



Research

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Are cyclooxygenase-2 and nitric oxide involved in the dyskinesia of Parkinson's disease induced by L-DOPA?[†]

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Inflammatory mechanisms are proposed to play a role in L-DOPA-induced dyskinesia. Cyclooxygenase-2 (COX2) contributes to inflammation pathways in the periphery and is constitutively expressed in the central nervous system. Considering that inhibition of nitric oxide (NO) formation attenuates L-DOPA-induced dyskinesia, this study aimed at investigating if a NO synthase (NOS) inhibitor would change COX2 brain expression in animals with L-DOPA-induced dyskinesia. To this aim, male Wistar rats received unilateral 6-hydroxydopamine microinjection into the medial forebrain bundle were treated daily with L-DOPA (21 days) combined with 7-nitroindazole or vehicle. All hemi-Parkinsonian rats receiving L-DOPA showed dyskinesia. They also presented increased neuronal COX2 immunoreactivity in the dopamine-depleted dorsal striatum that was directly correlated with dyskinesia severity. Striatal COX2 co-localized with choline-acetyltransferase, calbindin and DARPP-32 (dopamine-cAMP-regulated phosphoprotein-32), neuronal markers of GABAergic neurons. NOS inhibition prevented L-DOPA-induced dyskinesia and COX2 increased expression in the dorsal striatum. These results suggest that increased COX2 expression after L-DOPA long-term treatment in Parkinsonian-like rats could contribute to the development of dyskinesia.

1. Introduction

Parkinson's disease is an incurable neurodegenerative disease. The incapacitating motor symptoms are mainly due to the degeneration of dopaminergic neurons in substantia nigra compacta, leading to a loss of striatal dopaminergic fibres [1]. Dopamine is a key transmitter in the basal ganglia, yet dopamine transmission does not conform to several aspects of the classic synaptic doctrine [2]. In the striatum, axonal release sites are controversial, with evidence for dopamine varicosities that lack postsynaptic specializations. Instead, dopamine acts on extrasynaptic receptors and transporters. The dopamine precursor L-DOPA (L-3,4-dihydroxyphenylalanine) represents the most effective and best-tolerated compound for therapy for Parkinson's disease. Unfortunately, in many cases, L-DOPA treatment has to be suspended because of its side effects, which include motor fluctuations, dyskinesia (abnormal involuntary movements) and psychiatric problems [3]. Currently, the molecular events that underlie these adverse effects are poorly understood.

In vivo brain imaging of Parkinson's disease patients reveals an association between widespread microglial activation and the pathological process [4–6]

(see also [7]). In addition, both cellular and molecular studies of post-mortem human brain tissue show neuroinflammatory processes in the affected brain regions of these patients (for review, see [8–10]). Nonetheless, these studies do not indicate whether neuroinflammation is involved in the pathological process or is secondary to the neuronal degeneration. Moreover, because Parkinsonian patients are usually receiving L-DOPA or other anti-Parkinsonian medication at the time of death, it cannot be excluded that these treatments are also involved in the neuroinflammatory reaction [4,11–16]. According to this hypothesis, the excessive levels of dopamine in the striatal extracellular fluid following the administration of the dopamine precursor L-DOPA [17–20] would favour the development of a pro-inflammatory environment in the striatum [21].

We discovered that inhibition of the nitric oxide (NO) signalling pathway reduces L-DOPA-induced dyskinesia and the levels of associated molecular markers in hemi-Parkinsonian rodents [22–27]. This finding has been corroborated by studies from other laboratories in rats [28] and monkeys [29] and in the Pitx3-deficient aphakia mouse [30]. From a clinical standpoint, our behavioural analysis suggests that NO synthase (NOS) inhibitors could at least alleviate L-DOPA-induced dyskinesia in Parkinson's disease patients under chronic L-DOPA therapy without compromising its beneficial effect on akinesia [24,27]. NO is an interneuronal signalling molecule that is synthesized on demand from its precursor L-arginine by the NOS enzymes and freely diffuses out from the source cell [31,32]. Interestingly, NO also seems to participate in inflammatory processes observed in Parkinson's disease [33–35]. Hemi-Parkinsonian rats presenting L-DOPA-induced dyskinesia show increased expression in the striatum of neuronal NOS (nNOS) mRNA [24], nNOS and inducible NOS (iNOS) protein [27,36], FosB/ Δ FosB [25,27] and inflammatory markers (astrocytes, microglia [36]). These changes are decreased by administration of nNOS preferential inhibitor, raising the possibility that the anti-dyskinetic effects of these drugs would include interference in NO-mediated processes [37–39].

Evidence points to a positive effect of NO on cyclooxygenase-2 [40] (COX2-prostaglandin H-synthase) activity and/or expression. COX2 is a component of the inflammatory cascade in the periphery [41,42]. In the brain, COX2 is constitutive, expressed and regulated in neurons by synaptic activity [43]. NO and prostaglandin (the products COX2) have been proposed to function as retrograde messengers and to facilitate neurotransmitter release in the central nervous system. The beneficial or damaging role played by COX2 in brain pathologies is controversial [44,45].

Recently, non-neuronal factor such as inflammation have been suggested to be involved in L-DOPA-induced dyskinesia [36,46]. The anti-dyskinetic effects of anti-inflammatory treatments with either corticosterone [46] or IRC-82451 (a multitargeting molecule [47]) support the hypothesis. Moreover, there is evidence of an increased expression of inflammatory markers *in vivo* in human Parkinson's disease patients [4] and in animal models [36,48,49].

To further understand the potential role of the nitrergic system in L-DOPA-induced dyskinesia, this study is aimed at investigating the effect of L-DOPA-induced dyskinesia on the expression of COX2 in brain regions of hemi-Parkinsonian rats. We also analysed the impact of nNOS inhibition on COX2 expression.

2. Experimental procedures

(a) Subjects

Male Wistar rats (FMRP-USP, Ribeirão Preto, Brazil; 200–250 g body weight) were housed under 12 L : 12 D cycle with free access to food and water.

(b) 6-Hydroxydopamine lesion

All chemicals, if not specified, were purchased from Sigma-Aldrich, St Louis, MO, USA.

6-Hydroxydopamine (6-OHDA) was microinjected into the medial forebrain bundle as we previously described [27]. The rationale for our approach and the procedure used were provided in our previous papers [24,27,50]. In order to determine the degree of dopaminergic 6-OHDA lesion all rats were tested for apomorphine (0.5 mg kg⁻¹ subcutaneous (s.c.))-induced rotation 21 days after surgery. Only rats showing more than 90 full turns per 45 min (contralateral to the lesion) were selected for the study. The dopamine lesion was confirmed by analysis of tyrosine hydroxylase immunohistochemistry as described before [27] in the striatum and in the substantia nigra compacta (results not presented).

(c) Drug treatment and experimental groups

The dose regimen and route of administration were based on previously published studies [24,27] (electronic supplementary material, figure S1). Chronic treatment consisted of single daily injections for 21 days of: (i) 7-nitroindazole (7NI, a preferential nNOS inhibitor, 30 mg kg⁻¹) followed by L-DOPA (30 mg kg⁻¹ + benserazide 7.5 mg kg⁻¹ (Prolopa dispersive, Hoffman-LaRoche, Brazil; 7NI + L-DOPA, $n = 7$), (ii) vehicle (50% polyethyleneglycol–saline solution) followed by L-DOPA (VEH + L-DOPA, $n = 7$; electronic supplementary material, figure S1), (iii) 7NI followed by saline (7NI + SAL, $n = 3$), or (iv) VEH + SAL ($n = 6$), corresponding to daily injections of vehicle followed by saline. The dose of L-DOPA (30 mg kg⁻¹) was chosen for its ability to induce consistent abnormal involuntary movements throughout the chronic L-DOPA treatment [51–53].

(d) L-DOPA-induced abnormal involuntary movements

Rats were monitored for abnormal involuntary movements using a rat dyskinesia scale [54–56]. Experimental details were previously described by Bortolanza *et al.* [36] and Padovan-Neto *et al.* [24,26,27].

3. COX2 analysis

(a) Immunohistochemistry

Rats were deeply anaesthetized with urethane (25 mg kg⁻¹, i.p.) and sacrificed by transcardiac perfusion with physiological saline, followed by phosphate-buffered 4% paraformaldehyde (pH 7.4). The brains were removed and treated as described elsewhere [24]. Coronal sections of 25 μ m thickness were cut using a freezing microtome (LeicaR, model CM1850) throughout the striatum using the Paxinos & Watson [57] atlas.

The sections were processed using the procedure described previously [27,36]. Free-floating sections were incubated at 4°C with the primary antibodies (table 1) diluted in 0.1 M phosphate-buffered saline (pH 7.4), containing 0.15%

Table 1. Primary and secondary antibodies used in this study^a.

primary antibodies				secondary antibodies				
antigen	host	dilution	manufacturer	antigen	host	dilution	type	manufacturer
COX2	rabbit	1 : 300	Cayman Chemical	(H + L) rabbit	donkey	1 : 250	Alexa Fluor 488	Invitrogen
Cy3-GFAP	mouse	1 : 500	Sigma-Aldrich	—	—	—	—	—
OX-42	mouse	1 : 300	Abcam	(H + L) mouse	donkey	1 : 250	Cyanine (Cy3)	JIR
NeuN	mouse	1 : 60000	Chemicon	(H + L) mouse	donkey	1 : 250	Cy3	JIR
DARPP-32	mouse	1 : 500	Dr H. C. Hemmings	(H + L) mouse	donkey	1 : 250	Cy3	JIR
Calbindin	mouse	1 : 500	Sigma-Aldrich	(H + L) mouse	donkey	1 : 250	Cy3	JIR
nNOS	sheep	1 : 1000	Dr P. Emson	(H + L) sheep	donkey	1 : 250	Cy3	JIR
Calretinin	goat	1 : 500	Chemicon	(H + L) goat	donkey	1 : 250	Cy3	JIR
ChAT	goat	1 : 500	Chemicon	(H + L) goat	donkey	1 : 250	Cy3	JIR
Parvalbumin	mouse	1 : 500	Sigma-Aldrich	(H + L) mouse	donkey	1 : 250	Cy3	JIR

^aAntibodies used in immunohistochemistry and double fluorescence immunostaining procedure. COX2, cyclooxygenase-2; ChAT, choline-acetyltransferase; Cy3, cyanine-3; DARPP-32, dopamine and cAMP-regulated phosphoprotein of M_r 32 kDa; GFAP, glial fibrillary acidic protein; (H + L), heavy plus light chains; JIR, Jackson Immuno Research; NeuN, neuronal nuclei; nNOS, neuronal nitric oxide synthase; OX-42, CD11b/c equivalent protein.

Triton X-100. Biotinylated secondary antibodies (Vector Labs, Burlingame, CA, USA; diluted 1 : 400), followed by avidin–biotin–peroxidase complex (Vectastain ABC-kit, Vector Labs), were used for detection of the immunocomplexes (90 min for each step). To visualize the reaction, sections were incubated in 0.25 M Trizma base containing 3,3-diaminobenzidine (DAB).

Sections that had been processed for single-antigen with DAB colour reaction were examined to evaluate the overall pattern of COX2 immunoreactivity. All analyses were performed on both sides of the brain. Three rostrocaudal levels (rostral: 1.7 mm; medial: 0.7 mm; and caudal: –0.8 mm from bregma [57]) were examined within the striatum. An observer blinded to the treatment conditions acquired measurements from the dorsomedial, dorsolateral and ventrolateral striatal parts.

Adjacent or nearly adjacent sections were processed immunohistochemically by double immunolabelling. Distinct fluorescently tagged COX2-neuron-specific secondary antibody and antibodies were used against: NeuN (a neuronal nuclear protein), GFAP (glial fibrillary acidic protein of astrocyte), OX-42 (CD11b/c equivalent protein of microglia) and DARPP-32 (dopamine and cAMP-regulated phosphoprotein 32 kDa-striatum projection neurons) in order to analyse the cellular COX2 location in neurons or glial cells (table 1). The sections were processed using the procedure described previously [36,58].

Other series of striatal adjacent sections were double immunostained with COX2 and antigens to label striatal interneurons as described by Kawaguchi *et al.* [59] and Bennett & Bolam [60] as follows: (1) the large cholinergic interneurons identified by the presence of choline acetyltransferase (ChAT); (2) GABA (gamma-aminobutyric acid) interneurons identified individually by the presence of antigen against calcium-binding proteins (parvalbumin, calretinin and calbindin) or nNOS (table 1).

High-resolution optical sections were obtained by laser scanning microscopy. Overlapping neighbouring fields of view were acquired and merged with a Leica SP5 confocal laser scanning microscope (Leica Microsystems, Mannheim,

Germany) equipped with a motorized stage and hybrid detectors. The images were processed and reconstructed with FIJI [61] and IMARIS v. 7.5 software packages (Bitplane, Zurich, Switzerland).

(b) Western immunoblotting

To analyse the antibody specificity by Western immunoblotting, and to quantify striatal COX2-related protein expression, independent experimental groups were composed of rats receiving the following treatments: VEH + SAL, VEH + L-DOPA, 7NI + SAL and 7NI + L-DOPA ($n = 4$ rats in each group). The animals were decapitated, and the lesion-reactive (right) and the contralateral (left, control) striatum were microdissected on an ice-cooled dissection cover, with the help of a magnifying lens (Leica Zoom 2000), and immediately frozen in liquid nitrogen (–196°C). Tissue samples were stored at –80°C until use.

Left and right striata were processed separately. The homogenates were centrifuged at 10 000 r.p.m. for 25 min at 4°C. The supernatants were recovered for protein concentration measurements using Bradford assay (Bio-Rad Protein assay, Bio-Rad, Germany). Proteins (30 µg) were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% SDS-PAGE) and semi-dry transferred to a nitrocellulose membrane. Nitrocellulose membranes were incubated at 4°C overnight using the anti-COX2 antibody (1 : 500, table 1), with anti- α -tubulin as control (1 : 4000, table 1). Bound antibodies were detected with HRP-conjugated secondary anti-rabbit antibody (1 : 4000). Bands were visualized by enhanced chemiluminescence (ECL, Amersham, UK) and quantified with the software IMAGEJ [25]. Arbitrary units (arb. units) represent integrated density analysis extracted from band intensities of COX2 normalized by their respective tubulin band expression.

(c) Statistical analysis

Data were evaluated by repeated analysis of variance (rANOVA). Comparisons were done for treatment (VEH + SAL, VEH + L-DOPA, 7NI + SAL and 7NI + L-DOPA) and

brain side (ipsilateral and contralateral to the lesion). Tukey's test was used for post-hoc comparisons in cases of significant interactions. Data are presented as mean \pm s.e.m. and statistical significance level was set at $p < 0.05$. All statistical analysis was performed using SPSS for Windows (v. 8.0).

4. Results

(a) Behavioural observations and effect of 7NI on L-DOPA-induced dyskinesia

The total abnormal involuntary movements were scored every 60 min over a period of 180 min following L-DOPA administration.

Application of VEH + L-DOPA to 6-OHDA-lesioned rats induced contralateral rotation (day 1, locomotor: 4.85 ± 0.89) and abnormal, purposeless movements affecting cranial, trunk and limb muscles (day 1, axial, limb and orofacial: 22.14 ± 2.42) on the side of the body contralateral to the lesion. This score was maintained during the 21 days of treatment period (day 21, locomotor: 3.78 ± 0.76 ; axial, limb and orofacial: 20.85 ± 1.28).

7NI + L-DOPA-treated rats expressed less severe dyskinesia compared with L-DOPA alone over time (day 1, axial, limb, orofacial: 10.2 ± 4.03 ; locomotor: 1.71 ± 0.78). The scores progressively decreased on the subsequent days and at the end of the experiment (day 21) the abnormal involuntary movements scores were similar to control groups VEH + SAL and 7NI + SAL.

These results provide further evidence for the combination of chronic L-DOPA and 7NI ameliorating L-DOPA-induced dyskinesia.

(b) Effect of 6-hydroxydopamine lesion and L-DOPA treatment on COX2 expression

In accordance with the literature [43,62–64], analysis of constitutive COX2 immunoreactivity in the brain of control animals revealed positive neurons in the hippocampus (dentate gyrus granule cells, pyramidal cell neurons), piriform superficial cell layers of neocortex, the amygdala and, at a very low number, in the striatum, thalamus and hypothalamus (electronic supplementary material, figures S2 and S3). There were no positive cells in the substantia nigra, globus pallidus, entopeduncular and subthalamic nuclei.

Analysis of the dopamine-depleted (ipsilateral) striatum of the 6-OHDA-hemi-Parkinsonian rats receiving chronic L-DOPA treatment revealed depletion of tyrosine hydroxylase immunoreactivity (more than 80%—results not presented). Simultaneously, there was a remarkable immunopositive reaction for COX2 in the dorsal striatum. COX2 immunoreactivity presented a cytoplasmatic distribution with clean nuclei, at structures resembling neuronal cell bodies with ramifications (figures 1 and 3f',g').

COX2-positive labelling co-localized with immunoreactivity for NeuN (figure 1a–e), a 46/48 kD neuronal nuclear protein antigen widely used to identify neurons [65,66].

By contrast, COX2-positive immunoreactivity did not overlap either with GFAP-labelled astrocytes (figure 1i–k; electronic supplementary material, figure S4) or OX-42-labelled microglia (figure 1l–n). However, COX2 neurons

and GFAP-labelled astrocytes are in intimate proximity (electronic supplementary material, figure S4).

There was either very low or absent COX2 expression in the striatum contralateral to the lesion (figure 1a) or in the striatum of rats that received chronic 7NI + SAL or VEH + SAL, suggesting that COX2 induction does not reflect a general reaction of the neuronal cells within this brain region.

These results confirmed that the COX2 protein was found in neurons of lesioned striatum, which presented more than 80% absence of tyrosine hydroxylase-positive reaction and only after L-DOPA treatment.

(c) Characteristics of COX2 in striatal neurons of dyskinetic rats

In order to characterize COX2-positive neurons in the denervated striatum, we carried out double immunofluorescence staining reactions.

Confocal microscopy evaluation revealed that in the lesioned striatum of dyskinetic rats, striatal COX2 co-localizes with DARPP-32 (approx. 60% of the total neurons examined; figure 1f–h). DARPP-32-stained neurons are GABAergic medium-sized spiny striatal projection neurons and postsynaptic targets of convergent inputs from cortical glutamatergic neurons, cholinergic striatal interneurons and midbrain dopaminergic neurons [67].

Likewise, 100% of the acetylcholine-synthesizing enzyme ChAT-positive neurons analysed in the dorsal striatum co-expressed COX2 (figure 2a–c). ChAT stains cholinergic giant interneurons that can be recognized owing to their somatic size [59,60] (20–50 μ m). ChAT interneurons may be protected in several neurological conditions by their lack of AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor subunits [68,69], and enrichment in both forms of the superoxide dismutase free radical scavengers [70,71].

Approximately 60% of the calbindin-positive neurons in the dorsal striatum (figure 2d–f) co-expressed COX2. Because calbindin–COX2 double-immunostained neurons presented nuclear indentation, a well-established characteristic of striatal interneurons [60,72], they were identified as interneurons (see the electronic supplementary material, movie). However, because calbindin may also be detected in projection neurons, a more detailed analysis is required. Calbindin expression may offer to striatal neurons some protection against excitotoxicity [73]. Immunohistochemistry for calbindin heavily stained neurons in the matrix compartment of the striatum [74]. Cholinergic and DARPP-32-positive neurons at best play a key and selective role in L-DOPA-induced dyskinesia.

No double labelling between COX2 and nNOS or the two other calcium-binding proteins parvalbumin and calretinin was observed within the striatum (figure 2g–i; electronic supplementary material, figure S3).

(d) Analysis of COX2 expression in the striatum by Western blot and by immunohistochemistry

In agreement with a previous report [75], the COX2 antibody revealed a single protein band with a molecular weight of approximately 72 kDa corresponding to the non-glycosylated form of COX2 (figure 3a).

The Western blot analysis confirmed an increased COX2 protein content in the dopamine-depleted striatum of rats presenting L-DOPA-induced dyskinesia when compared with

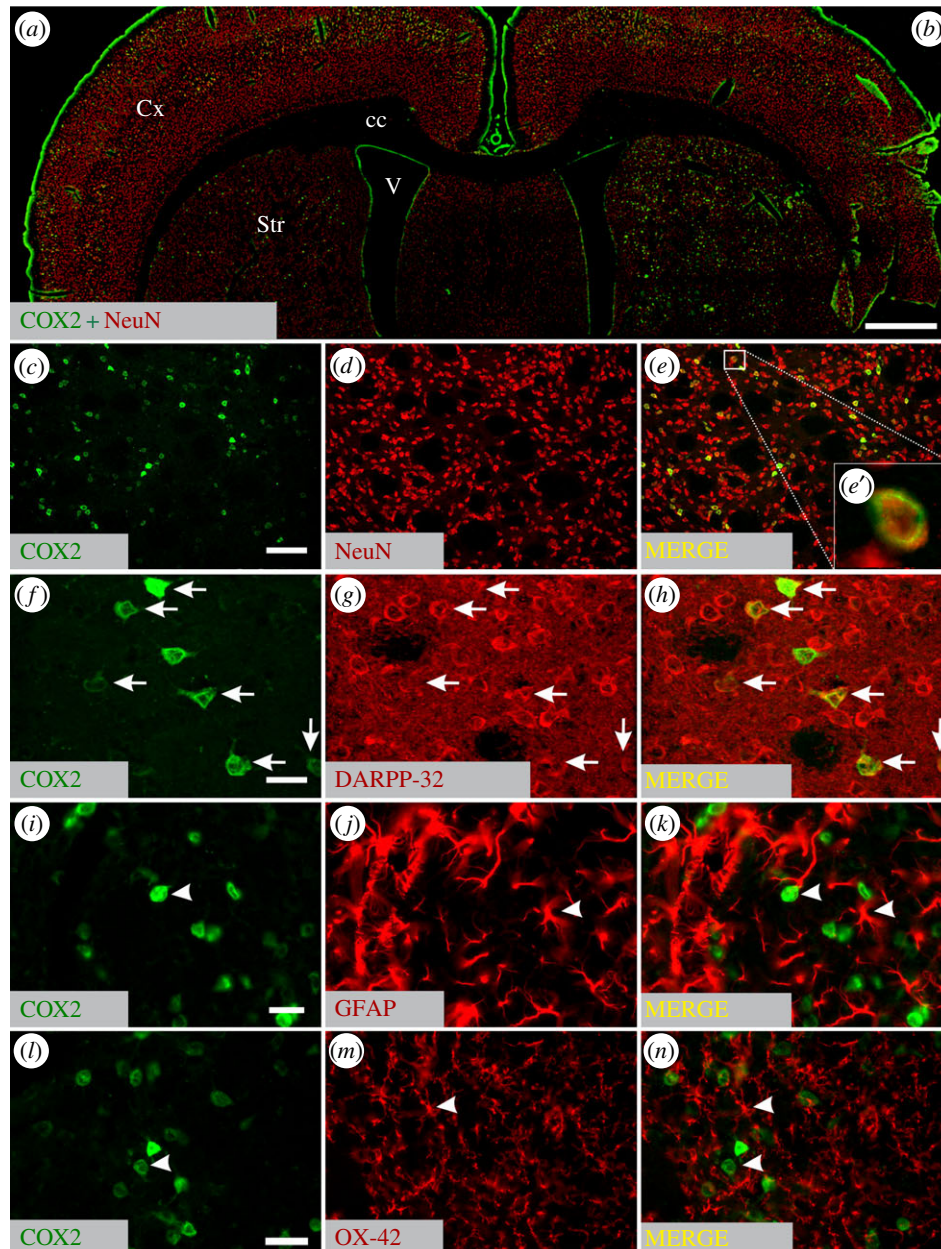


Figure 1. Striatal COX2 immunoreactivity in 6-OHDA-hemiparkinsonian rats with L-DOPA-induced dyskinesia. (a–b) Low-magnification photomicrograph showing NeuN (red) and COX2 immunoreactivity (green) in the striatum. There was COX2 immunoreactivity in the lesioned striatum (b, right) and occasional COX2 immunoreactivity in the contralateral one (a, left). (c–n) Photomicrographs of striatal sections showing immunofluorescence staining for COX2 antibody (green; c, f, j and l) in combination with antibodies (red) for neuronal protein (NeuN; d), dopamine-cAMP-regulated phosphoprotein-32 (DARPP-32; g), glial fibrillary acidic protein (GFAP; j), CD11b/c equivalent protein (OX-42; m). Co-localization with COX2 was observed for NeuN and DARPP-32. (e, h, k and n) correspond to merged images. Arrows indicate cells with co-localization. Arrowheads indicate cells with no co-localization. cc, corpus callosum; Cx, cortex; Str, striatum; V, ventricle. Fluorescent images were taken with the same setting. Scale bars: (a, b) = 100 μm ; (c, f, j, l) = 50 μm .

the contralateral side ($p < 0.05$) and to the lesioned striatum of rats receiving either VEH + SAL or 7NI + SAL treatment (treatment: $F_{3,23} = 3.132$, $p < 0.05$; figure 3b).

Analysis by immunohistochemistry supported the induction of COX2 in the striatum of Parkinsonian rats presenting abnormal involuntary movements elicited by L-DOPA treatment. It revealed an increase in the COX2 immunoreactivity (five- to sevenfold) in the dorsolateral, dorsomedial and ventrolateral striatum only after L-DOPA treatment (figure 3c, e, f and f') when compared with 6-OHDA-lesioned rats receiving either VEH + SAL or 7NI + SAL (interaction; dorsomedial: $F_{3,22} = 29.134$; dorsolateral: $F_{3,22} = 130.510$; ventrolateral: $F_{3,22} = 77.878$; $p < 0.05$; figure 3c).

Pre-treatment with 7NI reduced L-DOPA-induced dyskinesia and COX2 immunoreactivity in the dorsolateral and ventrolateral

striatum when compared with VEH + L-DOPA ($p < 0.05$ figure 3c, g and g'). There was also a trend ($p < 0.08$) for reduction in COX2 immunoreactivity in the dorsomedial striatum.

Finally, there was a significant correlation between the number of COX2-positive cells and abnormal involuntary movements (figure 3d; axial, limb, orofacial: dorsolateral and ventrolateral striatum $r = 0.9$, $p < 0.001$; locomotive: dorsolateral striatum $r = 0.7$, $p < 0.05$ and ventrolateral striatum $r = 0.9$, $p < 0.001$).

Taken together, these results show that during L-DOPA-induced dyskinesia, COX2 immunostaining is preferentially upregulated in neurons located in the dopamine-depleted striatum, which might influence L-DOPA-induced dyskinesia. The nNOS inhibitor 7NI mitigates dyskinesia and also COX2 neuronal expression.

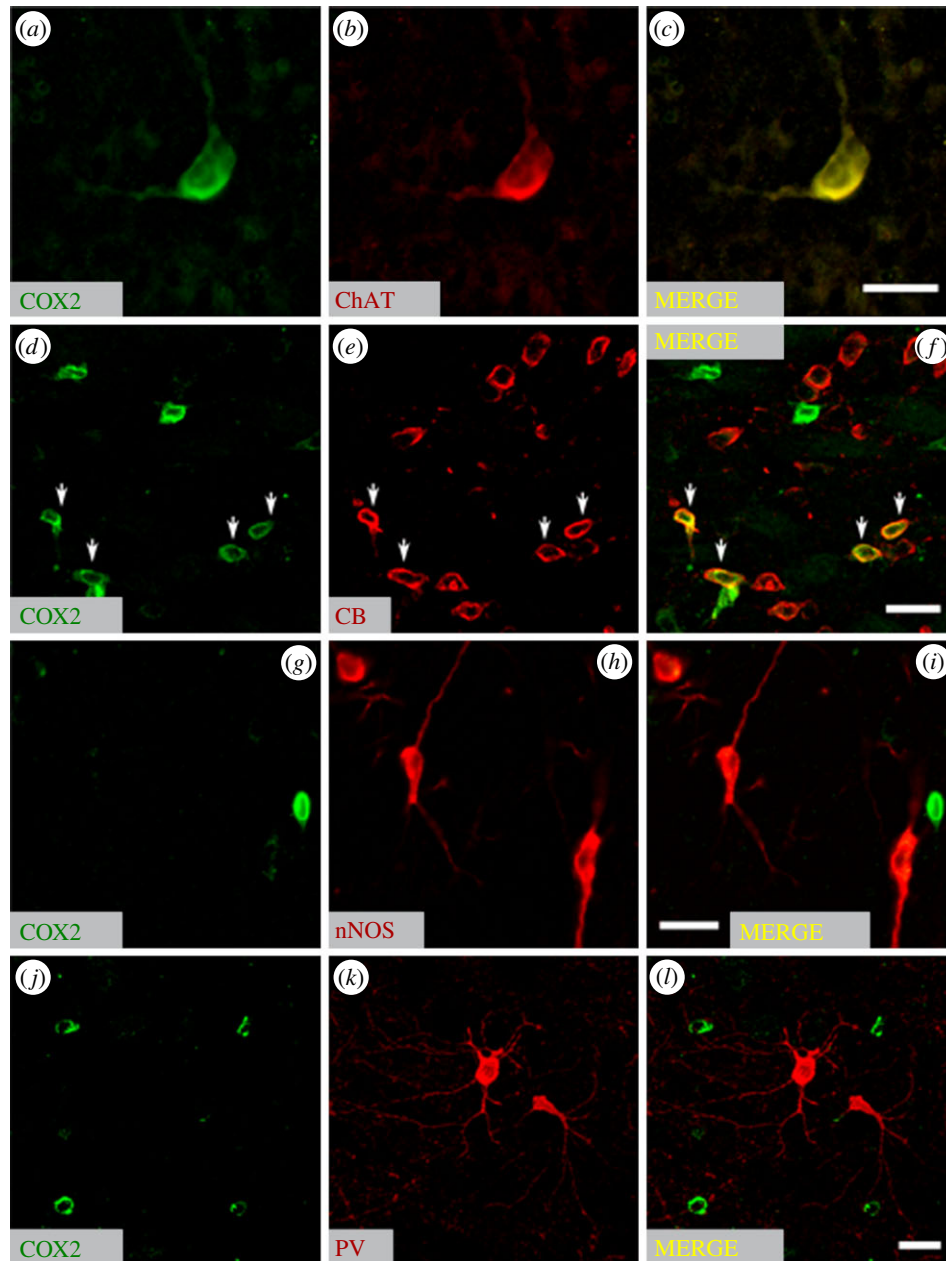


Figure 2. Expression of markers for striatal neurons co-localized with COX2-immunoreactive cells within the dopamine-denervated striatum following L-DOPA treatment. (*a–l*) Photomicrographs of striatal sections showing immunofluorescence staining with COX2 antibody (green; *a, d, g* and *j*) in combination with antibodies (red) for choline-acetyltransferase (ChAT; *b*), calbindin (CB; *e*), neuronal nitric oxide synthase (nNOS; *h*) and parvalbumin (PV; *k*). Co-localization with COX2 was observed for ChAT and CB. Arrows indicate cells with co-localization. (*c, f, i, l*) correspond to the merged images. Scale bars, 25 μ m.

5. Discussion

Here we show, we believe for the first time, a link between COX2 neuronal expression in the dopamine-depleted striatum that correlates with the severity of L-DOPA-induced dyskinesia. COX2 expression was prevented by co-treatment with the preferential nNOS inhibitor 7NI concurrently with L-DOPA-induced dyskinesia. L-DOPA administration did not induce COX2 expression in the non-lesioned striatum, suggesting that dopamine denervation is at the core of the mechanism leading to COX2 presence. We verified, using double-immunostaining that the COX2-positive immunoreactivity occurred in striatal neurons positive for DARPP-32, ChAT and calbindin. Cholinergic interneurons and DARPP-32 projection neurons play a key and selective role in L-DOPA-induced dyskinesia.

In Parkinson's disease, COX2 expression occurs mostly in glial cells in the substantia nigra compacta, being potentially

involved with neurodegeneration [76–80]. In agreement with this, COX2 knockout mice are more resistant to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-mediated intoxication [77,80,81]. On the other hand, evidence has been accumulated indicating that COX2 expression in Parkinson's disease does seem to derive from a mechanism distinct from the inflammatory properties of prostanoids [77]. In accordance, the inflammatory response associated with dopaminergic neurodegeneration in COX2 knockout mice did not differ from that observed in their wild-type counterparts [77]. Finally, non-steroidal anti-inflammatory drug treatment targeting COX2 has been studied in Parkinson's disease patients but with variable results [82].

(a) Mechanisms linking NO with COX2 expression

Compelling data have been generated supporting the concept that NO signalling is tightly coupled to the COX2-prostaglandin

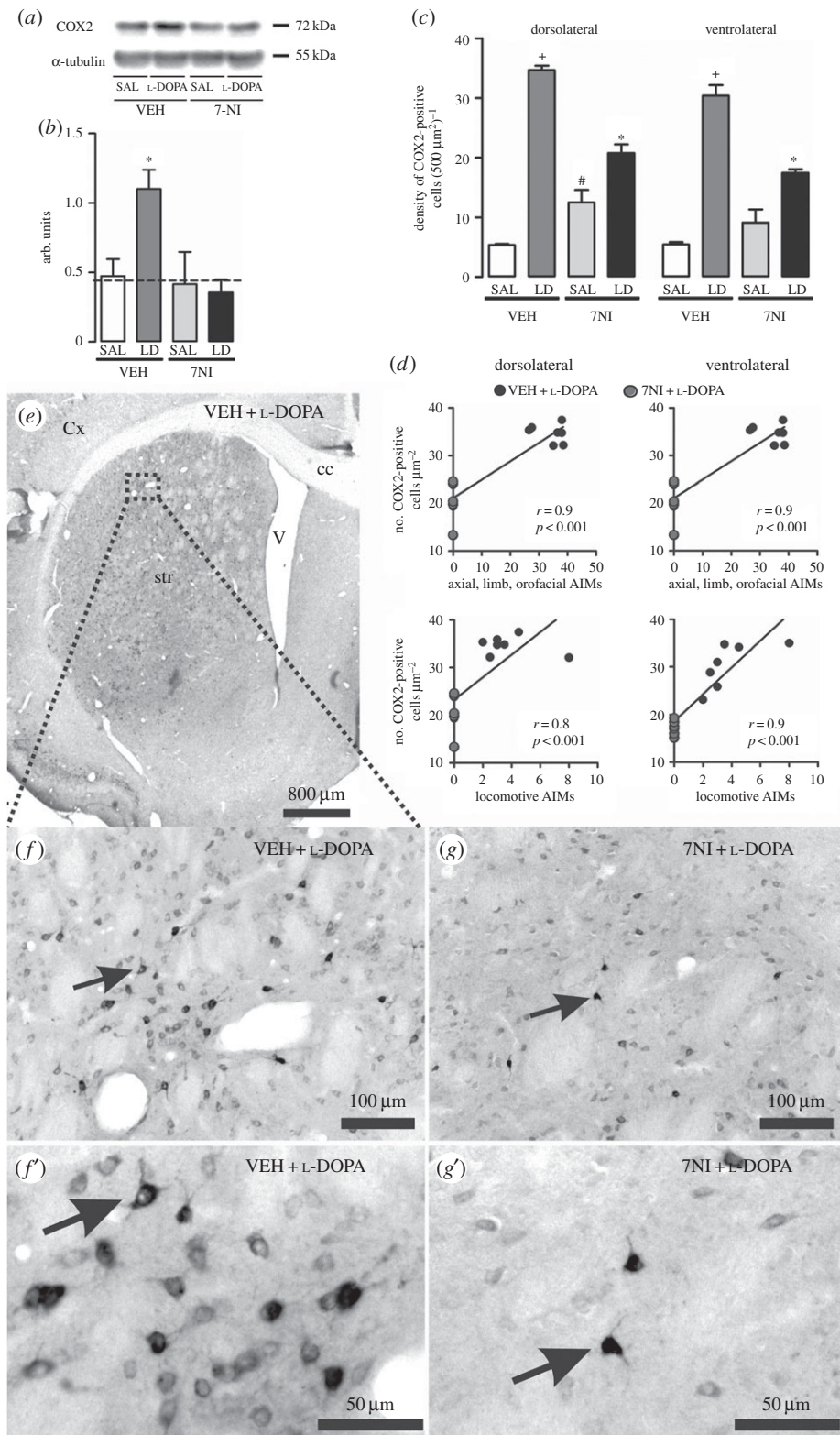


Figure 3. Analysis of L-DOPA and 7NI effect on COX2 expression in the 6-OHDA-lesioned striatum of rats. (a) Representative Western blots of striatal COX2 (72 kDa) and α -tubulin (50 kDa, control). (b) Western blotting quantification of striatal protein extracts obtained from 6-OHDA-lesioned rats chronically treated with either VEH + SAL, 7NI + SAL, VEH + L-DOPA or 7NI + L-DOPA. Dashed line indicates the mean of COX2 expression in the contralateral side to the lesion. (c) Quantification of COX2-immunostaining cells in the 6-OHDA-lesioned striatum. VEH + L-DOPA treatment significantly increased COX2 protein expression in the dopamine-denervated striatum ($p < 0.05$ if compared with (*) all other groups, with (+) VEH + SAL and 7NI + SAL, with (#) VEH + SAL). (d) Graphics showing positive correlation between COX2 expression and abnormal involuntary movements induced by L-DOPA treatment in the hemi-Parkinsonian rats. (e, f and g) Photomicrographs showing COX2-immunopositive neurons in the dopamine-depleted striatum of VEH + L-DOPA (e, f and f') and 7NI + L-DOPA (g and g')-treated rats. (f' and g') show high magnifications of a COX2 neuron. Arrows indicate an example of a COX2-positive cell. AIMs, abnormal involuntary movements; LD, L-DOPA; 7NI, 7-nitroindazole; SAL, saline; VEH, vehicle; cc, corpus callosum; Cx, cortex; str, striatum; V, ventricle.

pathway and vice versa [83,84]. In the central nervous system, nNOS-derived NO stimulates COX2 activity *in vivo* and *in vitro*.

7NI chronic treatment *per se* increases COX2-positive neurons in the dorsolateral striatum of 6-OHDA-lesioned rats. This may be interpreted as a NOS compensatory

mechanism. In agreement, chronic inhibition of NO synthesis has been reported to lead to an enhanced expression of COX2 in rat mesenteric arteries [85]. Therefore, another system in addition to the brain the induced absence of NO simulated by NOS inhibitor chronic treatment exhibit increase the expression of COX2 with less amounts of NO [85].

Overexpression of nNOS elicits S-nitrosylation of COX2 and activation of prostaglandin formation [86]. On the contrary, a large number of reports support the idea that NO can inactivate COX2 [87,88]. In several experimental models, COX2 has been linked to anti-inflammatory and neuroprotective properties [89,90]. In fact, prolonged overexpression of COX2 in the brain has been associated with the delayed cell death that occurs after many types of brain injuries [91]. These apparently contradictory results of NO influence on COX2 expression may be related to the levels of NO, the cell type and/or the state of activation of the cells [83,92].

Another possibility would be COX2 expression regulation of transcription, since COX2 end products can modulate gene expression [93]. Berke *et al.* [94] and Gerfen [95] proposed that COX2 mRNA induction in the striatum of hemi-Parkinsonian rat involves genetic adaptation mechanisms triggered by dopamine depletion. In general, COX2 expression under inductive stimuli follows the pattern of the so-called early genes that can directly modify cellular function [44,96]. Regulation of *cox2* gene transcription is controlled by consensus elements present in the *cox2* gene promoter. It contains a TATA box motif and a number of *cis*-acting elements, including a CREB-response element (cyclic AMP response element) nuclear factor interleukin-6, AP-1 (activator protein-1 transcription factor), and nuclear factor-kappa B (NF- κ B). Interestingly, transcription factors that are activated in the striatum in L-DOPA-induced dyskinesia [97] may bind to these consensus elements of the *cox2* gene [98]. For example, the mitogen-activated protein kinase extracellular signal-regulated kinase-1 (ERK1) and ERK2 in the cell nucleus, together with cAMP, trigger the activation of CREB, which drives the expression of COX2 [99] and of L-DOPA-induced dyskinesia proteins [100].

Finally, the capacity of NO and its effector cyclic GMP to modulate the function of several target proteins, including transcription factors such as NF- κ B and AP1, appears as the key pathway by which NO may regulate COX2 expression [101].

Whether or not these factors contribute to regulation of COX2 expression in the denervated striatum during L-DOPA action should be determined.

(b) COX2 expression in the DARPP-32, calbindin and cholinergic neurons

The main candidates for the mechanism underlying L-DOPA-induced dyskinesia are functional and structural alterations induced in the dopamine-depleted striatum [97,102–105]. Herein, besides an increase in COX2 expression in the dopamine-depleted striatum, we showed, we believe for the first time, that L-DOPA administration positively regulates COX2 expression in DARPP-32, acetylcholine and calbindin neurons.

There are higher levels of DARPP-32 in the striatum of dyskinetic rats compared with 6-OHDA rats that have not developed dyskinesia under L-DOPA treatment [106]. Most of the abnormal involuntary movements developed following chronic L-DOPA are associated with hyperactivation in

striatal medium spiny neurons of a signalling pathway comprising sequential phosphorylation of DARPP-32 [107]. L-DOPA-induced dyskinesia is associated with changes in dopamine-D1 receptor signalling in the dopamine-depleted striatum. The dopamine-D1 and the prostaglandin type 1 (EP1) receptors are co-expressed in striatal neurons [108]. In addition, EP1 prostaglandin signalling augments dopamine-D1-induced phosphorylation of DARPP-32 and facilitates hyperlocomotion induced by dopamine-D1 agonists [108].

Calbindin, a major calcium-binding protein in the cytosol, plays a critical role during intracellular Ca^{2+} homeostasis and is implicated in neuroprotection (when expressed at high levels) owing to its ability to buffer free intracellular Ca^{2+} [109–111]. Dopamine-D1 receptor signalling is modulated by calcium level and NO [2,67]. In many neurodegenerative diseases, intracellular Ca^{2+} homeostasis appears to be disrupted and might be a serious risk factor [112]. L-DOPA-induced toxicity in cultured midbrain neurons is also dependent on calcium regulation [113]. Considering that cellular degeneration is often accompanied by impaired calcium homeostasis, a protective role for calcium-binding proteins has been postulated [114].

Elimination of striatal cholinergic interneurons in the lesioned striatum of hemi-Parkinsonian mice attenuates the development of L-DOPA-induced dyskinesia without affecting the beneficial anti-Parkinsonian action of L-DOPA [100,115]. Cholinergic interneurons in the striatum establish intricate axonal projections that represent a widespread neurotransmission system [116–118]. Mechanistic studies revealed local regulation of dopamine release by acetylcholine as well as by proteins known to be disrupted in Parkinson's disease and other movement disorders [2,119]. Similarly, dopamine-D1 and -D2 receptor signalling is modulated by acetylcholine and NO [2,67]. Strategies reported to reduce striatal cholinergic tone after dopamine deregulation may represent a promising approach to decreasing L-DOPA-induced motor complications in Parkinson's disease.

We propose that L-DOPA induction of COX2 transcription in striatum of Parkinsonian-dyskinetic rats directly influences discrete striatal neurons containing DARPP-32, acetylcholine and calbindin, which play a significant role in L-DOPA-induced dyskinesia. Further studies are needed to analyse the role of COX2 in these neurons in L-DOPA-induced dyskinesia.

6. Extrasynaptic signalling/volume signalling

Because extrasynaptic transmission is modulated by NO, COX2 product prostaglandin, dopamine and acetylcholine modulate extrasynaptic transmission, our observation reveals a new mode of neurotransmitter action upon dopamine-depleted striatum submitted to L-DOPA chronic treatment. The role of extrasynaptic signalling in neurological diseases only recently has been described in cases of neurodegenerative disease. That there is evidence of cell death associated with neurodegenerative diseases may be partly due to an imbalance of synaptic and extrasynaptic signalling [120]. Also, functional recovery might be facilitated by non-synaptic neurotransmission [121].

The dense dopaminergic nerve terminal plexus in the striatal cellular networks mainly operates via extrasynaptic/volume transmission [122]. Various subtypes of extrasynaptic dopamine receptors [123–125] are located in projection neurons and interneurons as well as on the afferent nerve terminal networks.

The most extreme case of global volume signalling is that of gaseous NO. In the striatum, a single source of the NO signal can tune the activity of a set of nerve and glial cells by non-synaptic diffuse neurotransmission [126]. A close anatomical link between NO and dopamine-releasing neurons is proposed via the localization of NOS- and tyrosine hydroxylase-containing neuronal fibres and cell bodies in the nigrostriatal pathway [58].

The actions of acetylcholine on dopamine release do not conform to the synaptic doctrine, owing to the absence of direct synaptic contacts of cholinergic terminals on striatal dopaminergic axons. A widespread plexus of cholinergic nerve terminals exist especially in the striatum and thus mainly operate via extrasynaptic release and volume transmission [118] and may act by modulating the transmission of GABA-projection neurons.

COX2 and its products, especially prostaglandin E2 (PGE2), in addition to being involved in the inflammatory responses, have been reported to be important in modulating synaptic activity [127–130]. Teismann *et al.* [77] (for review, see [10]) suggested that the beneficial role of COX2 inhibition in the mouse model of Parkinson's disease is mediated by a mechanism that could involve facilitation of dopamine-mediated neurotransmission. For example, the COX2 products PGE2 are formed in response to dopamine receptor stimulation in the striatum and regulate signalling and function of dopamine-D1 and -D2 receptors [108].

COX2 is expressed predominantly in dendrites of glutamatergic neurons [76], localization consistent with its role in synaptic and extrasynaptic function [131] and neurovascular regulation [132]. Under pathological conditions, the most active NMDA-type glutamate receptor (NMDAR) subpopulation appears to be composed of extrasynaptic NMDAR activity and synaptic NMDAR activity [133]. Extrasynaptic-NMDARs may regulate COX2 expression or activity [134,135].

Given that COX2 expression in L-DOPA-induced dyskinesia may last for days [136], PGE2 might function as a long-term adaptive, paracrine-like mechanism to amplify dopamine signalling [108] and/or modify glutamatergic and cholinergic striatal neurotransmission.

7. Conclusion

Although additional studies are clearly needed to establish a causal relationship, our results suggest that NO-dependent changes in COX2 expression are correlated with the development of motor side effects after L-DOPA long-term treatment in Parkinson's disease. Because COX2-prostaglandin, NO, dopamine and acetylcholine modulate synaptic as well as extrasynaptic/volume transmission we postulate the intriguing possibility of extrasynaptic/volume transmission-mediated actions of these neurotransmitters in L-DOPA-induced dyskinesia [124].

Ethics statement. The experiments were conducted according to the principles and procedures described by the guidelines for the care and use of mammals in neuroscience and behavioural research (ILAR, USA). The local Ethical Committee approved the protocol.

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