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## Selective COX-2 inhibition prevents progressive dopamine neuron degeneration in a rat model of Parkinson's disease

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### Abstract

Several lines of evidence point to a significant role of neuroinflammation in Parkinson's disease (PD) and other neurodegenerative disorders. In the present study we examined the protective effect of celecoxib, a selective inhibitor of the inducible form of cyclooxygenase (COX-2), on dopamine (DA) cell loss in a rat model of PD. We used the intrastriatal administration of 6-hydroxydopamine (6-OHDA) that induces a retrograde neuronal damage and death, which progresses over weeks. Animals were randomized to receive celecoxib (20 mg/kg/day) or vehicle starting 1 hour before the intrastriatal administration of 6-OHDA. Evaluation was performed *in vivo* using micro PET and selective radiotracers for DA terminals and microglia. Post mortem analysis included stereological quantification of tyrosine hydroxylase, astrocytes and microglia. 12 days after the 6-OHDA lesion there were no differences in DA cell or fiber loss between groups, although the microglial cell density and activation was markedly reduced in animals receiving celecoxib ( $p < 0.01$ ). COX-2 inhibition did not reduce the typical astroglial response in the striatum at any stage. Between 12 and 21 days, there was a significant progression of DA cell loss in the vehicle group (from 40 to 65%) that was prevented by celecoxib. Therefore, inhibition of COX-2 by celecoxib appears to be able, either directly or through inhibition of microglia activation to prevent or slow down DA cell degeneration.

### Background

The role of microglia in the pathogenesis of neurodegenerative disorders is not clear [1]. Increasing evidence suggests that an inflammatory reaction accompanies the pathological processes seen in many neurodegenerative disorders, including Parkinson's disease (PD) [2-4]. Glial activation is part of a defense mechanism to remove debris and pathogens and promote tissue repair. How-

ever, inflammatory activation of microglial cells may contribute to the neurodegenerative process through structural invasion and the release of pro-inflammatory cytokines, reactive oxygen species (ROS), nitric oxide (NO) and excitatory amino acids at synapses and cell bodies. In cell culture and animal models, inflammation contributes to neuronal damage, and anti-inflammatory drugs have been shown to provide some neuroprotection

in different paradigms [5-7] including PD models [8,9]. Reactive microglia inhibit neuronal cell respiration via NO and cause neuronal cell death in vitro [10] and in vivo [11]. Interestingly, microglial cell activation by chronic infusion of lipopolysaccharide (LPS) appears to be capable of inducing a selective degeneration of nigral dopamine (DA) neurons [11]. Intranigral injection of LPS, but not of cytokines, induces DA degeneration [11,12]. LPS induces NO production and release from microglia and also release of pro-inflammatory cytokines such as IL-1 $\beta$  and TNF- $\alpha$ , which may also participate in cytotoxicity [13].

In PD there is evidence of an increase in oxidative and inflammatory nigral environment [2,14-16] that includes the presence of cyclooxygenase (COX)-immunoreactive activated microglial cells in the substantia nigra (SN) [17], elevated levels of TNF- $\alpha$  and other pro-inflammatory cytokines in the cerebrospinal fluid (CSF) [18,19]. DA neurons in the SN express TNF- $\alpha$  receptor 1 [3] which may contribute to the selective susceptibility of DA neurons to microglial toxicity. Supporting a role of inflammation in DA degeneration, mice deficient in TNF- $\alpha$  receptors are resistant to selective DA toxins [20]. In PD patients, a polymorphism in the TNF- $\alpha$  gene, leading to high production of TNF- $\alpha$ , was found to be more frequent than in matched healthy controls and to be related to earlier onset of the disease [21]. In addition, the results of a recent epidemiological study suggest that nonsteroidal anti-inflammatory drugs (NSAID) might delay or prevent onset of PD [22]. NSAIDs target p38 mitogen-activated protein kinase (MAPK) in addition to their main target COX [23] and inhibition of p38MAPK phosphorylation blocks NO release from activated microglial cells [24].

Selective COX-2 inhibitors lack the adverse effects of conventional NSAIDs, which inhibit both isoforms of COX (constitutive and inducible). COX-2 is induced by pro-inflammatory stimuli and cytokines [25]. Inhibition of the inducible form (COX-2) accounts largely for the therapeutic (anti-inflammatory) actions of NSAIDs whereas inhibition of the constitutively expressed form (COX-1) is responsible for the gastrointestinal side effects [25].

In this study we used the intrastriatal administration of 6-OHDA in the rat to evaluate the protective effect of selective COX-2 inhibition by celecoxib. Like other toxic and genetic models, this model has limitations, but it provides a time window to test neuroprotective strategies, as DA neurons die retrogradely over the course of several weeks [26-29]. We have previously shown that, in this model, DA cell death is accompanied by microglial cell activation [28].

## Methods

### 6-OHDA lesion model

To produce progressive and selective degeneration of the nigro-striatal DA system, Sprague Dawley rats (200 – 250 g, Charles River, Wilmington, MA) received unilateral intrastriatal stereotaxic injections of 6-OHDA (Sigma, St. Louis, USA) using a 10  $\mu$ l Hamilton syringe as previously described [28,30]. Acepromazine (3.3 mg/kg, PromAce, Fort Dodge, IA) and atropine sulfate (0.2 mg/kg, Phoenix Pharmaceuticals, St. Joseph, MO) were given i.m. 10 min before animals were anesthetized with ketamine/xylazine (60 mg/kg, Fort Dodge Animal Health, Fort Dodge, IA and 3 mg/kg, Phoenix, respectively, i.m.). A concentration of 3.0  $\mu$ g/ $\mu$ l free base 6-OHDA dissolved in 0.2% ascorbic acid/saline (Sigma) was injected into 3 locations (2.5  $\mu$ l/site, total dose 22.5  $\mu$ g) in the right striatum over 8 min per site at the following coordinates (calculated from bregma): site 1, AP +1.3, L -2.8, DV -4.5, IB -2.3; site 2, AP +0.2, L -3.0, DV -5.0, IB -2.3; site 3, AP -0.6, L -4.0, DV -5.5, IB -2.3 mm. Rate of injection was 0.5  $\mu$ l/min, leaving the needle in place for an additional 3 min before withdrawal. Following surgery, animals received 2 injections of buprenorphine (0.032 mg/kg, s.c., Sigma) 10 hours apart as post-operative analgesia. Rats were treated via oral intubation with a COX-2 inhibitor (celecoxib, 20 mg/kg/day, Pharmacia, Skokie, IL), n = 12 or vehicle (0.5 % methyl cellulose aqueous solution, Sigma), n = 13, beginning approximately one hour prior to lesion and continuing once per day for 14 or 21 days. To functionally evaluate the DA lesion, forepaw use was examined using the cylinder test 3 weeks after the striatal lesion. All animals showed a marked asymmetry ( $\sim$ 90% of the contacts were made using the ipsilateral paw).

### Histological and stereological procedure

Animals were terminally anesthetized by an i.p. injection of sodium pentobarbital (100 mg/kg) and perfused intracardially with heparin saline (0.1% heparin in 0.9% saline; 100 ml/rat) followed by paraformaldehyde (4% in phosphate buffer). The brains were removed and post-fixed for 8 hours in 4% paraformaldehyde solution. Following post-fixation, the brains were equilibrated in 20% sucrose in PBS, sectioned at 40  $\mu$ m on a freezing microtome, and serially collected in PBS.

All immunohistochemistry was performed on randomly selected series of sections that represented 1/6<sup>th</sup> of the total brain per primary antibody. Sections were treated for 10 minutes in 3% hydrogen peroxide (Humco, Texarkana, TX), washed 3 times in PBS, and incubated in 2% normal goat serum (NGS) and 0.1 % Triton X-100 for 30 minutes prior to overnight incubation at 4°C with the primary antibody diluted in 2% NGS and 0.1 % Triton X-100. The primary antibodies utilized were rabbit anti-tyrosine hydroxylase (TH) (Pel Freez, Rogers, AK; 1:300), mouse

anti-rat CD11b (OX42) (Accurate Chemical & Scientific Corporation, Westbury, NY; 1:100), and rabbit anti-gial fibrillary acidic protein (GFAP) (Dako A/S, Denmark; 1:500). After a  $3 \times 10$  minute rinse in PBS, the sections were incubated in biotinylated goat anti-mouse/rabbit secondary antibody (Vector Laboratories, Burlingame, CA; 1:300) diluted in 2% NGS in PBS at room temperature for 60 min. The sections were rinsed three times in PBS and incubated in streptavidin-biotin complex (Vectastain ABC Kit Elite, Vector Laboratories) for 60 min at room temperature. Following thorough rinsing with PBS, staining was visualized by incubation in 3, 3'-diaminobenzidine solution with nickel enhancement (Vector Laboratories). Controls with omission of the primary antibody were performed on selected sections that verified the specificity of staining. After immunostaining, floating tissue sections were mounted on glass slides and counterstained with cresyl violet before dehydrating, clearing and coverslipping.

Design based stereology was performed on the stained sections using an integrated Axioskop 2 microscope (Carl Zeiss, Thornwood, NY) and Stereo Investigator image capture equipment and software (MicroBrightField, Williston, VT). Quantification of TH fibers was performed utilizing a Cavalieri estimator probe. The quantification of GFAP and CD11b positive cells in separate section series was performed using an optical fractionator probe. The precision of the serial section analyses was assessed by the coefficient of error ( $p < 0.05$ ). TH positive cell bodies were counted utilizing the above system, which ensured cells were not omitted or counted twice. For TH fibers and cell counts results were expressed as percentage of contralateral (unlesioned) side. For GFAP and CD11b positive cell counts, estimation of total numbers was performed using the MicroBrightfield software and cell density was calculated for striatal and midbrain volumes. The striatum was outlined according to anatomical landmarks following the Paxinos atlas[31]. To avoid bias in the outline of the substantia nigra based on cresyl violet counterstain, the midbrain sections were divided in quadrants and the area ventral to the aqueduct was included for quantification of CD11b cell density and identified as ventral midbrain. Stereological analysis was performed by investigators blind to treatment group.

Group comparisons were performed using ANOVA to evaluate treatment, side and time effects. Post hoc analyses were performed whenever a significant effect ( $p < 0.05$ ) was found. Simple regression analyses were performed to evaluate correlation between fiber and cell density. Statistical analyses were made using Statview software (SAS Institute Inc, Cary, North Carolina).

### **PET imaging**

A total of 3 saline-injected and 11 6-OHDA lesioned rats were imaged by PET using  $^{11}\text{C}$ -CFT ( $2\beta$ -carbomethoxy- $3\beta$ -(4-fluorophenyl) tropane), a specific ligand for presynaptic DA transporters (DAT)[32,33]. To explore activation of microglia/macrophage function, imaging studies were conducted in the same rats with  $^{11}\text{C}$ -PK11195 (N-sec-butyl-1-(2-chlorophenyl)-N-methylisoquinoline-3-carboxamide), a specific ligand for activated microglia [28,34]. Imaging studies were performed 2 or 3 weeks after 6-OHDA injections using an in-house-built, super high-resolution rodent PET system[35].  $^{11}\text{C}$ -CFT was prepared according to previously published procedures [33,36].  $^{11}\text{C}$ -PK11195 was synthesized with a modified method of Camsonne et al [36]. Briefly, 1 mg of the precursor (N-sec-butyl-1-(2-chlorophenyl) isoquinoline-3-carboxamide) was dissolved in 500  $\mu\text{L}$  DMSO with 5–10 mg KOH, after trapping the C-11 methyl iodide, the vessel was heated at  $80^\circ\text{C}$  for 3 min and purified by HPLC system comprising a mobile phase pump (Hitachi), an automatic sample injector with 5 ml loop (Merck) and a radioactivity detector (in-house construction). Separation was performed on a  $\mu$ -Bondapak C-18 column (7.8–300 mm, Waters) using methanol and 0.01 M phosphoric acid (700 / 300, v/v) as the mobile phase with a flow of 8 ml/min. The radioactivity peak with a retention time of 5.6 min, similar to a reference standard was collected. After addition of 50  $\mu\text{L}$  5 M HCl, the collected fraction was evaporated and the residue was dissolved in saline buffer and sterilized by filtration through a 0.2- $\mu\text{m}$  filter (Millex<sup>®</sup>-GV). About 50% of the radioactivity was trapped in the filter because of the high lipophilicity of PK11195. The average yield of the final product was 20 mCi within 45 min.

For PET imaging studies, animals were anaesthetized with halothane (1 - 1.5%) using an oxygen flow rate of 3 L/min. Tail vein was catheterized for infusion of the labelled ligands. The animal was placed in the imaging position and the head was adjusted into an in-house-built stereotaxic head-holder. Imaging studies of microglia and DAT were conducted in the same imaging session.  $^{11}\text{C}$ -PK11195 (1–2 mCi iv.) was administered first and dynamic data were acquired at two different coronal brain levels for an hour. After an additional hour of decay time  $^{11}\text{C}$ -CFT (2 – 3 mCi iv.) was administered and data were acquired as above. Calibration of the positron tomograph was performed in each study session using a cylindrical plastic phantom (diameter of 3 cm) and  $^{18}\text{F}$ -solution. Imaging data were corrected for uniformity, sensitivity, attenuation, decay and acquisition time [32]. PET images were reconstructed using Hanning-weighted convolution backprojection and overlaid on atlas templates to confirm anatomical location. Regions of interest, including the left and right striatum and cerebellum were drawn and

activity per unit volume, percentage activity of injected dose and ligand concentration were calculated [32]. Binding ratios and left-right side differences were calculated as described previously[37].

## Results

Following the 6-OHDA intrastriatal infusion, rats received either celecoxib at 20 mg/kg (COXIB group, N = 12) or vehicle (N = 13) by oral gavage daily until the time of sacrifice at 12 (n = 4/5) or 21 (n = 8/8) days post surgery. First, we examined *in vivo* the effect of celecoxib compared to vehicle using micro PET and  $^{11}\text{C}$  PK11195, a peripheral benzodiazepine receptor ligand that binds to microglia [28] and  $^{11}\text{C}$  CFT, a cocaine analog that binds to the DAT. At 12 days we observed a decrease in CFT binding (~60% of contralateral BP) in the 6-OHDA lesioned striatum of both experimental groups (Fig. 1A). No striatal  $^{11}\text{C}$  PK11195 binding was present in the COXIB group (n = 3) while in the vehicle group (n = 2) there was binding ipsilateral to the 6-OHDA lesion, as described previously. No differences between COXIB (n = 3) and vehicle (n = 3) were observed at 21 days at the striatal level (Fig. 1A).

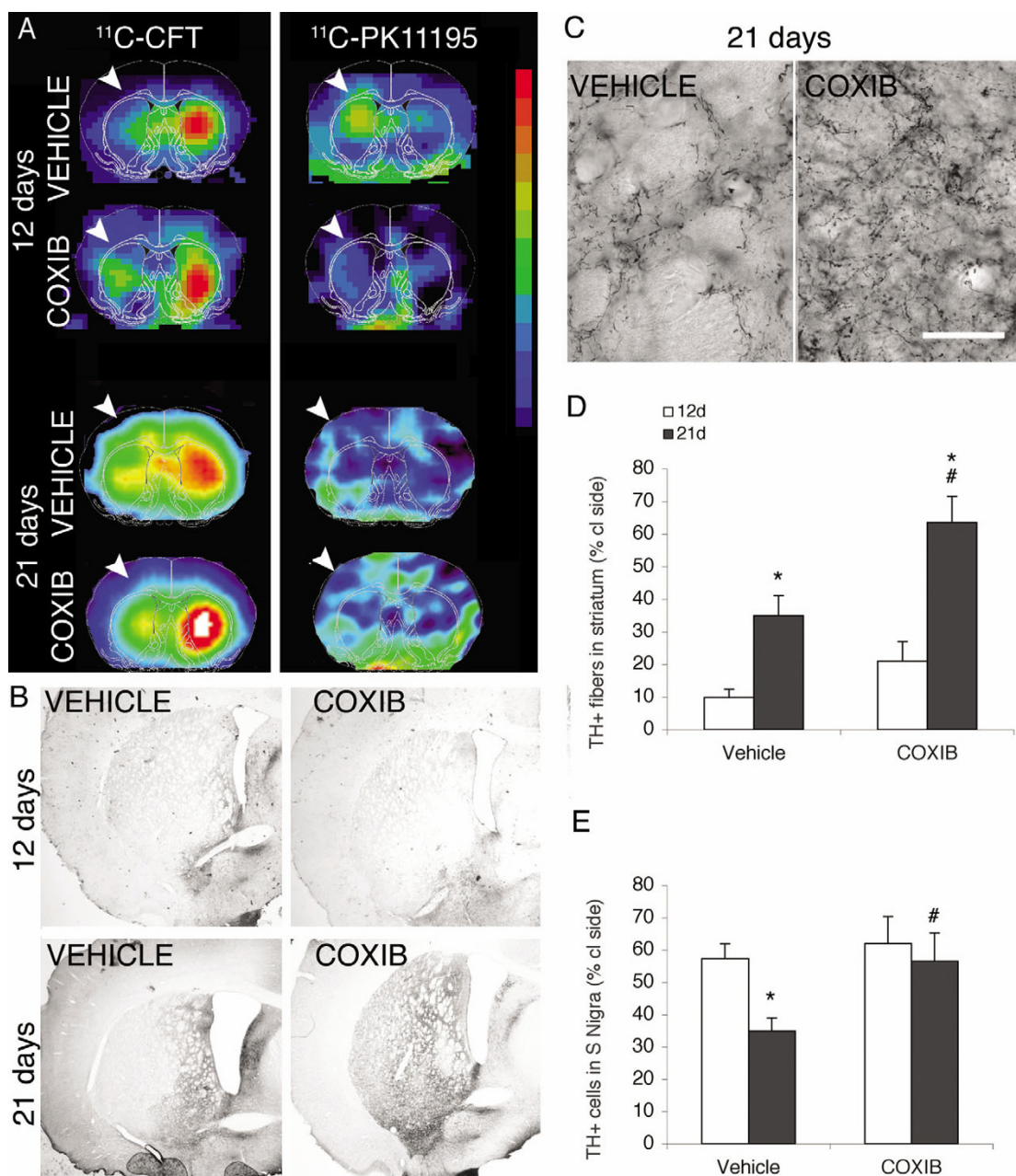
Next we examined the effect of celecoxib on the DA system. We analyzed the striatal volume of TH+ fibers (Fig. 1B,1C,1D) and quantified the number of TH+ cell bodies in the SN (Fig. 1E). At 12 days there was a severe (~85%) decrease in TH immunoreactive fibers in the striatum and less marked loss of TH+ cells (~40%) in the SN, and there were no differences between groups in either of these measures (Fig 1B,1D). However, at 21 days animals in the COXIB group displayed significantly larger volumes of DA terminal fibers in the striatal areas (> 50%,  $t = 7.8$ ,  $p = 0.01$ ) and corresponding larger number of TH+ cell bodies in the SN ( $t = 5$ ,  $p < 0.05$ , Fig. 1B,1C,1D). In the vehicle group there was a significant progression of TH+ cell loss from 12 to 21 days ( $t = 3.5$ ,  $p < 0.01$ ) (Fig. 1E), which is consistent with previous studies [26-29]. This progression did not occur in the COXIB group ( $p = 0.7$ ). In addition to the treatment effect, there was a significant recovery of striatal fiber density at 21 days in both groups. At this time point, the striatal TH fiber volume was directly correlated with the number of remaining DA cells in the SN ( $p < 0.01$ ).

Using a selective antibody directed against CD11b (C3R), we studied activated microglia by morphological analysis and performed stereological quantification in the striatum (Fig. 2) and ventral midbrain (Fig. 3). In the COXIB group, a significant reduction in the number of activated microglia was seen in both striata (treatment effect  $F_{1,32} = 7$ ,  $p = 0.01$ ). This effect of selective COX-2 inhibition was more pronounced in the striatum at the 12-day time point after the toxin injection than at 21 days after 6-OHDA (Fig 2A,2B,2C). In addition to a significant increase in cell

density, in the vehicle group the predominant morphology of microglial cells was amoeboid (activated) as opposed to ramified (resting) (Fig 2B and 2D). In the ventral midbrain microglial cell density was significantly higher ipsilateral to the 6-OHDA injection in the vehicle group at 21 days. ANOVA revealed a significant effect of time ( $F_{1,24} = 809$ ,  $p < 0.001$ ), lesion side ( $F_{1,24} = 17$ ,  $p < 0.001$ ) and treatment ( $F_{1,24} = 11.6$ ,  $p < 0.01$ ) on microglial density (Fig 3E,3F). Astrocytes immuno-labeled with GFAP were also quantified in the striatum. There was a significant astrogliosis ipsilateral to the 6-OHDA injection ( $F_{1,28} = 28$ ,  $p < 0.001$ , Fig 4). This lesion effect decreased but was still noticeable at 21 days in the vehicle group. Interestingly, no effect of treatment ( $p = 0.9$ ) was observed for astrocyte density (GFAP+ cells/mm<sup>3</sup>) (Fig 4E,4F). These results show that celecoxib produced a selective reduction of local microglial reaction in response to the neurotoxin.

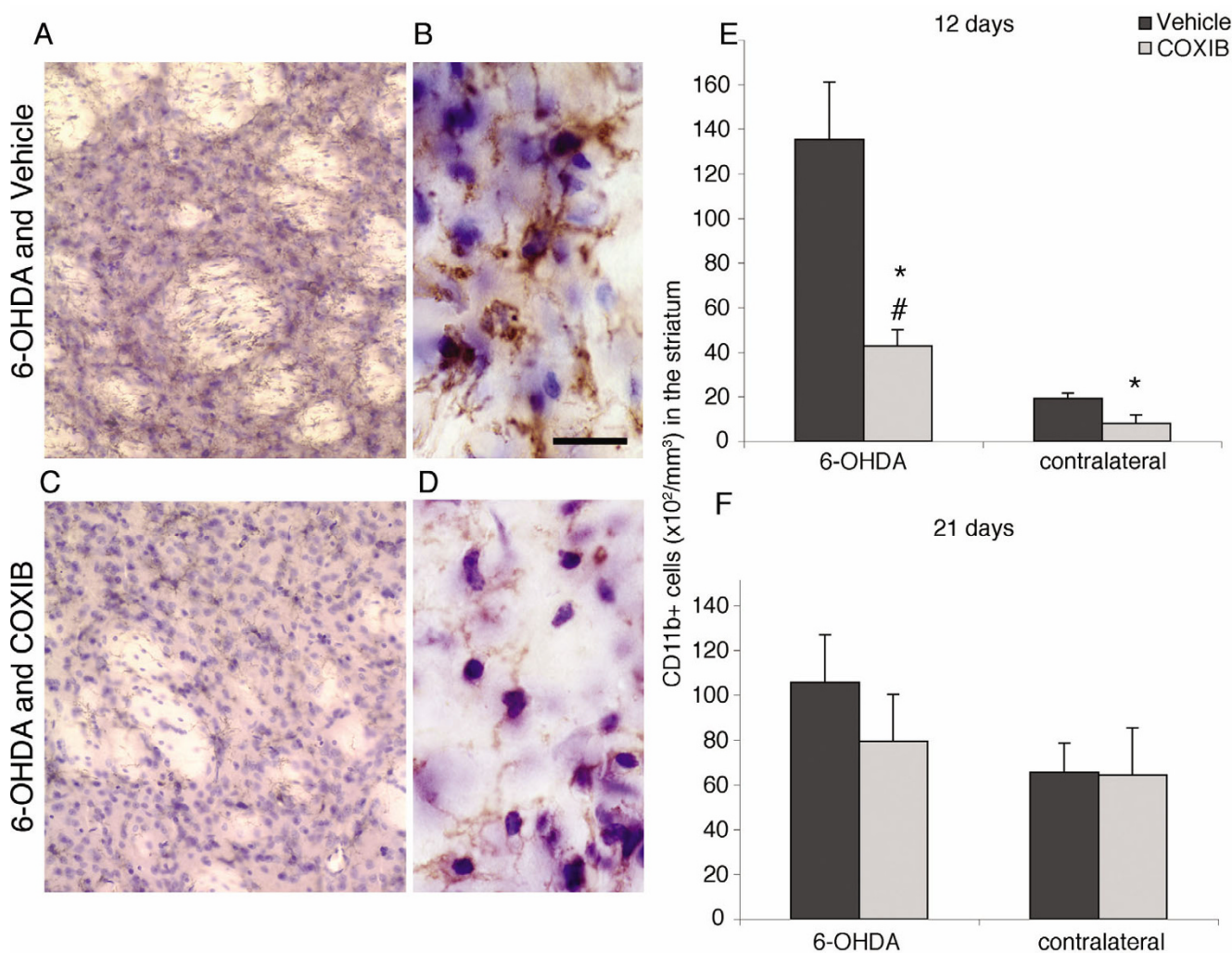
## Discussion

Regular intake of nonaspirin NSAIDs (and high dose of aspirin) has been reported to be associated to a 45% lower risk of PD in 2 large cohorts [22]. In this study we found that COX-2 inhibition by celecoxib decreased microglial activation and was associated with a prevention of the progressive degeneration seen in the 6-OHDA retrograde lesion model of PD. We used the striatal administration of 6-OHDA because neuronal damage and death, characteristically progress over weeks. This provides a close (although accelerated) model for the cascade of degenerative events that occurs in PD. Between weeks 2 and 3, DA cell loss progressed from 40 to 65 % in vehicle treated animals, as previously described for this model [26,27,29,38]. In contrast, we did not observe such a progression of DA neuronal cell loss in the COXIB group. It is worth noting that TH striatal fiber density was not correlated (more extensive) with DA cell numbers at 12 days, likely reflecting TH down-regulation in the acute stage of degeneration. Consistent with this explanation, there was a significant recovery of TH fibers in both groups at 21 days (Fig 1B,1C,1D) to levels that matched and corresponded to the number of DA neurons remaining in the substantia nigra. Based on this temporal pattern we propose that COX-2 inhibition protects a nigral neuronal cell population with reversible damage [15,39,40]. Such neurons are damaged and have reduced axonal TH expression at 2 weeks. Left to the natural evolution of the progressive degeneration half of these DA neurons will eventually die [26-29]. Our results show that COX-2 inhibition resulted in a complete protection of these damaged DA neurons. This effect can be dependent on specific intraneuronal effects of celecoxib and/or related to a classical anti-inflammatory mechanism, through microglial cell inhibition. Interestingly, the reduction of microglia activation by celecoxib was stronger at 12 days (Fig. 2E), while at this



**Figure 1**

A) Using micro-PET and selective radioactive tracers we measured *in vivo* the extent of dopamine terminal loss and inflammatory response 12 (top panel) and 21 (bottom panel) days after the 6-OHDA lesion. Color-coded images of <sup>11</sup>C-CFT ((2β-carbomethoxy-3β-(4-fluorophenyl) tropane, a dopamine transporter ligand) and <sup>11</sup>C-PK11195 (a peripheral-type benzodiazepine ligand that binds to microglia) in a representative animal of each group. As reported in our previous study [28] 6-OHDA injection resulted in a marked decrease of <sup>11</sup>C-CFT binding in the striatum and a parallel increase in <sup>11</sup>C PK-11195 binding in the control (vehicle) group. The increase in <sup>11</sup>C PK-11195 binding was absent in COXIB treated animals and in both groups at 21 days post lesion. B-C) Microphotographs of TH fiber density in the striatum in representative animals (same as shown in A). D) Volumetric analysis of fiber loss in the lesioned striatum showed a marked reduction at 12 days, that partially recovered at 21 days post-lesion (\*, p < 0.01). TH striatal volumes were significantly larger in COXIB treated than in the vehicle group (#, p < 0.01). E) At 12 days post-lesion, both treatment groups displayed a ~40% loss of TH positive cell bodies in the SN. The progressive loss of DA cell bodies between 12 and 21 days post-lesion in the vehicle treated rats was significant (\*p < 0.01) while there was no significant difference in DA cell bodies in the COXIB treated rats between 12 and 21 days. At 21 days the DA cell loss in the SN was significantly higher in vehicle treated animals (#, p < 0.05). Scale bar: 30 μm.



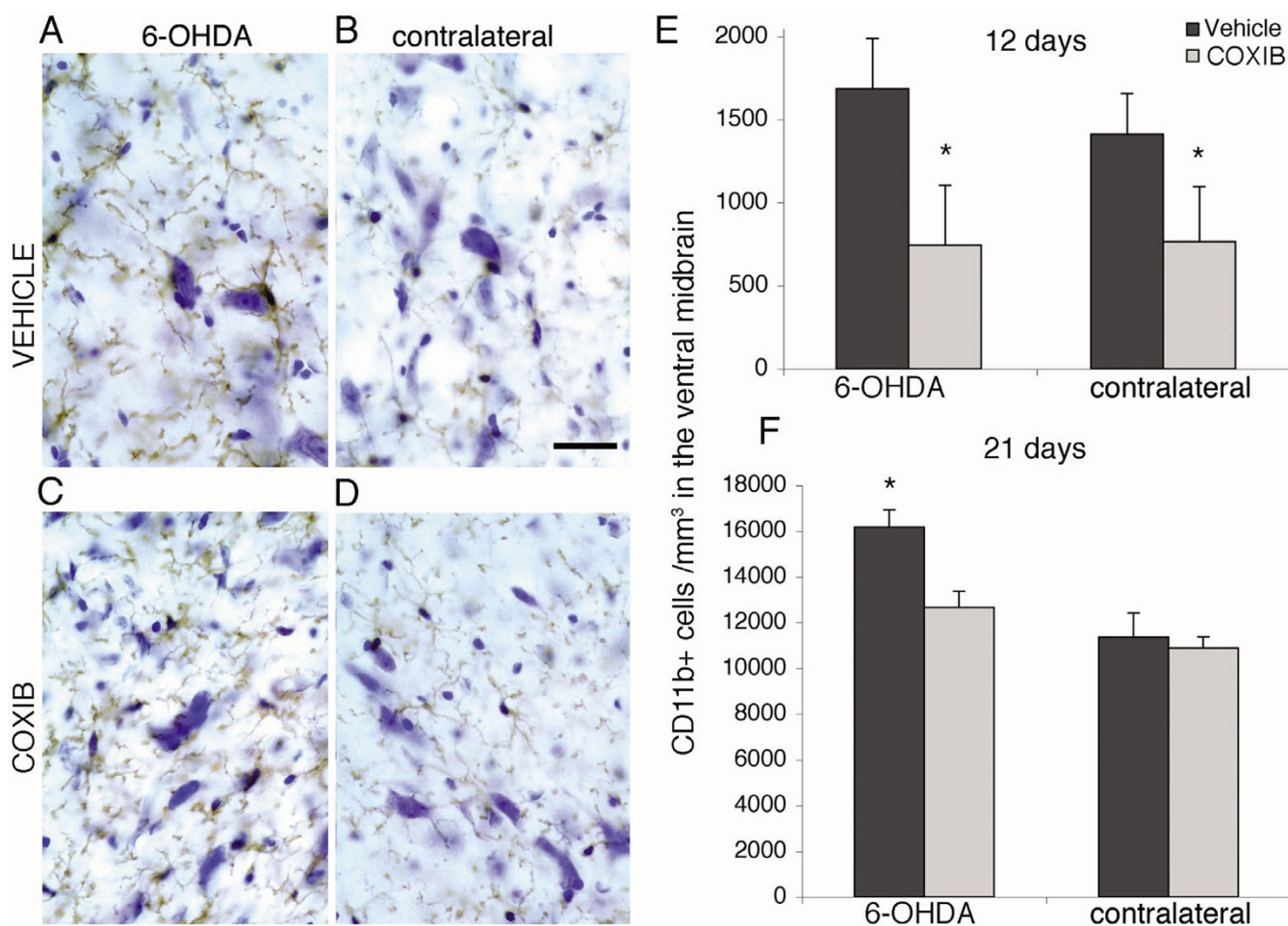
**Figure 2**

The microglial response to 6-OHDA injection was significantly attenuated in the striatum of COXIB treated animals. Photomicrographs of activated microglia immunohistochemistry in a representative striatal section of a vehicle (A, B) and a COXIB (C, D) treated animal 12 days after the injection of 6-OHDA. All images are ipsilateral to the injection side. E, F) Bar graphs showing the stereological quantification of activated microglia cell density at 12 (E) and 21 days (F). Microglial density was significantly reduced in the striatum of COXIB treated rats (treatment effect  $p < 0.05$ ) both in the lesioned and in the contralateral striata. However, the microglia response was not completely abolished in COXIB treated animals, as microglia density was significantly higher in the 6-OHDA injected striatum ( $p < 0.01$ ) and the density was higher in the lesioned/treated striatum than in the contralateral/untreated striatum ( $p < 0.05$ ). Scale bar: 100  $\mu\text{m}$  for A and C and 25  $\mu\text{m}$  for B and D.

time point there were no differences in DA markers between groups. The reduction in microglia was not accompanied by changes in astroglial reaction to the striatal injury (Fig. 4).

With infectious or tissue injury stimuli, including inflammatory or selective DA terminal lesions of the striatum, microglia can both proliferate and transform morphologically into reactive forms [28,41]. The reactive microglia's

amoeboid movement and activities in injured neural tissue include macrophage activity and presumed synaptic stripping along dendrites [42]. In the current PD model, microglial invasion and continued presence in the lesioned striatum and substantia nigra could contribute to long-term synaptic disconnection of the damaged DA terminal afferents. Such loss of normal neuron target interactions and trophic support can lead to DA neuronal vulnerability, atrophy or death [43,44]. In experimental *in*

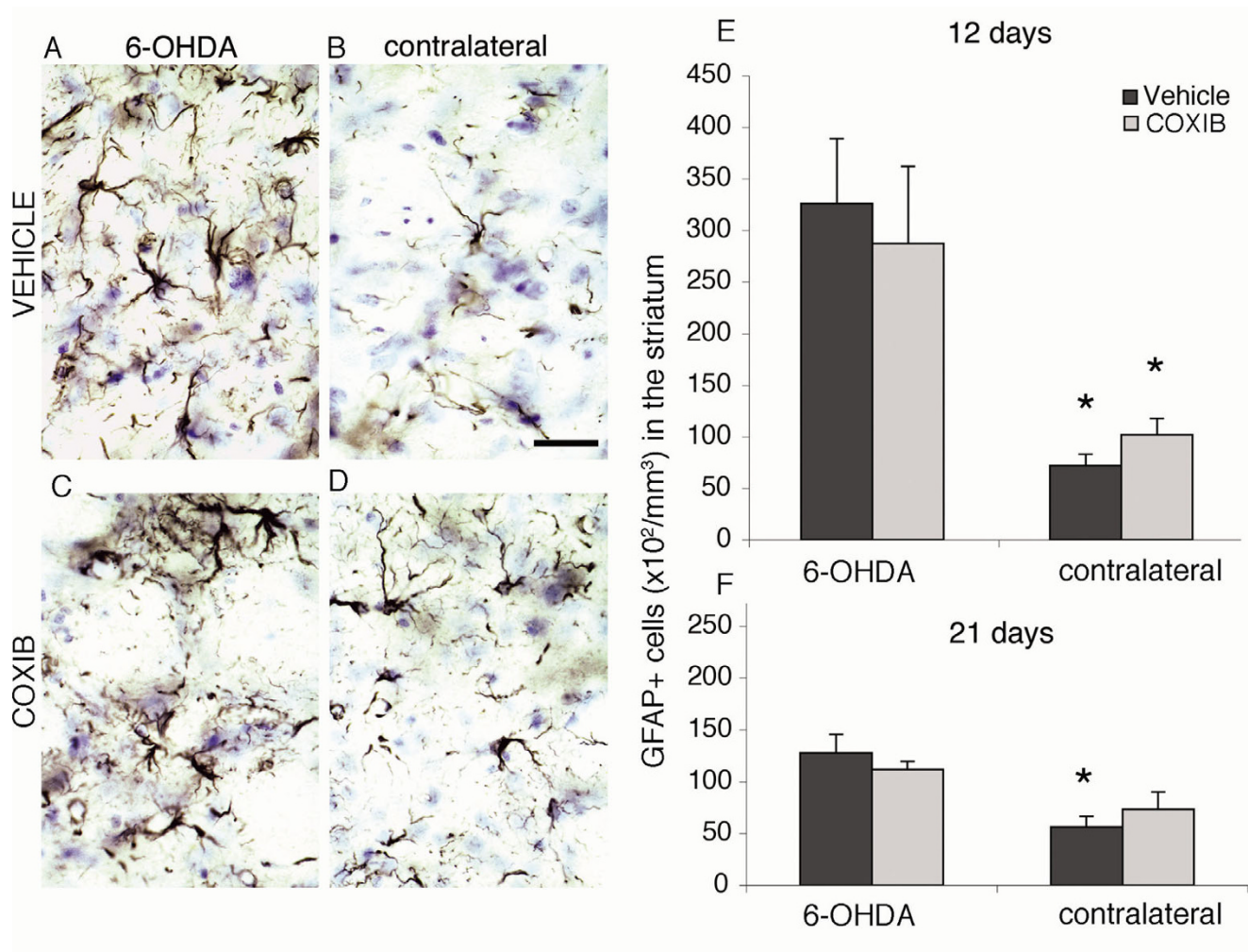


**Figure 3**

Microglial density in the ventral midbrain. Photomicrographs of activated microglia immunohistochemistry in a representative midbrain section of a vehicle (A, B) and a COXIB (C, D) treated animal 21 days after the injection of 6-OHDA. E, F) Bar graphs showing the stereological quantification of activated microglia cell density at 12 (E) and 21 days (F). Microglial density was significantly reduced in the ventral midbrain of COXIB treated rats (treatment effect  $F=6.28$ ,  $p < 0.05$ ) both in the lesioned and in the contralateral striata. Microglial density was higher at 21 days in all groups and was significantly higher in the vehicle group ipsilateral to the lesion ( $p < 0.05$ ). Scale bar: 25  $\mu\text{m}$ .

*in vivo* PD models with delayed DA neuronal death, various exogenous trophic factor support of DA neurons [29,43-46] or intracellular signalling related to neuroimmunophilins [30] can prevent long-term progressive DA degeneration to a similar degree to that seen by COX-2 inhibition in the present study. In chronic degenerative situations involving the striatum and midbrain and during marked fluctuations in neuronal ionic, metabolic and functional status, astroglia are thought to play a more homeostatic, trophic and protective role for DA neurons and terminals than microglia [47,48]. Our evidence clearly demonstrate that selective COX-2 inhibition did not reduce the typical astroglial response to injury in the

striatum, while to a large extent preventing expression of the morphologically activated microglial phenotype. In fact, COX-2 inhibition had by 3 weeks of treatment (compared to vehicle) caused a mild elevation of the number of reactive astrocytes in the striatum contralateral to the lesion (Fig. 4). In the period of progressive DA neuronal death between the 2<sup>nd</sup> and 3<sup>rd</sup> week after DA terminal injury by 6-OHDA, our data indicate that nigro-striatal DA terminals were restored in striatum, and a significant population of DA neurons were spared from cell death in the substantia nigra by COX-2 inhibition. These results point to an altered reactive astroglial to reactive microglial cell ratio by COX-2 inhibition that may provide insights



**Figure 4**

Astroglial response in the striatum. Photomicrographs of representative sections of the ipsilateral (A, B) and contralateral (C, D) striatum of an animal receiving COXIB at 21 days. E, F) Bar graphs showing the GFAP positive density in the striatum. E) Astroglial density was significantly higher in the lesioned striatum ( $P < 0.01$ ), with no significant differences between treatment groups. F). Scale bar: 25  $\mu\text{m}$ .

for how to create favorable conditions for prevention of progressive neurodegenerative cascades during and after neuronal injury similar to that seen in PD.

Microglial cells can also produce and release pro-inflammatory cytokines, in particular  $\text{TNF}\alpha$ , and cytotoxic molecules including ROS and NO [34] although such responses are non-specific to lesion type [41]. After 6-OHDA intra-striatal infusion, there is an acute increase in  $\text{TNF}\alpha$  in the striatum [49]. Pro-inflammatory cytokines IL-1 $\beta$  and  $\text{TNF}\alpha$  activate the p38MAPK cascade and NF $\kappa\text{B}$  translocation to the nucleus, resulting in transcriptional upregulation of COX-2.  $\text{TNF}\alpha$  activates COX-2 via the

JNK pathway [50] and induction of NF $\kappa\text{B}$  [51]. Importantly p38 MAPK stabilizes the mRNA of COX-2 and other pro-inflammatory factors [52,53]. Activated microglia cells release NO [54,55] and superoxide free radical [11]. DA neurons are particularly vulnerable to this type of inflammation induced oxidative stress as DA metabolism and DA autoxidation generate ROS [56]. Celecoxib (at low dose) [57,58] and other NSAIDs (and minocycline) inhibit p38MAPK leading to a decrease in COX-2 production, decreased mRNA stability and decreased  $\text{PGE}_2$  release. It is possible that celecoxib, by blocking COX-2 enzymatic activity and by inhibition of the p38MAPK pathway, constrained the inflammatory response induced



by striatal 6-OHDA thus limiting, to a certain extent, the progressive DA neuronal death. Similar protective effects of selective COX-2 inhibitors have been reported in excitotoxicity and ischemia models [7,59-61].

The benefit we observed can also stem directly from neuronal inhibition of COX-2, which is one of several enzymes capable of oxidizing DA to reactive DA quinone [62]. DA quinones can deplete the cells of antioxidants, inactivate enzymes and increase  $\alpha$ -synuclein protofibrils [56,63]. Induction of COX-2 results in an inflammatory cascade accompanied by formation of ROS. Recent work using the mouse MPTP model of PD suggests that an intraneuronal mechanism can be sufficient to achieve neuroprotection in that specific acute paradigm [64,65]. Therefore the reported effects could be attributed to a direct decrease of inflammatory mediators inside the neuron or to inhibition of release of proinflammatory and toxic factors from microglia [24,40]. The temporal relationship between glial activation and neurodegeneration suggests that microglial activation plays a key role in amplifying the toxic effect and thereby exacerbating DA cell loss, although it cannot be determined by these experiments whether microglia inhibition is absolutely required to achieve neuroprotection. Massive and prolonged microglial cell activation has been observed in aged mice exposed to MPTP, associated with a progressive loss of TH+ neurons [66]. Sugama et al. and our data strongly suggest that microglia activation prolongs an oxidative environment after the initial toxic insult, leading to the subsequent loss of neurons that have a reversible damage [39,40]. Specifically, in the retrograde 6-OHDA lesion paradigm that we used here, ~50% of the DA neurons with reversible damage will die between weeks 2 and 3 after the initial injury. In this study celecoxib treatment rescued this population. Therefore inhibition of COX-2 by celecoxib appears to be able, directly, and through inhibition of microglia activation to result in a reduction of DA cell degeneration.

The presence of activated microglia in the brain of PD patients [2] and after MPTP exposure both in humans [67] and monkeys [4] supports the existence of an ongoing inflammatory process that can contribute to the progression of the disease. The origin of neuroinflammation is unknown and is probably different for different individuals, being a common response to a variety of pathogenic insults. If indeed chronic neuroinflammation contributes to the progression of the degenerative process [15], anti-inflammatory drugs could prevent or slow down the disease, independently of the causative factors.

### Competing interests

None declared.

### List of abbreviations

- 6-OHDA: 6-hydroxydopamine
- ANOVA: analysis of variance
- BP: binding potential
- CFT: 2 $\beta$ -carbomethoxy-3 $\beta$ -(4-fluorophenyl) tropane
- COX: cyclooxygenase
- COX-2: cyclooxygenase type 2 isoform
- COXIB: COX inhibitor
- DA: dopamine
- DAT: dopamine transporters
- DMSO: dimethyl sulfoxide
- GFAP: glial fibrillary acidic protein
- HCl: hydrogen chloride
- HPLC: high performance liquid chromatography
- IL-1 $\beta$ : interleukin-1 beta
- LPS: lipopolysaccharide
- MAPK: mitogen-activated protein kinase
- MPTP: N-methyl 1,2,3,6 tetrahydropyridine
- NGS: normal goat serum
- NO: nitric oxide
- NSAID: nonsteroidal anti-inflammatory drugs
- OX42: CD11b
- PBS: phosphate buffered saline
- PD: Parkinson's disease
- PET: positron emission tomography
- PK-11195: N-sec-butyl-1-(2-chlorophenyl)-N-methylisoquinoline-3-carboxamide
- ROS: reactive oxygen species
- SN: substantia nigra

TH: tyrosine hydroxylase

TNF $\alpha$ : tumor necrosis factor alpha

### Authors' contributions

RSP participated in the design, surgical procedures, statistical analysis and manuscript preparation. AF participated in the surgeries and did all the treatments. OC carried out most of the histological and stereological procedures and analysis. MY carried out the HPLC and tracer synthesis procedures in the PET studies. ALB carried out and analyzed the PET studies. OI conceived the study and design analyzed the data and prepared the manuscript. All authors read, discussed and approved the final manuscript.

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