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Expression of Peroxisome Proliferator-Activated Receptor- γ in the substantia nigra of hemiparkinsonian nonhuman primates

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Abstract

OBJECTIVE—To characterize the distribution of Peroxisome proliferator-activated receptor-gamma (PPAR- γ) in the substantia nigra of normal and MPTP-treated hemiparkinsonian monkeys, in order to validate PPAR- γ as a target for neuroprotection.

METHODS—Immunohistochemical analysis of PPAR- γ expression was performed in the substantia nigra and other select brain regions of fifteen rhesus monkeys including controls ($n = 3$), hemiparkinsonian necropsied after 3 ($n = 5$) or 12 months ($n = 3$) after MPTP, and animals who received MPTP + 5 mg/kg of the PPAR- γ agonist pioglitazone ($n = 4$).

RESULTS—PPAR- γ expression was prominent in the subthalamic nucleus, oculomotor nucleus, ventral tegmental nucleus and to a lesser extent in the putamen; three or twelve months after MPTP, only the lesioned putamen had increased PPAR- γ . Stereological cell quantification in normal subjects showed that approximately 50% of neurons in the substantia nigra pars compacta (SNpc) expressed PPAR- γ . After MPTP there was a significant loss of dopaminergic (DA) neurons in the ipsilateral SNpc and the actual number of TH and PPAR- γ cells were not significantly different at either time point. Pioglitazone dosing protected TH positive neurons, closely matching the number of PPAR- γ expressing cells in the ipsilateral SNpc. Nigral immunofluorescence verified colocalization of PPAR- γ in neurons.

DISCUSSION—These results demonstrate that PPAR- γ is expressed in the SNpc and putamen of nonhuman primates and, that the DA nigral neurons expressing PPAR- γ are more likely to survive neurotoxin challenge after ligand activation by pioglitazone, therefore providing neuroanatomical validation for the use of PPAR- γ agonists in PD.

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Keywords

Parkinson's disease; substantia nigra; inflammation; basal ganglia; MPTP; non human primates; pioglitazone; PPAR- γ ; TH

Introduction

Peroxisome proliferator-activated receptors (PPARs) are part of the nuclear receptor super family and are ligand-dependent transcription factors¹. There are three PPARs isoforms: α , β/δ and γ , with splicing variants reported for all three subtypes^{2,3}. PPAR- γ in particular has received a great deal of attention by scientists and clinicians for its ability to be a “molecular sensor” in the body, binding to several types of molecules involved in metabolism¹.

The synthetic PPAR- γ agonists pioglitazone and rosiglitazone have shown anti-inflammatory and anti-oxidative properties, suggesting that PPAR- γ may be targeted to induce neuroprotection^{4,5}. The administration of either of these agonists to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mice and nonhuman primate (NHP) models of Parkinson's disease (PD) prevented dopaminergic nigral cell death, decreasing microglial response and nitric oxide-mediated toxicity^{6–9}. Both drugs induced neuroprotection in 6-hydroxydopamine (6-OHDA) parkinsonian rats with unilateral dopaminergic nigral cell loss¹⁰. The PPAR- γ agonists were also neuroprotective in preclinical models of ischemic stroke¹¹, spinal cord injury¹², Amyotrophic Lateral Sclerosis (ALS; ¹³), Alzheimer's Disease (AD; ¹⁴), and Multiple Sclerosis (MS; ¹⁵). The positive reports prompted a number of clinical trials for AD¹⁶, ALS¹⁷ and MS¹⁸ that presented different outcomes. Currently, a trial for early PD is ongoing¹⁹.

The increasing awareness of the potential of PPAR- γ activation to treat CNS disorders has generated a growing urgency to elucidate its receptor distribution in order to confirm therapeutic targeting and further understand mechanisms of action. While rodent studies have confirmed and mapped intracerebral PPAR- γ expression²⁰, information regarding the anatomical and cellular distribution of these receptors in NHP is currently not available. Their characterization will be a valuable tool for clinical translation.

Here we report our findings during the characterization of PPAR- γ expression in NHPs. Aiming to evaluate target validation for PD, we focused our analysis in select regions of the basal ganglia of normal and parkinsonian NHPs. We tested the hypothesis that PPAR- γ expression in the NHP substantia nigra (SN) is responsive to MPTP and the PPAR- γ agonist pioglitazone and examined whether changes subside over time.

Materials and Methods

Ethics Statement

The present study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (7th edition, 1996) in an AAALAC accredited facility (Wisconsin National Primate Research Center, University of Wisconsin - Madison). The experimental protocols of the projects

from which the tissue was obtained were approved by the Institutional Animal Care and Use Committees at the University of Wisconsin - Madison (permit no. G00492) and at the University of Illinois - Chicago (permit no. 00-073). All efforts were made to minimize the number of animals used and to ameliorate any distress.

Animals

Brain coronal sections from 15 rhesus monkeys were utilized for this study (*Macaca mulatta*, male, 5–7 yrs old). The animals, from which the tissue was obtained, were individually housed in Group 3 or Group 4 enclosures (cage floor area 4.3 ft.2 or 6.0 ft.2 per animal, height 30 or 32 in.) in accordance with the Animal Welfare Act and its regulations and the 7th edition of the Guide for the Care and Use of Laboratory Animals (1996) with a 12-hour light/dark cycle. Throughout the study, the animals were monitored twice daily by an animal research technician or veterinary technician for evidence of disease or injury (e.g., inappetence, dehydration, diarrhea, depression, lethargy, trauma, etc.) and body weight was monitored to ensure animals remained in properly sized cages. Animals were fed commercial nonhuman primate chow (2050 Teklad Global 20% Protein Primate Diet, Harlan Laboratories, Madison, WI) twice daily, supplemented with fruits or vegetables and a variety of forage items and received *ad libitum* water. Nonhuman primate chow soaked in a protein-enriched drink (Ensure®, Abbott Laboratories, Abbott Park, IL) was offered to stimulate appetite as needed.

The normal brain sections (n = 3) were obtained from the tissue bank at Wisconsin National Primate Center (WNPRC). Hemiparkinsonian brain sections were from two previously published studies. From our previous pioglitazone study⁹ we used tissue of monkeys that received MPTP and 24 hrs later, daily oral dosings of placebo (n = 5) or 5 mg/kg of pioglitazone (n = 4) and were necropsied after 3 months, as after 3 months there is significant degeneration of striatal terminals nigral neurons²¹. From an earlier study²² we used tissue of monkeys that received MPTP and subthalamic nucleus injections of adenoassociated viral vector 2 (AAV2) encoding for the marker gene green fluorescent protein (GFP) (n = 3) and were necropsied 12 months post neurotoxin in order to assess long term effects.

To induce parkinsonism in the aforementioned studies^{9,22}, monkeys received a unilateral intracarotid artery injection of 3 mg of MPTP-HCl (Sigma, St. Louis, MO) in 20 ml of saline (rate: 1.33 ml/min) in sterile surgical conditions under isofluorane anesthesia as previously described. Evaluation of the animals' parkinsonian state was done using a previously validated clinical rating scale⁹. The scale ranges from 0 to 32, with a score of 0 corresponding to normal behavior and 32 to extreme severe parkinsonian symptoms. A score of 9–13 points correspond to a stable hemiparkinsonian syndrome.

Necropsy and Tissue preparation

All animals were anesthetized with sodium pentobarbital (25 mg/kg iv) and transcardially perfused with heparinized saline, followed by 4% paraformaldehyde (PFA). All brains were postfixed in 4% PFA for 12–72 hours and cryoprotected by immersion in a graded (10–40%) sucrose/0.1 M phosphate buffered saline (PBS, pH 7.2) solution. The tissue was cut

frozen (40 μ m section thickness) on a sliding knife microtome. All sections were stored in a cryoprotectant solution until processing.

Immunohistochemistry

Brain coronal sections were stained with Nissl or by immunohistochemical methods according to our previously published protocols⁹. Briefly, endogenous peroxidase activity was removed with a 20-minute incubation in 0.1 M sodium periodate. After 3 $\times$ 10-minute washes in PBS plus 0.05% Triton-X (dilution media) background staining was blocked with a 1 hour incubation in a Tris buffered saline solution containing 3% normal horse serum, 2% bovine serum albumin, and 0.05% Triton X-100. The sections were then incubated with a primary antibody [mouse monoclonal anti-PPAR- γ 1:500 (MAB3872, Millipore, Billerica, MA) or mouse anti-TH at 1:20,000 (22941, Immunostar, Hudson, WI)] overnight at room temperature. Sections were then incubated for 1 hour in biotinylated secondary antibodies at 1:200 (horse anti-mouse BA-2000, Vector Laboratories, Burlingame, CA). After 12 $\times$ 10 minute washes in dilution media, the sections were placed in the avidin biotin (ABC, "Elite" kit, Vector Laboratories) substrate (1:1,000) for 75 minutes. Sections were then washed in a 0.1 M imidazole/1.0 M acetate buffer, pH 7.4, and then reacted in a chromagen solution containing 0.05% 3,3'-diaminobenzidine (DAB), and 0.05% H₂O₂. Nickel sulfate was added to the DAB chromagen reaction for PPAR- γ . Negative controls were performed in parallel by omitting the primary antibodies in the immunostaining procedures.

Immunofluorescence

Immunofluorescence to characterize PPAR- γ expression in cell type was performed in coronal brain sections according to our previously published protocols²³ with sequential day staining. After 6 $\times$ 10 minute washes in PBS nonspecific binding staining was blocked with 2 hrs incubation at room temperature in a blocking buffer containing dilution media with 5% normal serum, and 2% bovine serum albumin. To assess colocalization of PPAR- γ in neurons and microglia, the tissue was then incubated with the first primary antibody overnight at 4°C (rabbit anti-PPAR- γ 1:50, AB19481, Abcam, Cambridge MA). Tissues were then rinsed and incubated in PBS, secondary antibody-antigen reaction containing Alexa Fluor 594-conjugated donkey anti-rabbit antibody (1:1000; A21207, Invitrogen, Grand Island, NY) for 2 hrs at room temperature, rinsed and re-blocked for 2 hrs at room temperature and incubated overnight at 4°C in a second primary antibody (anti-human leukocyte antigen-DR clone LN3 (HLA-DR; 1:50, 693031, MP Biomedical, Santa Ana, CA). Sections were then rinsed again and incubated in the secondary antibody Alexa Fluor 488-conjugated donkey anti-mouse (1:1000; A21202, Invitrogen) for 2 hrs at room temperature. Subsequently, tissue sections were then rinsed again in PBS and incubated in fluorescent nissl for 1 hr at room temperature (1:500, NeuroTrace 21479, Invitrogen).

To assess colocalization of PPAR- γ and TH, after washing and blocking the sections were then incubated with the first primary antibody overnight at 4°C (anti-PPAR- γ 1:100, MAB3872, Millipore, Billerica, MA). Tissues were then rinsed and incubated in PBS, secondary antibody-antigen reaction containing Alexa Fluor 488-conjugated donkey anti-mouse antibody (1:1000; A21202, Invitrogen) for 2 hrs at room temperature, rinsed and re-

blocked for 2 hrs at room temperature and incubated overnight at 4°C in a second primary antibody (rabbit anti-TH 1:1000; P401011-0, Pel-Freez, Rogers, AR).

Sections were then rinsed again and incubated in the appropriate secondary antibody Alexa Fluor 594-conjugated donkey anti-rabbit for TH (1:1000; A21207, Invitrogen). Sections were also counterstained with DAPI as a nuclei marker. Negative controls were obtained by omitting the primary antibodies in the immunostaining procedures.

Semi-quantitative evaluation of PPAR- γ expression

PPAR- γ expression was assessed in four equally spaced coronal brain sections as well as two equally spaced sections at the level of the dorsal putamen (Bregma -0.675- 0.765 mm). Evaluated areas included the subthalamic nucleus (STN), red nucleus, ventral tegmental area (VTA), oculomotor nucleus and dorsal putamen. The nuclei were identified using a rhesus monkey stereotaxic atlas as reference²⁴. Immunostaining was scored using a previously validated rating scale²⁰ from 0 to 3, with 0 = none, 1 = weak, 2 = moderate, and 3 = strong, intense staining.

Stereological nigral cell quantification

The total number of TH and PPAR- γ positive cells in the right and left SN was calculated using unbiased stereological cell-counting methods described previously⁹. Six coronal brain sections were used from each animal. The optical dissector system consisted of a computer assisted image analysis system including a Zeiss Axioplan 2 imaging photomicroscope (Carl Zeiss, Inc) hard-coupled to a MAC5000 high precision computer-controlled x-y-z motorized stage, and a MicroFire CX9000 camera (Optronics, Goleta, CA). Neuronal counts were performed using Stereo Investigator Version 7.5 (MicroBrightField, Williston, VT). The SN was outlined under a low magnification (2.5 $\times$). Nigral sections were demarcated by the caudal edges of the mammillary bodies (rostrally) and the decussation of the superior cerebellar peduncle (caudally). Instruments were calibrated prior to each series of measurements. The number of cells was determined in six sections spaced 480 μ m apart in a series of coronal sections measuring 40 μ m thickness. The grid size used was $x = 600 \mu$ m and $y = 360 \mu$ m and the counting frame size was 150 μ m by 90 μ m. The total number of cells within the counting frame was counted using a 63x oil immersion objective with a 1.4 numerical aperture. The section thickness was empirically determined by first bringing the top of the section into focus and then stepping through the z-axis in 1- μ m increments. Cell counts were made according to stereological principles, and the z-plane was lowered at 1–2- μ m intervals. Care was taken to ensure that the bottom and top guard zones were never included in the analysis. The total number of cells was calculated by using the following formula: $N = \sum Q^- \cdot t/h \cdot 1/asf \cdot 1/ssf$. Gundersen's coefficients of error (CE) was determined to estimate the accuracy of the optical fractionator results. A CE of < 0.1 was used as the cutoff to ensure accurate results.

Statistical Analysis

All data was collected and analyzed by an investigator blind to treatment groups. A Wilcoxon Signed Rank Test was used to evaluate differences in PPAR- γ expression and

stereological cell quantification of PPAR- γ and TH between brain hemispheres. Semi-quantitative ratings were compared between groups using a Kruskal-Wallis non-parametric test with a Bonferroni correction for pairwise comparisons. Differences between treatment groups when examining stereological cell counts were evaluated with analysis of variance (ANOVA) followed by LSD *post hoc* analysis where appropriate. A paired T-test was used to assess differences within treatment groups between immunohistochemical markers and a Pearson correlation was applied to test associations. $p < 0.05$ was considered statistically significant. All statistics were performed using SPSS version 18.0 software (SPSS, Chicago, IL).

Results

Unilateral MPTP intracarotid artery dosing induced a hemiparkinsonian syndrome and reduced TH-ir nigral cell counts

Antemortem neurological evaluation of the animals using a clinical rating scale showed the presence of similar hemiparkinsonian symptoms in all MPTP-treated animals (see Table 1 for individual clinical rating scores).

To assess the extent of dopaminergic nigral cell loss after MPTP, stereological quantification of TH immunostained tissue was performed in all monkeys (Table 1). In normal animals, the number of TH positive neurons was in range with previous studies and no significant differences were found between the left and right side (Wilcoxon signed rank test $p = 0.109$). The MPTP-treated monkeys necropsied at 3 or 12 months post neurotoxin showed a similar (81% and 84%) and significant (Wilcoxon signed rank test $p = 0.043$ and $p = 0.003$ respectively) unilateral loss of TH-immunoreactive cells on the side ipsilateral to the lesion compared to the contralateral side. Comparison between normal controls and MPTP animals showed no difference in nigral cell counts in the side contralateral to the lesion (ANOVA $p = 0.626$), while on the ipsilateral side, they were significantly decreased (ANOVA $p < 0.001$; LSD *post hoc* $p < 0.001$) at 3 (82%) and 12 months (84%).

Stereological cell quantification of Nissl staining within the substantia nigra confirmed cell loss on the side ipsilateral ($33,974 \pm 3,275$) to MPTP administration compared to the contralateral side ($159,708 \pm 10,940$) at 3 months post-MPTP (Wilcoxon signed rank test, $p < 0.001$)⁹.

PPAR- γ was expressed in the monkey nigra and putamen nuclei and was affected by MPTP intoxication

Semiquantitative analysis in the subthalamic, red, and the oculomotor nuclei showed robust PPAR- γ -immunoreactivity (ir), while there was less PPAR- γ -ir in the VTA, all of which were not affected by treatment (Figure 1, Table 2). AAV2-GFP control treatment in the 12 months post MPTP monkeys did not affect PPAR- γ expression in the subthalamic nucleus or any other region evaluated. Within the dorsal putamen, PPAR- γ was expressed primarily in cells and to a lesser extent in fibers. PPAR- γ -ir within putamen nuclei increased in the side ipsilateral to the neurotoxin challenge (Kruskal-Wallis $p = 0.040$) at 3 (Bonferroni correction $p = 0.027$) and 12 months post-MPTP ($p = 0.023$) compared to controls. On the contralateral putamen PPAR- γ expression also increased (Kruskal-Wallis $p = 0.032$) at 3

months (Pairwise comparisons Bonferroni correction $p = 0.01$) and a trend was observed at 12 months ($p = 0.050$). PPAR- γ was expressed mostly in cells with neuronal morphology, independent of the region or treatment condition.

Qualitative observation of nigral tissue showed robust expression of PPAR- γ in the SNpc of normal monkeys, expressed largely in cells with neuronal morphology, with strong immunostaining in the ventral SNpc, and moderate expression in the dorsal SNpc. The presence of PPAR- γ in neurons but not in microglia was confirmed in the nigra by confocal microscopy immunofluorescence for PPAR- γ , fluorescent nissl (a neuronal marker), and HLA-DR (a marker of microglia; Figure 2). The substantia nigra *pars reticulata* (SNpr) also expressed PPAR- γ , albeit to a lesser degree. To assess whether the number of PPAR- γ positive cells was affected by treatment, we performed blind stereological nigral cell counts in all subjects in the SNpc. Normal monkeys had a similar number of PPAR- γ -ir cells in the left and right SN (Table 1; Wilcoxon signed rank test $p = 0.295$). In contrast, MPTP-treated animals presented an asymmetrical loss of staining in the lesioned SN compared to the contralateral one at 3 (65%) and 12 (66%) months post-neurotoxin (Wilcoxon signed rank test $p = 0.043$ and $P = 0.013$, respectively) (Figures 3 and 4).

Comparison between normal controls and MPTP-treated animals showed that, similar to TH-ir cell counts in the ipsilateral SN, there was a significant difference in the number of PPAR- γ -ir nigral cells between treatment groups (ANOVA $p < 0.001$). The number of PPAR- γ -ir nigral cells was significantly less at 3 (57%) and 12 months (58%) post MPTP (ANOVA LSD *post hoc* $p < 0.001$; $p < 0.001$, respectively). Interestingly, on the contralateral side, significant differences were also found (ANOVA $p < 0.001$), whereby animals treated with MPTP displayed significantly more PPAR- γ -ir nigral cells (+24% and 28% for 3 and 12 months; ANOVA LSD *post hoc* $p = 0.011$; $p = 0.008$, respectively) compared to controls.

PPAR- γ agonist pioglitazone prevented the hemiparkinsonian syndrome and modulated PPAR- γ expression in the SN

Oral daily dosing of 5 mg/kg of pioglitazone starting 24 hrs post-MPTP and continuing for 3 months was able to prevent the establishment of hemiparkinsonian signs, as well as TH-ir nigral cell loss (Table 1) as previously described⁹. In the SN contralateral to MPTP dosing TH cell count was significantly higher (ANOVA LSD *post hoc* $p = 0.002$) compared to 3 mo post-MPTP treated controls. Pioglitazone treated monkeys also had higher Nissl nigral cell counts on the side ipsilateral to MPTP ($57,297 \pm 7,033$) compared to 3 mo post-MPTP controls ($33,974 \pm 3,275$; ANOVA LSD *post hoc* $p = 0.017$). PPAR- γ expression in the dorsal putamen of pioglitazone-treated monkeys was not significantly different compared to normal controls (Kruskal-Wallis Bonferroni pairwise comparison ipsilateral $p = 0.52$; contralateral $p = 0.389$) or 3 months post-MPTP controls (Kruskal-Wallis Bonferroni pairwise comparison ipsilateral, $p = 0.191$, contralateral, $p = 0.382$).

In the SN the number of PPAR- γ -ir cells in the ipsilateral side was significantly less in pioglitazone-treated animals compared to normal controls (ANOVA LSD *post hoc* $p = 0.002$), but more when compared to 3 and 12 months post-MPTP controls, although due to individual variability it did not reach statistical significance (ANOVA LSD *post hoc* $p =$

0.052, $p = 0.090$, respectively; Table 1; Figure 3). Interestingly, like TH cell counts on the contralateral nigra, there were more PPAR- γ -ir cells in pioglitazone-treated monkeys compared to normal controls, 3 and 12 months post-MPTP subjects (ANOVA LSD *post hoc* $p < 0.001$; Figure 4).

Dopaminergic nigral cell population that survived MPTP expressed PPAR- γ

As the number of TH-ir and PPAR- γ -ir nigral cells was affected by MPTP treatment, we statistically compared cell populations within each treatment group to assess the extent of change and how it related (Table 1; Figure 5). In normal animals there were significantly fewer (approximately 50% less) PPAR- γ -ir nigral cells compared to TH-ir nigral cells for the ipsilateral (Paired T-test $p = 0.006$; Figure 5) and contralateral side (Paired T-test $p = 0.011$). After MPTP, the number of both PPAR- γ and TH positive cells decreased in the ipsilateral SN and, interestingly, the cell numbers were not significantly different 3 or 12 months post-neurotoxin (Paired T-test $p = 0.287$; $p = 0.145$, respectively). This was also observed in the pioglitazone + MPTP-treated monkeys in which the number of preserved TH-ir neurons closely matched the number of PPAR- γ -ir cells on the ipsilateral SN (Paired T-test $p = 0.270$). Similar to normal controls, the SN contralateral to MPTP at 3 and 12 months had fewer nigral PPAR- γ positive neurons compared to TH-ir cells on the same side (Paired T-test $p = 0.005$; $p = 0.044$, respectively). Pioglitazone + MPTP-treated monkeys also displayed fewer PPAR- γ vs. TH on the contralateral SN (Paired T-test $p = 0.003$). Pearson correlations further suggested a positive association between surviving the number of TH-ir neurons and PPAR- γ -ir cells in the MPTP treated SN, but not the in contralateral one for all treatment groups (Pearson correlation, right side- $r^2 = 0.891$, $p < 0.001$; left side- $r^2 = 0.300$, $p = 0.277$; Figure 5). The presence of PPAR- γ in dopaminergic neurons was confirmed by colocalization immunofluorescence of PPAR- γ and TH on nigral coronal sections (Figure 6). In the contralateral SNpc there were numerous TH positive neurons and in every field of view, approximately half of the cells colocalized with PPAR- γ . On the SNpc ipsilateral to MPTP treatment, both PPAR- γ and TH expression was decreased compared to the contralateral side, yet most cells colocalized the markers (Figure 6).

Discussion

These results demonstrate that PPAR- γ is expressed in the substantia nigra and putamen of nonhuman primates and, that the dopaminergic nigral neurons expressing PPAR- γ are more likely to survive neurotoxin challenge after ligand activation by pioglitazone. To the best of our knowledge, this is the first study to examine PPAR- γ expression in the CNS of NHP and its response to different treatments.

PPAR- γ expression in select regions of the basal ganglia seems to present some differences between rodents and rhesus monkeys. Using immunohistochemical methods, Moreno and colleagues²⁰ reported in normal, unaffected rats strong, nuclear PPAR- γ expression within the caudate/putamen and lesser in the SN, while in unaffected NHP brains, PPAR- γ staining intensity was stronger in the nigra than the putamen. In the oculomotor nucleus, the authors reported weak staining and none in the VTA, while in monkeys we observed strong staining in the first and, moderate in the latter. In the red nucleus, we found similar intensity in NHP

PPAR- γ to the adult rat²⁰. It should be noted that analysis of PPAR distribution in rats utilizing *in situ* hybridization, found no PPAR- γ reactivity in the red nucleus²⁵ and using RT-qPCR mRNA analysis in rat cell cultures, PPAR- γ was expressed at very low levels in all brain regions analyzed, including cerebellum, cortex, medulla oblongata and midbrain²⁶. In mice, low to undetectable levels of PPAR- γ mRNA expression were reported in brain areas such as the striatum and thalamus²⁷, and *in situ* hybridization detected no PPAR- γ expression within the hypothalamus and hippocampus. Taken together, these findings suggest there are species-specific differences with regard to PPAR- γ expression in the CNS.

It can be argued that differences in the methodology used for characterizing PPAR- γ expression in the CNS of rodents compared to our methods in NHPs may account for discrepancies in reported PPAR- γ distribution. Moreno and colleagues' immunohistochemistry methods in the adult rat CNS²⁰ included a tyramide amplification step in their DAB chromagen development. We did not use this step in our NHP tissue, as during preliminary optimization studies we found no difference in PPAR- γ immunoreactivity with its addition and, furthermore, recent reports suggest that endogenous biotin can increase false-positives during tyramide amplification²⁸. As we mentioned above, Braissant and colleagues²⁵ as well as Gofflot et al.²⁷ used *in situ* hybridization to characterize PPAR receptors in the adult rat CNS, while Cullingford et al.²⁶ used RT-qPCR to quantify PPAR- γ mRNA expression in CNS rat cultures. Both methods can detect mRNA expression of a transcript of interest, which may be different than protein expression. Yet, these methodological differences do not explain the lack of expression in the oculomotor nucleus and VTA described in rodents, nor differential staining patterns in the putamen and SN, but rather support a species difference in PPAR- γ expression in the CNS.

MPTP intoxication had different effects on PPAR- γ expression in the putamen vs. the nigra ipsilateral to the side of injection, as only the putamen had increased PPAR- γ immunoreactivity 3 or 12 months after administration of the neurotoxin. It can be argued that if we had sampled the brains in the first days post MPTP (before nigral cell death), we would have found PPAR- γ upregulation in dopaminergic nigral neurons. Interestingly, we have observed in rodents that 3 days post-MPTP mice have striatal but not nigral upregulation of PPAR- γ mRNA (quantified by RT-qPCR²⁹). Our monkey and mice findings conflict with a study by Breidert et al.⁶ who reported no significant differences in PPAR- γ protein expression in the striatum of mice 2, 5, or 8 days post-MPTP as measured by western blot. Species and methodological differences could account for discrepancies between the Breidert study and our current monkey study. It should be noted that PPAR- γ expression was upregulated in right and left putamen, suggesting a bilateral response to MPTP, which was not affected by pioglitazone. Breidert and colleagues⁶ found a similar effect of pioglitazone in MPTP-treated mice as measured by western blot.

Nigral cell counts and colabeling showed that after MPTP, more TH-ir than PPAR- γ -ir cells were lost in the nigra ipsilateral to the lesion and that when pioglitazone prevented dopaminergic nigral cell loss, the number of surviving TH-ir and PPAR- γ -ir nigral cells became a closer match. These results suggest that DA nigral cells expressing PPAR- γ are more resilient to MPTP and that ligand activation by pioglitazone facilitates PPAR- γ neuroprotective properties.

PPAR- γ -ir was increased in the contralateral SN of MPTP-treated animals, as well as MPTP and pioglitazone-treated monkeys compared to normal controls. Intracarotid artery infusion of MPTP is known to induce a unilateral lesion, but some effects on the contralateral side can occur³⁰. It is possible that a small amount of MPTP could have reached the contralateral side to the lesion through circulation by the circle of Willis^{21,30}. Although this was not enough to induce nigrostriatal degeneration, it may have sufficed to trigger an anti-inflammatory response, which could create an upregulation in PPAR- γ due to the plasticity of the nigra (and the putamen). PPAR- γ -ir nigral cell counts in MPTP + pioglitazone-treated monkeys were increased compared to MPTP alone but not significantly, yet it is noteworthy that in the contralateral SNpc TH cell number was significantly increased after MPTP + pioglitazone, suggesting that pioglitazone affects the expression of both proteins in nigral neurons.

Our results with PPAR- γ -ir and TH-ir nigral cell counts with and without MPTP, as well as a lack of colabeling of PPAR- γ and HLA-DR are in contrast with a study by Carta and colleagues³¹, where MPTP-treated mice displayed an increase in PPAR- γ -ir, as well as an increase in the ratio of PPAR- γ /TH-ir and PPAR- γ /cd11b-ir in the SN compared to vehicle-treated animals. Differences in species and experimental design may account for the disparate results. The authors utilized a chronic MPTP model with probenecid and mice were sacrificed 3 days post-MPTP. They reported a TH-ir nigral cell loss of 43%, while the Nissl cell number decreased 34%, suggesting their paradigm induced partial downregulation of the TH phenotype, instead of cell death, which could account for greater PPAR- γ in MPTP-treated mice. In comparison, in our model we have demonstrated through TH-ir and Nissl nigral stereological cell quantification that 3 months post-MPTP, the DA cell death is close to 80% compared to controls, presenting greater cell loss, and no downregulation of the TH phenotype⁹. It is possible that, as our animals were necropsied several months post-MPTP, we missed a time point to document an acute increase in PPAR- γ expression in neurons and glia after intoxication. As we mentioned before, we have observed upregulation in mRNA PPAR- γ expression 3 days post-MPTP, as well as an acute upregulation in cd11b in mice treated with a sub-chronic MPTP administration paradigm²⁹. It should be also noted that no differences in PPAR- γ expression were found between 3 and 12 months post MPTP, likely because the nigral lesion in the unilateral parkinsonian model stabilizes 4 weeks post-MPTP³² and persists for many years³³.

In conclusion, our finding of PPAR- γ expression in nigral and putaminal nuclei validates anatomical targeting of PPAR- γ agonists for the treatment of Parkinson's disease. The PPAR- γ response to neurotoxin challenge and agonist dosing implies a relationship between nigral neurodegeneration, PPAR- γ expression and ligand activation.

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Figure 1.

Microphotographs of coronal PPAR- γ immunostained sections within the Subthalamic Nucleus (STN), Red Nucleus, Oculomotor Nucleus, Ventral Tegmental Area (VTA), and Dorsal Putamen from normal controls, 3, 12 months post-MPTP, and pioglitazone + MPTP-treated monkeys. All MPTP-treated animals (3 and 12 mo post-MPTP) had significantly higher PPAR- γ expression in the dorsal putamen compared to normal controls. Scale bar: High magnification = 100 μ m; low magnification = 10 μ m.

Figure 2.

PPAR- γ was expressed in nigral neurons. Arrows indicate colocalization (D; indicated by yellow) of PPAR- γ -ir (A) and Nissl (B) immunofluorescence on the substantia nigra (SN), while arrowheads indicate a lack of colocalization with the microglia marker HLA-DR (C) on the side ipsilateral to MPTP. DAPI was used as a counterstain (indicated by blue nuclei). Scale bar = 25 μ m.

Figure 3.

Microphotographs of the substantia nigra on the side ipsilateral to MPTP dosing stained for PPAR- γ and TH immunostained of normal controls (A, E), 3 (B, F), 12 months post-MPTP (C, G), and 5 mg/kg of pioglitazone + MPTP-treated monkeys (D, H) the lesion. Rectangles in AH indicate the location of high magnification images a–h. Scale bar: A–H = 500 μ m; a–h = 10 μ m.

Figure 4. Microphotographs of the substantia nigra on the side contralateral to MPTP dosing stained for PPAR- γ and TH immunostained of normal controls (A, E), 3 (B, F), 12 months post-MPTP (C, G), and 5 mg/kg of pioglitazone + MPTP-treated monkeys (D, H) on the side contralateral to the lesion. Rectangles in A–H indicate the location of high magnification images a–h. Scale bar: A–H = 500 μ m; a–h = 10 μ m.

Figure 5.

Nigral PPAR- γ -ir vs. TH-ir nigral cell counts differed within treatment groups in nonhuman primates. On the right (ipsilateral) side (A), a significant difference between PPAR- γ -ir vs. TH-ir nigral cell populations was only detected within normal controls (* Paired t-test $p = 0.006$). On the left (contralateral) side (B), significantly less PPAR- γ -ir cells vs. TH-ir were found within normal controls (* $p = 0.011$), 3 (* $p = 0.005$), 12 months post-MPTP, (* $p = 0.044$), and pioglitazone + MPTP-treated animals (* $p = 0.003$). A significant association (Pearson correlation $r^2 = 0.891$, $p < 0.001$) between PPAR- γ -ir vs. TH-ir nigral cell counts was found on the nigral side ipsilateral to MPTP doing (C) but not ($r^2 = 0.300$, $p = 0.277$) on the contralateral side (D).

Figure 6. PPAR- γ was expressed in TH-ir nigral cells. Arrows indicate colocalization (D, F; yellow), of PPAR- γ -ir (A, D) and TH-ir (B, E) immunofluorescence on the substantia nigra (SN) ipsilateral (A, B, C) and contralateral (D, E, F) to MPTP. Arrowheads indicate lack of colocalization. DAPI (indicated by blue nuclei) was used as a counterstain. Scale bar = 25 μ m.

Clinical Rating (CR) and stereological cell quantification of tyrosine hydroxylase (TH) and PPAR-gamma immunoreactive (PPAR-γ-ir) nigral cells in nonhuman primates

Table 1

Ipsilateral (Ipsi) and contralateral (Contra) sides correspond to the hemisphere affected by MPTP dosing. Results displayed as mean ± SEM.

Subjects	CR	TH-ir Cell	Count	PPAR-γ-ir Cell Count	
Hemisphere		Contra	Ipsi	Contra	Ipsi
Normal					
Rh2308	0	198,777	185,167	84,889	90,456
Rh2293	0	179,864	172,225	84,331	91,366
R01046	0	170,806	163,176	91,779	90,456
MEAN	0	183,149	173,523	87,000	90,759
SEM	0	8,239	6,381	2,395	303
3 mo post-MPTP					
R02047	9.00	133,619	27,120	95,342	31,185
R02070	9.31	153,970	22,285	122,228	47,905
R02134	9.58	158,488	25,538	92,866	39,577
R03014	9.83	183,864	31,566	111,660	26,702
R03003	10.19	192,776	25,638	118,458	44,684
MEAN	9.58	166,091	26,429 ^a	108,111 ^a	38,011 ^a
SEM	0.21	12,810	1,508	5,976	3,995
12 mo post-MPTP					
Rh6851	9.63	155,443	23,501	120,337	34,864
Rh6837	12.31	193,123	34,201	114,546	36,757
Rh6840	10.50	171,670	25,251	101,138	43,861
MEAN	10.81	173,412	27,651 ^b	112,007 ^b	38,494 ^b
SEM	0.79	10,912	3,313	5,685	2,738
3 mo post-MPTP + pio					
R02077	8.38	187,845	33,305	161,776	40,125
R03032	7.50	195,795	48,135	162,017	68,223
R02015	5.96	176,638	58,879	155,351	36,863

Subjects	CR	TH-ir Cell		Count		PPAR- γ -ir Cell Count	
		Contra	Ipsi	Contra	Ipsi	Contra	Ipsi
R03113	8.65	183,974	74,619	162,333	74,544		
MEAN	7.62	186,063 ^c	53,734 ^c	160,369 ^d	54,938 ^e		
SEM	0.61	3,990	8,715	1,676	9,604		

Significances for ANOVA LSD post hoc:

- ^a₃ months (mo) post-MPTP vs. normal, $p < 0.001$;
- ^b₁₂ mo post-MPTP vs. normal, $p < 0.001$;
- ^cMPTP + pioglitazone vs. normal, $p = 0.002$. MPTP + pioglitazone vs. 3 mo post-MPTP, Contra and Ipsi TH, $P = 0.002$;
- ^dContra PPAR- γ -ir, $p < 0.001$;
- ^eIpsi PPAR- γ -ir, $p = 0.052$.

Table 2

PPAR-γ cell immunoreactivity rating

PPAR-gamma (PPAR-γ) expression evaluated in the basal ganglia using a scale that ranges from 0–3, where 0 = no staining present, 1 = weak staining, 2 = moderate staining, and 3 = intense staining.

Region	STN		Red N		Oculomotor N		VTA		Putamen	
	Contra	Ipsi	Contra	Ipsi	Contra	Ipsi	Contra	Ipsi	Contra	Ipsi
Normal										
Mean	2.06	1.89	1.67	1.67	2.58	2.58	0.33	0.33	1.00	1.00
SEM	0.39	0.20	0.19	0.19	0.22	0.22	0.33	0.33	0.00	0.00
3 mo post-MPTP										
Mean	1.90	1.80	1.55	1.40	2.57	2.57	0.85	0.75	2.2*	2.0*
SEM	0.20	0.29	0.16	0.23	0.19	0.19	0.25	0.19	0.37	0.35
12 mo post-MPTP										
Mean	2.39	2.17	2.33	2.05	2.67	2.67	0.89	0.89	2.17*	2.33*
SEM	0.20	0.42	0.33	0.19	0.19	0.19	0.11	0.11	0.17	0.17
3 mo post-MPTP + pio										
Mean	2.20	2.56	1.85	1.85	2.79	2.79	1.30	0.98	2.00	1.63
SEM	0.52	0.26	0.09	0.09	0.13	0.13	0.15	0.12	0.20	0.24

Regions evaluated included the Subthalamic Nucleus (STN), Red Nucleus (Red N), Oculomotor Nucleus (Oculomotor N), Ventral Tegmental Area (VTA), and Putamen. Results are displayed as the mean ± SEM.

* Significantly different than normal controls, Kruskal-Wallis with Bonferroni pairwise comparisons, $p < 0.05$.