behavior is widely used to evaluate the level of DA lesion, a more sensitive test (as we use in our model) should be used due to the sensitization effects of apomorphine. In our rats, this small DA depletion was reliably detected by rotational behavior and by a significant contralateral biased-swing

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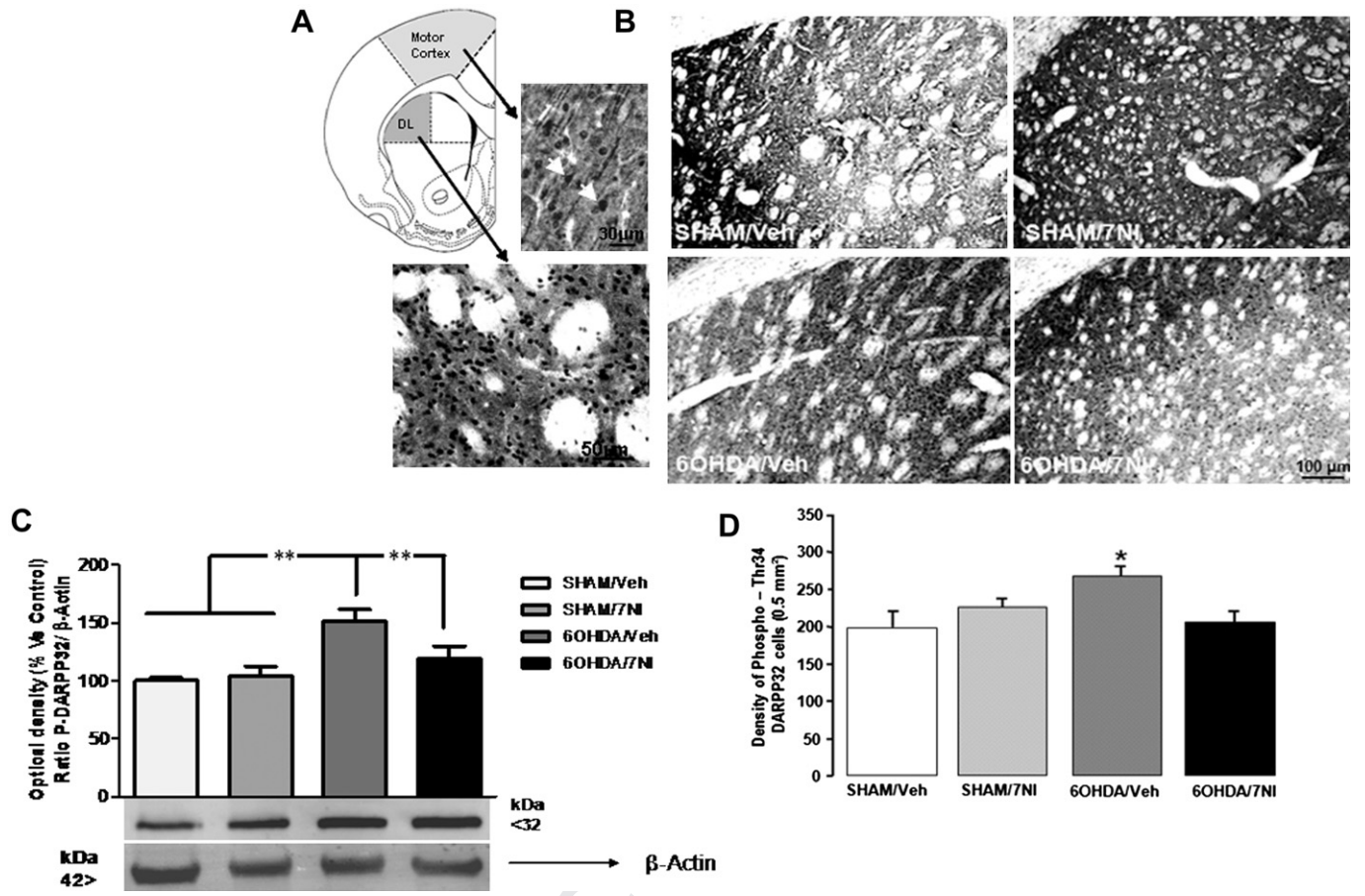

Fig. 4. Intrastriatal 6-OHDA injections increases phospho-Thr34-DARPP-32 in the dorsolateral striatum and is reverted by 7-NI. (A) Photomicrograph showing cytosolic *Phospo-Thr34*-DARPP-32 labeling (arrowheads) in Motor Cortex, and Caudate–Putamen. (B) Photomicrographs show an increased expression in 6-OHDA/Veh group compared with the other experimental groups. Quantifications of the Phospho-Thr34-DARPP-32 revealed a significant increase in 6-OHDA/Veh group that is prevented by 7-NI treatment (D). $^{*}P < 0.05$ 6-OHDA *vs* the other groups (ANOVA followed by the Duncan test). (C) Normalized values with β-actin were compared with those of Sham or 6-OHDA treatment groups. $^{**}P < 0.05$ 6-OHDA/Veh *vs* Shams groups and 6-OHDA/Veh *vs* 6-OHDA/7-NI (One way ANOVA followed by the Newman–Keuls post-hoc test).

activity. Therefore, the EBST seems to be a good drug-free measurement for asymmetrical motor behavior (Borlongan and Sanberg, 1995). These asymmetries were detected in body swing behavior revealing a small imbalance of DA output that other analyses may not be sensitive enough to detect (Yuan et al., 2005; Baluchnejadmojarad and Roghani, 2004). Like the injections of 6-OHDA in the medial forebrain bundle or the SNpc, unilateral intrastriatal injections of 6-OHDA in rats could also be considered a good model for describing progressive loss of DA-containing structures in PD (Przedborski et al., 1995; Kirik et al., 1998; Debeir et al., 2005) and hence, a model for studying nNOS regulation and its effects on DA neurodegenerative processes (Gomes et al., 2008).

As regards the increase in nNOS, our results agree with previous studies showing that a striatal 6-OHDA lesion causes an increase in nNOS positive cells in the anterior and middle striatum, with a significant decrease of nNOS density in cells in the SNpc, an effect that is modified by NOS inhibitors (Gomes et al., 2008). This increase of nNOS may be responsible for the increase of NO that contributes to DA neurodegeneration, but also continuing to the signaling of projecting neurons of the striatum.

As previously reported, we observed that the administration of 7-NI prevented the loss of TH^{+} fibers in the striatum and neurons of the SNpc. This could be explained by the fact that DA depletion in the striatum increased the oxidative stress exerted by peroxynitrite, which is recognized as an important mediator of neuronal degeneration in the DA system (Smith and Cass, 2007). Subsequently, NOS inhibitors, such as 7-NI, could protect against oxidation/nitration processes, reducing the levels of peroxynitrite itself (Denicola and Radi, 2005), and putatively attenuating 6-OHDA toxicity.

Besides inhibiting nNOS, 7-NI is also a good inhibitor of MAO-B, and indeed Di Monte and coworkers have attributed to this mechanism the neuroprotective effect of 7-NI against MPTP in mice (Di Monte et al., 1997). However, the mechanism of action of 6-OHDA differs from that of MPTP, and so inhibition of MAO-B is unlikely to affect 6-OHDA toxicity. Nevertheless, when MAO-B inhibitors are sub-chronically administered neurorestorative and neuroprotective effects are observed (Blandini et al., 2004; Sagi et al., 2007).

On the other hand, this protective effect of NOS inhibition could also be due to the effect of NO on dopamine transporter (DAT) and/or to its biochemical interaction with the 6-OHDA. In fact, since DAT activity is modulated by NO, and 6-OHDA is a substrate for DAT (Blum et al., 2001), it is reasonable to propose that NOS inhibition may lead to a reduction of DAT activity and hence prevent the access of 6-OHDA to DA terminals (Volz and Schenk, 2004). Besides, in cultured mesencephalic neurons and organotypic culture models (Gomez-Urquijo et al., 1999), it was demonstrated that some dopaminergic neurons also express nNOS giving further support for a pre-synaptic interaction between both systems, and suggesting a possible effect of nitric oxide on DA uptake, previously verified through NO donors and an indirect DA agonist, amphetamine (Salum et al., 2008).

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Table 1
Quantification analysis in dorsolateral striatum region in the right and left hemisphere.

	TH[a]		nNOS		Total DARPP-32		Thr34DARPP-32	
	R	L	R	L	R	L	R	L
SHAM/Veh	64.2 ± 6.9	60.3 ± 4.6	24.4 ± 1.5	27.5 ± 2.2	181.9 ± 13.3	192.3 ± 13.4	199.2 ± 22.7	214.1 ± 12.6
SHAM/7NI	61.1 ± 7.9	62.1 ± 5.3	28.6 ± 2.5	28.4 ± 2.0	190.9 ± 13.1	182.2 ± 4.9	231.0 ± 10.1	203.0 ± 20.5
60HDA/Veh	12.1* ± 2.0	60.1 ± 5.4	38.4* ± 2.8	29.7 ± 0.4	105.9* ± 11.6	177.4 ± 6.0	274.1* ± 13.8	200.9 ± 30.1
60HDA/7NI	26.8* ± 4.8	58.0 ± 5.1	32.1# ± 1.0	29.6 ± 4.5	295.4* ± 20.8	188.6 ± 2.3	207.1 ± 12.6	215.5 ± 2.7

Other data are expressed as numbers of labeled cells/0.5 mm^2 and represented with mean ± S.E.M. Further specifications as in results. R = right side and L = left side. Q3

[a] TH was measured by optical density analysis.

NO has been shown to diffuse into medium spiny neurons and activate soluble guanylyl cyclases (sGC), resulting in the formation of transient increases in intracellular cGMP levels that can affect to medium spiny neuron (MSN) activity via activation of PKG and downstream targets (e.g., cyclic nucleotide gated channels) (West and Grace, 2004; West and Tseng, 2011), which then causes the increased phosphorylation of DARPP-32 in threonine residue 34 (Thr-34). This is because DARPP-32 at Thr-34 is an excellent substrate for PKG as well as PKA and therefore the activated PKG induces the phosphorylation of DARPP-32 at Thr-34 (Hemmings et al., 1984; Tsou et al., 1993; Greengard et al., 1999).

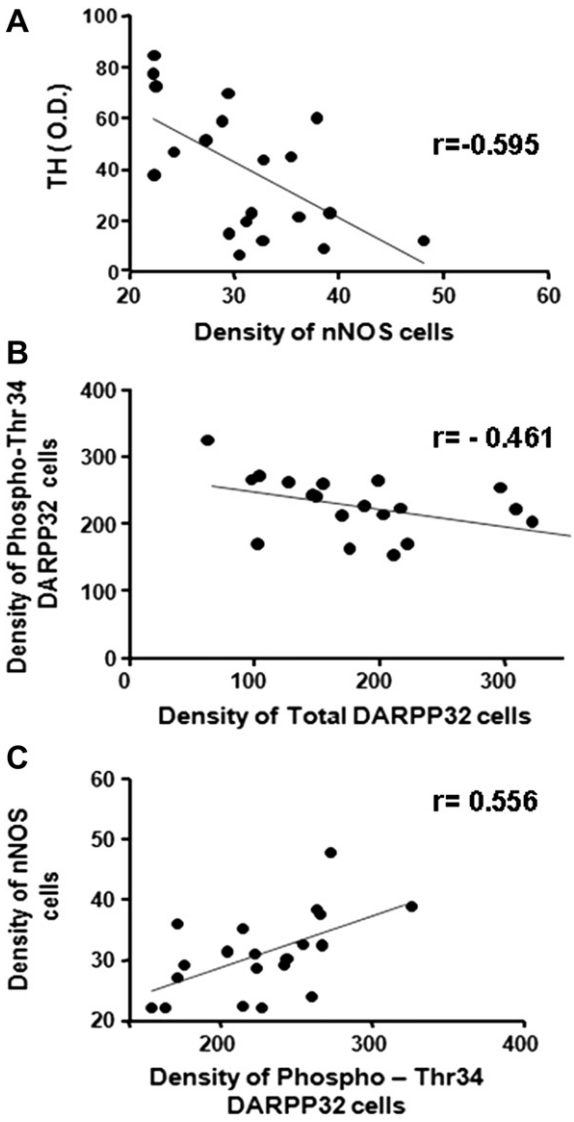

Fig. 5. Correlations of the changes in the dorsolateral striatum after 6-OHDA neurodegeneration. (A) Negative correlation between nNOS *vs* TH ($r = -0.595$, $P = 0.0056$, Pearson r correlation). An increased nNOS expression is associated with a decrease in TH positive neurons. (B) Negative correlation between Total DARPP-32 *vs* phospho-Thr34-DARPP-32 ($r = -0.4611$, $P = 0.041$, Pearson r correlation). An increase of total DARPP-32 expression is associated with a decrease in DARPP-32 phosphorylated positive neurons. (C) Positive correlation between Phospho-Thr34-DARPP-32 *vs* nNOS ($r = 0.5562$, $P = 0.011$, Pearson r correlation). An increase of phospho-Thr34-DARPP-32 expression is associated with an increase in nNOS positive cells.

Remarkably consistent outcomes have been reported in studies examining the impact of DA D1 and D2-like receptor agonists/antagonists on striatal NOS activity and NO production via cGMP (Di Stefano et al., 2005; Sammut et al., 2006; Siuciak et al., 2006; Park and West, 2009; Hoque et al., 2010).

Together, these studies confirm that both D1 and D2-like receptor activation strongly regulates striatal nNOS activity, albeit in opposing manners (i.e., D1 receptor activation facilitates the activity, while, D2 receptor activation is inhibitory), and identify a critical role of nNOS–NO signaling in the generation of striatal cGMP.

Glutamate, by activating NMDA and AMPA receptors, stimulates the synthesis of NO in nNOS interneurons in the neostriatum (Tsou et al., 1993), and the synthesized NO regulates the function of MSNs (Calabresi et al., 2000). Glutamate also induces a rapid and transient increase in DARPP-32 Thr-34 phosphorylation. It is abolished by inhibitors of nNOS (as well 7-NI) and soluble guanylyl cyclase, indicating that glutamate activates nNOS in GABAergic interneurons, where nNOS is expressed in the neostriatum (Tsou et al., 1993).

Once phosphorylated, the DARPP-32 at Thr-34 inhibits protein phosphatase-1 (PP-1) activity (Svenningsson et al., 2004), acting as a cAMP-dependent kinase inhibitor (Svenningsson et al., 2004; Nishi et al., 2005). In fact, in the dorsolateral striatum, phosphorylation mechanisms play a physiological role in motor control, and the induction of both long-term depression (LTD) and long-term potentiation (LTP), two opposing forms of synaptic plasticity. Both forms of plasticity (LTP/LTD) are also critically linked to the activation of DA receptors, supporting the idea that in the striatum, a close interplay among DA receptors, DARPP-32 state phosphorylated/de-phosphorylated, and glutamatergic transmission might underlie the functional role of this structure in motor control and cognitive activities. (Calabresi et al., 2000, 2007).

In the context of experimental parkinsonism, our findings show that following dorsolateral striatal lesion with 6-OHDA, there is an increase in nNOS that may induce the phosphorylation of DARPP-32 in Thr-34, with a concomitant decrease of total DARPP-32, suggesting that NO mediates a regulatory effect in experimental parkinsonism (Fig. 6). Importantly, DARPP-32, located in medium-sized spiny neurons in most cytosolic subcellular compartments including dendrites, axons and axon terminals, may provide the anatomical framework for the action of multiple outputs of the striatum regulated by NO in PD (Lindskog et al., 2002; Nairn et al., 2004). In this context, the regulation of DARPP-32 via nNOS may

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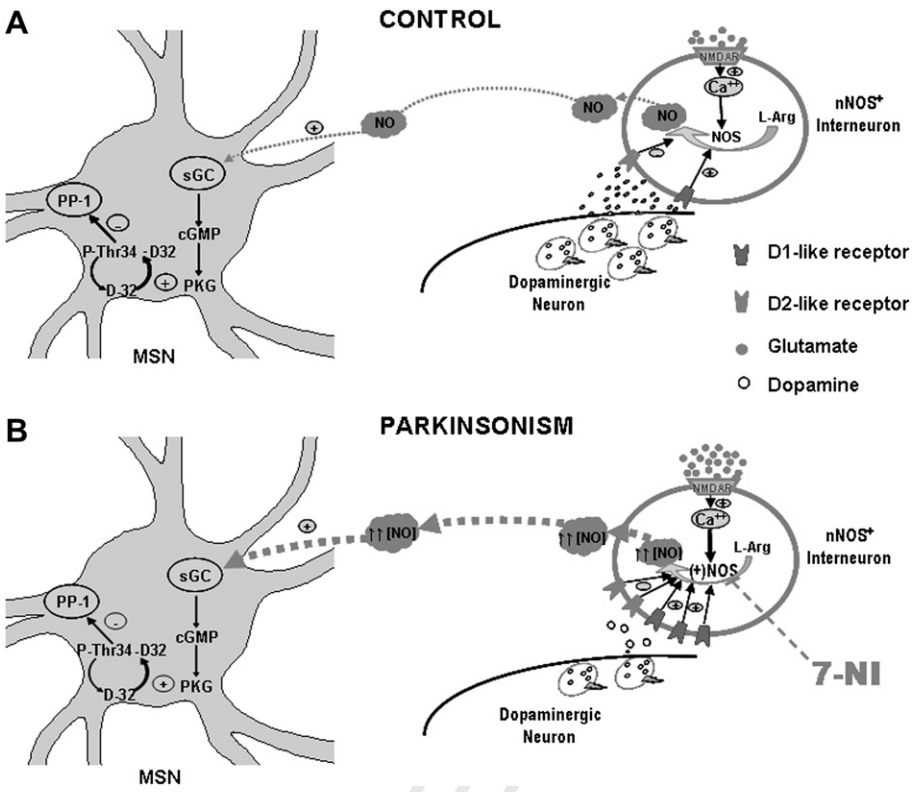

Fig. 6. Model of the role of NO signaling in the short-term facilitation of dopaminergic transmission in control and parkinsonism. (Adapted from Nishi et al., 2005 and Calabresi et al., 2007). (A) Schematic diagram of NO mechanism in a striatal MSN and nNOS interneuron. In the nitrergic neurons released NO leads to the activation of PKG in MSN (via cGMP) and the phosphorylation of DARPP-32 in Thr-34. Phospho-Thr34 DARPP-32 causes PP-1 inhibition, which controls the excitatory synaptic potentials in the neostriatum (Calabresi et al., 2007). (B) In Parkinsonism, the loss of DA modulation overexpress D1- and D2-like receptors, it could lead to overproduction of NOS and increased concentration of NO. Also, an altered enhanced stimulation corticostriatal signaling (glutamate inputs) could lead to NO-dependent facilitation (via $Ca2^{++}$), with a PKG activated via sGC and producing DARPP-32 phosphorylated in Thr-34 (Calabresi et al., 2007). When an inhibitor nNOS is administered (e.g., 7-NI) it can reverse the situation and produce an increase of total DARPP-32 and a decrease of DARPP-32 phosphorylated. MSN: medium-sized spiny neuron. NMDAR: NMDA receptor.

represent a therapeutic target for controlling the disorganized activity of medium-sized spiny neurons in DA-depleted striatum in parkinsonism.

5. Conclusions

This study shows that a mild dorsolateral striatal 6-OHDA lesion, which provokes a significant increase of nNOS, is correlated with a positive balance of phospho-Thr34-DARPP-32 expression in the neostriatum. Importantly, treatment with 7-NI, apart from providing a neuroprotective effect, restores the striatal levels of phosphorylation DARPP-32 and increases the level of total DARPP-32. These results throw light on the role of NO in regulating the activity of the striatum, suggesting that nNOS inhibitor may have a beneficial effect in restoring the neurotransmitter imbalance of the basal ganglia circuitry in parkinsonism.

Acknowledgments

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