Dose-related biphasic effect of the Parkinson's disease neurotoxin MPTP, on the spread, accumulation, and toxicity of α -synuclein

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Abstract

Background: 1-methyl, 4-phenyl, 1,2,3,6 tetrahydropyridine (MPTP), a mitochondrial neurotoxin, has been used previously to generate a PD mouse model. However, MPTP does not induce Lewy bodies or α -syn aggregation in mice. In the present study, we evaluated the effect of different doses of MPTP (10 mg/kg.b.wt and/or 25 mg/kg.b.wt) on the spread, accumulation, and toxicity of endogenous α -syn in mice administered an intrastriatal injection of human α -syn PFF

Methods: We inoculated human WT α -syn PFF in mouse striatum. At 6 weeks post PFF injection, we challenged the animal with two different doses of MPTP (10 mg/kg.b.wt and 25 mg/kg.b.wt) once daily for five consecutive days. At 2 weeks from the start of the MPTP regimen, we collected the mice brain and performed immunohistochemical analysis, and Rotarod test to assess motor coordination and muscle strength before and after MPTP injection.

Results: A single injection of human WT α -syn PFF in the mice striatum induced the propagation of α -syn, occurring as phosphorylated α -synuclein (pS129), towards the SNpc, within a very short time. Injection of a low dose of MPTP (10 mg/kg.b.wt) at 6 weeks post α -syn PFF inoculation further enhanced the spread, whereas a high dose of MPTP (25 mg/kg.b.wt.) reduced the spread. Majority of the accumulated α -syn were proteinase K resistant, as recognized using a conformation-specific α -syn antibody. Injection of α -syn PFF alone caused 12% DA neuronal loss while α -syn PFF + a low dose of MPTP caused 33% loss, compared to the control mice injected with saline. Interestingly, a low dose of MPTP alone did not cause any significant DA cell death compared to saline treatment. Animals that received α -syn PFF and a high dose of MPTP showed massive activation of glial cells and decreased spread of α -syn, majority of which were detected in the nucleus.

Conclusion: Our results suggest that a combination of human WT α -syn PFF and a low dose of MPTP increases the pathological conversion and propagation of endogenous α -syn, and neurodegeneration, within a very short time. Our model can be used to study the mechanisms of α -syn propagation and screen for potential drugs against PD.

1. Background

Parkinson's disease (PD) is a progressive multifactorial neurodegenerative disorder that is characterized by the progressive loss of a selective population of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc), which results in the depletion of dopamine neurotransmitters in the striatum. The pathological hallmark of PD is the accumulation of cytoplasmic proteinaceous aggregates known as Lewy bodies (LBs). The major constituent of LBs are α -synuclein (α -syn), a protein with a molecular weight of 14 kDa. The vast majority of PD cases appear to be sporadic; however, several familial forms of PD cases have been identified in which *α -syn*, *Parkin*, *DJ-1*, *LRRK2*, *PINK1* genes, and other candidate loci have been implicated [1]. Mutations in α -syn gene (*SNCA*) have been found in familial PD cases suggesting that α -syn protein plays an important role in the progression of PD pathogenesis, not only in

sporadic, but also in familial PD cases. Indeed α -syn pathology is present in virtually all sporadic and familial PD patients [2], and its distribution is correlated with motor/cognitive dysfunction. Transgenic mice with ectopic expression of WT or mutant α -syn have also been shown to cause an age-associated reduction in dopamine neurotransmitters and behavioral defects [3].

Recent studies have demonstrated that the intracerebral injection of α -syn preformed fibrils (PFF) in mice brain induces the propagation and spread of endogenous α -syn in anatomically interconnected brain regions far from the injection site [4, 5]. It is well evidenced that the pathological form of α -syn self-propagates and spreads progressively throughout interconnected brain regions, thereby supporting the staging of clinical Parkinsonian symptoms [6, 7]. The mechanisms by which the pathological α -syn is taken up and transported to adjacent cells are poorly understood. However, based on current evidence, it is hypothesized that the pathological species of α -syn spread from cell-to-cell as a result of template misfolding and aggregation of nascent or properly configured α -syn, which are subsequently transferred to neighboring cells [8–10]. Further evidence in support of this transcellular spread of pathological α -syn is derived from the study conducted by Luk *et al.* [2012], which demonstrated that the intrastriatal injection of synthetic α -syn PFF in WT mice induced α -syn pathology, which propagated to anatomically interconnected brain regions. A more recent study reported that the intrastriatal injection of mouse α -syn PFF into rats accelerated α -syn pathology and bilateral nigrostriatal degeneration [5]. It is noteworthy to mention both studies have demonstrated the robust α -syn pathology observed at 60–90 days post injection in different interconnected regions of the brain. However, certain investigators have failed to observe similar spreading since it is suggested that the nature of α -syn fibril, site of injection, and amount of fibril are critical factors for the successful spread and propagation of α -syn [11].

The mitochondrial neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), has been well known to produce Parkinsonian features in mice, monkeys, and humans. These features are strikingly similar to PD [12] and include a reduction in the density of dopamine transporter (DAT) in the striatum, and death of DA cell bodies in the SNpc [13]. MPTP is a lipophilic organic molecule with the ability to cross the blood brain barrier. It is transported to the glial cells for metabolism. The enzyme monoamine oxidase B present in the glial cells metabolizes MPTP to its active metabolite, 1-methyl-4-phenylpyridinium (MPP^+), which enters into DA neurons using dopamine transporter (DAT). Once inside the DA neurons, MPP^+ blocks complex I of the mitochondrial respiratory chain [14] and subsequently generates free radicals that lead to the oxidative nitration of α -syn [15]. The overexpression of α -syn by itself causes oxidative stress and increases inclusion formation and mitochondrial structural abnormalities in cultured neurons [16]. Additionally, it has also been reported that the targeted over-expression of α -syn by rAAV2/1 vector in the presence of MPTP in mice induces neurotoxicity, suggesting that MPTP also plays an important role in enhancing α -syn-mediated neurodegeneration [17]. It has been reported that the silencing of α -syn provides neuroprotection against MPTP [18]. Interestingly, MPTP exposure in mice does not produce LB-like protein aggregates or is not associated with α -syn spread or aggregation. Therefore, the aims of the present study are: 1. To examine the effect of MPTP on the propagation, spread, and formation of pathological aggregates of α -syn in mice administered an intrastriatal injection of human wild type (WT)

α -syn PFF 2. To establish a model that depicts α -syn spread, the formation of the pathological aggregates of α -syn, and the loss of DA neurons within a short period of time.

2. Materials And Methods

2.1 Animals

Male C57BL/6 mice were obtained from the United Arab Emirates University Animal House Facility. For the experiment, 2-3-month-old mice weighing approximately 25–30 grams were used. Mice were housed in a 12-hour light/dark cycle and had access to food and water during the entire duration of study. All experimental procedures were conducted in accordance with the approval of the UAEU Animal Ethics committee guidelines.

2.2 Stereotaxic injections

α -syn PFF was stereotaxically injected into the striatum (coordinates from bregma: anterior-posterior, + 0.5 mm; medial-lateral, – 2.2 mm; dorsal-ventral, – 3.4 mm) [19] at an infusion rate of 0.5 μ l/min, using a syringe connected to an automatic pump (World Precision Instruments, USA). Five micrograms of WT recombinant human α -syn PFF in 2.5 μ l phosphate buffered saline (PBS) was injected at a constant rate, over 5 minutes, followed by a one minute interval to allow proper diffusion of the α -syn PFF. The control mice received an equal volume of PBS. Injected mice were housed in individual cages and monitored on a routine basis.

2.3 MPTP administration

At 6 weeks post α -syn PFF injection, mice were challenged with two different doses of MPTP [10 mg/kg.b.wt. or 25 mg/kg.b.wt., measured in terms of free base; MPTP-HCL; Sigma-Aldrich] or an equal volume of saline (control) once daily, for five consecutive days, through intraperitoneal injection. MPTP was first dissolved in normal saline to obtain the 10 mg/kg.b.wt. and 25 mg/kg.b.wt. concentrations for injection [20–22]. The brains of the mice were collected at 2 weeks from the start of the MPTP regimen and assessed for the survival of the DA neurons.

2.4 Brain collection

At the end of the experiments, the animals were anaesthetized. Cardiac perfusion was performed using normal saline to wash out the blood followed by a wash with PBS containing 4% PFA. The brain of the animals was quickly removed and placed in a container containing 4% PFA overnight at 4°C. PFA solution was replaced with 10% sucrose in 0.1 M PB and 0.02% sodium azide, twice a day, for 3 days. The brain was then placed on tissue paper to remove water, frozen, and stored at -80°C for future use.

2.5 Behavior study

2.5.1 Rotarod

Mice were pre-trained to obtain stable performance on an automated four lane rotarod unit (Rotamex-5, Columbus Instruments, Columbus, OH; lane width, 95 mm, rod diameter, 30 mm). We used a speed

accelerating protocol where mice were pre-trained to stay on the rod while increasing the speed from 1 to 40 rpm over a period of 5 min. On day one of the training, mice were exposed to speeds of 2 to 20 rpm gradually increased over a period of 5 min. On day two, mice were exposed to speeds of 3 to 30 rpm, gradually increased over a period of 5 min. On day three, mice were trained to stay on a rotarod accelerated gradually from a speed of 4 to 40 rpm over a period of 5 min. At the end of the training period, each animal underwent four trials with a minimum interval of 15 min between trials. Their performance was measured as the average of three best runs. The trials were performed one day prior and one week after the MPTP challenge. Mice were placed on an accelerating rotating spindle of 3 cm diameter for testing. The speed of the rotarod was gradually increased from 4 to 40 rpm over a 5 min period. The mean latency to fall was recorded for three trials and the data obtained was used for analysis [23].

2.5.2 Grip test

Grip test was performed one day before the start of MPTP treatment and one week after MPTP exposure. Forelimb Grip strength was measured using an apparatus obtained from Ugo Basile, Comerio, Italy. Mice were held by the tail above the base plate of the apparatus and lowered toward the apparatus until the mice grasped a triangular bar. The mice were then gently pulled away, along the horizontal axis, and the maximal grip force achieved was recorded. The procedure was repeated five times for each animal and the mean score of the middle three trials was used for analysis [24].

2.6 Preparation of brain samples for immunohistochemical analysis

For assessment of DA neuronal death in the SNpc area, the brain was cut into sections of 40 µm thickness from the caudal to the rostral end, covering the whole SNpc area. Analysis of dopamine transporter integrity was performed using striatal sections of 40 µm thickness. For immunostaining, the DA neurons and dopamine nerve terminal sections were stained with Tyrosine Hydroxylase (TH) and Dopamine transporter (DAT) antibodies, respectively.

2.6.1 Tyrosine hydroxylase immunostaining of SNpc neurons

Every sixth section of seven coronal serial sections from the rostral to the caudal end, covering the region from - 2.18 to -3.80 mm of bregma, from each brain, were used for immunostaining by the free floating technique. The sections were washed thrice, for 10 minutes each time, with 0.01 M PBS, pH 7.4, and incubated with 10% normal goat serum in PBS containing 0.3% Triton-X 100 as a blocking reagent for 1 h, at room temperature. The sections were then washed once with 0.01 M PBS and incubated with primary antibody against TH (TH, immunostar, 1:1000) for 48 h at 4°C. Biotin streptavidin donkey anti-mouse antibody was used as the secondary antibody to increase sensitivity (Biotin-sp-conjugated, Jackson Immuno Research, 1:1000). Following PBS wash, the brain sections were incubated for 1 hour with streptavidin conjugated with horseradish peroxidase (Sigma, Streptavidin-HRP Conjugate, 1:200), and the immunoreactivity was visualized using 3,3'-Diaminobenzidine (DAB) reaction. Subsequently, the sections were mounted and defatted, and the number of DA neurons was counted using stereological technique, as reported previously [21, 22].

2.6.2 Dopamine transporter staining of striatum

Striatal sections were collected and washed thrice, for 10 minutes each time, with 0.01 M PBS, pH 7.4. The sections were then incubated with 10% normal goat serum in PBS containing 0.3% Triton-X 100 as a blocking reagent for 1 h, at room temperature. Subsequently, the sections were washed once with 0.01 M PBS and incubated with polyclonal rat antibody against DAT (1:1000) for 48 h at 4°C. The sections were then incubated for overnight with the corresponding secondary antibody and visualized using the avidin-biotin complex peroxidase reaction of DAB, as described previously [21, 22]. To assess the loss of striatal fibers in the striatum, the optical density of the dopamine fiber was measured using Image J software (NIH, Bethesda, MD, USA). The optical density of three different fields of striatal sections of equal area were used for analysis.

2.7 Immunofluorescence and confocal microscopy

Brain sections were washed with 0.01 M PBS, pH 7.4, and incubated with 10% normal goat serum in PBS containing 0.3% Triton-X 100 for 1 h, at room temperature. The sections were washed once with 0.01 M PBS, incubated at 4 degree with mouse primary monoclonal antibody against TH (TH, immunostar, 1:1000), and double labeled with rabbit polyclonal antibody detecting endogenous levels of phosphorylated α -syn (α -synuclein (phospho S129) abcam, 1:1000) or anti- α -syn filament specific rabbit polyclonal antibody (α -syn filament, Abcam, 1:2500) or rabbit polyclonal anti-GFAP antibody (GFAP, Abcam, 1:1000). Iba1 was stained using rabbit primary polyclonal anti-Iba1 antibody (Iba1, Wako, MA, USA 1:1000). The sections stained with α -syn filament, GFAP and Iba1 were previously treated with antigen retrieving agent (Proteinase K, 5 μ g/ml, for 30 min at 25°C). After two days, the sections were washed with 0.01 M PBS several times, and incubated at 4 degree overnight with corresponding secondary anti-mouse Alexa 488 antibody (1:1000), anti-rabbit Alexa 594 antibody (1:1000). Following PBS wash, the sections were mounted on a slide using Fluoroshield mounting medium (Sigma) and the images were acquired using a confocal microscope (Nikon EZC1). For detecting the pathological accumulation of α -syn within the TH neurons, three sections were selected from the following anatomical region: -2.82 mm, - 3.1 mm, and - 3.38 mm, relative to the bregma of each animal.

2.8 Stereology

Stereology is the method of choice for quantifying the number of cells in histological samples. It provides a three dimensional (3D) unbiased interpretation of two dimensional (2D) cross sections. To estimate the number of TH neurons in the SNpc area, we used an optical fractionator of the stereo investigator system (version 2018). Briefly, seven serial coronal sections from the SNpc area of each animal were counted following the manufacturer's instruction. The thickness of the sections was measured using a z-axis microcreator. The counting contour was outlined using a 5x objective lens and the count was obtained using a 63x objective lens [21, 22]. The results are presented as the number of DA neurons in the SNpc.

2.9 Expression and purification of recombinant human α -synuclein

The expression vector pT7-7 wt- α -synuclein (pT7-7 wt- α -synuclein was a gift from Hilal Lashuel, Addgene plasmid #36046) was transformed in *E. coli* BL-21 DE-3 cells, and expression was induced by the addition of isopropyl D-thiogalactopyranoside (IPTG). Cells were harvested, resuspended in non-

denatured lysis buffer (PBS containing 5 mM EDTA and 0.02% Sodium Azide), homogenized using a glass homogenizer, and subjected to sonication for 10 min. The resuspended cells were then boiled for 10 min and cooled on ice for 30 min. Subsequently, the cell lysate was centrifuged at 15000 rpm for 20 min, and the supernatant was subjected to dialysis in the gel filtration buffer (10 mM Tris (pH 7.6), 50 mM NaCl, 1 mM EDTA, 1 mM PMSF). The lysate was then filtered on 0.22 µm filters and concentrated (2–4 mL/L of cell culture) using protein concentration columns (MWCO 7K). The concentrated lysate was injected into a gel filtration column (Superdex 200) and the fractions were collected and examined by SDS gel separation. The selected fractions were pooled, and the concentration of the pooled fractions was estimated by the BCA assay.

2.10 Generation of α-synuclein pre-formed fibrils (PFF)

About 100 µM of α-syn was incubated at 37°C, with continuous shaking at 800 rpm, in a Thermomixer (Eppendorf, Germany), for 7 days. Samples were collected at different time points for thioflavin-S assay, to monitor the fibril formation. Subsequently, the samples were centrifuged at 18,000xg for 10 min and the insoluble fibrils were collected. These fibrils were fragmented on ice by ultra-sonication in a Sonic raptor 250 equipped with a fine tip (pulse – 5 s, output – 40 W, time – 5 min) [25]

2.11 Statistical analysis

The Graph Pad Prism software (InStat software, La Jolla, CA, USA) was used for data analysis. The data were analyzed using one-way ANOVA followed by Dunnett's Multiple comparison test. Two-way ANOVA was used for the analysis of data from the behavior study. T-test was used to estimate the difference between (PBS + MPTP low dose) versus (PFF + MPTP low dose) and (PBS + High dose) versus (PFF + MPTP High dose). All data are presented as mean ± SEM. Significance is denoted as * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

3. Results

3.1. A low dose of MPTP (10 mg/kg.b.wt) enhances the spread of endogenous α-syn from the PFF injection site to the SNpc while a high dose (25 mg/kg.b.wt) decreases the spread

MPTP has been known to cause DA neuronal death and has been used as a neurotoxin in animal models for the study of PD. However, MPTP does not contribute to LB-like pathology in mice. The reason for this inconsistency is not clear at the moment. To examine whether MPTP has any role in the enhancement of α-syn accumulation and spread in PFF inoculated mice, we undertook the current experiment. We found that pS129 synuclein was detected at the site of injection, in significant amounts, in the animal injected with wild type α-syn PFF in the presence of saline or MPTP (Fig. 1). However, we did not observe pS129 α-syn in the contralateral side or in mice inoculated with PBS (Fig. 1). This result suggests that PFF induces the expression of endogenous α-syn irrespective of MPTP exposure. Interestingly, we observed that the pS129 α-syn antibody in mice inoculated with α-syn PFF and challenged with a low dose of MPTP showed a consistently more severe and widely distributed immunoreactivity. However, mice administered an intrastriatal injection of PBS and challenged with any dose of MPTP or saline did not show any

detectable pS129 α -syn immunoreactivity within the experimental time period, suggesting that the induction and expression of endogenous α -syn requires PFF as seeding material.

SNpc neurons project their nerve terminals towards the striatum where they release the neurotransmitter dopamine. In this study, we examined if exogenous α -syn PFF self-triggered its spread from the injection site to the cell body, and the effect of different doses of MPTP on this spread. We challenged the α -syn PFF injected mice with MPTP to examine if MPTP had the ability to further accelerate the rapid propagation and robust α -syn pathology in the interconnected brain areas such as the DA neurons of SNpc. We performed double immunofluorescence staining of TH and pS129 α -syn. In mice injected with a combination of α -syn PFF and saline or a low dose of MPTP, we detected the accumulation of pS129 α -syn in the cytoplasm surrounding the nucleus of the neurons positively stained by TH (Fig. 2). Surprisingly, we noticed that the pattern of α -syn spread was significantly diminished in animals that received a combination of α -syn PFF and a high dose of MPTP (Fig. 2). Moreover, the accumulation of α -syn was detected only in the SNpc in the ipsilateral side in the animals of all groups administered PFF (Fig. 2). Furthermore, we performed a count of the number of TH neurons in the SNpc area, in a similar anatomical location that stained positive for pS129- α -syn. We observed a significantly high level of co-localization and accumulation of pS129- α -syn in the TH neurons of PFF injected animals challenged with a low dose of MPTP (Fig. 2B/C) compared to a high dose of MPTP. Collectively, our results suggest that a low dose of MPTP enhances the spread and accumulation of α -syn.

3.2. A low dose of MPTP enhances the cytoplasmic aggregation of proteinase-k resistant α -syn whereas a high dose of MPTP enhances nuclear translocation in α -syn PFF injected mice

To further characterize if the observed accumulation of α -syn in the SNpc was a true representation of the pathogenic form of α -syn, we treated the brain section with proteinase K for 30 min at 25°C, and stained it with an α -syn conformation-specific antibody recognizing the α -syn filament. Interestingly, we found significant accumulation of α -syn within the TH neurons of the animals inoculated with PFF and further treated with saline or a low dose of MPTP (Fig. 3). Similar to pS129 α -syn staining, we found that the PFF inoculated animal injected with a low dose of MPTP showed greater accumulation of α -syn within the TH positive neurons compared to the saline treated animal. However, PFF inoculated animals injected with a high dose of MPTP showed significantly low levels of α -syn accumulation in the TH positive neurons compared to the animals treated with saline (Fig. 3). As expected, accumulation of conformation/filament-specific α -syn was not detected in the SNpc in the contralateral side of animals of all groups injected with PFF (Fig. 3) or in the ipsilateral side of animals injected with PBS (Fig. 3). Interestingly, using α -syn conformation-specific antibody, we detected two different types of synuclein signals: the cytoplasmic signal, which accumulated around the nucleus, and the stain accumulated in the nucleus (co-localized with the nucleus-specific stain, To-PRO-3). In the group of animals inoculated with α -syn PFF and treated with saline, majority of the signal identified was localized in the cytoplasm surrounding the nucleus (Fig. 4). However, in the animals inoculated with α -syn PFF and further administered a low dose of MPTP, two types of signals were detected: the nucleus-accumulated signal and the cytoplasmic signal that surrounded the nucleus (Fig. 4). In contrast to the group of animals

administered a low dose of MPTP, majority of the signals in the high dose group appeared in the nucleus (Fig. 4).

3.3 Activation of astrocytes and microglial cells in animals inoculated with PFF and treated with MPTP

Previous research suggests that neuroinflammation plays a critical role in the pathophysiology of PD. The activation of astrocytes and microglial cells has been considered as a marker of neuroinflammation progressing to neurodegeneration. Hence, we examined the activation of astrocytes and microglial cells by immunostaining with GFAP and Iba-1, respectively. We found that a high dose of MPTP caused a significant increase in the number of activated astrocytes and microglial cells in the SNpc area irrespective of whether the animals were inoculated with α -syn PFF or not. Moreover, this activation was more profound when α -syn PFF inoculated mice were challenged with a high dose of MPTP. However, we observed a subtle increase in the activation of astrocytes in animals that received a low dose of MPTP with or without α -syn PFF injection (Fig. 5). We also observed that the mice inoculated with PFF showed higher activation of microglial cells, as identified by the cell count following Iba-1 staining, compared to the PBS injected control animals. However, this activation did not increase further in the presence of a low dose of MPTP (Fig. 6).

3.4 MPTP enhances neurodegeneration in nigrostriatal pathways in mice inoculated with PFF

We counted the number of DA neurons in the SNpc area to examine if they played any role in the spread of α -syn, to augment the toxicity induced by MPTP injection. We used an unbiased stereo investigator system to count the total number of DA neurons in the SNpc area. A high dose of MPTP (25 mg/kg.b.wt) has been known to cause DA neuronal death (usually 45–50% DA neurons) [21, 22]. The PFF injected animals treated with a high dose of MPTP showed more DA neuronal death compared to animals injected with only MPTP (Fig. 7). As expected, a low dose of MPTP had a very subtle effect on DA neuronal death, which was non-significant compared to saline treated animals (8%). Interestingly, PFF inoculated animals challenged with a low dose of MPTP showed significant loss of DA neurons (33%). Collectively, our findings suggest that PFF significantly enhances the toxicity of MPTP. However, a high dose of MPTP did not enhance the pattern of spread of endogenous α -syn. The DA neurons in the SNpc area projected their nerve endings towards the striatum. Thus, the neurodegeneration seen in the SNpc area must be correlated with the loss of nerve terminals in the striatum. To confirm this, we stained the dopamine transporters in the striatum. The results are presented in terms of the intensity of staining (Fig. 8). Following treatment with a high dose of MPTP, we observed a significant decrease in the level of DAT in the striatum. This decrease was further augmented in the presence of PFF. These findings correlate with the results obtained in the TH count. We also observed a significant reduction in the intensity of DAT staining in animals that received both α -syn PFF and a low dose of MPTP. However, a low dose of MPTP or α -syn PFF alone caused only a very mild reduction in the intensity of DAT (Fig. 8).

3.5 Mice administered MPTP and α -syn PFF showed reduced motor activity

We expanded our study to examine the motor activity of animals pre-inoculated with α -syn PFF and treated with MPTP. We performed the rotarod test to assess the motor coordination of the mice and grip

test to assess the muscle strength before and after the MPTP injection. Animals that received a high dose of MPTP along with α -syn PFF or PBS showed a significantly faster to fall from the rotarod. Animals that received a low dose of MPTP or α -syn PFF alone did not show any defective performance in the rotarod test (Fig. 9). However, mice that received both α -syn PFF and a low dose of MPTP showed a significant deficit in the performance in the rotarod test (Fig. 9). However, none of the animals in all the groups tested showed any detectable changes in performance in the grip test (data not shown).

4. Discussion

α -Syn is a 14 kDa, soluble, naturally unfolded protein, mainly present in the presynaptic terminal of the neurons. The cytoplasmic accumulation of misfolded α -syn as LB forms the pathological hallmark of both sporadic and familial PD [26]. Point mutations as well as duplication and triplication of the α -syn gene, *SNCA*, have been reported in PD patients of several families. The physiological function of α -syn is not fully understood. However, it has been shown that the germ line deletion of α -syn in mice impairs neurotransmitter release. Moreover, α -syn deficient mice are resistant to the mitochondrial neurotoxin, MPTP, while mice presenting the ectopic expression of α -syn are sensitive to MPTP. siRNA mediates the knockdown of α -syn in mice, thereby making them resistant to MPTP toxicity [18, 27]. The above observation suggests that the oxidative stress generated in the mitochondria due to MPTP injection augments the pathological nature of α -syn in producing toxic species. However, the data showing α -syn aggregation in mice injected with MPTP are conflicting [28–30]. It has been reported that the exposure of mice to MPTP does not cause any LB-like pathology, although profound DA neurodegeneration has been observed [30]. One report demonstrated the increased expression of syn mRNA in mice that received prolonged MPTP treatment [29]. Moreover, Meredith *et al.* [28] provided evidence that MPTP injection causes the accumulation of granular and filamentous α -syn inclusions in DA neurons. However, other researchers negated the claim and reported that MPTP causes neurodegeneration without α -syn inclusion [30]. It is noteworthy to mention that different doses of MPTP have been used in mice models of acute, sub-chronic, and chronic PD. Interestingly, only a chronic dose of MPTP has the ability to increase the expression of α -syn mRNA; an acute dose does not. Although only monkeys injected with MPTP have shown intraneuronal inclusions suggestive of LBs [31, 32], the major drawback of this mouse model was the lack of α -syn inclusion in MPTP treated mice. Moreover, in monkeys, a low dose of MPTP has been shown to cause a greater loss of DA nerve terminals in the putamen than in the caudate nucleus [33, 34]. Thus, one of the arguments raised is that this discrepancy in α -syn inclusion generation is a result of the animal species used, the dosage, and duration of MPTP administration in these models.

Growing number of experimental data suggest that the inoculation of a pathological form of α -syn, such as PFF, in mice brain, can induce the progressive propagation and spread of endogenous α -syn throughout the interconnected brain regions, through cell-to-cell transmission mechanisms [4, 5, 35, 36]. However, this α -syn propagation requires the presence of endogenous α -syn. A previous study reported that α -syn knockout mice failed to show any such spread and aggregation [4]. However, transgenic mice expressing WT or mutant α -syn did not self-initiate the propagation, thereby indicating that PFF injection, which acts as a seeding material, is a prerequisite. Thus, the α -syn fibrils mediating the seeding process

are very critical for the initiation and propagation of endogenous α -syn. It is noteworthy to mention that α -syn propagation has only been observed using a pS129 α -syn-specific antibody. Therefore, the hyperphosphorylation of α -syn at serine 129 residue is considered to be a pathological consequence which induced by α -syn PFF and plays a critical role in the aggregation of α -syn, and in PD progression [5]. Previous reports have shown that the minimum time required to observe the spread of α -syn in the SNpc area is at least a month or more after the inoculation of α -syn PFF at multiple sites. However, DA neuronal death does not appear before three months post the injection of PFF.

In the current study, we examined to see if the injection of different doses of MPTP (one low and one high dose) in mice pre-inoculated with α -syn PFF had any effect on the spread, toxicity, and pathology of endogenous α -syn. To accomplish this, we administered a single stereotaxic injection of α -syn PFF in the striatum of mice, and challenged them with two different doses of MPTP: a high dose of 25 mg/kg.b.wt and a low dose of 10 mg/kg.b.wt. A high dose of MPTP has been known to cause the rapid destruction of DA neurons in the nigrostriatal pathway, with concomitant loss of DAT, and has been commonly used in PD mouse models. A low dose of MPTP has been reported to induce mild effects in DA neurons without significant destruction of these neurons [20]. MPTP, following conversion to MPP⁺, inhibits complex 1 of the mitochondrial respiratory chain complex and selectively generates reactive oxygen species (ROS) in the DA neurons [37]. We observed that the exposure of PFF inoculated mice to a low dose of MPTP significantly increased the spread and accumulation of endogenous α -syn, from the striatum to the SNpc, as detected by pS129 α -syn antibody. Surprisingly, we found that the exposure of PFF inoculated mice to a high dose of MPTP reduced the spread of α -syn from the striatum to the SNpc, and the localization of α -syn in the cytoplasm. An *in vitro* study demonstrated that α -syn, owing to its chemical structure, had the tendency to become less soluble and form insoluble, high molecular weight aggregates when exposed to an oxidizing agent [38]. MPTP can generate reactive oxygen species (ROS) and increase the amount of insoluble α -syn aggregates. Thus, MPTP intensifies this aggregation process [39] and results in more detrimental effects on neurons. In our current study, we observed a higher DA neuronal death in PFF injected mice exposed to a high dose of MPTP, leading to the lower availability of healthy DA neurons for the active spread of α -syn.

A growing body of evidence has shown that the proteinase K-resistant α -syn species formed in the brain of human PD patients have the characteristics of intracytoplasmic inclusions of LBs [40]. To examine this phenomenon, prior to immunostaining, we treated the brain section with proteinase K to detect proteinase K-resistant aggregates of α -syn using a conformation-specific antibody, as reported previously [41]. In addition to removing the soluble synuclein, proteinase K has also been extensively used to retrieve the antigen [42, 43]. In α -syn PFF injected mice with or without MPTP challenge, we found that the proteinase K-resistant α -syn were accumulated in the SNpc area of the brain. However, in α -syn injected mice challenged with a low dose of MPTP, we found an abundant amount of proteinase K-resistant α -syn species. In addition, we did not observe α -syn positive staining in the SNpc region of control mice or mice treated with MPTP alone following proteinase-k treatment. Surprisingly, we observed significant nuclear localization of α -syn in α -syn PFF injected animals challenged with a high dose of MPTP.

In transgenic fruit flies and tissue culture systems, it has been reported that α -syn is translocated to the nucleus, where it promotes cell death by inhibiting histone acetylation. However, the administration of histone deacetylase inhibitor was found to rescue the cells from α -syn toxicity [44]. Recently, it has been demonstrated that TRIM28 regulates the nuclear accumulation and toxicity of α -syn [45]. Transgenic mice expressing human α -syn have been reported to show nuclear translocation with distinct cytoplasmic inclusion [46]. Another study revealed that α -syn promotes cell death by activating nitric oxide synthase (NOS), which leads to the damage of DNA and activation of polyadenosine 5'-diphosphate-ribose polymerase-1 (PARP-1), leading to parthanatos [47]. In tissue culture, it has been found that the oxidative stress induced by H_2O_2 increases the nuclear translocation of α -syn, leading to increased cell death [48]. In our current study, we observed that the exposure of PFF inoculated mice to a high dose of MPTP led to the nuclear translocation of proteinase K-resistant α -syn, and the highest degree of DA neuronal death. Our results are in agreement with the aforementioned findings, and support further that α -syn mediates the pathological effect leading to neurodegradation by increasing its nuclear translocation rather than spread.

Neuroinflammation significantly contributes to neurodegeneration not only in animal models of PD [49] but also in PD patients [50]. α -Syn aggregation elicits microglial cell and astrocyte response, which mediate neuroinflammation by secreting inflammatory mediators such as reactive oxygen species, cytokines, and chemokines that eventually contribute to enhanced DA neuronal death in the SNpc area [51, 52]. Mice over-expressing human α -syn show an increased level of activated microglial cells and TNF- α in the striatum and SNpc area [53]. It has been reported that extracellular α -syn released from the neuron elicits microglial inflammatory response by acting as an endogenous agonist for toll-like receptor 2 (TLR2) [54]. In mice, MPTP induces glial cell response by activating microglial cells and astrocytes. In MPTP treated mice, activated microglial cells have a large cell body with short and thick processes while activated astrocytes show a large cell body with long and thick processes. Consistent with the above report, in mice that received a high dose of MPTP along with α -syn PFF, we found a significant increase in the number of activated astrocytes and microglial cells in the SNpc area. In the same group of animals, we also observed the highest DA neurons loss with nuclear translocation of α -syn within the TH neurons. The prior hypothesis of α -syn stated that α -syn could be taken up by neurons, where it tends to act as a seed and enhance the aggregation of endogenous α -syn and release it into the extracellular space. These α -syn aggregates then enter the neighboring neurons, microglial cells or astrocytes [55–57]. α -Syn, upon endocytosis by astrocytes, causes a change in gene expression, leading to an increase in the induction of pro-inflammatory cytokines and chemokines which lead to an inflammatory response which mediates the pathological effect [57]. Compared to the control, in α -syn injected mice, we observed an increase in the number of activated astrocytes in the SNpc area. This activation became higher when we combined α -syn PFF with a high dose of MPTP, a mitochondrial complex I inhibitor that plays a key role in the massive release of free radicals following its intraperitoneal administration in rodents. Collectively, aggregated α -syn and a high dose of MPTP have the ability to enhance the activation of glial cells and amplify the cascade of nigrostriatal degeneration.

Finally, we performed behavioral studies on our experimental mice. In PFF inoculated mice challenged with MPTP, in addition to the effect on the neurons in the SNpc, we also found compromised behavior, as measured by the rotarod test. However, we did not observe any significant change in the grip test performance of the experimental animals compared to the PBS injected and saline treated control animals. In summary, through this study, we have demonstrated the significant spread of α -syn and the moderate DA neuronal loss in mice injected with a low dose of MPTP in conjunction with α -syn PFF. Thus, the administration of a low dose of MPTP to PFF inoculated mice enables the use of these animal models in future mechanistic studies and for novel drug development.

Conclusion

Our current study indicates that the injection of a low dose of MPTP in mice pre-inoculated with α -syn PFF enhances the spread of endogenous α -syn from the injection site to interconnected brain regions such as the SNpc. This α -syn spread has been considered as an early event in PD. Surprisingly, we observed a significant reduction in the spread of endogenous α -syn when a high dose of MPTP was employed in mice administered an intrastriatal injection of α -syn PFF. However, we observed a significantly higher level of DA neuronal death in the SNpc area, altered motor coordination, and higher activation of glial cells (microglia and astrocytes) in this group of animals. Owing to the enhancement of α -syn spread and moderate DA neuronal death in PFF inoculated mice administered a low dose of MPTP, this animal model may be useful for the study of the mechanisms underlying α -syn-mediated toxicity and for the screening of potential drugs against PD, by tracking the spread of α -syn and the neurotoxicity.

List Of Abbreviations

α -Syn: α -Synuclein

DA: Dopaminergic

MPTP: 1-methyl, 4-phenyl, 1,2,3,6 tetrahydropyridine

PD: Parkinson's disease

PFF: Pre Formed Fibril

TH: Tyrosine Hydroxylase

WT: Wild type

Declarations

Ethics approval

All experimental procedures were conducted in accordance with the approval of the UAEU Animal Ethics committee guidelines.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Authors contributions

All the authors provided important intellectual content, reviewed the content and approved the final version for the manuscript. Contributed significantly, read and approved the manuscript: MMM, MT, TK, MEH. Conceived and designed the experiments: MEH. Performed the experiments: MMM, MT. Analyzed the data: MMM, MEH, Contributed reagents/materials/analysis tools: MEH. Wrote the paper: MMM, MEH.

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Figures

Figure 1

Intrastriatal injection of human WT α -syn preformed fibrils induced spreading of endogenous α -syn. Mice were subjected to intrastriatal injection of human wild type α -syn preformed fibrils (PFF) or phosphate buffered saline (PBS). Then mice were challenged with MPTP (low and high dose) or saline six weeks after post injection of PFF. Two weeks after MPTP injection, Mice were sacrificed and brains were collected for immunohistochemical analysis. Coronal section of striatum tissue immunostained with antibody to detect endogenous level of pS129 α -syn only in the injected side. The image showing detectable level of pS129 α -Syn at the injected site of the animal that received intrastriatal wild type α -Syn PFF with or without MPTP. Scale bar 50 μ m

Figure 2

Low dose of MPTP administration enhances the spreading of endogenous α -syn in human WT α -syn FFP injected mice in the SNpc area. A. Coronal section of SNpc stained with pS129 α -syn to detect endogenous level of pS129 α -syn (green) and TH antibody (red). The image showing spreading of pS129 α -syn with saline, low and high doses of MPTP. High dose of MPTP decreases the spreading of endogenous α -syn in the SNpc area. Scale bar 30 μ m. B. High magnification of the immunostained image of coronal section of mouse showing at SNpc stained with TH antibody (red) and antibody detect the pS129 α -syn (green). Scale bar 50 μ m. C. Number of TH positive neurons that co-localizes pS129 α -syn was counted and presented as percentage in the bar graph. Data represent mean value $\pm$ SEM. Statistical analysis was performed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. ** $P < 0.01$ and *** $P < 0.001$.

Figure 3

Low dose of MPTP administration enhances the spreading of filamentous specific α -syn in human WT α -syn FFP injected mice in the SNpc area. A. Coronal section of SNpc stained with filament specific α -syn antibody to detect filamentous α -syn (green) and TH antibody (red). The image showing spreading of filamentous specific α -syn with saline and low dose of MPTP treated group of animals. High dose of MPTP decreases the spreading of filament specific α -syn species in the SNc area. Scale bar 30 μ m . B. High magnification of the immunostained image of coronal section at SNpc area showing TH immunopositive neurons (red) and filament specific α -syn (green). Scale bar 50 μ m . C. Number of TH positive neurons that co-localizes with filament specific α -syn was counted and presented as percentage in the bar graph. Data represent mean value $\pm$ SEM. Statistical analysis was performed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. * $P < 0.05$, ** $P < 0.01$.

Figure 4

Low Dose of MPTP Enhances the Proteinase-k Resistant Cytoplasmic aggregation prone α -syn where High Dose of MPTP Enhances Nuclear Translocation in mice injected with WT-PFF syn. Coronal section of brain section containing SNpc area immune-stained with TH antibody (red), TO PRO-3 (blue) for nucleus and filament specific α -syn (green) antibody. Thick arrow pointing the nuclear localization of filamentous α -syn. Thin arrow pointing the cytoplasmic accumulation surrounding the nucleus by the filament specific α -syn. Scale bar 50 μ m

Figure 5

Activation of Astrocyte in the Animal Inoculated with α -syn-PFF followed by MPTP injection. A. Coronal section containing SNpc stained with GFAP antibody (green) to detect activated astrocytes and TH antibody to detect TH immune-positive neurons (red). Significant increase in activation of astrocyte in the SNpc area was observed in high MPTP dose group. Scale bar 30 μ m. B. Number of activated astrocytes at the SNpc per field is counted and presented in the graph. Data represent mean value $\pm$ SEM. Statistical analysis was performed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. ** $P < 0.01$ and *** $P < 0.001$

Figure 6

Activation of Microglia in the Animal Inoculated with α -syn-PFF and injected with MPTP. Coronal section containing SNpc area stained with antibody Iba-1 (green) to detect activated microglia and TH antibody to detect dopamine neurons (red). Animal injected wt- α -syn PFF and high dose of MPTP showing significant increase in the number of activated microglia. Arrow pointing amoeboid shape of microglia as activated. Scale bar 50 μ m. B. Number of activated microglia at the SNpc per field was counted and presented. Data represent mean value $\pm$ SEM. Differences were estimated using one-way ANOVA followed by Dunnett's Multiple Comparison Test. **P < 0.01

A

B

Figure 7

Immunohistochemistry of tyrosine hydroxylase (TH) to assess the loss of TH-immuno-positive (TH+) dopamine neurons in the substantia nigra pars compacta (SNpc). A. Representative images of coronal section of SNpc showing tyrosine hydroxylase (TH) positive neurons. Decreased immunoreactivity of TH positive neurons observed in ipsilateral side of SNpc that received α -syn PFF along with low/high dose of MPTP (α -syn+MPTP) injected mice as compared to (PBS+MPTP) injected mice at the similar dose of MPTP. Scale Bar represent 400 μ m. B. The number of TH neurons at the SNpc area counted using unbiased stereo-investigator system. MPTP increases the neurotoxic effect of α -syn PFF. Data represent mean value $\pm$ SEM. Significant difference was calculated and analyzed using one-way ANOVA followed by using Dunnett's Multiple Comparison Test. ***P < 0.001

Figure 8

Immunohistochemistry of dopamine transporter (DAT) to assess the density of dopamine neurons nerve ending in the striatum. A. Representative images of DAT showing immunostaining of nerve ending at the striatum. Decreased immunoreactivity of DAT observed in ipsilateral side of striatum with low/high dose of MPTP challenged mice that received α -syn preformed fibrils (α -syn+MPTP) injected mice as compared to (PBS +MPTP) injected mice with similar dose of MPTP. Scale Bar represent 400 μ m. B. The intensity of DAT signal at the striatum was estimated using NIH Image J and presented in the graph. MPTP exposure further increases the neurotoxic effect among the mice that received α -syn PFF. Data represent mean value $\pm$ SEM. Differences were estimated using one-way ANOVA followed by Dunnett's Multiple Comparison Test. *P < 0.05, ***P < 0.001

Figure 9

Rotarod test performance of mice from different groups. MPTP exaggerate the toxic effect of α -syn PFF. Data represent mean value $\pm$ SEM. Differences were estimated using Two-way ANOVA. *P < 0.05