RESEARCH ARTICLE

Dopaminergic Positron Emission Tomography Imaging in the Alpha-Synuclein Preformed Fibril Model Reveals Similarities to Early Parkinson's Disease

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ABSTRACT: Background: Positron emission tomography (PET) imaging in early Parkinson's disease (PD) subjects reveals that increased dopamine (DA) turnover and reduced dopamine transporter (DAT) density precede decreases in DA synthesis and storage. The rat α-synuclein preformed fibril (α-syn PFF) model provides a platform to investigate DA dynamics during multiple stages of α-syn inclusion-triggered nigrostriatal degeneration.

Objectives: We investigated multiple aspects of in vivo dopaminergic deficits longitudinally and similarities to human PD using translational PET imaging readouts.

Methods: Longitudinal imaging was performed every 2 months in PFF and control rats for 7 months. [^{18}F]-Fluoro-3,4-dihydroxyphenyl-*L*-alanine (FDOPA) imaging was performed to investigate DA synthesis and storage (K_{occ}) and DA turnover, estimated by its inverse, the effective distribution volume ratio (EDVR). ^{11}C-Methylphenidate (MP) was used to estimate DAT density (BP_{ND}).

Results: Early DA turnover increases and DAT binding decreases were observed in the ipsilateral striatum of PFF rats, progressing longitudinally. EDVR decreased 26%, 38%, and 47%, and BP_{ND} decreased 36%, 50%, and 65% at the 2-, 4-, and 6-month time points, respectively, compared to ipsilateral control striatum. In contrast, deficits in DA synthesis and storage were not observed in the ipsilateral striatum of PFF rats compared to control injections and were relatively preserved up to 6 months (K_{occ} decreased 20% at 6 months).

Conclusions: The relative preservation of DA synthesis and storage compared to robust progressive deficits in DAT density and increases in DA turnover in the rat α-syn PFF model display remarkable face validity to dopaminergic alterations in human PD. © 2022 The Authors. *Movement Disorders* published by Wiley Periodicals LLC on behalf of International Parkinson and Movement Disorder Society

Key Words: alpha-synuclein; synucleinopathy; rodent model; PET imaging; nigrostriatal system

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Parkinson's disease (PD) is characterized by the degeneration of the nigrostriatal system within the context of accumulation of pathological alpha-synuclein (α-syn) inclusions (Lewy bodies and Lewy neurites). Using [^{18}F]-fluoro-3,4-dihydroxyphenyl-*L*-alanine (FDOPA) positron emission tomography (PET) imaging we have shown that increased dopamine (DA) turnover, estimated as reduction of its inverse, the effective dopamine distribution volume (EDV), precedes decreases in DA synthesis and storage in early PD.[1] Similarly, asymptomatic carriers of leucine-rich repeat kinase 2 (*LRRK2*) exhibit normal DA synthesis and storage; however, DA turnover is increased and dopamine transporter (DAT; ^{11}C-methylphenidate [MP])

and vesicular monoamine transporter type 2 (VMAT2; ^{11}C-(6)-α-dihydrotetrabenazine [DTBZ]) are reduced.[2,3] In general, as the disease progresses, deficits in DA synthesis and storage remain relatively modest compared to other dopaminergic PET markers.[4,5] These findings underscore that nigrostriatal degeneration in PD is dynamic, with disease stage-dependent events impacting dopaminergic transmission. Understanding the DA dynamics of PD progression will inform the development of dopaminergic therapies.

PET imaging has been applied to neurotoxicant and α-syn overexpression rodent models that result in rapid, severe degeneration of the nigrostriatal system.[6-10] In general, these studies demonstrate that decreased DAT, VMAT2, DA synthesis and storage, and DA turnover mirror lesion status as defined by histological markers of nigrostriatal neuron survival. However, none of these models recapitulate the prolonged stage of Lewy body (LB) accumulation associated with PD and thus cannot inform the impact of this phenomenon on DA dynamics. Recently, we have characterized the impact of intrastriatal injection of preformed α-syn fibrils into rats.[11-14] In this model, intraneuronal LB-like phosphorylated α-syn inclusions are triggered to form within structures innervating the striatum, including the nigrostriatal system. Nigral α-syn inclusion formation peaks 2 months after injection, with significant loss of dopaminergic phenotype observed at 4 months and overt degeneration occurring 5–6 months following injection.[12,13] Thus, the rat α-syn PFF model provides a platform to investigate DA dynamics during multiple stages of α-syn inclusion-triggered nigrostriatal degeneration. In the present study we used longitudinal multitracer PET imaging to investigate DA synthesis and storage, DA turnover, and DAT binding in the rat α-syn PFF model. Our results indicate that the degenerative cascade of events triggered by intrastriatal PFF injections into rats results in progression of dopaminergic dysfunction similar to that observed in early PD.

Methods

Rats were unilaterally injected with either preformed α-syn fibrils (PFF, n = 8) or Dulbecco's phosphate buffered saline (PBS) (control, n = 8) into two sites in the dorsal striatum. PET scans with ^{18}F-FDOPA (effective distribution volume ratio [EDVR, the tissue input equivalent of EDV[7,15]] for turnover and K_{occ} for DA synthesis)[1] and ^{11}C-MP (BP_{ND}) were performed at 2, 4, and 6 months (PFF rats) or 3, 5, and 7 months (control). At the conclusion of PET scanning all rats were perfused intracardially and brains analyzed at 7.5 months following surgery. Fig. 1A illustrates the experimental design.

Rats

Male, 3-month-old Fischer 344 rats (Charles River, Wilmington, MA) were housed in the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) -approved MSU Grand Rapids Research Center vivarium and the UBC Animal Resource Unit. All procedures were conducted in accordance with guidelines set by the Institutional Animal Care and Use Committee (IACUC) of MSU and the UBC Animal Care Committee.

Preparation and Instratriatal Injection of α-syn PFFs

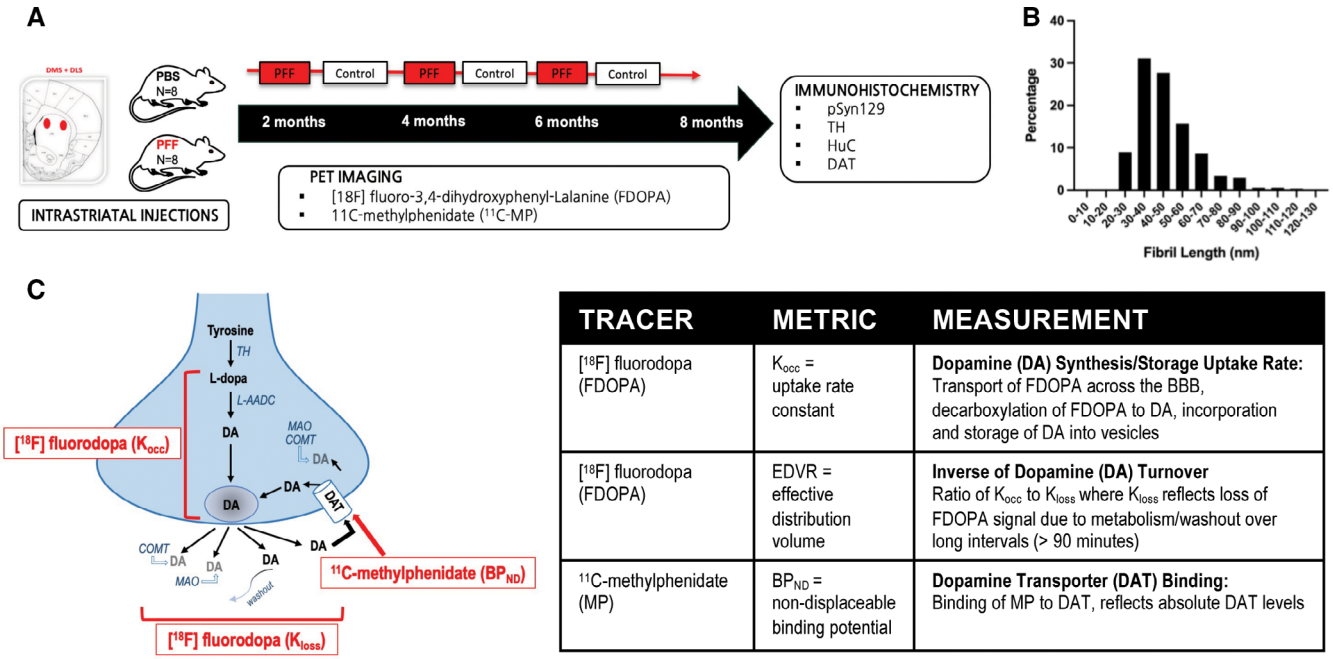
Recombinant, full-length mouse α-syn PFFs were prepared.[16,17] Mouse α-syn was selected to generate PFFs due to the superior efficiency of this protein species in seeding pathological inclusions in rodents.[18,19] Prior to injection, PFFs were thawed, diluted to (4 μg/μL), and sonicated at room temperature. A sample of the PFFs was imaged on a JEOL JEM-1400 transmission electron microscope. The length of ~500 fibrils per sample was measured to validate that average fibril size approximated 50 nm (Fig. 1B). Intrastriatal α-syn PFF injections were conducted as described previously.[12,14,16] Rats were anesthetized before surgery with isoflurane and each rat received two unilateral, intrastriatal injections (AP +1.0 mm, ML +2.0 mm, DV –4.0 mm and AP +0.1 mm, ML +4.2 mm, DV –5.0 mm, AP and ML relative to bregma and DV relative to dura, injection rate 0.5 μL/min,) of sonicated α-syn PFFs (4 μg/μL, 2 × 2 μL injections) or an equal volume of Dulbecco's PBS.

Scanning

The injected activity, administered as bolus, was 4.5 ± 0.2 MBq (mean ± SD) per 100 g of body weight for ^{18}F-fluoro-3,4-dihydroxyphenyl-L-alanine (FDOPA)[20] and 4.4 ± 0.2 MBq per 100 g of body weight for ^{11}C-methylphenidate (MP).[21] The specific activity at injection was 18 ± 10 MBq/micromole (mean ± SD) for FDOPA and >150 GBq/micromole for MP.

Rats were scanned with both tracers at 2, 4, and 6 months after surgery (PFF rats) or 3, 5, or 7 months after surgery (control rats). A minimum of 5 days was allowed between two subsequent scans on the same animal to allow for full recovery between scans (with the exception of two animals at a single time point due to technical reasons). All studies were performed on a Siemens/Concorde microPET Focus 120.

The scanning procedure was similar for both tracers: the rats were anesthetized and maintained with a 2% isoflurane/O_2 gas mixture. Each rat was positioned in a stereotactic head-holder mounted to the scanner bed, allowing accurate and reproducible positioning. After a 6-min transmission scan with a ^{57}Co source, emission scans were acquired dynamically starting at injection. MP data were



TRACER	METRIC	MEASUREMENT
[18F] fluorodopa (FDOPA)	K_{occ} = uptake rate constant	**Dopamine (DA) Synthesis/Storage Uptake Rate:** Transport of FDOPA across the BBB, decarboxylation of FDOPA to DA, incorporation and storage of DA into vesicles
[18F] fluorodopa (FDOPA)	EDVR = effective distribution volume	**Inverse of Dopamine (DA) Turnover** Ratio of K_{occ} to K_{loss} where K_{loss} reflects loss of FDOPA signal due to metabolism/washout over long intervals (> 90 minutes)
11C-methylphenidate (MP)	BP_{ND} = non-displaceable binding potential	**Dopamine Transporter (DAT) Binding:** Binding of MP to DAT, reflects absolute DAT levels

FIG. 1. Experimental overview of surgical design, positron emission tomography (PET), and postmortem outcome measures. (**A**) Rats received unilateral striatal injections of alpha-synuclein (α-syn) preformed fibrils (PFFs) or an equal volume of phosphate-buffered saline (PBS). PET imaging with [^{18}F]-fluoro-3,4-dihydroxyphenyl-*L*-alanine (FDOPA) and ^{11}C-methylphenidate (MP) was conducted longitudinally in all rats at either 2,4, 6 (PFF rats) or 3,5,7 months (control PBS rats) following surgery. At 7 months post-surgery rats were euthanized for various immunohistochemical assessments. (**B**) Distribution of mouse α-syn PFF fibril lengths post-sonication. A total of 546 individual fibrils were measured; the average fibril size was 49.38 nm (SD = 15.07 nm). (**C**) Schematic of PET tracers used. FDOPA imaging of the striatum was used to determine the dopamine (DA) synthesis and storage rate (K_{occ}) and the effective distribution volume ratio (EDVR), the inverse of DA turnover. MP was used to determine dopamine transporter (DAT) binding. Abbreviations: pSyn129, α-syn phosphorylated at serine 129; TH, tyrosine hydroxylase; HuC, pan neuronal marker; L-AADC, aromatic L-amino acid decarboxylase; MAO, monoamine oxidase; COMT, catechol-O-methyltransferase. [Color figure can be viewed at wileyonlinelibrary.com]

acquired over 61 min and framed into 17 frames (6 × 30 s; 2 × 60 s; 5 × 300 s; 2 × 450 s; and 2 × 480 s). Ninety minutes prior to FDOPA injection, entacapone (20 mg/kg), a catechol-O-methyltransferase (COMT) inhibitor, was administered intraperitoneally (IP), followed by IP administration of the aromatic L-amino acid decarboxylase (AADC) inhibitor benseraside (10 mg/kg) 30 min prior to tracer injection to minimize peripheral FDOPA metabolism. Data were acquired for 150 min and framed into 24 frames (6 × 30 s; 2 × 60 s; 5 × 300 s; 3 × 400 s; 6 × 700 s; and 2 × 900 s). Heart rate, blood oxygen saturation level, and temperature were continuously monitored to ensure normal range. Details and validation of the FDOPA imaging and analysis are as published previously.[6,7]

Image Analysis

Time–activity curves (TACs) were extracted from the images using predefined regions of interest (ROIs) using the ASIPro software (CTI Concorde Microsystems, Knoxville, TN). For each scan, ROIs were placed on each striatum and on the cerebellum, used as reference region, on the three axial planes where the structures were visually best defined. The ROI volumes were 0.043 cm^3 (cerebellum) and 0.022 cm^3 (striatal). The TACs from the FDOPA images were used to estimate K_{occ} and EDVR with in-house software using Matlab (The Mathworks, Natick, MA). The tissue input Logan graphical approach was used to estimate MP BP_{ND}, a measure of DAT density.

Immunohistochemistry and Unbiased Stereology

Seven months following surgery all rats were deeply anesthetized and perfused intracardially with heparinized normal saline followed by 4% paraformaldehyde. Free-floating brain sections (40 μm) were immunolabeled with primary antibodies: mouse anti-phosphorylated α-syn at serine 129 (pSyn, 81A; Abcam, Cambridge, MA; AB184674; 1:10000), rabbit anti-tyrosine hydroxylase (TH; Millipore, Temecula, CA; AB152, 1:4000), or mouse anti HuC (Invitrogen, Waltham, MA; A-21271, 1:2000) followed by the appropriate biotinylated secondary antibodies, amplified and visualized by development in 3,3′-diaminobenzidine (DAB; Sigma-Aldrich, St. Louis, MO; D5637-10G) and 0.03% H_2O_2. The number of tyrosine hydroxylase immunoreactive (THir) and HuC immunoreactive neurons (HuCir; pan neuronal marker) in the ipsilateral and contralateral substantia nigra pars compacta

(SNpc) was estimated using unbiased stereology with the optical fractionator principle.[12,13]

Immunofluorescence

Striatal sections were immunolabeled using rabbit anti-dopamine transporter (DAT; Millipore, Temecula, CA; D6944, 1:1000) followed by secondary labeling with near-infrared secondary antibody (donkey anti-rabbit 800CW IRDye, LiCor #926-32213, 1:500) and imaged on a LiCor Odyssey CLx flatbed scanner as described previously.[12,22] The near-infrared immunofluorescent intensity signal for DAT was quantified in every sixth 40 μM coronal section through the caudate-putamen.

Statistical Analysis

Statistical tests were completed using either GraphPad Prism software (version 9, GraphPad, La Jolla, CA) or in R (version 4.0.3) using the *lme4* package. Two-way analysis of variance (ANOVA) with repeated measures was used to determine significant differences between the injected hemispheres or between the uninjected hemispheres, or between time points within hemispheres of PFF- or PBS-injected rats, using Sidak's multiple comparison tests. Simple linear regression modeling was used to examine relationships at individual time points whereas a linear mixed effects regression model was used to examine relationships of repeated measures across multiple time points.

Results

Impact of PFF-Induced Pathology on DA Synthesis and Storage is Relatively Modest and Delayed

Rats were imaged with FDOPA to estimate the DA synthesis and storage uptake rate (K_{occ}; Fig. 1C) in both the contralateral uninjected striatum and ipsilateral striatum that received PFF injection. In the uninjected striatum of either PFF or control rats, no significant effect of treatment or postsurgical interval on K_{occ} was observed ($P > 0.05$; Fig. 2A). In order to control for the effect of intrastriatal surgical injection artifact, primary comparisons were made between the ipsilateral striata of control- and PFF-injected rats (Fig. 2B). No significant differences in K_{occ} were observed between the ipsilateral striatum of PFF and control injected rats at any of the time points ($P > 0.05$). Within the PFF-injected striatum a significant decrease of ~20–27% K_{occ} was observed over time (4–6 months) compared to the 2-month time point ($P < 0.05$), with no progression observed between 4 and 6 months. These results suggest that DA synthesis and storage is modestly impacted by PFF injection-associated toxicity, and only at relatively later time points.

PFF Injection-Induced Degeneration Impacts DA Turnover earlier and to a Greater Degree than DA Synthesis and Storage

FDOPA data were also evaluated for the effective dopamine distribution volume ratio (EDVR) (Fig. 1C); a decrease in EDVR reflects increased DA turnover.[1] In the uninjected striatum of either PFF or control rats, no significant effect of treatment or postsurgical interval on EDVR was observed ($P > 0.05$; Fig. 2C). However, a significant reduction of EDVR was observed in the ipsilateral striatum of PFF-injected rats compared to control injections at all time points examined (Fig. 2D; $P < 0.01$). Specifically, we observed a ~26%, ~38%, and ~47% reduction at the 2-, 4-, and 6-month time points, respectively. Repeated measures analysis revealed that the PFF-associated decrease of EDVR progressed significantly between 2 months and 4 months following injection ($P < 0.05$), with PFF rats at both 4 and 6 months exhibiting significantly lower EDVR than at 2 months ($P < 0.05$). Collectively, these results suggest that the pathology produced by PFF injection results in early and progressive increases in DA turnover following PFF injection.

PFF Injection-Induced Pathology Results in Early and Progressive Reduction in DAT Density in the Ipsilateral Striatum

Using ^{11}C-methylphenidate (MP) we examined DAT density rats across a 7-month time span. In the uninjected striatum of either PFF or control rats, no significant effect of treatment or postsurgical interval on BP_{ND} was observed ($P > 0.05$; Fig. 2E). However, comparisons between the ipsilateral striata of control and PFF-injected rats (Fig. 2F) revealed that PFF injection resulted in a significant reduction in BP_{ND} compared to control injections at all time points ($P < 0.0001$). Specifically, we observed a ~36% and ~50% reduction in BP_{ND} at the 2- and 4-month time points, progressing to ~65% at the 6-month time point (Fig. 2F,G). Repeated measures analysis revealed a significant progressive decrease of BP_{ND} in the ipsilateral striatum of PFF rats over time, with PFF rats at 4 months exhibiting significantly lower BP_{ND} than at 2 months ($P < 0.001$) and PFF rats at 6 months significantly lower than at 4 months ($P < 0.05$). Collectively, these results demonstrate that the synucleinopathy-induced degeneration following intrastriatal PFF injection results in significant and progressive loss of DAT binding in the ipsilateral striatum.

Decreased DAT and Enhanced DA Turnover Induced by PFF Injection-Induced Pathology are Significantly Associated

We next examined the relationship between DAT (MP BP_{ND}) and DA turnover ratio (EDVR) and

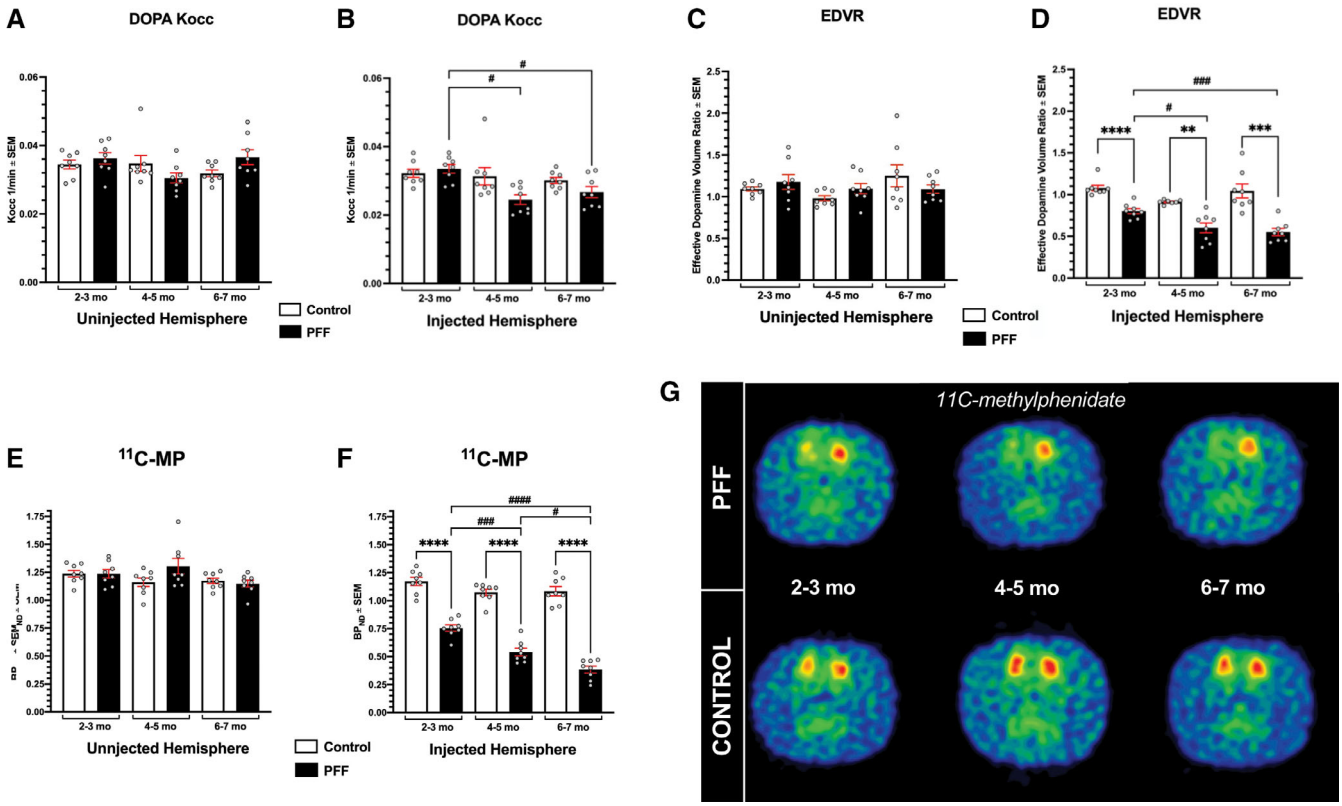


FIG. 2. Longitudinal changes in dopamine (DA) synthesis/storage, DA turnover, and dopamine transporter (DAT) binding in preformed fibril (PFF) and control rats. **(A–B)** K_{occ} in the uninjected striatum of control (**A**) and α-synuclein PFF (**B**) injected rats. (**A**) No significant effect of either surgical treatment was observed on K_{occ} in the uninjected striatum. (**B**) In PFF-injected rats, no significant differences in K_{occ} were observed in the ipsilateral striatum compared to the ipsilateral striatum of control-injected rats. Within the PFF-injected striatum, K_{occ} was significantly decreased at the 4 and 6 month time points compared to the 2-month time point. **(C–D)** Effective distribution volume ratio (EDVR) in the uninjected striatum of control (**C**) and α-syn PFF (**D**) injected rats. (**C**) No significant effect of either surgical treatment was observed on EDVR in the uninjected striatum. (**D**) In PFF-injected rats, EDVR was significantly reduced in the ipsilateral striatum compared to the ipsilateral striatum of control-injected rats 2, 4, and 6 months after surgery. Within the PFF-injected striatum, EDVR was significantly decreased between the 2- and 4-, and 2- and 6-month time points. **(E–F)** DAT density (BP_{ND}) in the uninjected striatum of control (**E**) and α-syn PFF (**F**) injected rats. **(E)** No significant effect of either surgical treatment was observed on BP_{ND} in the uninjected striatum. **(F)** In PFF-injected rats, BP_{ND} was significantly reduced in the ipsilateral striatum compared to the ipsilateral striatum of control-injected rats 2, 4, and 6 months after surgery. Within the PFF-injected striatum, BP_{ND} was significantly decreased between all time points. (**G**) Representative image of DAT binding in a PFF-injected and control rat over time demonstrating progressive loss of BP_{ND} signal in the striatum over time. Values represent the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$ between PFF and control rats; $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$, $\#\#\#\#P < 0.0001$ between PFF rats at different time points. [Color figure can be viewed at wileyonlinelibrary.com]

between DAT and DA synthesis/storage (K_{occ}) within individual rats. In control rats, no significant relationship was observed in either hemisphere at any time point ($P > 0.05$). In contrast, we observed a significant relationship between MP BP_{ND} and EDVR within the PFF-injected hemisphere at both 2 and 6 months (2 months: $P = 0.0116$, $R^2 = 0.68$; 6 months: $P < 0.05$, $R^2 = 0.51$; Fig. 3A). We also observed a significant relationship between MP BP_{ND} and EDVR within the contralateral hemisphere of PFF-injected rats at 6 months ($P < 0.05$, $R^2 = 0.50$; Fig. 3A). No significant relationship was observed between MP and K_{occ} in either control or PFF-injected rats in either hemisphere at any specific time point ($P > 0.05$; Fig. 3B) or when examined across all time points ($P > 0.05$, data not shown). Thus, we observed that in addition to the ipsilateral striatum of PFF-injected rats exhibiting reduced DAT and increased DA turnover relative to the contralateral hemisphere, within the ipsilateral striatum at 2 and 6 months decreased DAT was associated with increased DA turnover. Further, within the contralateral striatum of PFF-injected rats at 6 months, decreased DAT also correlated with increased DA turnover.

DAT Immunofluorescence Intensity is Significantly Reduced in the Ipsilateral Striatum 7 Months after PFF Injection

After completion of the longitudinal study, rats were analyzed for immunofluorescent DAT intensity in the striatum. Decreased DAT signal spanned the striatum ipsilateral to PFF injection in all rats with no alterations in DAT signal observed ipsilateral to PBS injection (Fig. 4A). Quantification revealed that PFF injection resulted in a significant ~58% reduction in DAT immunofluorescence compared to control (Fig. 4B; $P < 0.01$).

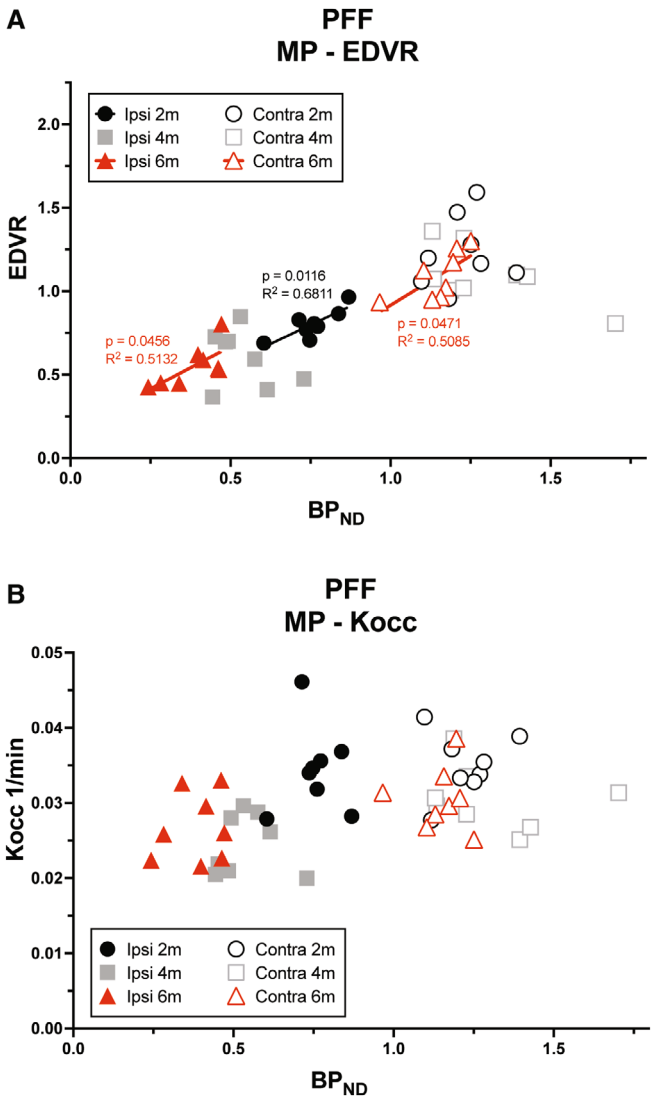


FIG. 3. Decreased dopamine transporter (DAT) binding in the striatum of α-synuclein preformed fibril (PFF)-injected rats is positively associated with increased dopamine (DA) turnover. (**A**) Relationship between DAT density (^{11}C-methylphenidate [MP] BP_{ND}) and DA turnover (effective distribution volume ratio [EDVR]) and (**B**) between DAT density and DA synthesis/storage (K_{occ}) within individual PFF-injected rats. (**A**) A significant relationship is observed between MP BP_{ND} and EDVR within the PFF-injected hemisphere at both 2 and 6 months (2 months: $P = 0.0116$, $R^2 = 0.68$; 6 months: $P < 0.05$, $R^2 = 0.51$) revealing that decreased DAT binding is associated with increased turnover of DA. A significant relationship between MP BP_{ND} and EDVR within the contralateral hemisphere of PFF-injected rats is also observed at 6 months ($P < 0.05$, $R^2 = 0.50$). Correlation lines highlight significant associations. Filled black circles, grey squares, and red triangles depict the ipsilateral striatum at 2, 4, or 6 months, respectively. Open black circles, grey squares, and red triangles depict the contralateral striatum at 2, 4, or 6 months, respectively. (**B**) In PFF-injected rats no relationship was observed between MP-K_{occ} in either hemisphere at any time point. [Color figure can be viewed at wileyonlinelibrary.com]

This reduction was similar to the ~65% reduction in MP BP_{ND} in the ipsilateral striatum of PFF rats observed at the 6-month time point (Fig. 2F). To examine the relationship between in vivo PET data and postmortem immunofluorescent analyses, we compared striatal DAT immunofluorescence intensity at 7 months to MP BP_{ND}-derived estimates of DAT density for the ipsilateral striatum of the PFF-injected rats at 6 months and that obtained for PBS-injected rats at 7 months. A strong, significant correlation (Fig. 4C; $R^2 = 0.8251$, $P < 0.0001$) was found. These results suggest that postmortem immunofluorescent labeling of DAT agrees well with the BP_{ND} of MP.

Intrastriatal PFF Injection Resulted in Accumulation of Phosphorylated α-Syn (pSyn) Inclusions

All rats injected with PFFs exhibited numerous inclusions immunoreactive for phosphorylated α-syn (pSyn) within neurons in various cortical regions and the striatum (Fig. 4D,E). Some pSyn inclusions also were observed in the SNpc ipsilateral to α-syn PFF injection (Fig. 4F). No pSyn inclusions were observed in control rats. This pattern of pSyn accumulation replicates what we have previously observed 6 months following intrastriatal PFF injections to rats in which pSyn inclusion formation in the SNpc and cortex peaks at earlier time points (1–2 months); pathology is maintained in the cortex over time but decreases in the SNpc as pSyn-containing nigrostriatal DA neurons degenerate.[12] In addition, accumulation of pSyn-ir neurons in the striatum peaks at later intervals after intrastriatal PFF injection at approximately 6 months and was present in abundance at the 7-month time point.

Intrastriatal PFF Injection Results in Marked Nigrostriatal Degeneration in the Ipsilateral Hemisphere

Examination of DAT immunoreactivity in the striatum revealed a marked denervation in the ipsilateral striatum in PFF-injected rats unlike in the ipsilateral striatum of controls (Fig. 4A,B). Similarly, in PFF-injected rats a marked loss (~ 60%) of SNpc THir neurons was observed ipsilaterally ($P < 0.0001$; Fig. 4H) with no loss observed in the ipsilateral SNpc of control rats ($P > 0.05$; Fig. 4H). Stereological quantification of HuCir neurons revealed a significant reduction in neurons in the ipsilateral SNpc of PFF-injected rats ($P < 0.0001$; Fig. 4I) with no loss of SNpc neurons observed in controls ($P > 0.05$; Fig. 4I). The average total number of SNpc THir neurons lost (compared to the contralateral hemisphere) in PFF-injected rats was 10,450 compared to 9450 HuCir neurons lost, indicating that the overwhelming majority of loss represented overt neuronal degeneration and not simply a downregulation of TH phenotype.

Discussion

Previously, we have demonstrated that intrastriatal injection of α-syn PFFs to rats results in peak

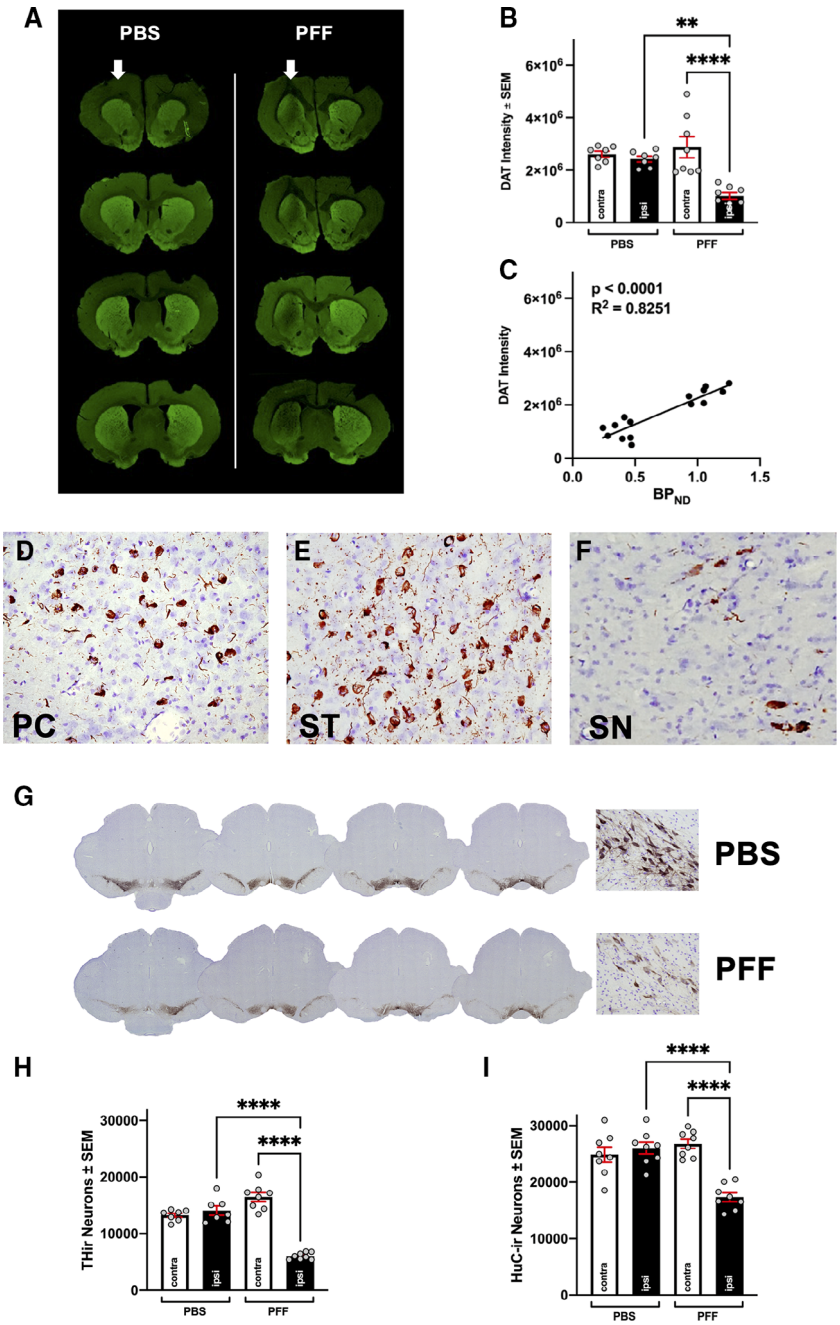


FIG. 4. Postmortem immunohistochemical assessments at 7 months confirm nigrostriatal degeneration in the α-synuclein preformed fibril (PFF) model. (**A**) Representative dopamine transporter (DAT) immunofluorescence in PFF and control striatum. (**B**) Quantification revealed that PFF injection resulted in a significant reduction in striatal DAT immunofluorescence compared to control injection. (**C**) Striatal ^{11}C-methylphenidate DAT density (MP BP_{ND}) at 6 months and DAT immunofluorescence are significantly correlated ($R^2 = 0.8251$, $P < 0.0001$). (**D–F**) Representative α-syn inclusions in the pyriform cortex (PC) (**D**), striatum (ST) (**E**), and substantia nigra pars compacta (SNpc) (**F**) identified using antisera against α-syn phosphorylated at serine 129 (pSyn). (**F**) Reduced pSyn immunoreactive neurons within the SNpc reflects loss of pSyn containing neurons at the 7-month time point. (**G**) Representative tyrosine hydroxylase (TH) immunoreactivity in the SN of PFF and control rats. (**H**) Stereological quantitation reveals significant degeneration of THir neurons in the ipsilateral SNpc of α-syn PFF-injected rats with no loss observed in the ipsilateral SNpc of control rats. (**I**) Stereological quantitation of HuC immunoreactive neurons (pan neuronal marker) reveals significant neuronal loss in the ipsilateral SNpc of α-syn PFF-injected rats with no loss observed in the ipsilateral SNpc of control rats, validating loss of THir neurons due to PFF injection is not limited to loss of TH phenotype. Values represent the mean ± SEM. $^{**}P < 0.01$, $^{****}P < 0.0001$. PBS, phosphate-buffered saline. [Color figure can be viewed at wileyonlinelibrary.com]

phosphorylated α-syn (pSyn) inclusion formation in the SNpc at 2 months, followed by loss of TH phenotype and ultimately nigrostriatal degeneration between 4 and 6 months.[12-14] The later degenerative phase is also associated with significant deficits in motor function and striatal DA.[11,12] In the present study we used multitracer PET imaging to address two different questions: (i) Are other aspects of dopaminergic alterations

detectable in the α-syn PFF rat model? and (ii) Do their relative magnitude and time courses compare to what is observed in early human PD? We used an imaging-based readout similar to that which we previously used in sporadic and LRRK2 mutation-related human PD.[1,2] Overall, a striking similarity between the human PD studies and the PFF-induced synucleinopathy model was observed. The PFF injection resulted in an early reduction in DAT binding that progressed significantly over the 6-month study interval. This decrease in DAT was paralleled by a significant and progressive increase in DA turnover (decrease in EDVR); whereas DA synthesis and storage exhibited later and modest reductions that remained relatively stable over time. Similarly, early PD subjects, as well as asymptomatic LRRK2 mutation carriers, exhibit increased DA turnover and decreased DAT[1,2] with relatively preserved DA synthesis and storage.[1,4,5,23] Our human data led to the interpretation that a functional role of DAT is to maintain relatively constant synaptic DA levels and to preserve DA in nerve terminals[24] and that clinical motor symptoms manifest when the surviving neurons become unable to maintain levels of DA synthesis and storage; at that time K_{occ} decreases by approximately 30–40% with DAT binding decreasing by ~55–60%.[2] In the present α-syn PFF model study, we did not observe a decrease in K_{occ} until 4 months after PFF injection, with the reduction in K_{occ} relatively smaller compared to reductions in DAT binding and increased DA turnover (20–27% reduction in K_{occ} compared to ~65% and ~47% reduction in DAT binding and EDVR, respectively). In the context of human disease, it is thus not completely surprising that the PFF-injected rats do not exhibit observable motor deficits until 6 months after injection.[12] Overall, our rat α-syn PFF model study indicates that the pattern of dopaminergic dysfunction resulting from PFF-triggered nigrostriatal synucleinopathy accurately recapitulates the progression of dopaminergic dysfunction observed in early PD.

The specific pathogenic relationship between intraneuronal pSyn immunoreactive inclusions and the ultimate degeneration of the nigrostriatal system remains unclear. pSyn inclusions colocalize with markers commonly observed in LB (p62 and ubiquitin), consist of α-syn oligomers and fibrils, and are also thioflavin-S positive and proteinase-K resistant.[11-13] Inclusion-bearing neurons selectively degenerate in the α-syn PFF model[25]; however, the mechanistic link between the presence of pSyn immunoreactive aggregates and neuronal toxicity remains unknown. α-Syn aggregates and/or oligomers may be directly toxic,[26] depletion of monomeric α-syn may have toxic consequences,[27] inclusion-associated neuroinflammation may contribute to toxicity,[13] and/or other mechanisms not yet fully understood may contribute to degeneration of inclusion-bearing neurons. Thus, in the present study, pSyn immunoreactivity in the SNpc predicts, but does not necessarily directly cause, pathogenic consequences.

Previous studies have used PET imaging of DAT, vesicular monoamine transporter 2 (VMAT2), DA synthesis/storage, and DA turnover in either the 6-hydroxydopamine (6-OHDA) or viral vector-mediated α-syn overexpression model in rats. In all of these studies severe loss of nigrostriatal neurons was documented within 1–2 weeks post-lesion, with PET imaging tracking with lesion severity.[6-10] The rat α-syn PFF model offers the ability to examine DA dynamics over a protracted interval when nigrostriatal DA neurons remain alive but increasingly more dysfunctional. Using model parameters identical to the present study, we observe peak α-syn inclusion formation in nigral neurons for a period of 2 months after PFF injection with SNpc THir neurons maintained to near control levels.[12,14] At 4 months after PFF injection significant loss of TH phenotype is observed; however, no significant degeneration of SNpc (neuronal loss) is observed until at least 5 months.[12,13] Thus, the α-syn PFF model provides insight into the DA dynamics of inclusion-bearing nigrostriatal neurons at distinct phases of degeneration. At the 2-month time point we observe that α-syn inclusion-bearing SNpc neurons maintain DA synthesis and storage; however, DA turnover is increased and DAT density is reduced. At this same time point we have recently observed a significant reduction in DAT mRNA in the pSyn inclusion-seeded SNpc.[28,29] The loss of MP BP_{ND} at the 2-month time point might therefore reflect either a loss of DA terminal density and/or a loss of DAT density in the absence of terminal loss. α-Syn and DAT are known protein partners, cell surface recruitment of DAT is modulated by α-syn, serine 129 phosphorylation of α-syn reduces normal facilitation of DAT function, and in the PD striatum DAT/α-syn complexes are redistributed and reduced.[30-33] At the 4-month time point, when significant loss of TH phenotype is observed,[12,13] DA synthesis and storage becomes compromised, DA turnover increases further, and DAT is further reduced. Finally, at the 6-month time point, increased DA turnover is maintained, DAT decreases further, and DA synthesis and storage remains only modestly impacted (Fig. 5). In the only other study to date that applied PET imaging to the rat α-syn PFF model,[34] VMAT2 ([^{11}C]-dihydrotetrabenazine) was significantly reduced compared to control-injected striatum 4 months after PFF injections. The slower time course of VMAT2 reduction likely reflects the lower quantities of PFFs used compared to that which we used in the present study,[12] resulting in a less efficient seeding of pSyn inclusions. No additional dopaminergic imaging measures were included in the Thomsen et al 2021 report.[34] Thus, through simultaneous incorporation of K_{occ}, EDVR, and MP imaging, our study is the first to provide

OVERVIEW OF PROGRESSIVE ALPHA-SYNUCLEIN INCLUSION-INDUCED DOPAMINERGIC DYSFUNCTION			
Timepoint	**2 month**	**4 months**	**6 months**
Striatal TH	little to no change	↓	↓
SN TH	little to no change	↓	↓↓
SN Neurons	no change	no change	↓↓
DAT Binding	↓	↓↓	↓↓↓
DA Turnover	↑	↑↑	↑↑
DA Synth/Storage	no change	↓	↓

FIG. 5. Time course of dopaminergic alterations in the rat α-synuclein preformed fibril (PFF) model as revealed by immunohistochemistry and positron emission tomography imaging. Changes in striatal tyrosine hydroxylase (TH) immunoreactivity, substantia nigra pars compacta (SN) TH neuron number, and total SN neuron number are based on previous studies using the same intrastriatal α-syn PFF parameters at 2, 4, and 6 months after surgery.[12] Two months: intrastriatal α-syn PFF injection results in little to no change in striatal TH immunoreactivity, SN TH immunoreactive (THir) neurons, or dopamine (DA) synthesis or storage; however, striatal dopamine transporter (DAT) binding is significantly reduced and DA turnover is significantly increased. This time point corresponds to peak accumulation of pathological α-syn inclusions in the SN.[12-14] Four months: decreased striatal TH, reductions in SN THir neurons, and reduced DA synthesis and storage are detectable for the first time. SN neurons have lost TH phenotype but have not yet degenerated. Reductions in striatal DAT binding and increased DA turnover progress further. Six months: further loss of SN THir neurons, now representing overt degeneration. Maintained reductions in striatal TH immunoreactivity and increases in DA turnover. Modest reductions in striatal DA synthesis and storage are maintained, and reductions in striatal DAT binding progress further.

insight into progressive DA dynamic alterations associated with synuclein-inclusion-triggered nigrostriatal degeneration.

In the present study we observed a similar pattern and time course of reductions in DAT binding and reductions in EDVR. EDVR is a measure of the ratio of K_{occ} to k_{loss}, in which k_{loss} reflects the rate of loss of FDOPA signal over time which is dependent on multiple factors, including DA release, DA metabolism, and DA washout. It is not clear from our study which of these processes contributed to the observed increase in DA turnover. However, our observation that reductions in EDVR mirrored reductions in DAT binding, both between surgical treatment groups and within individual PFF-injected striatum, suggests that decreased DAT contributed in decreased DA reuptake, leading to increased DA in the synaptic compartment and thus more vulnerable to metabolism and washout, increasing DA turnover. These results also mirror findings in human PD[1,2] as well as similar studies performed in a 6-OHDA rat model of PD,[7,35] suggesting a consistent functional role of DAT in the presence of dopaminergic deficit. Further studies will be required to directly examine the impact of PFF-seeded nigrostriatal synucleinopathy on synaptic DA levels prior to overt degeneration.

In conclusion, we observe that the nigrostriatal synucleinopathy and degenerative cascade of events triggered by intrastriatal α-syn PFF injections into rats results in a progression of dopaminergic dysfunction similar to that observed in early PD. The formation of LB-like α-syn inclusions in nigrostriatal DA neurons is associated with decreased DAT density and an increase in DA turnover in the striatum. The progression of inclusion-seeded nigrostriatal neurons to ultimate degeneration is associated with a decrease in DA synthesis and storage in the striatum; however, this is relatively spared earlier in the time course of the lesion. The face validity of the rat α-syn PFF model provides a clinically relevant platform to investigate the early consequences of pathological α-syn inclusions and for the development of therapeutic strategies to optimize dopaminergic therapies for PD. ●

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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